Cladosporium fulvum effector proteins and their role in pathogen virulence

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Chapter 1:
Cladosporium fulvum (syn. *Passalora fulva*), a highly specialized plant pathogen as a model for functional studies on plant pathogenic Mycosphaerellaceae

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Introduction

Cladosporium fulvum [syn. *Passalora fulva* (Braun et al., 2003)] is the causal organism of tomato leaf mold, a fungal disease first described by Cooke (1883). Generally, foliage is the only tissue affected by the fungus, although occasionally also stems, blossoms, petioles and fruit are attacked (Butler and Jones, 1949; Jones et al., 1997). Conidia of the fungus can infect successfully if they settle on the abaxial side of a leaf, germinate, and subsequently enter through open stomata. Initial disease symptoms occur at the earliest one week after the start of infection as pale green or yellowish diffuse spots on the upper leaf surface, which later enlarge, turning into distinctive yellow spots (Fig. 1A). This appearance is the effect of cell death in the palisade parenchyma. The abaxial side of the leaf shows the most distinct symptoms: patches of white to olive–green mold that turn brown once sporulation commences (Fig. 1B). In advanced stages of disease development stomata do not function properly, because they are blocked by aggregations of conidiophores (Fig. 1E) that use the stomata to exit the leaf and liberate conidia. These subsequently contribute to spread of the disease. As a result of stomatal clogging, plant respiration is severely hampered (Butler and Jones, 1949). This can result in wilting of leaves, partial defoliation and, in severe infections, death of the host (Jones et al., 1997).

Although *Solanum esculentum* (tomato) is susceptible to the fungus, many other *Solanum* species are often resistant (Butler and Jones, 1949). About 100 years ago it was discovered that resistance against *C. fulvum* is genetically determined by the presence of *Cf* resistance genes (Lind, 1909; Norton, 1914). Later it was found that the relationship between host and pathogen is governed by a so–called 'gene–for–gene' relationship. The gene–for–gene hypothesis states that each dominant pathogen avirulence (*Avr*) gene confers recognition to a corresponding dominant host resistance (*R*) gene (Flor, 1942, 1946; Oort, 1944). Although *C. fulvum* most likely originates from the natural habitat of *Solanum* species in South America, greenhouse cultivation has also generated favorable conditions for the pathogen in temperate climate areas. As a result, for decades yearly outbreaks of the disease occurred also in these regions in greenhouses and tomato leaf mold became a persistent disease. However, the introduction of resistance loci from related wild species of tomato (*Cf–1* to *Cf–5*) into cultivated tomato has resulted in efficient containment of the pathogen (Boukema and Garretsen, 1975; Boukema, 1977; Hubbeling, 1978; Kerr et al., 1971; Langford, 1937). Since the introduction of the *Cf–9* resistance locus in the late 1970s in currently grown tomato cultivars in the late 1970s, *C. fulvum no* longer poses a serious threat to commercial tomato cultivation. Despite its limited agronomic importance, the *C. fulvum*–tomato interaction has become a model to study plant–pathogen interactions after intensive studies by the research groups of Drs. Higgins (Higgins et al., 1998), Oliver (Oliver et al., 2000) and de Wit (Joosten and de Wit, 1999).

This review will mainly focus on the pathogenic properties of *C. fulvum* and the mechanisms deployed by the fungus to establish pathogenicity. In addition, the properties of this interaction to serve as a model system for the interaction between plants and other members of *Mycosphaerella* will be discussed. As recent advances of the research on the

tomato *Cf* resistance genes and homologous genes that act in pathogen defense from other plant species have been extensively reviewed (Kruijt et al., 2005; Rivas and Thomas, 2002), we will not address *Cf*–gene structures and Cf–mediated downstream defense signaling.

The infection cycle on susceptible plants: The compatible interaction

The conidia of *C. fulvum* are generally spread by wind or water splash. If conidia land on the abaxial side of a leaf, successful infection can occur. At high relative humidity (over 85%) conidia germinate and form thin runner hyphae that grow randomly (undirectional) over the leaf surface (Bond, 1938; de Wit, 1977; Lazarovits and Higgins, 1976a). After approximately three days, a main germ tube or a lateral branch of the hyphae enters the tomato leaf upon encountering an open stoma (Fig. 1C). From this stage onward, the diameter of fungal hyphae enlarges at least two–fold. Subsequently, hyphal growth continues from the substomatal cavity into the intercellular space between the spongy mesophyll cells (apoplast) by the formation of long, branched hyphal structures (Bond, 1938; de Wit, 1977; Lazarovits and Higgins, 1976a). Fungal growth appears to be preferentially directed towards the vascular tissues, probably triggered by a sucrose gradient around the phloem (van den Ackerveken et al., 1994; Wubben et al., 1994). Sometimes, but only in later stages of the infection, the palisade parenchyma is invaded (Lazarovits and Higgins, 1976a).

Although no obvious feeding structures such as haustoria can be observed, growth of the fungus appears to depend on maintenance of close contact between fungal hyphae and host cells (Fig. 1D). This can sometimes be observed as slight indentations where fungal hyphae touch host cells (de Wit, 1977). This close contact suggests that the pathogen actively withdraws nutrients from the host (Bond, 1938; Lazarovits and Higgins, 1976b). No visible reaction of the host cells other than occasional callose deposition on the mesophyll cell walls can be observed during these stages of infection (de Wit, 1977; Lazarovits and Higgins, 1976a, b). However, several ultrastructural changes have been described, including the occurrence of endoplasmic reticulum parallel to the plasmamembrane at the site of fungal contact, and cytoplasmic lipid bodies and microbodies containing crystalline inclusions (Lazarovits and Higgins, 1976b). In mature lesions, mesophyll cells display various signs of degeneration of cell organelles, more specifically the mitochondria and chloroplasts (Lazarovits and Higgins, 1976b). Occasionally, the release of cytoplasmic contents due to damage to the plasmamembrane and sometimes even the tonoplast has been observed (Lazarovits and Higgins, 1976b). Nine to ten days after the onset of the infection, hyphal aggregations (stromatic bodies) are produced by the fungus in the substomatal spaces. Subsequently, aerial mycelium is formed from which conidiophores protrude through the stomata to the exterior where they produce chains of mostly two–celled conidia (Fig. 1E). After dispersal these conidia can contribute to the spread of the disease (Bond, 1938).

The infection on resistant plants: The incompatible interaction

No differences are generally observed between compatible and incompatible interactions with regard to conidial germination, formation of runner hyphae and stomatal penetration (de Wit, 1977; Lazarovits and Higgins, 1976a). Although the initial stages of incompatible and compatible interactions are very similar, occasionally in incompatible interactions the fungus grows out of the stoma again after having entered it (de Wit, 1977). This suggests that runner hyphae entering the apoplast through an open stoma elicit host defense responses that successfully repel the fungus. However, the majority of hyphae does not grow out of the stomata, and host defense results in arrest of fungal growth one or two days after penetration (de Wit, 1977). By then, the fungus has hardly grown from the stomatal cavity into the apoplast and hyphae appear swollen and curled. Hyphal cells that are in close contact with host mesophyll cells often collapse. Cell wall depositions containing callose are formed, leading to increased cell wall thickness, and deposits of extracellular material in the vicinity of fungal hyphae (de Wit, 1977; Lazarovits and Higgins, 1976a). At the molecular level, phytoalexins as well as pathogenesis–related (PR)–proteins accumulate (de Wit and Flach, 1979; de Wit and Kodde, 1981; de Wit and van der Meer, 1986; Joosten and de Wit, 1989). Although the accumulation of PR–proteins also occurs in compatible interactions, in incompatible interactions the accumulation usually is faster. Chitinases and β–1,3–glucanases were found to accumulate in vacuolar protein aggregates and in extracellular material surrounding mesophyll cells (Wubben et al.,1992). In addition, accumulation of PR–proteins near the stomata is observed, although this phenomenon also occurs in compatible interactions (Wubben et al., 1993). Therefore, it can be concluded that the accumulation of PR–proteins by itself does not contain the fungus, although the speed at which the accumulation takes place might influence the outcome of the interaction (Wubben et al., 1993).

The most striking feature of host defense in the incompatible interaction is the hypersensitive response (HR), in which mesophyll cells adjacent to the intracellular hyphae (and in addition occasionally guard cells and some epidermal cells) collapse in a manner that is reminiscent of apoptosis. As a result of this defense response the fungus is contained in a limited area of infection sites, exposed to components that are released upon host cell disruption, and thus cannot establish a successful infection (Joosten and de Wit, 1999).

The infection on non-host plants: Basic incompatibility

As mentioned before, the host range of *C. fulvum* is restricted to *Solanum* species and thus species from other plants are non–hosts (Bond, 1938). As early as 1938, experiments were described measuring the growth of *C. fulvum* on host plants, resistant plants and diverse ‘inappropriate hosts’ (Fig. 1F–H). Although no visual symptoms were recorded upon inoculation of non–host plants, stomatal penetration occurred in almost all species, although usually less frequent than on tomato. Maximal growth was recorded in a number of Solanaceous species that allowed some growth on young tissues, whereas mature tissues

allowed almost no fungal growth outside substomatal cavities, and often necrosis was observed (Fig. 1G). The most restricted fungal growth was reported in *Callistephus* sp. (aster), *Antirrhinium majus* (snapdragon) and *Bryonia dioica* (white bryony) where hyphae were hardly able even to enter the substomatal cavity (Fig. 1H). It was noted that in those interactions, fungal mycelium was confined to single peg–like branches and that host cell death did not occur (Bond, 1938).

Even at present, non–host resistance is a poorly understood defense mechanism (Mysore and Ryu, 2004). When assessing HR–associated recognition of extracellular *C. fulvum* components in non–host plants it was noted that Ecp2 displayed elicitor activity in several *Nicotiana* species (de Kock et al., 2004; Laugé et al., 2000). This observation justifies the question of whether Ecp2 recognition establishes non–host resistance in those species. However, this appeared not to be the case. On non–host *Nicotiana* species *C. fulvum* conidia did germinate and produce runner hyphae. Subsequent stomatal penetration was observed in rare cases, but hyphal growth always arrested very soon thereafter; by no means was the fungus able to grow further than the substomatal cavity. No differences were observed between Ecp2–recognizing and non–recognizing accessions and it is unclear what controls fungal arrest (de Kock et al., 2004). It is speculated that this is due to lack of production of the essential pathogenicity factors by the pathogen or due to the production and accumulation of effective defense components by the plant (Bond, 1938; de Kock et al., 2004).

Taxonomy of *Cladosporium fulvum*

C. fulvum is an asexual fungal species. The genus *Cladosporium* is extremely heterogeneous, containing more than 700 names, and consisting of close to 20 distinct, as yet undescribed genera (P. W. Crous, unpublished data). The genus *Cladosporium s.s.*, which has teleomorphs in *Davidiella* (Mycosphaerellaceae), contains saprobic as well as pathogenic taxa. *C. fulvum* (syn. *P. fulva*) is a typical species of *Passalora*, belonging to *Mycosphaerella s.s*. As *C. fulvum* is a biotrophic fungus of the non–obligate type, it can be cultured *in vitro* on simple media. The colonies that appear are strongly pigmented, greenish to black, and relatively slow–growing. The one– or two–celled, pigmented conidia are present in long, branched chains, arising from pigmented conidiophores. The superficial mycelium of *C. fulvum* is well developed, and consists of branched, septate hyphae, with cell walls consisting mainly of glucan and chitin polysaccharides (Joosten and de Wit, 1999).

As is often the case for asexual fungal species, classification is ambiguous, as it has in the past mostly been based on the phenotype, which was rarely supported by DNA phylogeny, or links to known teleomorph states. Earlier attempts to reduce heterogeneity of the genus *Cladosporium* by placing taxa in genera such as *Fulvia* or *Mycovellosiela* (Ciferri, 1952; von Arx, 1983) never gained broad acceptance. The introduction of a more phylogenetic approach has resulted in a simplification in many of these anamorph generic

concepts in the Mycosphaerellaceae (Crous et al., 2000). Based on phylogenetic analysis of internal transcribed spacer (ITS) regions from ribosomal DNA (rDNA) it was anticipated only a decade ago that *Cladosporium* species, including *C. fulvum*, comprised a monophyletic group (Curtis et al., 1994). In addition, *C. fulvum* was found by molecular data to belong to the genus *Mycosphaerella*, the most numerous genus of the Ascomycetes with more than 2000 described species (Crous et al., 2001; Goodwin et al., 2001). Recently, it has again been questioned whether *C. fulvum* should indeed be assigned to *Cladosporium*, as morphological and molecular data did not clearly support this link (Wirsel et al., 2002). Furthermore, as part of a taxonomic revision of *Cladosporium*, Braun et al., (2003) restricted *Cladosporium* to *C. herbarum* and its allies, and placed their teleomorphs in the newly formed teleomorph genus *Davidiella*. The genus *Passalora* is distinguished from *Cladosporium* by having conidial hila that are darkened, thickened and refractive, but not protuberant as in the case of *Cladosporium*, and having *Mycosphaerella* teleomorphs, while those of *Cladosporium* belong to *Davidiella* (Braun et al., 2003; Crous & Braun 2003). By resolving *C. fulvum* to be a species of *Passalora*, and thus a true *Mycosphaerella* anamorph, it also suggests that many of the host–pathogen mechanisms resolved in this pathosystem should also be active in other *Mycosphaerella* pathosystems. An extremely high percentage of DNA similarity (ITS1, 5.8S, ITS2) is observed between the DNA sequences of *C. fulvum* and other well–known *Mycosphaerella* pathogens lodged in GenBank, such as those causing *Mycosphaerella* leaf blotch of Eucalyptus (*M. aurantia*, *M. ellipsoidea*, *M. kensiensis*, 96–98%) (Crous et al., 2004a), leaf spot of grapevines (*P. dissiliens*, 97%), red band needle disease of pines (*Dothistroma* spp., 96%) (Barnes et al., 2004; see Fig. 1M), leaf and sheath red spot of sugarcane (*P. vaginae*, 97%), purple seed stain and leaf blight of soybean (*Cercospora kikuchii*, 92%), crassicarpa leaf blight of acacia (*P. perplexa*, 95%) (Beilharz et al., 2004; see Fig. 1N), leaf spot of cassava (*P. henningsii*, 97%), ivy (*M. hedericola*, 97%), lupin (*M. lupini*, 95%) (Kaiser and Crous, 1998), peanuts (*P. arachidicola*, 94%), sugarbeet (*Cercospora beticola*, 92%) and Acacia (*C. acaciae–mangii*, 92%) (Crous et al., 2004b).

The genus *Mycosphaerella* contains numerous economically important non–obligate hemi–biotrophic plant pathogens. These include *M. fijiensis*, *Cercospora zeae–maydis* and *M. graminicola*, the causal agents of black Sigatoka on banana (Fig. 1J), grey leaf spot disease on maize (Fig. 1L) and Septoria leaf blotch on wheat (Fig. 1K), respectively (Balint–Kurti et al., 2001; Palmer and Skinner, 2002; Ward et al., 1999) to name but a few. Plant pathogenic Mycosphaerellaceae species seem to share a number of characteristics: penetration through natural openings like stomata, extracellular growth between mesophyll cells without forming obvious feeding structures, and lack of obvious disease symptoms until re–emergence of conidiophores from stomata to release conidia. In all cases active penetration by appressoria and formation of haustoria has never been observed; colonization is strictly intercellular and mainly restricted to the mesophyll. Intriguingly, plant pathogenic Mycosphaerellaceae species have narrow host ranges, their hosts are highly divergent plant species, and they are found on all continents. The genetic

relationship between different Mycosphaerellaceae species and the high degree of host specialization suggests an evolutional lineage from a common fungal ancestor. This ancestor might have been a pathogen of an ancestral plant species that existed before the divergence into the many different plant species that are attacked by the different Mycosphaerellaceae species today. Co–evolution between host and pathogen has since then resulted in the high degree of specialization among species that all have a narrow host range.

Uptake of nutrients by *Cladosporium fulvum*

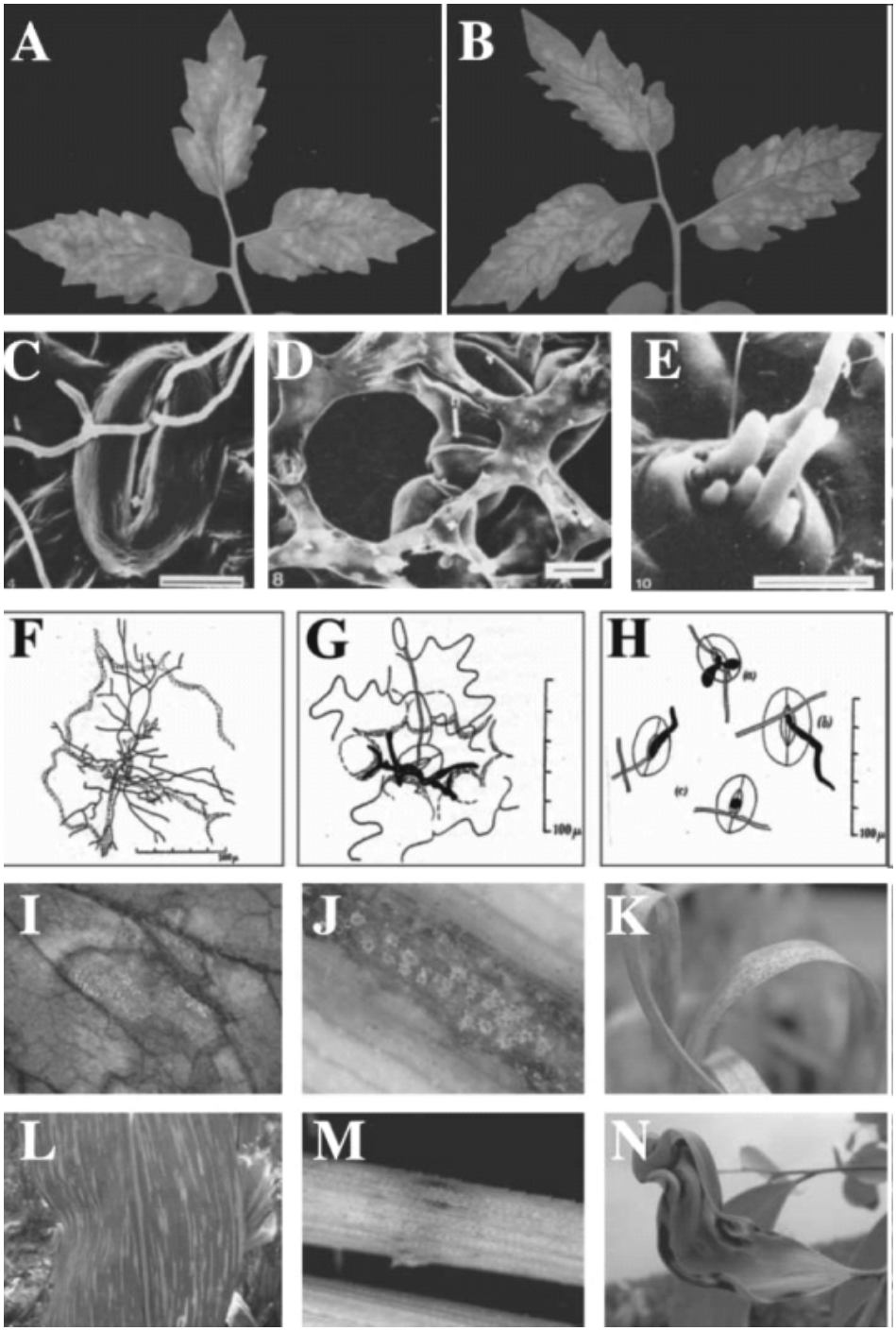
C. fulvum prefers to colonize a well–nourished host. On weak, starved, chlorotic or senescent plants growth of the fungus is severely limited (Butler and Jones, 1949). In advanced infections, most fungal biomass is concentrated around vascular tissues (van den Ackerveken et al., 1994; Wubben et al., 1994). In most plant species sucrose is the major sugar translocated in the phloem, and thus the concentration of fungal biomass around the vascular tissues is most likely caused by the gradient of apoplastic sucrose, of which the highest concentrations can be found near the phloem cells. *C. fulvum* is able to convert apoplastic sucrose into the hexose monomers glucose and fructose using a cell–wall–bound invertase and store it as mannitol (Joosten et al., 1990a). Furthermore, it has been demonstrated that during colonization of the leaf the concentration of apoplastic sucrose decreases (Joosten et al., 1990a; Noeldner et al., 1994), indicating that there is an increase of invertase activity. It is not known whether the increased invertase activity in the *C. fulvum*–tomato interaction is solely due to fungal invertases because host cell invertase activity is also generally found to increase upon pathogen infections (Berger et al., 2004; Roitsch et al., 2003; Sturm and Chrispeels, 1990). The increase in extracellular invertase activity by the host is a common response to pathogen challenge (Hall and Williams, 2000; Roitsch et al., 2003). Because the activation of plant defense responses triggered upon pathogen detection requires energy, the local increase of invertase activity could meet the increased demand for carbohydrates in tissues invaded by pathogens (Roitsch et al., 2003). Furthermore, an increase in carbohydrates generates a metabolic signal for the expression of defense–related genes (Roitsch et al., 2003). In turn, fungi can take up and convert hexose monomers such as glucose and fructose into polyhydroxy alcohols (polyols) such as mannitol (the predominant polyol stored by *C. fulvum*), glycerol or sorbitol. As many plants (including tomato) are not able to metabolize sugar alcohols, the accumulation of polyols allows fungi to store carbon in such a way that it is inaccessible to the host (Lewis and Smith, 1967). *C. fulvum* displays mannitol dehydrogenase activity, leading to a significant increase of mannitol concentrations during infection (Joosten et al., 1990a; Noeldner et al., 1994). Polyols have been implicated in diverse roles in fungi, including contribution to the osmotic balance, antioxidants (quenchers of host–produced reactive oxygen species), facilitation of carbon transportation through the hyphae and storage (Jennings, 1984; Lewis and Smith, 1967). The observation that mannitol is found in fungal conidia where it is metabolized at a very early stage of germination and the finding that polyols are

metabolized under starvation conditions has strengthened the view that polyols are indeed used as storage compounds (Dijkema et al., 1985; Horikoshi et al., 1965; Witteveen and Visser, 1995). In some cases a role in fungal virulence has been shown for polyols. For instance, in *Magnaporthe grisea*, the polyol glycerol is required to build up the osmotic pressure in the appressorium that is required for epidermal penetration (de Jong et al., 1997). In *C. fulvum* mannitol most likely accumulates as a carbon storage compound and a role in fungal virulence has not been established yet.

Virulence of *Cladosporium fulvum*

C. fulvum is a pathogen that does not penetrate host cells at any stage of its life cycle. Although hyphae are observed to grow in close contact with mesophyll cells, all communication and exchange of components between pathogen and host occurs in the apoplastic space and the extracellular matrices of both pathogen and host. Because the apoplastic fluids can be harvested by vacuum infiltration of infected tomato leaves with water or buffer followed by low–speed centrifugation, these components can be identified fairly easily (de Wit and Spikman, 1982). Considerable efforts have been made to isolate fungal components that contribute to virulence in this way.

Figure 1 (next page): Physiology of the *Cladosporium fulvum* infection on host and non-host plants (A–H) and typical symptoms on host plants caused by other plant pathogenic Mycosphaerellaceae as found in nature (I–N) (see page 198 for full color version). (A) Adaxial side of a tomato leaf (MoneyMaker *Cf–0*) 18 days after inoculation with a compatible race of *C. fulvum.* Distinctive yellow spots can be seen as a result of dead palisade parenchyma cells. (B) Abaxial side of a tomato leaf *(*MoneyMaker *Cf–0)* 18 days after inoculation with a compatible race of *C. fulvum*. White mold can be seen developing into light brown patches where sporulation takes place. (C–E) SEM images from *C. fulvum*–infected tomato leaves in a compatible interaction at different timepoints after inoculation (pictures are taken from: de Wit, P.J.G.M. Light and scanning–electron microscopic study of infection of tomato plants by virulent and avirulent races of *Cladosporium fulvum* Neth. J. Plant Pathol. (1977) 83, 109–122, with permission). (C) *C. fulvum*–infected tomato leaf in a compatible interaction 2 days post inoculation with fungal hyphae entering a stoma. (D) *C. fulvum*–infected tomato leaf in a compatible interaction 7 days post inoculation. In the spongy mesophyll hyphae (h) grow in close contact with the plant cells. (E) *C. fulvum*–infected tomato leaf in a compatible interaction 12 days post inoculation. Young conidiophores emerging from the stomata are observed. (F–H) Drawings upon microscopic analysis of lactophenol–stained leaf material of several plant species upon inoculation with *C. fulvum* (drawings are reproduced from: Bond, T.E.T. Infection experiments with *Cladosporium fulvum* Cooke and related species.Ann. Appl. Biol. (1938) 25, 277–307, by permission of Oxford University Press). (F) Growth of *C. fulvum* mycelium in the tomato cultivar 'Giant red' 7 days after inoculation. The growth is characterized by long runner hyphae that pass between spongy mesophyll cells to send out ascending branches. (G) Limited growth of mycelium in *Hyoscyamus niger* (Solanaceae) 6 days after inoculation. Fungal growth does not go further than the substomatal cavity and a ring of discolored cells is observed. (H) Penetration of *C. fulvum* in so–called inappropriate hosts (or non–hosts) 6 days after inoculation: *Anthirrhinum majus* (a), *Bryonia dioica* (b) and *Callistephus* sp. (c). Mycelium is confined to single peg–like branches. (I) *Cercospora beticola* sporulating on sugarbeet leaves (*Beta vulgaris*). (J) Fasciculate conidiophores of *Pseudocercospora fijiensis* on banana (*Musa*) leaves. (K) Pycnidia of *Mycosphaerella graminicola* on wheat. (L) Angular leaf spots of *Cercospora zeae–maydis* on maize (*Zea mays*). (M) Conidiomata of *Dothistroma septospora*, causing red band needle disease of *Pinus* sp.(N) *Passalora perplexa* causing Crassicarpa leaf blight on *Acacia crassicarpa*.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The role of nitrogen in *Cladosporium fulvum* pathogenicity

Although knowledge of nitrogen metabolism of plant pathogens is limited, nitrogen seems to play an important role in pathogenesis (Snoeijers et al., 2000). A large proportion of genes that exhibit *in planta*–induced expression are also expressed *in vitro* under nutrient–deprived conditions both in *C. fulvum* and in other fungi (Coleman et al., 1997; Pieterse et al., 1994; Talbot et al., 1993; van den Ackerveken et al., 1993b). For instance, the race–specific elicitor gene *Avr9* is highly induced *in planta* and was found to be induced by nitrogen limitation *in vitro* (van den Ackervekenet al., 1993b). This suggests that during *in planta* growth, limited nitrogen is available for the colonizing pathogen (Snoeijers et al., 2000). Several studies have shown that plant pathogens have found ways to alter their host's nitrogen metabolism to their own benefit (Hall and Williams, 2000; Snoeijers et al., 2000). In *C. fulvum* there are indications for such a mechanism with respect to production of γ–aminobutyric acid (GABA), a non–protein–type amino acid that is produced in organisms ranging from microbes to plants and mammals (Bouché and Fromm, 2004). It is a predominant metabolite in plants and is expected to be involved in many processes. It is suggested that GABA, similar to its neurotransmitter role in animals, acts as a signaling molecule. In addition, GABA has been suggested to play a role in osmoregulation, pH regulation, nitrogen metabolism, and in defense against insects and oxidatve stress (Bouché and Fromm, 2004). In uninfected plants GABA is already the most abundant non–protein amino acid in the tomato apoplast, and during infection its concentration rises three– to four–fold (Solomon and Oliver, 2001). A GABA transaminase involved in metabolizing GABA has been isolated from *C. fulvum* and was found to be induced by the addition of GABA *in vitro*. In addition, the tomato GABA biosynthetic enzyme glutamate decarboxylase is induced during infection (Solomon and Oliver, 2002). It was suggested that *C. fulvum* manipulates the host metabolism to release nutrients because the presence of *C. fulvum* in the apoplast leads to enhanced GABA production by the plant and *in vitro* assays indicate that *C. fulvum* can utilize GABA both as a nitrogen and as a carbon source (Oliver and Solomon, 2004; Solomon and Oliver, 2002). In addition, both GABA and mannitol can act as a protection agent for plant cells against oxidative damage caused by the oxidative burst that is elicited as a defense response against the invading pathogen (Bouché et al., 2003; Coleman et al., 2001). In a compatible interaction, however, the oxidative burst is not effective as a defense response and the fungus may have developed means to utilize the secreted GABA as a nutritional source.

Nitrogen-controlled pathogenicity genes

As mentioned above, the avirulence gene *Avr9* is induced both *in planta* and *in vitro* during nitrogen starvation (Snoeijers et al., 1999; van den Ackerveken et al., 1994). Analysis of the *Avr9* promoter showed the presence of 12 (TA)GATA boxes (Snoeijers et al., 1999). These are known to act as binding sites for GATA type regulators such as AREA in Aspergillus nidulans or NIT2 in *Neurospora crassae* (Chiang and Marzluf, 1995; Punt et al., 1995).

Indeed, it has been shown using a reporter construct in *A. nidulans* that the *C. fulvum Avr9* promoter is induced during nitrogen starvation, but remains inactivated in an areA null mutant (Snoeijers et al., 1999; van den Ackerveken et al., 1994). Subsequently, from *C. fulvum* the *AREA/NIT2* homologue *Nrf1* (for Nitrogen response factor 1) was isolated (Pérez–García et al., 2001). As expected, this transcription factor was found to regulate *Avr9* transcription as *C. fulvum* transgenes deleted for *Nrf1* show severely reduced *Avr9* induction *in vitro* under nitrogen limitation and *in planta* during infection. Nevertheless, residual production of Avr9 in the *Nrf1* knockout line suggests that additional regulators of *Avr9* exist (Pérez–García et al., 2001). Although initial data suggested that deletion of *Nrf1* did not affect pathogenic capacity (Pérez–García et al., 2001), recent results indicate that virulence of *Nrf1* knockout strains is actually decreased (Thomma et al., 2006). In addition, the virulence of *Nrf1* knockout strains was compared with a strain in which only the *Avr9* gene is deleted. The results show that *Avr9* deletion lines, in contrast to the *Nrf1* knockouts, show a level of virulence that is similar to the parental lines (Thomma et al., 2006). This leads to the conclusion that *Nrf1* is a virulence factor that controls, in addition to *Avr9*, other fungal components that are involved in the establishment of successful colonization.

Seven (TA)GATA consensus sequences are present in the promoter of the avirulence gene *Avr4E* (Westerink et al., 2004), suggesting that *Avr4E* expression might be controlled by *Nrf1* in a similar fashion. It has been noted that overlapping TAGATA sequences contribute to the inducibility of the *Avr9* promoter (Snoeijers et al., 2003). However, in contrast to the two overlapping TAGATA boxes present in the *Avr9* promoter, the *Avr4E* promoter lacks overlapping boxes. At present is unclear whether the *Avr4E* promoter is induced under low nitrogen conditions. None of the promoters of other known genes encoding secreted elicitor peptides carries (TA)GATA boxes. *Avr9* is the only elicitor gene for which there is evidence that it is induced by nitrogen starvation. This also suggests that factors other than nitrogen depletion are involved in regulation of *C. fulvum* pathogenicity.

Several other starvation–induced genes, in addition to *Avr9*, include an alcohol dehydrogenase (*Adh1*), an alcohol oxidase (*Aox1*) and an acetaldehyde dehydrogenase (*Aldh1*) (Coleman et al., 1997; Oliver and Solomon, 2004). *Aox1* was found to be inducible by carbon starvation but repressed by nitrogen starvation *in vitro* (Segers et al., 2001). Remarkably, *in planta Aox1* is highly expressed, which could mean that either sucrose levels are depleted at sites of fungal growth or that factors other than carbon starvation trigger expression of *Aox1*. Targeted disruption of *Aox1* resulted in decreased growth *in planta* and reduced sporulation. Currently, the role of alcohol oxidases in pathogenicity is not clear. In general these enzymes catalyse the conversion of ethanol or methanol to hydrogen peroxide and acetaldehyde or formaldehyde, respectively. Contribution to pathogenicity could be due to its contribution to carbon metabolism, the removal of (m)ethanol present in tomato leaves, or the production of H_2O_2 (Segers et al., 2001). Although disruption of the acetaldehyde dehydrogenase 1 gene (*Aldh1*) was not found to affect pathogenicity, its expression was also found to be highly induced *in planta* (Segers et al., 2001). Possibly, the acetaldehyde that is generated by Aox1–mediated oxidation of

ethanol is oxidized to acetate by the ALDH1– enzyme or, alternatively, reduced to ethanol by alchohol dehydrogenase (ADH1).

Other putative virulence proteins: Avrs and Ecps

A number of proteins have been identified that are secreted by *C. fulvum* in the apoplast of susceptible tomato leaves. Apparently, tomato has built at least part of its surveillance system on recognizing these peptides as resistance depends on the perception of the presence or activity of these proteins mediated by the *Cf* resistance genes (Kruijt et al., 2005). The proteins secreted by *C. fulvum* are divided into extracellular proteins (Ecps) and avirulence proteins (Avrs) based on the observation that some of them are produced by all strains (Ecps) whereas others are race–specific (Avrs). However, this largely is a matter of semantics as Ecps, like Avrs, are specific elicitors that are recognized only by a few plants (Laugé et al., 1998a). All currently known *Avr* and *Ecp* genes are highly expressed *in planta* but hardly any expression is detected *in vitro*. This has led to the idea that these proteins play a central role in the establishment of disease and all have been recognized by some genotypes that occurred during the tomato population evolution.

Although the degree of sequence conservation is very limited between individual Avrs and Ecps, the proteins are small (varying between 3 and 15 kDa) and contain an even number of cysteines (varying between four and eight). These cysteines are connected by disulphide bridges that contribute to the stability and activity of these proteins in the harsh protease–rich environment of the host apoplast (Kooman–Gersmann et al., 1997; Luderer et al., 2002a; van den Burg et al., 2003; van den Hooven et al., 2001). For Avr9 it has indeed been shown that the three–dimensional structure of the 28 amino acid peptide contains three anti–parallel beta–sheets with two solvent–exposed loops, which are stabilized by three disulphide bridges (Mahé et al., 1998; van den Hooven et al., 2001; Vervoort et al., 1997). This overall structure is typical for cystine–knotted peptides, which, although structurally related, share very little sequence homology and display very diverse biological functions (Pallaghy et al., 1994).

Despite the absence of clear homology between *Avr* and *Ecp* genes and absence of sequence homology with other proteins in public databases, some of their properties point towards putative intrinsic functions. *Avr9* encodes a 63 amino acid protein that is C– and N–terminally processed by fungal as well as plant proteases, leading to a 28 amino acid peptide containing six cysteine residues (van den Ackerveken et al., 1993b; van Kan et al., 1991). Based on length, cysteine spacing and beta–sheet character, homology of Avr9 with peptidase inhibitors was suggested and indeed a high structural homology to a carboxypeptidase inhibitor was found (van den Hooven et al., 2001; Vervoort et al., 1997). Functional assays, however, could not show inhibition of carboxypeptidases by Avr9 (van den Hooven et al., 2001). Nevertheless, it was demonstrated that Avr9 can bind to a component that is present in the plasma membrane of tomato and other Solanaceous plants (Kooman–Gersmann et al., 1996). The binding is probably independent of expression of the *Cf–9* resistance gene, as experiments to establish binding between Cf–9 and Avr9 were

unsuccessful (Luderer et al., 2001). Despite many efforts, the nature of this binding site is not known yet. Possibly, Avr9 acts as a blocker of specific membrane channels as has been found for other cystine–knotted peptides.

Like Avr9, Avr4 was found to attach to membrane components. However, unlike Avr9, Avr4 binds to those of fungal rather than of plant origin (Westerink et al., 2002). Nevertheless, because Avr4 triggers an HR in *Cf–4*–carrying plants, it can be anticipated that Avr4 also binds to a component of plant origin. *Avr4* encodes a 135 amino acid pre–pro–protein, which is C– and N–terminally processed upon secretion in the apoplast, resulting in an 86 amino acid mature protein carrying eight cysteine residues (Joosten et al., 1994, 1997; Laugé et al., 1997; Vervoort et al., 1997). Based on the disulphide pattern of Avr4, a homologous sequence designated as an invertebrate chitin–binding domain (inv ChBD, Shen and Jacobs–Lorena, 1999) was identified. Binding of Avr4 to chitin was confirmed experimentally (van den Burg et al., 2003, 2004). Interestingly, Avr4 was found to protect effectively the cell wall of the fungi *Trichoderma viride* and *Fusarium solani* against antifungal activity by basic chitinases *in vitro* (van den Burg et al., 2003). Although the chitin–binding domain of plant chitinases (also called the Hevein domain) and the inv ChBD are sequentially unrelated, they do show strong structural homology. Remarkably, and in contrast to plant chitin–binding proteins, positive allosteric interactions were observed between chitin–binding Avr4 molecules (van den Burg et al., 2004). During growth *in vitro C. fulvum* does not produce Avr4 and its chitin is inaccessible. However, during infection of tomato, chitin in the fungal cell walls is accessible and Avr4 is produced (van den Burg et al., 2006). This all suggests that Avr4 shields fungal cell walls against activated host enzymes during infection. Apparently, some tomato plants have developed means (i.e. *Cf–4*) to recognize Avr4, recognition of which results in HR. Natural isoforms of Avr4 that are no longer recognized by plants carrying the resistance gene *Cf–4* exist that are still able to bind chitin. This shows that in some mutant alleles the intrinsic function of Avr4 seems to be preserved while unstable and protease–sensitive Avr4 variants still show chitin binding capability (van den Burg et al., 2003). Despite this, the absence of functional Avr4 in a mutant carrying a single nucleotide deletion does not lead to a compromised virulence phenotype, indicating that *Avr4* is dispensable for full virulence (Joosten et al., 1997).

Dispensability for full fungal virulence also holds true for Avr9, as fungal strains in which the *Avr9* gene is either absent or replaced do not display markedly decreased virulence (Marmeisse et al., 1993; van Kan et al., 1991). It is therefore not unlikely that functional redundancy occurs for *Avr* genes as they are not uniformly present throughout all *C. fulvum* strains.

Another avirulence protein for which there are leads towards a function is Avr2. The corresponding *Avr2* gene was cloned and found to encode a 58 amino acid mature protein that contains eight cysteine residues (Luderer et al., 2002b). The expression of *Avr2* leads to an HR in plants carrying the resistance gene *Cf–2* (Dixon et al., 1996; Luderer et al., 2002b). In addition, a gene has been identified that is required for *Cf–2*–mediated resistance

called *Rcr3* (Dixon et al., 2000; Krüger et al., 2002). As *Rcr3* only plays a role in *Cf–2*–mediated resistance, and not in resistance mediated by other *Cf* genes, it is anticipated that this component functions upstream of the signaling cascade that leads to the HR (Dixon et al., 2000). Moreover, the predicted apoplastic localization of Rcr3 suggests that this protein is involved in the interaction between Avr2 and Cf–2, perhaps mediating the actual perception of the Avr protein by the Cf protein (Krüger et al., 2002; Luderer et al., 2002b). This would be in agreement with the 'guard hypothesis'. This hypothesis suggests that *R* gene products can act as guards that sense the modification of specific plant components that are targets of pathogen virulence components (van der Biezen and Jones, 1998). *Rcr3* was cloned and it was found to encode a cysteine protease (Krüger et al., 2002). Recent evidence indeed points towards a function of Avr2 as a cysteine protease inhibitor (Rooney et al., 2005). How Avr2 enhances virulence of the fungus in susceptible tomato plants that do not carry the *Cf–2* resistance gene still remains to be determined.

Five Ecps have been isolated from the apoplast of *C. fulvum* colonized tomato leaves and four of the corresponding genes have been cloned (Laugé et al., 2000; van den Ackerveken et al., 1993a). In contrast to *Avr* genes, all *Ecp* genes are consistently present throughout the *C. fulvum* isolates. This observation, in addition to the finding that these genes are highly expressed *in planta* (Wubben et al., 1994), has led to the idea that *Ecp* genes are essential for virulence. This has indeed been shown for *Ecp1* and *Ecp2* as virulence assays on six–week–old soil–grown plants showed a significant decrease in fungal growth of *Ecp1*– and *Ecp2*–disruptants (Laugé et al., 1997; Marmeisse et al., 1994). For Ecp4 and Ecp5 a contribution to virulence needs yet to be established.

Structural analysis showed that the cysteine spacing of Ecp1 has remarkable similarity to the cysteine spacing of tumor necrosis factor receptors (TNFRs) (Bazan, 1993). One of the functions of TNFR family proteins is to initiate programmed cell death (Itoh et al., 1991). This is typically achieved by signaling through a ligand passing mechanism, meaning that a first accessory receptor recruits the ligand and regulates the association with the second receptor (Tartaglia et al., 1993). Pathogen–derived TNFRs that have been found in mammalian viruses interfere in the function of mammalian cytokines by mimicking their receptors (the endogenous TNFRs) and thus preventing the cytokines from reaching their endogenous targets and eliciting defense (Alcami and Smith, 1992). Interestingly, receptor molecules that share homology with mammalian TNFR molecules have also been identified in plants (Becraft et al., 1996). Experimental evidence establishing this particular function for Ecp1 is still lacking.

Intriguingly, in contrast to *Avr* encoding genes, no significant sequence variation has been found in *Ecp* genes of *C. fulvum* isolates gathered from tomato fields and greenhouses. The high mutation frequency for *Avr* genes is thought to be due to selection pressure imposed by the use of Cf resistance genes in commercial tomato cultivation. Although *Cf–Ecp* resistance genes have been identified (Laugé et al., 2000), they have not been used on a large scale in tomato cultivars, resulting in absence of selection pressure on *Ecp* genes.

Because *Avr* genes are not ubiquitously represented throughout the *C. fulvum* species, it can be argued that none of the individual *Avr* genes is absolutely required for the establishment of disease. It is likely that redundancy occurs within the total pool of *Avr* genes present in the fungal genome, making individual *Avr* genes dispensable. As a consequence, fungal pathogenicity could rely on a set of virulence factors that are partially dispensable, although their combination is required for full virulence. Possible intrinsic functions could be the induction of nutrient leakage, the suppression of defense responses or the establishment of protection against host defense. In addition, it cannot be excluded that some of these factors have an important function for survival or competition in a specific habitat outside the natural host, although the specific plant–induced expression patterns suggest differently. This plant–induced expression could, however, also be explained as an induction that is caused upon monitoring the presence of specific antagonists of *C. fulvum* in the apoplast of tomato leaves, a phenomenon that has not yet been studied. It is expected that the use of *Arabidopsis thaliana* can greatly facilitate investigations into the intrinsic function of these secreted proteins and can help to determine the effects of these proteins on plants that do not carry corresponding *Cf* resistance genes. This would be facilitated even more with the availability of *Mycosphaerella* species that are able to infect Arabidopsis.

Other putative virulence proteins: Hydrophobins

Many if not all filamentous fungi produce cell wall proteins that confer a water repellent nature to conidia and mycelium called hydrophobins. They are relatively small proteins that display a low level of sequence conservation but share similar hydropathic profiles and contain eight cysteine residues arranged in a strictly conserved manner (Whiteford and Spanu, 2002). They cover the surface of fungal structures by spontaneous polymerization into amphipathic bilayers. Hydrophobins are involved in various developmental processes such as the formation of aerial mycelium, sporulation, formation of infection structures, formation of fruit bodies and dispersal of conidia (Whiteford and Spanu, 2002; Wösten, 2001). In some cases it has also been demonstrated that hydrophobins contribute to fungal virulence. The rice blast fungus *Magnaporthe grisea*, for instance, requires the hydrophobin MPG1for attachment and appressorium formation (Talbot et al., 1993;1996). In addition, a hydrophobin appears to act as a virulence factor by increasing pathogen fitness in the causal agents of Dutch elm disease, *Ophiostoma ulmi* and *O. novo–ulmi* (del Sorbo et al., 2000; Temple and Horgen, 2000).

In *C. fulvum* six hydrophobin genes (*HCf–1* to *–6*) have been identified, each showing different expression profiles (Nielsen et al., 2001; Segers et al., 1999; Spanu, 1997). *HCf–1* appears to be specifically expressed after emergence of the conidiophores from the plant and during the start of the production of conidia, and was found to play a role in water–mediated dispersal of conidia (Whiteford and Spanu, 2001; Whiteford et al., 2004). *HCf–6* is specifically expressed in runner hyphae that enter stomata and it is speculated that HCf–6

may act as a primer for hydrophilic molecules that help the fungus to attach to the leaf surface. Alternatively, HCf–6 could be involved in preventing elicitation of host defense responses by helping to mask the presence of the pathogen (Whiteford et al., 2004). Single deletion mutants of the *C. fulvum* hydrophobins *HCf–1*, *HCf–2* or *HCf–6* did not display a reduction in virulence (Spanu, 1998; Whiteford and Spanu, 2001; Whiteford et al., 2004). This suggests that a high degree of functional redundancy exists between different hydrophobins although a double knock–out of *HCf–1* and *HCf–2* did not show altered virulence (Whiteford and Spanu, 2001). Nevertheless, redundancy with other hydrophobin genes or functional homologues can occur or the role of hydrophobins in virulence is indeed not imperative.

The *Cladosporium fulvum*-tomato pathosystem as a future experimental model for studying the Mycosphaerellaceae

Despite the study of many pathosystems, there is still little insight into what determines pathogenicity of a filamentous fungus, which are the required virulence factors and what determines its host range. *C. fulvum* is no exception. Through selection pressure new *C. fulvum* strains have emerged that have overcome the introgressed resistance traits and thus regained virulence by modification of *Avr* genes (Day, 1957). Although in these virulent *C. fulvum* strains *Avr* genes were sometimes found to be absent (van Kan et al., 1991), others contained point mutations (Joosten et al., 1994) or transposon insertions (Luderer et al., 2002b). In addition, mutagenesis experiments have yielded large sets of *C. fulvum* mutants that display reduced virulence, but the affected genes have not been characterized (Kenyon et al., 1993). The study of virulence mechanisms of fungal pathogens should greatly be facilitated with the increasing availability of fungal genome sequences. With sequencing and annotation of microbial genomes becoming more and more common practice, establishment of the *C. fulvum* genome sequence will also become more feasible. In the meantime, research on *C. fulvum* will benefit from genome sequences that are currently generated for the Mycosphaerellaceae species *M. graminicola* and *M. fijiensis* and *vice versa* when functional analysis of the latter species will have to be carried out.

Based on phylogenetic data, *C. fulvum* is found to be closely related to a number of economically important *Mycosphaerella* pathogens (Braun et al., 2003; Crous et al., 2001; Goodwin et al., 2001). This phylogenetic relationship is supported by morphological observations on the interactions of these pathogens with their respective host plants. For instance, cytological studies of the interaction between *M. fijiensis* (the causal agent of the devastating black Sigatoka disease; Fig. 1J) and *Musa spp*. (banana and plantain) revealed that *M. fijiensis*, like *C. fulvum*, behaves as a biotrophic pathogen, entering the leaf through open stomata and exclusively colonizing the intercellular space between mesophyll cells without forming haustoria (Beveraggi et al., 1995). In susceptible cultivars the interaction is characterized by a long biotrophic stage before morphological distortions are observed; in resistant cultivars depositions of fluorescent materials near the entry sites of the fungus are

observed as early as seven days post inoculation and early necrosis of guard cells also occurs, reminiscent of an HR mediated by a gene–for–gene relationship (Beveraggi et al., 1995). Another example is the Septoria wheat blotch pathogen, *M. graminicola*, which is a major foliar wheat pathogen (Fig. 1K) in temperate and subtropical regions, and employs similar infection mechanisms: no active penetration, purely extracellular growth, a lack of feeding structures and eventually the fungus causes, like *C. fulvum*, wilting as a consequence of non–functioning stomata (Palmer and Skinner, 2002).

Cercospora leaf spot disease (Fig. 1I) is considered to be the most important foliar disease of sugar beet (*Beta vulgaris*) worldwide (Weiland and Koch, 2004). The disease is caused by the asexual fungus *Cercospora beticola* that, apart from species of the genus *Beta*, also infects a number of Chenopodiaceae species. Although this fungal species appears to have a less narrow host range than many of the other *Mycosphaerella* pathogens, again, in addition to the taxonomic relationship, the cytology of infection of *C. beticola* resembles that of *C. fulvum*. The fungus penetrates the abaxial side of the leaf through stomata and grows within the intercellular space of the leaf during the biotrophic stage of its infection cycle. After intense colonization of the leaf tissue, the parenchyma and epidermal cells collapse in the vicinity of the fungal hyphae and the final necrotic zone appears, causing typical sporulating leaf spots (Feindt et al., 1981; Steinkamp et al., 1979).

Another interesting feature that many of these pathogens have in common is their appearance as epi– or endophytes that become pathogenic only under certain conditions. Endophytic growth of *C. beticola* has been reported upon root–inoculation of sugar beet seedlings prior to the pathogenic stages (Vereijsen et al., 2004). In addition, such an endophytic lifestyle has been demonstrated for *M. buna*, which colonizes foliage of Japanese beech (*Fagus crenata*), and also for the type species of *Mycosphaerella*, *M. punctiformis*, which was isolated from asymptomatic living oak (*Quercus robur*) leaves (Kaneko and Kakishima, 2001; Verkley et al., 2004).

Despite the lack of a genome sequence, *C. fulvum* is a baseline *Mycosphaerella* pathogen that provides an ideal model to investigate basic pathogenicity mechanisms. As a result of the limited contact between pathogen and host, the lack of complicated feeding structures, and because host cells stay intact during the major part of the interaction, communication signals of the two interacting organisms present in the apoplast can easily be isolated by harvesting intercellular washing fluids. This has led to the identification of many secreted proteins and the corresponding genes as discussed above. Although one major disadvantage of *C. fulvum* is the lack of a sexual stage and thus the inability to generate the crossings that are imperative for gene mapping studies, a number of important genomics tools have been developed in recent years.

Although genomic transformation has long been possible in *C. fulvum*, recently an *Agrobacterium tumefaciens*–mediated transformation protocol was established facilitating transformation procedures and reducing artefacts as protoplasting is no longer required. In addition, RNAi–technology has been established which, in combination with

Agrobacterium–mediated transformation, should facilitate the study of putative pathogenicity genes.

Another important advantage is the considerable effort that has been made to unravel disease resistance signaling in the interaction between *C. fulvum* and its host (Rivas et al., 2004; Rowland et al., 2005). C. *fulvum* was the first biotrophic fungus for which not only the first *Avr* genes were isolated but also the first corresponding *R* gene was cloned (Jones et al., 1994) and by now quite a number of *Cf* genes, and even complete *Cf*–clusters of genes, have been isolated (Kruijt et al., 2005). In total, four *Avr* genes and their corresponding plant *Cf* genes have been cloned. Apart from the *C. fulvum*–tomato interaction, for most other interactions between Mycosphaerellaceae and their hosts, conclusive evidence for gene–for–gene relationships is lacking. Nevertheless, recently such an interaction was demonstrated for resistance of wheat against a specific isolate of *M. graminicola* (Brading et al., 2002). For the other interactions, although suggested, such a relationship has never been proven, probably because of the poor availability of genetic tools for these plant–pathogen interactions (Harelimana et al., 1997; Lewellen and Whitney, 1976; Weiland and Koch, 2004).

More recent efforts on the *C. fulvum*–tomato interaction are directed towards downstream signaling that establishes the final resistance. The sequencing of the tomato genome by an international consortium (http://www.sgn.cornell.edu/about/tomato_sequencing.pl) will greatly facilitate this research. Tomato will most likely be the first dicotyledenous crop plant for which a genome sequence is available and is therefore likely to develop even more into a model plant for the Solanaceae than it is today. In light of these advancements, we anticipate that *C. fulvum* can act as a model for many fungus–pathogen interactions in general and *Mycosphaerella* – plant interactions more specifically.

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Thesis outline

Cladosporium fulvum (syn. *Passalora fulva*) is a biotrophic fungal pathogen that causes leaf mold of tomato (*Solanum esculentum*). The fungus exclusively colonizes the tomato leaf apoplast where it secretes several relatively small, cysteine–rich effector proteins that elicit a defense response in tomato plants that carry the cognate *Cf*–resistance genes. However, in susceptible host plants that lack cognate *Cf*–resistance genes, these effectors are thought to play a role in disease establishment. In **chapter 2**, a targeted proteomics approach to investigate the *in planta* role of *C. fulvum* effectors and to identify their *in planta* targets is described. *C. fulvum* proteins were expressed as recombinant fusion proteins carrying various affinity–tags at either their C– or N–terminus. However, the stability of the *in planta*–expressed recombinant fusion proteins proved to be insufficient in the tomato apoplast. This resulted in removal of the affinity–tag from the fusion proteins. A similar removal of affinity tags from recombinant effector fusion proteins was also observed when the fusion proteins were expressed in other Solanaceous species, but not when they were expressed in Arabidopsis. In **chapter 3**, a detailed study addressing the intrinsic biological function of Avr4 is presented. In this chapter it is demonstrated that heterologous Avr4 expression in Arabidopsis results in increased virulence of several fungal pathogens with exposed chitin in their cell walls, whereas the virulence of a bacterial and an oomycete pathogen, both without exposed chitin in their cell walls, remained unaltered. Furthermore, it was found that heterologous expression of Avr4 in tomato increases the virulence of the vascular pathogen *Fusarium oxysporum* f. sp. *lycopersici*. Tomato GeneChip analysis was used to show that Avr4 expression in tomato resulted in the induced expression of only few genes in the absence of pathogen challenge. Finally, silencing of the *Avr4* gene in *C. fulvum* significantly decreased its virulence on tomato. This chapter presents the first example of fungal effector protein of which the intrinsic biological function is known and is shown to be required for full pathogen virulence. In **chapter 4**, a study on the intrinsic biological function of Avr2 is presented. Previous studies have demonstrated that Avr2 inhibits the tomato cysteine protease Rcr3, and that this interaction is required for *Cf–2*–mediated signaling. In this chapter it is demonstrated that Avr2 expression in Arabidopsis results in increased virulence of several fungal pathogens. Cysteine protease profiling revealed that Avr2 specifically inhibits several extracellular Arabidopsis cysteine proteases. Furthermore, microarray analysis was performed to show that *Avr2*–expressing Arabidopsis undergoes transcriptional reprogramming that is comparable to the reprogramming observed upon pathogen challenge. Subsequently, it is shown that Avr2 expression in tomato results in increased virulence of race 2 *C. fulvum* strains, and also increases the virulence of several other fungal pathogens. In tomato, cysteine protease profiling revealed that Avr2 specifically inhibits several extracellular cysteine proteases in addition to Rcr3. Finally, silencing of the *Avr2* gene in *C. fulvum* resulted in clearly compromised virulence on tomato. Overall, **chapter 4** demonstrates that Avr2 is a virulence factor that inhibits extracellular cysteine proteases that are essential for basal host defense. **Chapter 5**

describes the identification of three novel *C. fulvum* proteins; PhiC, Ecp6, and Ecp7. While PhiC shows homology to fungal phialides, Ecp7 encodes a small, cysteine rich protein with no homology to known proteins. Ecp6 contains LysM domains that may be involved in chitin binding and can be found in many fungal and non–fungal species. By RNAi–mediated gene silencing it is demonstrated that *Ecp6* is required for full *C. fulvum* virulence on tomato. Furthermore, heterologous over–expression of *C. fulvum Ecp6* in *Fusarium oxysporum* f. sp. *lycopersici* enhanced the virulence of this fungus on tomato. In **chapter 6**, a microarray study is presented that compares a compatible and an incompatible interaction of both *C. fulvum* and *V. dahliae* on tomato. Both pathogens have fundamentally different infection strategies as *C. fulvum* is a narrow host–range foliar pathogen, while *V. dahliae* is a soil–borne vascular pathogen of over 200 host plants. Despite these different infection strategies, both pathogens also share a number of important characteristics as they invade their host through natural openings and grow strictly extracellular without the formation of feeding structures such as haustoria. Furthermore, in incompatible interactions recognition of these pathogens is mediated by plasmamembrane–anchored extracellular receptor proteins that belong to the receptor–like protein class of resistance proteins. In this chapter, establishment of the transcriptomes of susceptible and resistant tomato lines upon challenge by *C. fulvum* and *V. dahliae* is described. In addition, a custom script was used to assign GO annotations to the gene sets, enabling the identification of the major differentially regulated biological processes. Some of these processes were studied in detail with the use of pathway reconstruction. These data were used to identify differences and similarities in compatible and incompatible interactions of both pathogens. In **chapter 7** the implications of the data presented in this thesis are discussed for the use of *C. fulvum* as a model. Also, the use of heterologous expression systems to study fungal effectors is briefly discussed. In addition, it reflects on the use of microarrays in plant biology. In plant biology, many genes have unknown functions. Furthermore, many plant gene sequences do not have clear homologues in other model organisms. Therefore, interpretation of transcriptional profiles is challenging. Over the past five years, various *in silico* tools have been developed that assist plant scientists in the reconstruction of cellular (metabolic, biochemical and signal transduction) pathways based on plant gene expression. This reconstruction is very useful since it enables researchers to identify cellular processes that might otherwise be obscured by the large amount of primary data. In this chapter, an evaluation is made of the currently available *in silico* tools based on plant gene expression datasets. Furthermore, it is shown how expression profile comparison at the level of these various cellular pathways can contribute to the postulation of novel hypotheses which, after experimental verification, can provide further insight into decisive elements that play a role in cellular processes.

Chapter 2:
Affinity-tags are removed from *Cladosporium fulvum* effector proteins expressed in the tomato leaf apoplast

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Abstract

Cladosporium fulvum (syn. *Passalora fulva*) is a biotrophic fungal pathogen that causes leaf mold on tomato (*Solanum esculentum*). The fungus grows exclusively in the tomato leaf apoplast where it secretes several small (<15 kDa) cysteine–rich proteins that are thought to play a role in disease establishment. To investigate the role of these proteins, and to identify their *in planta* targets, a targeted proteomics approach was undertaken. *C. fulvum* proteins were expressed as recombinant fusion proteins carrying various affinity–tags at either their C– or N–terminus. Although these fusion proteins were correctly expressed and secreted into the leaf apoplast, detection of affinity–tagged C. *fulvum* proteins failed, and affinity–purification did not result in the recovery of these proteins. However, when using *C. fulvum* effector protein–specific antibodies, specific signals were obtained for the different proteins. It is concluded that the stability of the *in planta* expressed recombinant fusion proteins is insufficient, which results in removal of the affinity–tag from the fusion proteins, irrespective of the C– or N–terminal fusion or the nature of the affinity–tag. Similar phenomena were observed when the fusion proteins were expressed in other Solanaceous species, but not when expressed in *Arabidopsis thaliana*.

Introduction

Cladosporium fulvum (syn. *Passalora fulva*) is a biotrophic fungal pathogen that causes leaf mold on tomato (*Solanum esculentum*) (Thomma et al., 2005). The fungus grows exclusively in the tomato leaf apoplast without forming any known feeding structures like haustoria. This implies that during the infection process, molecular components that play a role in the interaction between pathogen and host need to pass the host apoplast. Eight *C. fulvum* effector proteins, secreted by the fungus during infection, have been characterized in detail and their corresponding genes have been cloned (Thomma et al., 2005). These comprise the race–specific avirulence proteins Avr2, Avr4, Avr4E, and Avr9, and the extracellular proteins Ecp1, Ecp2, Ecp4, and Ecp5, of which the corresponding genes are thus far found in all *C. fulvum* strains (Thomma et al., 2005). All effector proteins are relatively small (ranging between 3–15 kDa) and contain a high number of cysteine residues that are involved in disulphide bridge formation (Kooman–Gersmann et al., 1997; van den Burg et al., 2003). These bridges provide a compact tertiary structure for the *C. fulvum* effector proteins in the tomato apoplast which is reported to be rich in proteases (Tornero et al., 1997; Jorda et al., 1999; Krüger et al., 2002).

Tomato resistance against *C. fulvum* is genetically determined by the presence of *Cf* resistance genes in a so called 'gene–for–gene' relationship (Kruijt et al., 2005). For all four cloned *C. fulvum Avr* genes, the corresponding *Cf* resistance genes have also been cloned (Jones et al., 1994; Dixon et al., 1996; Thomas et al., 1997; Takken et al., 1998). Furthermore, *Cf* resistance genes that confer recognition of Ecp genes have been described (Laugé et al., 1998a; de Kock et al., 2005; Kruijt et al., 2005). Since *Avr* and *Ecp* genes have been maintained within the *C. fulvum* population they are likely to provide a specific

fitness benefit to the fungus, either *in planta* during the infection, or in the absence of the natural host (Thomma et al., 2005). The observation that they are highly expressed *in planta* at the onset of the infection suggests they play a role in disease establishment (van Kan et al., 1991; van den Ackerveken et al., 1993a; Joosten et al., 1997). This hypothesis is supported by the observation that the Avr4 and Avr2 proteins display biological activities that suggests they are true virulence proteins (Krüger et al., 2002; van den Burg et al., 2003; Rooney et al., 2005).

In this study, an attempt was made to find virulence targets for *C. fulvum* effector proteins. As it was desired to address the virulence function of *C. fulvum* effector proteins, these studies were carried out in the absence of *Cf* resistance genes (*Cf–0*) to mimic the situation in a compatible interaction. In this targeted proteomics approach, affinity–tagged *C. fulvum* effector proteins were used as bait to fish for their *in planta* targets. However, affinity–purification of the affinity–tagged effector protein fusions failed, and subsequent experiments demonstrated that no, or only very low amounts of affinity–tagged proteins could be detected in the apoplast of tomato leaves. These data suggest that, irrespective of the sequence of the affinity–tag or the C– or N–terminal fusion, the affinity–tags are removed when the fusion proteins are deposited into the apoplast of Solanaceous species, but not in the apoplast of *Arabidopsis thaliana*. This phenomenon will have great implications for the *in planta* use of affinity–tagged apoplastic proteins in Solanaceous species.

Results

To investigate the intrinsic function of *C. fulvum* proteins secreted during infection, a search for their plant interactors that may act as virulence targets was initiated. To this end, fusion proteins consisting of the mature Avr or Ecp proteins and an affinity–tag were expressed (Table 1; Fig. 1), in principle allowing affinity–purification of the Avr or Ecp protein together with possible interactors. To allow systemic expression throughout the whole plant, and thus boost the total amount of protein produced, a binary potato virus X (PVX)–based expression system was used (Fig. 1A), permitting *Agrobacterium tumefaciens*–mediated inoculation of the virus (Luderer et al., 2002b; Takken et al., 2000; Westerink et al., 2004).

Initially, two sets of binary PVX constructs were produced; the first encoding N–terminally His_6–FLAG–tagged Avr and Ecp effector proteins and the second set encoding N–terminally His_6–FLAG–StrepII–tagged effector proteins (Fig. 1B). In all cases the sequence encoding the tobacco PR1a signal peptide was used for targeting of the fusion proteins towards the apoplast, where the effector proteins are also secreted by the fungus during infection.

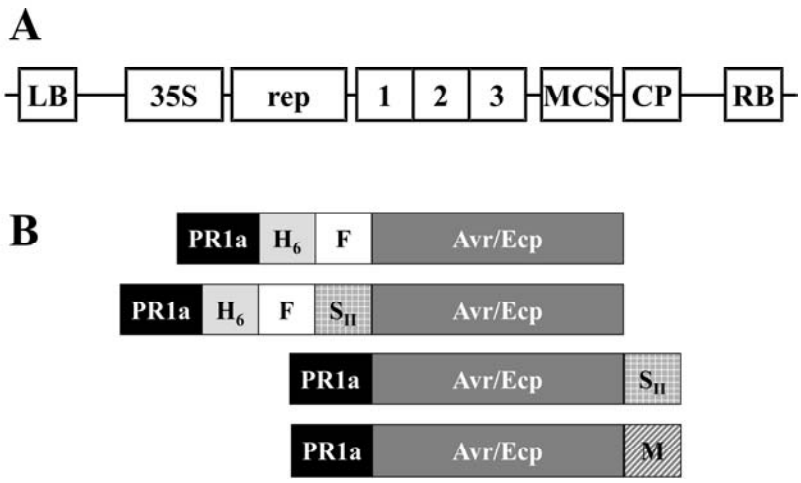

Figure 1. Constructs used in this study. (A) Schematic representation of the T–DNA region of the binary PVX–based expression vector. The T–DNA from left border (LB) to right border (RB) consists of a CaMV 35S promoter (35S), the PVX replicase gene (rep) and triple block (1, 2, 3), the duplicated coat protein promoter (black arrows) with a multiple cloning site (MCS) and the PVX coat protein. (B) Fusion constructs for PVX–mediated expression of affinity–tagged *C. fulvum* effector (Avr/Ecp) proteins. All constructs carry the tobacco PR1a signal sequence (PR1a) for extracellular targeting of the fusion proteins. The affinity–tags used are His_6 (H_6), FLAG (F), StrepII (S_{II}) and c–*myc* (M). The relative sizes of the different components presented do not reflect actual relative sizes.

To test whether expression of the constructs *in planta* results in biologically active proteins, *A. tumefaciens* strains carrying the various constructs were inoculated on two–week–old MoneyMaker tomato plants carrying the corresponding *Cf* resistance genes. About two–three weeks post–inoculation, a systemically spreading HR appeared (Fig. 2A). This response was not observed when the constructs were expressed in tomato plants without any functional resistance gene (*Cf-0* plants) (Fig. 2B), nor on tomato plants carrying non–corresponding *Cf* resistance genes (data not shown). This indicated that biologically active proteins were produced. To determine whether the PVX–expressed recombinant fusion proteins were correctly targeted towards the leaf apoplast, *Cf-0* plants were inoculated with individual *A. tumefaciens* strains harboring the different constructs. At 15 days post inoculation (DPI), AF was isolated, and subsequently injected into leaves of tomato plants carrying the corresponding *Cf* resistance genes. This resulted in triggering of effector specific, HR–like symptoms in the injected sectors (Fig. 2C, D).

Table 1. Affinity-tags, matrices and detection chemistries used in this study

Affinity-tag	Size (kDa)	Fusion[1]	Matrix[2]	Detection[3]
Poly–His	0.84	N	Ni^{2+}–NTA	–
FLAG	1.01	N	–	anti–FLAG bioM2
StrepII	1.06	N/C	StrepTactin sepharose	StrepTactin–HRP
c–myc	1.20	C	–	anti–*myc*–HRP

[1]N and C indicate use as an N–terminally or C–terminally fused affinity–tag, respectively.
[2]Matrix used for affinity purification.
[3]Chemistry used for detection of affinity–tags

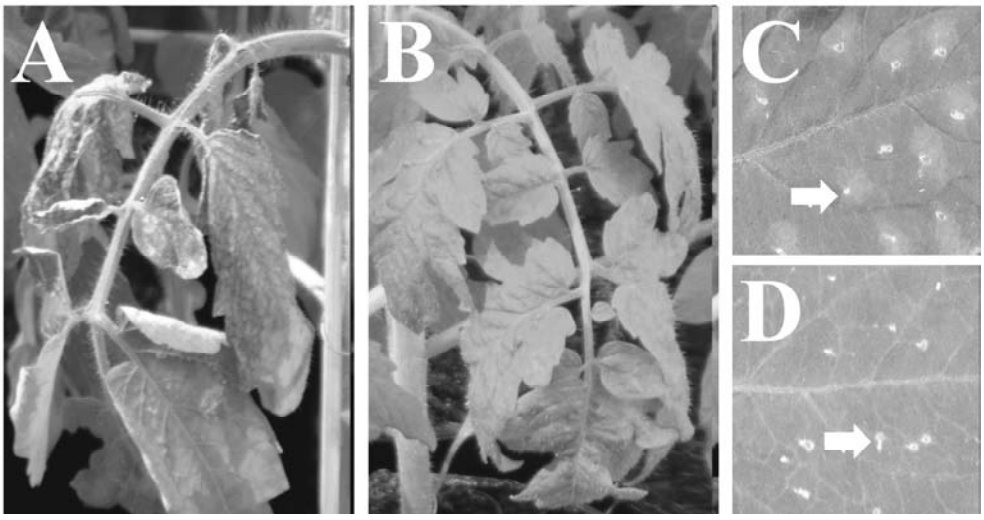

Figure 2. PVX-mediated expression of affinity-tagged *Cladosporium fulvum* effector protein leads to production of biologically active proteins (see page 200 for full color version). His_6–FLAG–tagged Ecp2 is expressed and targeted towards the apoplast of tomato leaves by making use of a binary potato virus X (PVX)–based expression system. (A) Typical spreading necrosis phenotype in a tomato plant carrying the corresponding *Cf–Ecp2* resistance gene 14 days post inoculation(DPI). (B) Phenotype of a tomato *Cf–0* plant, not carrying *Cf–* resistance genes, 14 days post PVX–inoculation. (C) Injection of AF isolated from a tomato *Cf–0* plant inoculated with a binary PVX vector encoding His_6–FLAG–tagged Ecp2 in a leaf of a *Cf–Ecp2* tomato plant. (D) Injection of AF solated from a tomato *Cf–0* plant inoculated with a binary PVX vector encoding His_6–FLAG–tagged Ecp2 in a leaf of a tomato *Cf–0* plant.

It was therefore concluded that upon expression of affinity–tagged Avr and Ecp proteins by PVX, biologically active effector proteins are produced and secreted into the leaf apoplast.

Having verified the correct expression and targeting of the recombinant fusion proteins, it was attempted to recover the expressed affinity–tagged Avr and Ecp proteins from PVX–inoculated tomato *Cf–0* plants. To this end, AF, as well as total leaf extract, was isolated. Subsequent affinity–purification using Ni^{2+}–NTA magnetic agarose beads (with high affinity for the His_6–tag) or StrepTactin sepharose (with high affinity for the StrepII–tag),

however, did not result in recovery of detectable amounts of affinity–tagged effector proteins (data not shown).

Two additional sets of binary PVX constructs were generated to investigate whether the N–terminal fusion, or the biochemical nature of the affinity–tags, interfered with the stability or the level of production of the recombinant fusion proteins. These sets consisted of C–terminally StrepII–tagged and C–terminally c–myc–tagged fusions of Avr2, Avr4E and Ecp2 (Fig. 1B). Expression, biological activity, and localization were confirmed in a similar fashion as for the N–terminal fusion proteins. However, the C–terminal c–myc or StrepII fusions could also not be detected using an anti–c–myc antibody or StrepTactin–HRP, neither in AF, nor in total leaf extract from tomato *Cf–0* plants expressing the different fusion constructs (data not shown).

As all attempts to purify or detect affinity–tagged *C. fulvum* effector proteins from tomato AF failed, an attempt was made to determine whether (part of) the affinity–tags were still attached to the secreted effector proteins. To this end, AF isolated from *Cf–0* tomato plants expressing the various constructs was denatured, separated on Tricine gels, and blotted to PVDF membranes. Subsequent western blot analysis using anti–FLAG antibodies, anti–c–myc antibodies, or StrepTactin–HRP did not result in detection of PVX–expressed effector proteins (see Fig. 3 for detection using anti–FLAG as an example). However, when using polyclonal antibodies raised against the individual *C. fulvum* effector proteins themselves (no antibodies were available for Ecp1 and Avr9), specific signals were detected for all effector proteins, except for Avr4, showing that the presence of most effector proteins could be detected in the AF from inoculated *Cf–0* tomato plants (Fig. 3). The inability to detect Avr4 can be attributed to the characteristics of the polyclonal antibodies, or to the level of production of stable Avr4 protein in tomato AF.

It cannot be excluded that the ability to detect the proteins with effector–specific polyclonal antibodies, but not with affinity–tag–specific antibodies, is caused by superior sensitivity of the effector–specific polyclonal antibodies. To test this hypothesis, a five–fold dilution series of *P. pastoris*–produced His_6–FLAG–tagged Avrs and Ecps was dot–blotted and used in a western analysis to compare the sensitivity of the anti–FLAG antibody to the effector–specific polyclonal antibodies. This demonstrated that the anti–FLAG antibody generally displays a higher sensitivity compared with the antibodies raised against the individual *C. fulvum* effector proteins (Fig. 4). These data altogether suggest that the affinity–tags, irrespective of whether they are fused to the N– or the C–terminus of the effector proteins, are removed from the recombinant fusion proteins in the tomato apoplast.

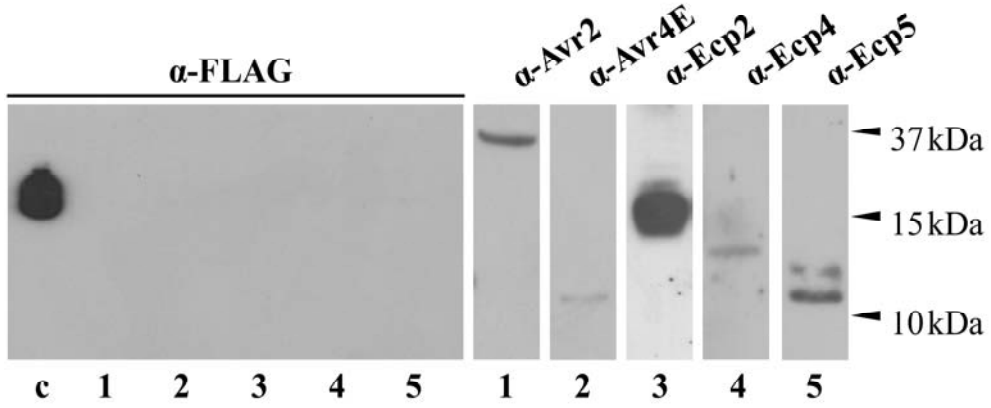

Figure 3. Detection of PVX-expressed *Cladosporium fulvum* effector proteins in apoplastic fluid (AF) from inoculated tomato leaves. Western blot analysis of AF from tomato plants expressing different affinity–tagged *C. fulvum* effector proteins, isolated 14 days post inoculation. Signals are obtained using antibodies raised against the FLAG–tag (α–FLAG) or against the individual *C. fulvum* effector proteins (α–Avr/Ecp). The samples loaded in each lane are indicated at the bottom of the figure. The c indicates a sample of *P. pastoris* produced His6–FLAG–tagged Ecp2 as a control for detection based on the FLAG–tag, 1 = Avr2, 2 = Avr4E, 3 = Ecp2, 4 = Ecp4, 5 = Ecp5. Detection using anti–c–*myc* antibodies or StrepTactin–HRP yielded similar results as the detection using α–FLAG (not shown).

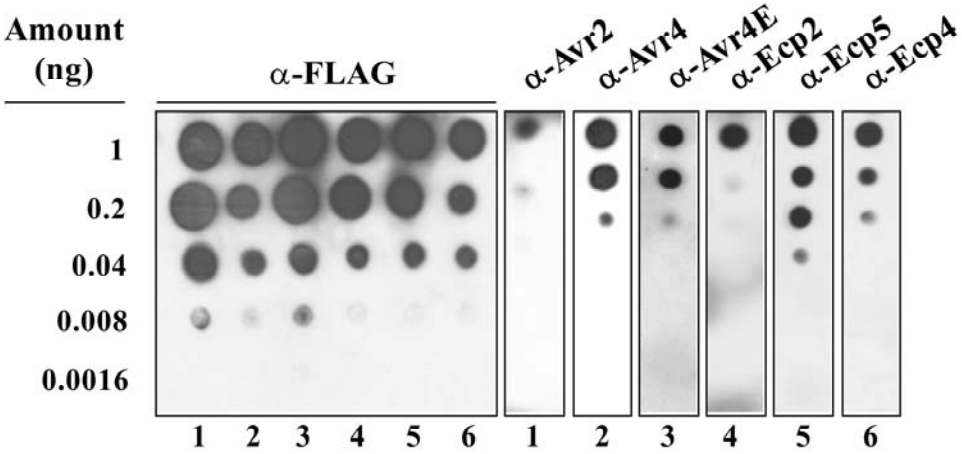

Figure 4. Comparison of the sensitivity of the anti-FLAG antibody with antibodies raised against the individual *C. fulvum* effector proteins. Dot blots were prepared from a 5–fold dilution series for each of the *P. pastoris*–produced His_6–FLAG–tagged *C. fulvum* effector proteins starting at 1 ng. The samples spotted in each lane are indicated at the bottom of the figure with 1 = Avr2, 2 = Avr4, 3 = Avr4E, 4 = Ecp2, 5 = Ecp4, 6 = Ecp5. Spotted protein quantities are indicated on the left of the figure. Signals were obtained using the antibody raised against the FLAG–tag (α–FLAG) as well as the antibodies raised against the individual *C. fulvum* effector proteins (α–Avr/Ecp).

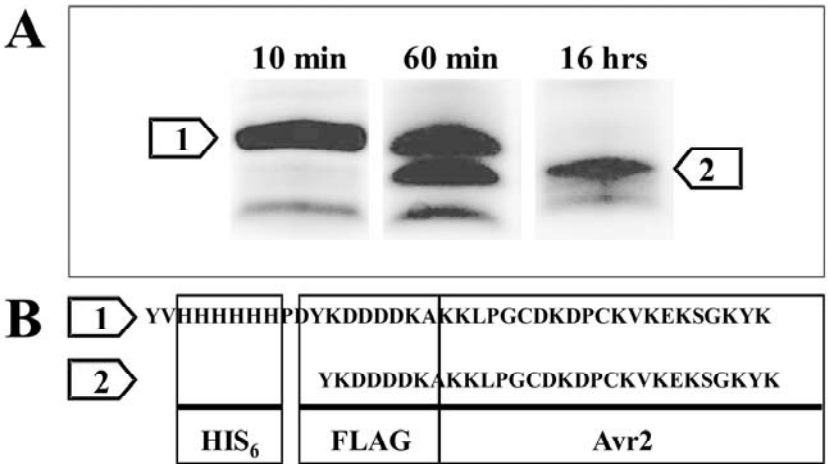

Figure 5. Incubation of *Pichia pastoris*-produced His_6-FLAG-tagged Avr2 in tomato apoplastic fluid (AF) results in cleavage of the affinity-tag. (A) Coomassie brilliant blue–stained Tricine gel loaded with *Pichia pastoris*–produced His_6–FLAG–tagged Avr2 that was incubated in AF of healthy tomato *Cf–0* plants for 10 min, 60 min or 16 hours prior to gel electrophoresis. (B) N–teminal sequences of products indicated by the arrows in panel A. The sequences of the His_6–tag, FLAG–tag, and N–terminus of the Avr2 protein are indicated.

The tomato leaf apoplast is known to contain many proteases. Furthermore, it has been shown that upon *C. fulvum* infection the protease activity in the apoplast increases (Solomon and Oliver, 2001; Krüger et al., 2002; Rooney et al., 2005). To test the stability of affinity–tagged *C. fulvum* proteins in tomato AF, an *in vitro* assay was performed using N–terminally His_6–FLAG–tagged *P. pastoris*–produced Avr2 protein. Twelve µg of Avr2 protein was incubated in 300 µl of AF isolated from leaves of four–week–old MoneyMaker *Cf–0* tomato plants. At regular intervals, subsamples were taken that were separated on a Tricine gel, and stained. The appearance of a band of a lower molecular weight than the His_6–FLAG–tagged Avr2 indicated that degradation of the tagged Avr2 protein already occurs 1 h after incubation in AF (Fig. 5). N–terminal sequencing of the smaller protein band demonstrated that the affinity–tag was cleaved from the intact Avr2 protein. Although the major part of the FLAG–tag is still attached to the Avr2 protein, the removal of the N–terminal aspartic acid destroys the core epitope for the FLAG–specific antibody (Hopp et al., 1988; Miceli et al., 1994).

To determine whether removal of the affinity–tags from PVX–expressed *C. fulvum* proteins also occurs in other Solanaceous species, His_6–FLAG–tagged Avr2 was expressed in several *Nicotiana* species (*N. benthamiana*, *N. clevelandi*, *N. glutinosa*, *N. tabacum*). At 14 DPI, AF was isolated, denatured, and analysed on western blots. The *N. tabacum* plants severely suffered from the PVX infection such that it was impossible to obtain sufficient amounts of AF and were, therefore, discarded from the assay. Similar as for tomato, for *N. benthamiana*, *N. clevelandi*, and *N. glutinosa*, detection of Avr2 using the FLAG–specific

antibodies failed while detection using Avr2–specific antibodies resulted in specific signals (data not shown).

In addition, an attempt was made to assess the stability of affinity–tagged *C. fulvum* effector proteins in a non–Solanaceous plant species. To this end the model plant *Arabidopsis thaliana* was chosen. Since PVX infection cannot be employed in Arabidopsis, *P. pastoris* produced His_6–FLAG–tagged Avr2 was incubated for 1, 2, or 4 h in AF isolated from Arabidopsis as well as tomato (Fig. 6). This experiment clearly demonstrated that after incubation for 1 h in tomato AF, the His_6–FLAG–tagged Avr2 was already partly degraded. This degradation did not occur upon incubation in Arabidopsis AF (Fig. 6).

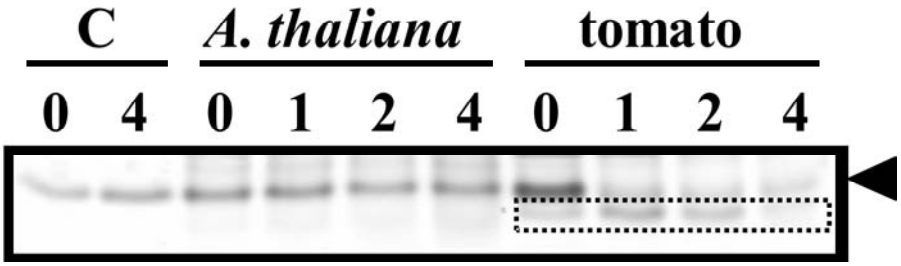

Figure 6. Stability of His6-FLAG-tagged Avr2 in apoplastic fluid (AF) from Arabidopsis and tomato. *P. pastoris* produced His6–FLAG–tagged Avr2 (24 µg) was incubated in 300 µl Arabidopsis or tomato AF for 1, 2 or 4 hours, respectively. After incubation, 7.5 µl was loaded on a Tricine gel, separated, and silver stained. As a control (C), His6–FLAG–tagged proteins were incubated for 4 hours in water. The boxed area shows degradation products for His6–FLAG–tagged Avr2 that appear already after incubation for 1 hour in tomato AF, whereas affinity–tagged Avr2 remains stable in the AF of Arabidopsis. The arrow indicates the intact recombinant effector protein fusion.

Since affinity–tagged effectors appear stable upon incubation in Arabidopsis AF, an attempt was made to perform affinity–purification in Arabidopsis. Arabidopsis plants were stably transformed (Clough and Bent, 1998) allowing expression of His_6–FLAG–tagged Ecp2 driven by the constitutive CaMV 35S promoter and using the tobacco PR–1a signal sequence for extracellular targeting. First generation transformants were selected on 50 µM kanamycin and subsequently transferred to soil. These plants were used for the isolation of AF as well as total leaf extract. Affinity–purification based on the presence of the His_6–tag indeed resulted in successful recovery of affinity–tagged Ecp2 in amounts that are clearly visible on silver–stained Tricine gel (Fig. 7A). The identity of the band was confirmed in western analysis using anti–FLAG to detect His_6–FLAG–tagged Ecp2 (Fig. 7B). This experiment clearly shows that the affinity–tag remains on the fusion proteins expressed in Arabidopsis in contrast to fusion proteins expressed in Solanaceous plants.

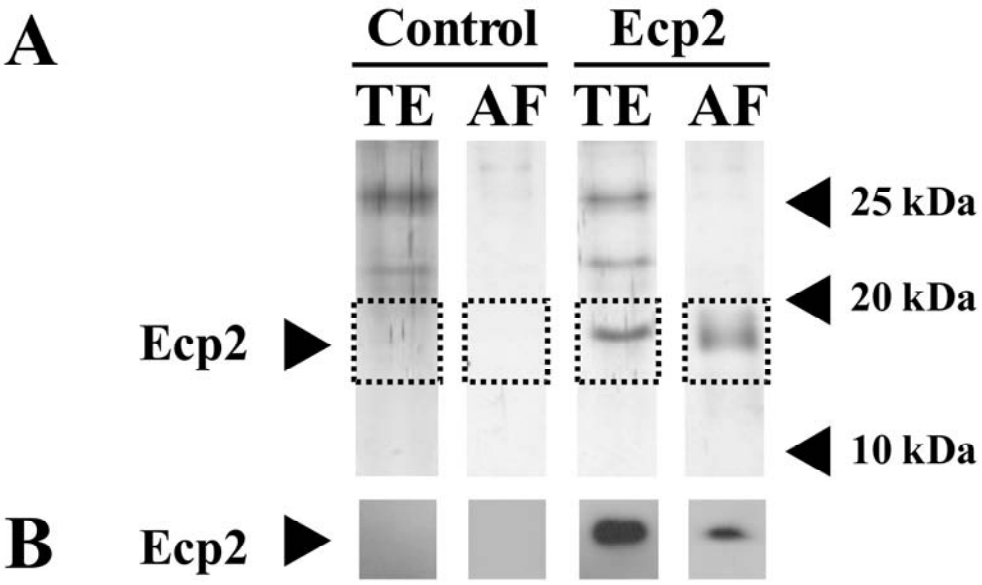

Figure 7. His_6-FLAG-tagged Ecp2 in extracts of transgenic *Arabidopsis thaliana*. (A) Total leaf extracts (TE) and apoplastic fluid (AF) was prepared from leaves of Arabidopsis plants constitutively expressing His_6–FLAG–tagged Ecp2 (Ecp2) and from control plants (control). After purification using Ni^{2+}–NTA agarose beads, extracts were eluted, electrophoresed on Tricine gel and silver–stained. The arrow indicates the affinity–tagged Ecp2 protein. (B) Western blot analyses of the samples shown in A. Total extracts were probed with an antibody raised against the FLAG–tag and apoplastic fluids were probed with an antibody raised against Ecp2. The region corresponding to the boxed areas in panel A are shown in panel B.

Discussion

Now that full–genome sequences are increasingly becoming available for more and more organisms, the major focus in research has shifted from the presence and transcription of specific genes towards the functions of their encoded products. Therefore, the interest in biochemical functions of proteins, their interacting partners, and their post–translational modifications is growing. Several methods are available to study proteins *in vitro*, but the question remains whether results obtained with *in vitro* methods represent the *in vivo* situation. Therefore, in vivo experiments are preferred above *in vitro* experiments. The production of recombinant proteins carrying epitope–tags that allow proteins of interest to be detected and purified through affinity–purification are frequently used to find interacting partners within crude extracts of complex biological materials (Hearn and Acosta, 2001; Terpe, 2003; Witte et al., 2004; Lichty et al., 2005).

To identify *in planta* virulence targets of the effector proteins that are secreted by *C. fulvum* during infection of its host, recombinant *C. fulvum* effector proteins were expressed *in planta* as fusions with an affinity–tag. Many protein–tags are available for such studies, each with their own characteristics. Because the *C. fulvum* effector proteins are rather small (ranging between 3 and 15 kDa), peptide affinity–tags were chosen (Table 2) that are likely to exert minimal or no effect on the tertiary structure and biological activity of the effector proteins. This is a major concern as in some cases it has indeed been reported that an affinity–tag can interfere with a biologically relevant target binding site (Goel et al., 2000).

To study effector proteins without the interference of additional *C. fulvum* effectors, *in planta* expression of single effector proteins was pursued rather than exploiting *C. fulvum* itself to express recombinant effectors. A systemic PVX expression system was chosen, allowing expression of the recombinant affinity–tagged fusion proteins throughout the whole plant, thus anticipating on low abundant interactors. In several studies, this PVX expression system was successfully employed for the *in planta* expression of secreted *C. fulvum* proteins (Luderer et al., 2002b; Takken et al., 2000; Westerink et al., 2004). Also in this study, inoculation with PVX constructs resulted in the production of biologically active proteins that were correctly targeted towards the tomato leaf apoplast (Fig. 2).

Based on the results obtained in this study, it can be concluded that the stability of the affinity–tag that is fused to the *C. fulvum* effector proteins is rather low due to cleavage of the tag in the tomato apoplast. The *C. fulvum* effector proteins themselves are very stable due to their disulphide bridges that render them less prone to degradation by plant proteases (Kooman–Gersmann et al., 1997; Luderer et al., 2001; van den Hooven et al., 2001; van den Burg et al., 2003). Indeed, for Avr4 it has been shown that disruption of individual disulphide bridges causes the protein to be sensitive to proteolysis (van den Burg et al., 2003). Interestingly, it has been demonstrated for the *C. fulvum* effector peptide Avr9 that proteolytic processing by plant factors leads to the trimming of the 34 amino acid Avr9 precursor into the 28 amino acid mature Avr9 peptide that acts as an elicitor of plant defense (van den Ackerveken et al., 1993b). This trimming stops in close proximity to the

first structural cysteine residue that is involved in a disulphide bridge (van den Hooven et al., 2001).

Since the protection of proteins by a compact tertiary structure seems to be crucial in the tomato leaf apoplast, it is anticipated that any stretch of linear amino acids that is added to an extracellular protein is sensitive to proteolytic degradation. This might explain why all the different tags that were tested, and both N–terminal of C–terminal fusions, are proteolytically removed from the fusion protein. To overcome this problem, several strategies could be pursued. One such strategy could be to include the affinity–tag at such a position in the protein that it is protected by the tertiary structure. However, it is not unlikely that the addition of a number of amino acids in the core of a protein would interfere with its biological activity. An alternative strategy could be to develop affinity–tags for N– or C–terminal fusions that adopt a tertiary structure and thus might be more resistant to proteolytic cleavage. However, it is anticipated that due to their size such tags might interfere with the biological activity of the protein of interest.

Interestingly, the stability of the affinity–tagged effector proteins does not seem to be a major issue in the apoplast of Arabidopsis. Affinity–purification using stable Arabidopsis transformants expressing His_6–FLAG–tagged Ecp2 resulted in successful recovery of the tagged effector protein. Although *C. fulvum* is not a pathogen of Arabidopsis, it can be anticipated that part of the host defense responses that are targeted by its effector proteins might also exist in this non–host species. Therefore, an attempt will be made to isolate targets for *C. fulvum* effector proteins in Arabidopsis by exploiting overexpression of recombinant affinitytagged fusions of *C. fulvum* effector proteins. Once virulence targets in Arabidopsis are identified, homologues of these targets might be found in the tomato genome. These homologues can be used for detailed functional analysis.

Materials and methods

Heterologous production of His_6-FLAG-tagged *C. fulvum* effector proteins in *Pichia pastoris*

Plasmids for the expression of affinity–tagged *C. fulvum* proteins in the yeast *Pichia pastoris* were generated as described (Rooney et al., 2005). Briefly, vector pPIC–9 (Invitrogen, Carlsbad, CA, USA) was modified by inserting an adaptor, encoding the His_6–tag and *Sma*I, *Apa*I, and *Sac*II restriction sites, resulting in vector pPIC–9His. To create His_6–FLAG–tagged effector proteins, cDNA for each effector was amplified (see primers for *Pichia pastoris* expression; Table 1) and cloned into pPIC–9His using the *Sma*I (blunt) and *Eco*RI restriction sites. Subsequently, *P. pastoris* strain GS115 (Invitrogen, Carlsbad, CA, USA) was transformed.

Fermentation was performed as previously described (van den Burg et al., 2001; Rooney et al., 2005). Proteins in the culture supernatant were separated on a Tricine SDS–PAGE gel, and stained with Coomassie Brilliant Blue or analysed on western blots. After removal of cells and concentration of the supernatant, the His_6–FLAG–tagged proteins were purified using a Ni^{2+}–NTA Superflow column (Qiagen, Leusden, the Netherlands), according to the manufacturer's protocol. The eluted protein fractions were pooled and dialysed using Milli–Q water. Protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). In addition, proteins were tested for their hypersensitive response (HR–) inducing activity by injection into leaves of tomato plants carrying the corresponding *Cf*–resistance genes.

PVX-mediated expression of affinity-tagged *C. fulvum* effector proteins *in planta*

The binary PVX vector pGr106 (Jones et al., 1999) was used as a backbone for all PVX expression constructs used in this study. From left border to right border the T–DNA of this vector consists of a CaMV 35S promoter–driven PVX sequence containing the replicase gene, the triple gene block, the duplicated coat protein promoter, and the coat protein gene (Fig. 1A). The multiple cloning site is located directly downstream of the duplicated coat protein promoter. The *P. pastoris* expression vectors described above were used to amplify the His_6–FLAG–tagged effector proteins. The cloning strategy for the various constructs is described below (see Table 2 for primer sequences). Constructs for N–terminally His_6–FLAG–tagged effector proteins: First, the *N. benthamiana* PR1a signal sequence was amplified introducing a 5' *Cla*I restriction site (primers PR1a–*Cla*I and PR1a– His1). In addition, all His_6–FLAG–tagged effector proteins were amplified from the *P. pastoris* expression vectors using a forward primer annealing to the His_6–tag (primer PR1a–His2) and a reverse primer annealing to the pPIC–9 backbone (primer 39 AOX1). Subsequently, utilizing overlap extension PCR, the coding region for the PR1a signal sequence was fused to that of the His_6–FLAG–tagged effector proteins (primers PR1a–ClaI and 3' AOX1).

Constructs for N–terminally His_6–FLAG–StrepII–tagged effector proteins

The coding sequence for the effector proteins was amplified from the *P. pastoris* expression vectors using gene–specific forward primers including a 5' StrepII coding sequence overhang and a reverse primer annealing to the pPIC–9 backbone (primer 3' AOX1). In addition, constructs encoding the N–terminally His_6–FLAG–tagged effector proteins were used to amplify the PR1a signal sequence fused to the His_6–FLAG–tag, including a 39 StrepII coding sequence overhang using the primers PR1a–*Cla*I and FLAG–Strep. For each construct, both PCR products were fused by overlap extension PCR with the primers PR1a–*Cla*I and 3' AOX1. Constructs for C–terminally StrepII– or c–myc–tagged effector proteins: First, the coding sequence for the effector proteins was amplified from the *P. pastoris* expression vectors using gene–specific forward primers including a 5' overhang for the PR1a coding sequence, and gene–specific reverse primers with an overhang to include the coding sequence for the StrepII or c–myc affinity–tag followed by a stop codon and a *Not*I restriction site. In addition, the PR1a signal sequence was amplified using the primer PR1a–*Cla*I and a set of reverse primers containing effector gene–specific overhangs. Subsequently, PCR products were fused by overlap extension PCR with the PR1a–*Cla*I forward primer and the gene–specific reverse primers that were used to add the StrepII or c–myc affinity–tag.

In all cases, PCR fragments were purified from agarose gel (QIAquick, Qiagen, Leusden, the Netherlands) and cloned into the pGEM–T easy vector (Promega, Mannheim, Germany). After DNA sequencing, inserts were obtained using the *Cla*I and *Not*I restriction enzymes and ligated into pGr106. The resulting plasmids were transformed into *Agrobacterium tumefaciens* (GV3101) by electroporation.

A. tumefaciens strains containing PVX constructs for the expression of *C. fulvum* effector proteins were cultured on plates containing modified LB medium (10 g/L bacto–peptone; 5 g/L yeast extract; 2.5 g/L NaCl; 10 g/L mannitol) for 48 h at 28 °C. Subsequently, colonies were selected and inoculated on 2–week–old tomato plants by toothpick inoculation.

Western blot analyses

Apoplastic fluid (AF) was isolated from leaf material, using demineralized water for vacuum infiltration (de Wit and Spikman, 1982). Total leaf extracts were prepared by homogenizing leaf material in demineralized water. Both total extracts and AF were denatured by boiling for 5 min in an equal volume of denaturing solution (0.0625 M TRIS–HCl, pH 6.8, 2% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) β–mercaptoethanol, and 0.001% (w/v) bromophenol blue). Proteins separated on Tricine gels were transferred to polyvinylidene difluoride (PVDF) membrane (Bio–Rad, Hercules, CA, USA) and blocked overnight at 4 °C with phosphate buffered saline, pH 7.3, 3% (w/v), and BSA, 0.1% (v/v) Tween 20). Subsequently, blots were incubated for 2 h at room temperature with primary (rabbit) antibody (10 µl in 10 ml antibody buffer: PBS, pH 7.3, 0.3% (w/v) BSA, and 0.1% (v/v) Tween 20). After washing in antibody buffer, blots were incubated (2 h at room temperature) with secondary horseradish peroxidase (HRP)–conjugated antibody. After washing, the HRP–conjugate was activated (SuperSignal, Pierce, Rockford, IL, USA) and detected on film. Western blot analysis with StrepTactin–HRP (IBA, Göttingen, Germany), monoclonal anti–FLAG bioM2 (Sigma, St Louis, MO, USA) and anti–myc–HRP antibody (Invitrogen, Carlsbad, CA, USA) was performed according to the manufacturer's specifications. Polyclonal antibodies raised against the individual *C. fulvum* effector proteins were produced upon immunization of rabbits according to standard procedures (Eurogentec, Seraing, Belgium). For immunization, enterokinase–treated (for affinity–tag removal), *P. pastoris*–produced *C. fulvum* effectors were used.

Affinity-purification using the His_6-tag

For His_6–based affinity–purification, AF was isolated from leaf material by vacuum infiltration (de Wit and Spikman, 1982) using a buffer (50 mM NaH_2PO_4, pH 7.0, 300 mM NaCl, 10 mM imidazole, and 0.005% (v/v) Tween 20) compatible with subsequent purification steps using Ni^{2+}–NTA magnetic agarose beads (Qiagen, Leusden, the Netherlands). The same buffer was used for the preparation of total protein extracts. Total protein extracts were prepared by homogenizing 2 g of leaf material frozen in liquid nitrogen after which 2 ml of buffer was added. Subsequently, 50 µl of Ni^{2+}–NTA magnetic agarose beads suspension was added to 2 ml of AF or total protein extract and incubated for 60 min at 21 °C. The beads were recovered by using a magnetic separator and washed four times in 2 ml washing buffer (50 mM NaH_2PO_4, pH 7.0, 300 mM NaCl, 20 mM imidazole, and 0.005% (v/v) Tween 20). Elution was performed in 50 µl of elution buffer (50 mM NaH_2PO_4, pH 7.0, 300 mM NaCl, 250 mM imidazole, and 0.005% (v/v) Tween 20). Eluates were separated on a 16% Tricine gel and visualized by silver–staining (Blum et al., 1987).

Affinity-purification using the StrepII-tag

AF was isolated from leaf material by vacuum infiltration (de Wit and Spikman, 1982) using demineralized water containing a protease inhibitor cocktail (complete Protease Inhibitor Cocktail Tablets, Roche, Basel, Switzerland). AF was concentrated 8× by using a 5 kDa cut–off filter (Vivaspin 4, Vivascience GA, Germany). Purification of the protein was carried out essentially as described by Witte et al., (2004). To 200 µl of concentrated AF, 60 µl of a 5× buffer [500 mM TRIS, pH 8.0, 25 mM EGTA, 25 mM EDTA, 750 mM NaCl, 50mM DTT, 2.5 mM AEBSF (4–(2–aminoethyl)benzenesulphonylfluoride hydrochloride), and 2.5% (v/v) Triton X–100] was added plus 40 µl of avidin (1 mg/ml) (Witte et al., 2004). After 15 min of incubation at 4 °C, 50 µl StrepTactin sepharose (IBA, Göttingen, Germany) was added. After 30 min of incubation at 4 °C the slurry was transferred to a glass wool column (200 µl void volume) and washed twice with 1 ml and four times with 0.5 ml wash buffer (50 mM TRIS, pH 8.0, 2.5 mM EDTA, 150 mM NaCl, 2 mM DTT, and 0.05% (v/v) Triton X–100). Elution was performed by filling the void volume of the column with elution buffer (10 mM TRIS, pH 8.0, 10 mM desthiobiotin, 2 mM DTT, and 0.05% (v/v) Triton X–100). Subsequently, seven fractions of 50 µl were collected by stepwise adding 50 µl of elution buffer. As a final elution step, the glass–wool was removed from the column, taking care that the sepharose was also transferred, and boiled in 200 µl 23 denaturing buffer (0.0625M TRIS–HCl, pH 6.8, 2% (w/v)

SDS, 10% (w/v) glycerol, 5% (v/v) β–mercaptoethanol, and 0.001% (w/v) bromophenol blue). Washing and eluted fractions were separated on a 16% Tricine gels and were visualized by silver–staining (Blum et al., 1987).

Plant cultivation

All tomato plants were grown under standard greenhouse conditions: at 21/19 °C over the 16/8 h day/night period, 70% relative humidity and 100W/m^2 supplemental light when the sunlight influx intensity was below 150 W/m^2. Similarly, Arabidopsis plants were also grown under greenhouse conditions of 21/18 °C during the 16/8 h day/night, 60% relative humidity and 100 W/m^2 supplemental light when the sunlight influx intensity was below 150 W/m^2.

Table 2. Primers used in this study, grouped based on their specific use

Name	F/R[1]	Primer sequence (5'–3')[2]	Description
***Pichia pastoris* expression**			
Avr2–N	F	GACTACAAGGACGACGATGACAAG GCCAAAAAACTACCTGGCTG	FLAG–tag at 5' end of *Avr2*
Avr2–C	R	CGC**GAATTC**TACGTATCATCAACC GCAAAGACCAAAACAG	*Eco*RI site at 3' end of *Avr2*
Avr4–N	F	GACTACAAGGACGACGATGACAAG AAGGCCCCCAAAACTCAACC	FLAG–tag at 5' end of *Avr4*
Avr4–C	R	CGC**GAATTC**TACGTATCATTGCGG CGTCTTTACCGGACACG	*Eco*RI site at 3' end of *Avr4*
Avr4E–N	F	GACTACAAGGACGACGATGACAAG GATTTCTCGCGCGATTGCC	FLAG–tag at 5' end of *Avr4E*
Avr4E–C	R	CGC**GAATTC**TACGTACTATCTGTTT GCCATCCTCTC	*Eco*RI site at 3' end of *Avr4E*
Avr9–N	F	GACTACAAGGACGACGATGACAAG TACTGTAACTCAAG	FLAG–tag at 5' end of *Avr*9
Avr9–C	R	CGC**GAATTC**TACGTATCACTAGTG GACACATTGTAGCT	*Eco*RI site at 3' end of *Avr*9
Ecp1–N	F	GACTACAAGGACGACGATGACAAG TTCGCAAAAAAGTTCAACC	FLAG–tag at 5' end of *Ecp*1
Ecp1–C	R	CGC**GAATTC**TACGTATCATTAAAG GCACTTGGGGTTTG	*Eco*RI site at 3' end of *Ecp*1
Ecp2–N	F	GACTACAAGGACGACGATGACAAG AACGCTGGCAACTCGCCC	FLAG–tag at 5' end of *Ecp2*
Ecp2–C	R	CGC**GAATTC**TACGTATCACTAGTC ATCGTTGGACGGGT	*Eco*RI site at 3' end of *Ecp2*
Ecp4–N	F	GACTACAAGGACGACGATGACAAG GACCCTTCCTTCCGCTTCAG	FLAG–tag at 5' end of *Ecp4*
Ecp4–C	R	CGC**GAATTC**TACGTATCATTACGG GCAAGTGACCTG	*Eco*RI site at 3' end of *Ecp4*
Ecp5–N	F	GACTACAAGGACGACGATGACAAG AGGGGCGACAATAAGCCC	FLAG–tag at 5' end of *Ecp5*
Ecp5–C	R	CGC**GAATTC**TACGTATCACTATCC AGAACTCTGACACCAGT	*Eco*RI site at 3' end of *Ecp5*
PVX expression			
N-terminal His_6-FLAG-tagging			
PR1a–ClaI	F	TTTCC**ATCGAT**ATGGGATTTGTTCT CTTTTCACAATTG	*Cla*I site at 5' end of PR1a
PR1a–His1	R	GGGATGATGATGATGATGATGATT TTGGGCACGGCAAGAGTG	Overlap PR1a and His_6–tag
PR1a–His2	F	CACTCTTGCCGTGCCCAAAATCATC ATCATCATCATCATCCC	Overlap PR1a and His_6–tag
3'AOX1	R	CAAATGGCATTCTGACATCC	Reverse primer for pPIC9–

His

N-terminal His_6-FLAG-StrepII-tagging

Avr2–N–Strep	F	TGGAGCCACCCACAATTCGAGAAG GCCAAAAAACTACCTGGCTGC	StrepII–tag at 5' end of *Avr2*
Avr4–N–Strep	F	TGGAGCCACCCACAATTCGAGAAG AAGGCCCCCAAAACTCAACC	StrepII–tag at 5' end of *Avr4*
Avr4E–N–Strep	F	TGGAGCCACCCACAATTCGAGAAG GATTTCTCGCGCGATTGCCC	StrepII–tag at 5' end of *Avr4E*
Avr9–N–Strep	F	TGGAGCCACCCACAATTCGAGAAG TACTGTAACTCAAGTTGTACTAGGG	StrepII–tag at 5' end of *Avr9*
Ecp1–N–Strep	F	TGGAGCCACCCACAATTCGAGAAG TTCGCAAAAAAGTTCAACCAGAAC TG	StrepII–tag at 5' end of *Ecp1*
Ecp2–N–Strep	F	TGGAGCCACCCACAATTCGAGAAG AACGCTGGCAACTCGCCC	StrepII–tag at 5' end of *Ecp2*
Ecp4–N–Strep	F	TGGAGCCACCCACAATTCGAGAAG GACCCTTCCTTCCGCTTC	StrepII–tag at 5' end of *Ecp4*
Ecp5–N–Strep	F	TGGAGCCACCCACAATTCGAGAAG AGGGGCGACAATAAGCCCG	StrepII–tag at 5' end of *Ecp5*
FLAG–Strep	R	CTTCTCGAATTGTGGGTGGCTCCAC TTGTCATCGTCGTCCTTGTAG	Overlap extension FLAG–StrepII

C-terminal StrepII- and c-*myc*-tagging

PR1a–Avr2	F	CACTCTTGCCGTGCCCAAAATGCCA AAAAACTACCTGGCTG	PR1a overhang at 5' end of *Avr2*
PR1a–Avr4E	F	CACTCTTGCCGTGCCCAAAATGATT TCTCGCGCGATTGCC	PR1a overhang at 5' end of *Avr4E*
PR1a–Avr9	F	CACTCTTGCCGTGCCCAAAATTACT GTAACTCAAG	PR1a overhang at 5' end of *Avr9*
PR1a–Ecp2	F	CACTCTTGCCGTGCCCAAAATAACG CTGGCAACTCGCCC	PR1a overhang at 5' end of *Ecp2*
PR1a–Avr2	F	CAGCCAGGTAGTTTTTTGGCATTTT GGGCACGGCAAGAGTG	*Avr2* overhang at 3' end of PR1a
PR1a–Avr4E	F	GGCAATCGCGCGAGAAATCATTTT GGGCACGGCAAGAGTG	*Avr4E* overhang at 3' end of PR1a
PR1a–Avr9	F	CTTGAGTTACAGTAATTTTGGGCAC GGCAAGAGTG	*Avr9* overhang at 3' end of PR1a
PR1a–Ecp2	F	GGGCGAGTTGCCAGCGTTATTTTGG GCACGGCAAGAGTG	*Ecp2* overhang at 3' end of PR1a
Avr2–C–Strep	R	CCC**GCGGCCGC**TACGTATCATCAC TTCTCGAATTGTGGGTGGCTCCAAC CGCAAAGACCAAAACAGCAAAG	*Not*I site and StrepII–tag at 3' end of *Avr2*
Avr4E–C–Strep	R	CCC**GCGGCCGC**TACGTACTACTTC TCGAATTGTGGGTGGCTCCATCTGT TTGCCATCCTCTCAGG	*Not*I site and StrepII–tag at 3' end of *Avr4E*
Avr9–C–Strep	R	CCC**GCGGCCGC**TACGTATCACTAC TTCTCGAATTGTGGGTGGCTCCAGT GGACACATTGTAGCTTATGAAAG	*Not*I site and StrepII–tag at 3' end of *Avr9*
Ecp2–C–Strep	R	CCC**GCGGCCGC**TACGTATCACTAC TTCTCGAATTGTGGGTGGCTCCAGT CATCGTTGGACGGGTTGTACG	*Not*I site and StrepII–tag at 3' end of *Ecp2*
Avr2–C–myc	R	CCC**GCGGCCGC**TACGTATCATCAG AGGTCCTCCTCGCTGATGAGCTTTT GCTCACCGCAAAGACCAAAACAGC AAAG	*Not*I site and c–*myc*–tag at 3' end of *Avr2*
Avr4E–C–myc	R	CCC**GCGGCCGC**TACGTACTAGAGG TCCTCCTCGCTGATGAGCTTTTGCT CTCTGTTTGCCATCCTCTCAGG	*Not*I site and c–*myc*–tag at 3' end of *Avr4E*
Avr9–C–myc	R	CCC**GCGGCCGC**TACGTATCACTAG	*Not*I site and c–*myc*–tag at 3'

		AGGTCCTCCTCGCTGATGAGCTTTT GCTCGTGGACACATTGTAGCTTATG AAAG	end of *Avr9*
Ecp2–C–myc	R	CCC**GCGGCCGC**TACGTATCACTAG AGGTCCTCCTCGCTGATGAGCTTTT GCTCGTCATCGTTGGACGGGTTGTA CG	*Not*I site and c–*myc*–tag at 3' end of *Ecp2*

[a] Orientation of the primer. F = forward primer, R = reverse primer.

[b] Restriction sites are indicated in bold. The coding sequence for affinity–tags is underlined.

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Chapter 3:
The chitin-binding *Cladosporium fulvum* effector protein Avr4 is a virulence factor

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Abstract

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

Introduction

The biotrophic fungal pathogen *Cladosporium fulvum* (syn. *Passalora fulva*) is the causal agent of tomato leaf mold (de Wit, 1992; Thomma et al., 2005). Colonization of leaves by the fungus is restricted to the apoplast and no haustoria or other feeding structures are produced (Bond, 1938; de Wit, 1977; Lazarovits and Higgins 1976). Therefore, all molecular components of both plant and fungus that are involved in the interaction are present in the apoplast. Eight fungal proteins that are secreted by *C. fulvum* during infection have been characterized and the corresponding genes have been cloned (Joosten et al., 1994; Laugé et al., 2000; Luderer et al., 2002b; Thomma et al., 2005; van den Ackerveken et al., 1993a; van Kan et al., 1991; Westerink et al., 2004). This set consists of four race–specific avirulence proteins (Avr2, Avr4, Avr4E and Avr9), and four extra–cellular proteins (Ecp1, Ecp2, Ecp4, Ecp5) that, in contrast to the Avrs, show much less polymorphisms since the cognate *Cf–Ecp* genes have not, or rarely been used in commercial tomato lines (Stergiopoulos et al., 2007; Thomma et al., 2005). Despite a number of common features shared by all *C. fulvum* effector proteins such as their small size and even number of cysteine residues, they display no sequence similarity to each other or any other protein sequence present in public databases.

Resistance in tomato against *C. fulvum* is governed in a gene–for–gene manner as initially proposed by Flor (1942) and Oort (1944) for the Flax–*melampsora lini* and the wheat–*Ustilago tritici* pathosystems, respectively. It is conferred by dominant *Cf* (for *C. fulvum*) resistance genes that activate a defense cascade eventually leading to a hypersensitive response (HR) and host immunity (Kruijt et al., 2005). The fungus can evade this immunity if it lacks the cognate *Avr* gene or carries mutant *avr* alleles that are no longer recognized by the plant. For all four *Avr* genes, the cognate *Cf* resistance genes have

been cloned (Dixon et al., 1996; 1998; Jones et al., 1994; Takken et al., 1998; Thomas et al., 1997), while *Cf* loci that confer recognition of the Ecps have also been described (de Kock et al., 2005; Laugé et al., 1998a). Although the *Solanum* genus has developed Cf proteins to mediate recognition of *C. fulvum* effector proteins, the effector genes are maintained within the fungal population. Furthermore, the effector genes are highly expressed *in planta* during colonization of the host (de Wit, 1992; Joosten et al., 1997; Thomma et al., 2006; van den Ackerveken et al., 1994; van Kan et al., 1991). Altogether these observations suggest that *C. fulvum* effectors play a role in pathogen virulence (Thomma et al., 2005).

At present, the intrinsic functions of a growing number of pathogen effectors, mostly those of bacterial origin, are being uncovered. However, the intrinsic functions of only a few fungal and oomycetous plant pathogens have been described. For *C. fulvum* it has been shown that the secreted effector protein Avr2 inhibits the extracellular tomato cysteine protease Rcr3 which is guarded by the tomato extracellular, membrane–anchored resistance protein Cf–2 in resistant tomato varieties (Rooney et al., 2005). When Avr2 binds and inhibits Rcr3, this interaction is somehow perceived by the Cf–2 protein, resulting in an HR and resistance (Rooney et al., 2005). Several secreted effectors from the oomycetous pathogens *Phytophthora infestans* have been identified that also display protease–inhibitory activity, and may thus suppress host defense responses (Tian et al., 2004; 2005; 2007). Nevertheless, it remains to be demonstrated that these effectors indeed are required for full pathogen virulence. In contrast, it was recently shown that two avirulence proteins from the barley powdery mildew fungus increase fungal infectivity on susceptible host plant cells, but their intrinsic functions remain to be discovered (Ridout et al., 2006). Similarly, from *C. fulvum* two novel *in planta* secreted proteins have recently been identified that are required for full virulence, but also for these proteins their intrinsic functions are not known yet (Bolton et al., 2007).

In addition to the *C. fulvum* Avr2 effector described above, a putative intrinsic function has been ascribed to the Avr4 effector. Initially, *in vitro* experiments showed that this effector specifically binds to fungal cell walls, but not to those of tomato (Westerink et al., 2002). Subsequently, based on structural analysis, Avr4 was found to harbor an invertebrate chitin–binding domain (van den Burg et al., 2003; 2004). Chitin is a major component of fungal cell walls and is targeted by plant chitinases as part of the plant's defense response (Grison et al., 1996; Jongedijk et al., 1995; Mauch et al., 1988; Schlumbaum et al., 1986; Sela–Buurlage et al., 1993). Avr4 was found to specifically bind to chitin present in fungal cell walls *in planta* (van den Burg et al., 2006) and it has been suggested that through this binding, Avr4 can protect fungal hyphae against hydrolysis by plant chitinases. This has indeed been demonstrated *in vitro* for the fungal species *Fusarium solani* and *Trichoderma viride* (van den Burg et al., 2006). Chitin in the cell walls of *in vitro* grown *C. fulvum* was found to be inaccessible to either chitinases or Avr4 due to the presence of a matrix of glucans and proteins covering the chitin present in cell walls of *C. fulvum* grown *in vitro* (de Wit and Kodde, 1981; de Wit and Roseboom, 1980). However, it was demonstrated that

Avr4 does bind to the hyphae of *C. fulvum* present in the apoplast of infected tomato plants. This suggests that Avr4 may protect *C. fulvum* hyphae from the activity of chitinases that accumulate in the tomato apoplast during infection (van den Burg et al., 2006; Wubben et al., 1993). Interestingly, natural strains of *C. fulvum* have been identified that produce Avr4 isoforms which are degraded by proteases in the tomato apoplast, but have retained their chitin–binding ability (van den Burg et al., 2003). In this way, Cf–4–mediated recognition of mutant isoforms of Avr4 by the Cf–4 protein in the cell wall of tomato is evaded but possibly without loss of the intrinsic function, suggesting that both native and mutant forms of Avr4 contribute to fitness of *C. fulvum* (van den Burg et al., 2003). However, experimental proof for Avr4 being a genuine virulence factor for *C. fulvum* is still lacking.

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

Results

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

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

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

Since no obvious macroscopically visible phenotypic differences could be observed, we subsequently analyzed whether the presence of Avr4 in the apoplast of tomato leaves causes significant transcriptional changes in the plant. To this end we used a custom–designed Affymetrix tomato GeneChip array (Syngenta Biotechnology, Inc., Research Triangle Park, NC, USA) that contains probe sets representing 22,721 tomato gene transcripts. Recently, cDNA–AFLP analysis revealed that expression of Avr4 in tomato plants carrying the cognate resistance gene *Cf–4* leads to extensive transcriptional reprogramming during synchronously induced host cell death (Gabriëls et al., 2006). Expression profiles from leaves of four–week–old Avr4–producing transgenic tomato lines were compared to those of leaves of the parental MM–*Cf–0* line. In total, only seven differentially expressed genes were identified that showed at least a two–fold change in expression level between the two tomato lines (Table 1). This differential expression was confirmed with real–time PCR analysis for all genes except for the chitinase gene (data not shown). Interestingly, of the six remaining differentially expressed genes, three were also up–regulated in transgenic tomato lines expressing the *C. fulvum Avr9* gene (data not shown). Apart from the presence of a high number of cysteine residues that are all involved in disulfide bridges, there is no homology between Avr4 and Avr9. Therefore, it can be concluded that so far only three tomato genes display Avr4–specific down–regulated expression; one gene encodes a vacuolar sorting receptor protein and the other two encode polyphenol oxidases (Table 1). These results show that the presence of Avr4 in the apoplast of tomato causes remarkably small effects on the plant transcriptome in absence of the cognate *Cf–4* resistance gene.

Table 1. Differentially expressed genes in four-week-old Avr4-producing transgenic tomato plants when compared to control plants as revealed by Affymetrix tomato GeneChips.

Array fold change	Annotation
–8.21	similar to *Solanum tuberosum* vacuolar sorting receptor protein
–4.15	polyphenol oxidase
–3.96	polyphenol oxidase
–2.59 §	chitinase
3.02 *	similar to *Arabidopsis thaliana* RNA–binding protein–like
3.42 *	induced in tomato root during/after fruit set
4.83 *	hypothetical protein

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

§ This differential could not be confirmed by quantitative real–time PCR analysis.

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

The model plant *Arabidopsis thaliana* is well–characterized with respect to pathogen defense responses (Thomma et al., 2001). To further investigate the activity of Avr4 *in planta*, transgenic Arabidopsis plants that constitutively produce Avr4 were generated. A binary construct with cDNA encoding the mature Avr4 protein fused to the coding region for the tobacco PR1a signal sequence for extracellular targeting and driven by the constitutive CaMV 35S promoter was transformed into the Arabidopsis ecotype Col–0 (Clough and Bent, 1998). Several independent homozygous single–integration lines were obtained and screened for Avr4 production by western analyses (Fig. 1) of total protein extracts with Avr4–specific polyclonal antibodies (van Esse et al., 2006). The three lines that displayed the highest levels of Avr4 production were chosen for further analysis. None of the Avr4–producing Arabidopsis plants showed any macroscopically visible phenotypic anomalies when grown under standard greenhouse conditions.

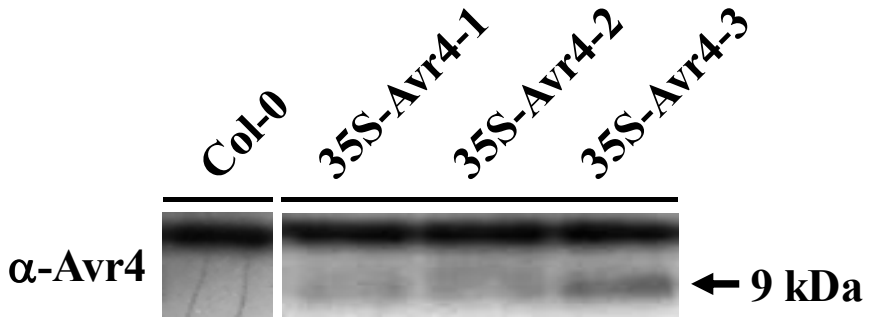

Figure 1. Detection of Avr4 in apoplastic fluid isolated from transgenic Arabidopsis plants. Western analyses of apoplastic fluids isolated from three independently transformed Arabidopsis lines that constitutively express the *C. fulvum* effector gene *Avr4* and from the parental Col–0 line, using an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

As *C. fulvum* is not a pathogen of Arabidopsis, we used typical Arabidopsis pathogens to challenge the Avr4–producing Arabidopsis transgenes (Thomma et al., 1998; 2000; 2001). We found that four–week–old greenhouse–grown control and Avr4–producing plants inoculated with conidia of the necrotrophic fungal pathogen *Botrytis cinerea* showed a clear difference in disease progression. Avr4–producing Arabidopsis lines showed significantly stronger disease symptoms compared to both the parental control line, and Avr9–producing Arabidopsis (Fig. 2). On the Avr4–producing plants lesions developed faster, showed a larger diameter and displayed chlorotic halos that were contained in a later stage. A similar increase in disease susceptibility was observed for Avr4–producing Arabidopsis plants when inoculated with the necrotrophic fungus *Plectosphaerella cucumerina* (Fig. 2), although assays with the necrotrophic fungus *Alternaria brassicicola* did not convincingly reveal increased disease susceptibility of Avr4–producing plants. However, in contrast to *B.*

cinerea and *P. cucumerina*, *A. brassicicola* behaves as an incompatible pathogen on wild–type Arabidopsis plants (Thomma et al., 1998; 2003) which may be due to other host factors than chitinases. To investigate whether production of Avr4 also increases virulence of pathogens other than fungi, the symptoms caused by the hemibiotrophic oomycete *Phytophthora brassicae* and the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 were also analyzed. Interestingly, no increase in virulence was observed for either of these two pathogens (Fig. 2). For *P. syringae* we measured the bacterial colonization using real–time PCR quantification (Brouwer et al., 2003) confirming the lack of significant differences between wild–type and Avr4–producing Arabidopsis genotypes (results not shown). Taken together our data suggest that on Arabidopsis Avr4 promotes the virulence of fungal pathogens, but not of oomycetous or bacterial pathogens.

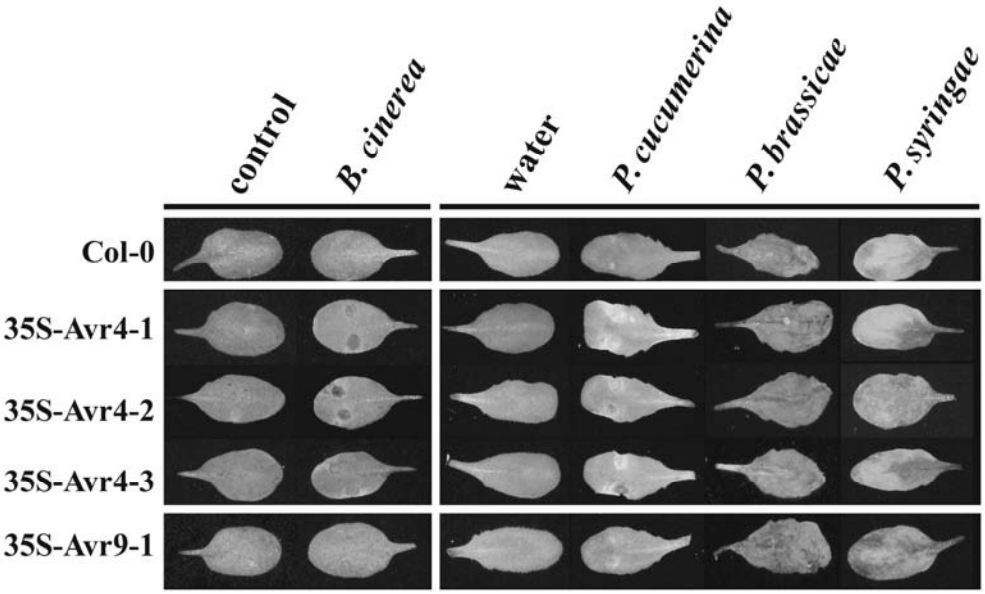

Figure 2. Avr4-producing Arabidopsis is more susceptible to several fungal pathogens (see page 201 for full color version). Typical symptoms caused by *Botrytis cinerea, Plectosphaerella cucumerina, Phytophthora brassicae* and *Pseudomonas syringae* on four–week–old plants of three independent Avr4–producing Arabidopsis lines at four days post inoculation. Disease progression by *B. cinerea* and *P. cucumerina* is faster on Avr4–producing Arabidopsis than on the parental Col–0 line and Avr9–producing Arabidopsis. No differences in disease progression by the oomycetous pathogen *P. brassicae* or the bacterial pathogen *P. syringae* are observed on the same set of Arabidopsis lines.

Avr4 protects various fungi against chitinases *in vitro*

It has previously been demonstrated that Avr4 protects *Fusarium solani* f. sp. *phaseoli* and *Trichoderma viride* against the deleterious effects of plant chitinases *in vitro* (van den Burg et al., 2006). To test whether Avr4 is also able to protect the Arabidopsis pathogens *B. cinerea* and *P. cucumerina* against chitinase activity, we performed similar *in vitro* assays for these two fungi, using *C. fulvum* as a control. Conidia were germinated, incubated with *Pichia pastoris*–produced Avr4, and subsequently treated with a crude extract of tomato leaves containing intracellular, basic chitinases. Growth of *P. cucumerina* was clearly inhibited by the hydrolytic enzymes present in this extract, and Avr4 was able to protect the fungus against the deleterious effects in a dose–dependent manner (Fig. 3). However, growth of *B. cinerea* was not inhibited by the treatment with chitinases, thus rendering it impossible to test whether Avr4 can protect this fungus against the activity of tomato chitinases. Similarly, it has previously been shown that *in vitro* growth of *C. fulvum* is not inhibited by chitinases, and that Avr4 does not bind to hyphae of *in vitro*–grown *C. fulvum* (Joosten et al., 1995; van den Burg et al., 2006). In contrast, during colonization *in planta* chitin in the hyphae of this fungus appeared to be highly accessible (van den Burg et al., 2006). A similar situation may be true for *B. cinerea*.

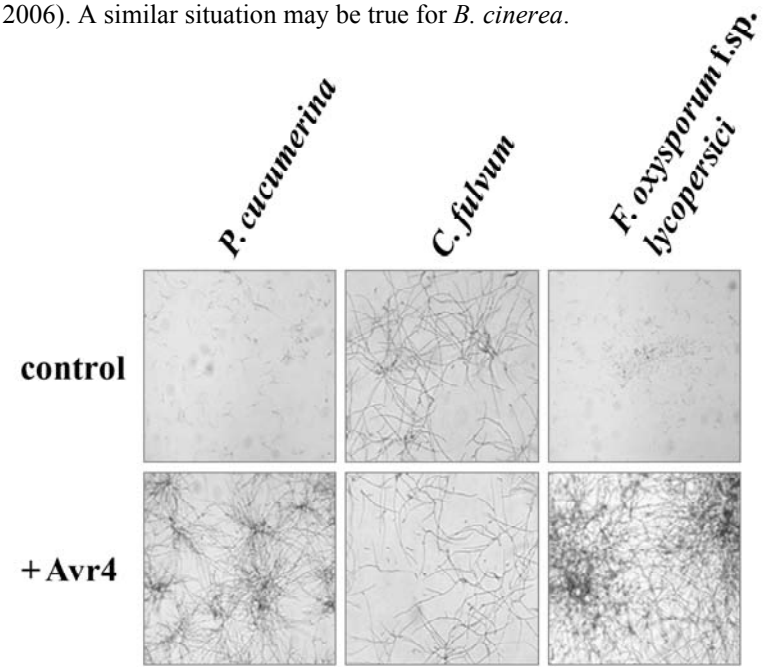

Figure 3. Avr4 protects Arabidopsis and tomato fungal pathogens against chitinases *in vitro.* Micrographs of *Plectosphaerella cucumerina, Cladosporium fulvum* and *Fusarium oxysporum* f. sp. *lycopersici* taken 24 h after addition of a crude extract of tomato leaves containing intracellular, basic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

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

Species of the genus *Fusarium* are generally known to be sensitive to chitinases. We therefore investigated whether transgenic Avr4–producing tomato plants show increased susceptibility to *F. oxysporum* f. sp. *lycopersici*. After inoculation of *F. oxysporum* f. sp. *lycopersici* on four–week–old transgenic Avr4–producing tomato plants and the parental MM–*Cf–0* line, disease progression was monitored. Interestingly, disease symptoms developed earlier and were more pronounced on the transgenic Avr4–producing tomato plants compared to the control, exemplified by a faster disease progression, earlier wilting, and chlorosis. In addition, inoculated Avr4–transgenic tomato plants were significantly shorter than inoculated control plants (Fig. 4). Furthermore, pre–incubation of germinated *F. oxysporum* f. sp. *lycopersici* conidia with Avr4 was found to protect the fungal hyphae against the deleterious activity of tomato chitinases (Fig. 3).

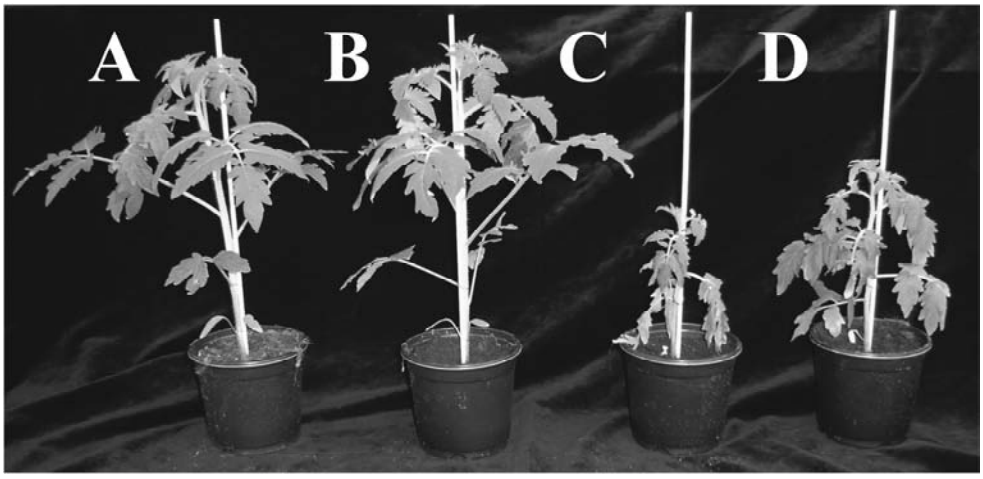

Figure 4. Heterologous expression of *Avr4* in tomato results in increased susceptibility to *Fusarium oxysporum* f. sp. *lycopersici* (see page 202 for full color version). Typical symptoms of disease after inoculation of four–week–old Avr4–producing tomato plants with *Fusarium oxysporum* f. sp. *lycopersici* at 14 days post inoculation. (A) Mock–inoculated Avr4–producing MM–Cf–0 tomato. (B) Mock–inoculated control tomato. (C) *F. oxysporum*–inoculated Avr4–producing tomato. (D) *F. oxysporum*–inoculated control MM–*Cf–0* tomato. Disease symptoms are more pronounced on Avr4–producing MM–Cf–0 tomato plants when compared to MM–*Cf–0* controls.

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

Our results obtained with tomato and Arabidopsis plants expressing *Avr4* show that Avr4 increases virulence of chitin–containing fungal pathogens. Therefore, we investigated the role of Avr4 in virulence of *C. fulvum* itself by employing *Avr4*–specific gene silencing. A binary vector was generated containing an inverted repeat fragment of the *Avr4* gene, driven by the *ToxA* promoter of the fungal wheat pathogen *Pyrenophora tritici*–repentis for

constitutive expression (Ciuffetti et al., 1997). Using *A.. tumefaciens*–mediated transformation (Bolton et al., 2007) this construct was introduced into a race 4 strain of *C. fulvum*, race 4(2), which produces an Avr4 isoform containing a Tyr67–to–His amino acid substitution (Joosten et al., 1997). Several *Avr4* inverted repeat transformants were obtained, four of which were used for further analysis. To determine whether the introduction of the *Avr4* inverted repeat construct resulted in *Avr4* silencing, four–week–old MM–*Cf–0* tomato plants were inoculated with the four transgenic *C. fulvum* strains. The *in planta* expression levels of *Avr4* were determined relative to the constitutively expressed *C. fulvum* actin gene using real–time PCR. It appeared that *Avr4* expression was strongly reduced in each of the four transformants when compared to *Avr4* expression level in the parental *C. fulvum* strain (Fig. 5A). Virulence assays on MM–*Cf–0* tomato plants showed that the *Avr4*–silenced transformants were significantly compromised in their ability to colonize tomato leaves when compared to the parental *C. fulvum* strain (Fig. 5B). Also the biomass produced by the *C. fulvum* transformants was significantly lower than that produced by the parental strain as could be shown by real–time PCR quantification of *C. fulvum* actin transcripts (Fig. 5C).

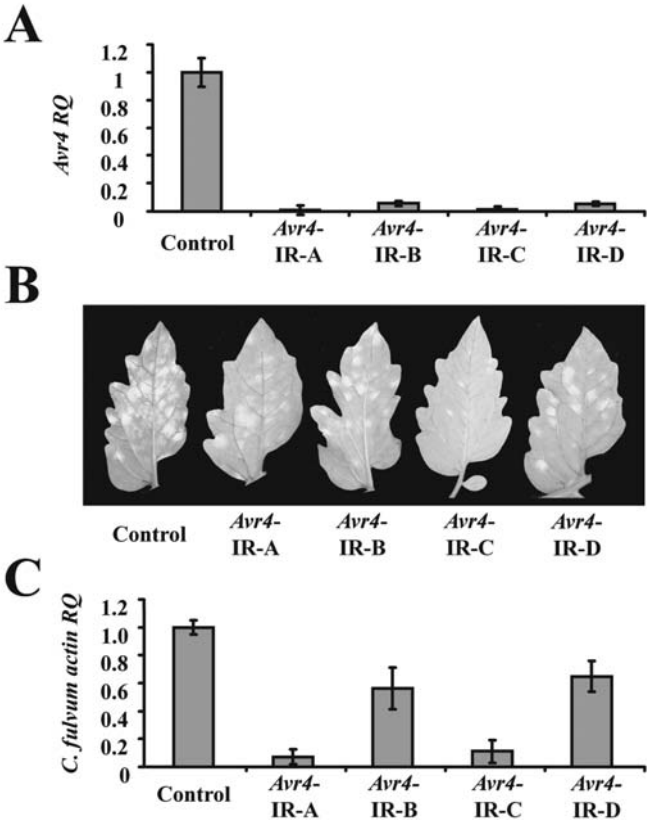

Figure 5 (previous page). Silencing of *Avr4*-expression in *Cladosporium fulvum* decreases its virulence on its host tomato. (A) Quantitative real–time PCR of *Avr4* transcript levels during a compatible interaction with MM–*Cf–0* tomato. *Avr4* transcript levels are significantly reduced in four independent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

Discussion

The *C. fulvum* Avr4 protein provides the first example of a fungal avirulence protein whose intrinsic biological function is demonstrated to contribute to pathogen virulence. It has been demonstrated previously that Avr4 is a lectin with chitin–binding activity that can protect fungal cell walls against the deleterious activity of plant chitinases (van den Burg et al., 2003; 2004; 2006). By generating transgenic plants that heterologously express the *C. fulvum Avr4* gene we now demonstrate that the presence of apoplastic Avr4 promotes the virulence of several fungal pathogens in Arabidopsis and tomato. Furthermore, *Avr4* silencing in *C. fulvum* clearly results in compromised virulence, demonstrating that Avr4 is an effector protein that contributes to pathogen virulence. This observation may also comply with the current view that pathogen effector molecules are mainly evolved to counteract pathogen–associated molecular pattern (PAMP)–triggered basal immune responses in the plant (Chisholm et al., 2006; Jones and Dangl, 2006). Chitin oligosaccharides are known to act as PAMPs that trigger strong host defense responses, including the accumulation of chitinases (Ramonell et al., 2002; 2005; Shibuya and Minami, 2001), and recently a plasma membrane receptor for chitin oligosaccharides has been identified in rice (Kaku et al., 2006). The *in planta* production of Avr4 by *C. fulvum* not only protects chitin in the fungal cell wall against hydrolysis by host chitinases, but may also reduce the release of chitin fragments that could trigger host immune responses. In *Cf–4* tomato plants, detection of Avr4 produced by *C. fulvum* results in an effector–triggered Cf–4–mediated immune response, which may be overcome by the fungus by the production of protease–sensitive Avr4 isoforms that are quickly degraded in the apoplast and are thus no longer recognized (Joosten et al., 1994; 1997).

Initial experiments with a natural race 4 *C. fulvum* strain that does not produce functional Avr4 (strain 38) failed to demonstrate increased virulence of this strain in transgenic Avr4–producing tomato lines. This may be explained by functional redundancy among pathogen effector molecules, implying that *C. fulvum* has additional chitin–binding effectors that are different from Avr4. Recently, a novel *C. fulvum* effector, Ecp6, has been identified that carries three lysin motifs (LysM domains) (Bolton et al., 2007). Interestingly, these motifs are found in a wide variety of proteins that are involved in oligosaccharide binding, including chitinases and the rice chitin–binding plasma membrane receptor that acts in

innate immunity (Joris et al., 1992; Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; Amon et al., 2000; Ponting et al., 1999; Kaku et al., 2006). Furthermore, a glycoprotein with LysM domains has been identified in the plant pathogenic fungus *Colletotrichum lindemuthianum*, that was found to bind to fungal hyphae *in planta* (Perfect et al., 1998) in a similar way as observed for Avr4 of *C. fulvum* (van den Burg et al., 2006). This all suggests that Ecp6 may be a functional, chitin–binding homologue of Avr4 (Bolton et al., 2007).

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

We also assessed whether Avr4 could have a direct function as host defense modulator. It has previously been shown that the progeny of a cross between MM–*Cf–0* tomato plants that express *C. fulvum Avr4* and MM–*Cf–4* tomato plants generates viable *Cf–4*/*Avr4* seeds that are able to germinate. However, after germination, the progeny displays lethality at the seedling stage due to the initiation of an HR (Thomas et al., 1997). This HR–initiation is temperature–sensitive, and can be prevented by growth of the *Cf–4*/*Avr4* seedlings at 33°C and high relative humidity (Thomas et al., 1997; de Jong et al., 2002). Subsequent transfer of these seedlings from 33°C to 20°C leads to a synchronous and systemic initiation of the HR. A cDNA–AFLP transcriptome analysis on *Cf–4*/*Avr4* seedlings after transfer from 33°C to 20°C has shown an extensive reprogramming of gene transcription, including many genes that are thought to act in primary metabolism and in stress signaling cascades (Gabriëls et al., 2006). Remarkably, production of *C. fulvum* Avr4 in tomato plants that do not carry the *Cf–4* resistance gene only results in very few and rather slight transcriptional

changes (Table 1). The three genes whose expression was found to be three– to five–fold induced are similarly up–regulated in *Avr9*–expressing tomato plants, suggesting that their induced expression is not caused by intrinsic Avr4 activity. The chitinase gene that was found to be slightly repressed in the array could not be confirmed by real–time PCR analyses. That leaves only three genes of which expression is specifically altered upon expression of *Avr4*, and remarkably their expression is repressed. Two of those genes encode a polyphenol oxidase, proteins of which the exact function is still unclear (Mayer et al., 2006). In some cases a positive correlation has been found between polyphenol oxidase activity levels and pathogen resistance (Li and Steffens, 2002; Rai et al., 2006; Thipyapong et al., 2004), although it is not clear whether this relationship is causal (Mayer et al., 2006). Nevertheless, the Avr4–specific repression of both tomato genes is quite modest and unlikely to account for the observed enhanced pathogen susceptibility. The strongest Avr4–specific reduction of expression is eight–fold and was observed for a gene encoding a vacuolar sorting receptor protein. These receptor proteins select proteins in the trans–Golgi for sorting to clathrin–coated vesicles and delivery to the vacuole (Kirsch et al., 1994). In this way, it has been demonstrated that seed storage proteins such as 2S albumin are delivered into the vacuoles of seeds (Jolliffe et al., 2004; Shimada et al., 2003). A direct link between vacuolar sorting receptor proteins and defense against pathogens has not been demonstrated, although plant vacuoles are rich in antimicrobial components that are likely to be transported through a similar mechanism. In addition, also for seed storage proteins like 2S albumins *in vitro* antimicrobial activity has been demonstrated (Terras et al., 1992; 1993). In our study, it has not been investigated whether the tomato genes of which expression is specifically altered upon expression of *Avr4* play a role in resistance against *C. fulvum*, nor whether expression of their Arabidopsis homologues is similarly altered upon *Avr4*–expression.

Altogether, the chitin–binding activity is likely to be the sole intrinsic function of the Avr4 effector protein, which is supported by the observation that the virulence of pathogens that do not contain chitin in their cell walls, such as the bacterium *P. syringae* and the oomycete *P. brassicae*, is not enhanced in Avr4–producing plants. In accordance with the "guard hypothesis", resistant plants recognize cognate pathogen avirulence proteins indirectly by sensing the manipulation of their host targets (guardees) by R proteins (guards) which subsequently triggers an immune response (Axtell and Staskawicz 2003; Mackey et al., 2003; Rooney et al., 2005). Such an indirect interaction has been proposed for *C. fulvum* Avr9 (Luderer et al., 2001) and has indeed been demonstrated for *C. fulvum* Avr2, that targets the tomato cysteine protease Rcr3 which is guarded by the Cf–2 protein (Rooney et al., 2005). However, examples of a direct interaction between plant resistance proteins and pathogen avirulence proteins have also been demonstrated in the pathosystems *Magnaporthe grisea*–rice (Jia et al., 2000), *Melampsora lini*–flax (Dodds et al., 2006), *Ralstonia solanacearum*–Arabidopsis (Deslandes et al., 2003) and tobacco mosaic virus–tobacco (Ueda et al., 2006). Avr4 presents a strong candidate that may be recognized directly by the tomato Cf–4 resistance protein since we, and others (Westerink et al., 2002),

have not been able to detect a target in the host plant tomato that could possibly act as a guardee. Future experiments will show whether Avr4 indeed interacts with the Cf–4 protein *in planta*.

Materials and methods

Cultivation of micro-organisms and plants

C. fulvum strain 38 (Bailey and Kerr, 1964), and *F. oxysporum* f. sp. *lycopersici* (kindly provided by Dr. B. Lievens, Scientia Terrae, Belgium) were cultured at room temperature on half–strength potato dextrose agar (Oxoid, Basingstoke, England) supplemented with 7 g/l technical agar (Oxoid, Basingstoke, England). *A. brassicicola*, *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* isoolate CBS686.95 were 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.

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/m2. 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/m2 supplemental light when the intensity dropped below 150 W/m^2.

Plant inoculations

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) on each leaf. Inoculation with *A. brassicicola* and *P. cucumerina* was performed in a similar way, using an aqueous suspension containing 5×10^5 conidia/ml). For all pathogens, plants were incubated at 20°C, 100% RH and a 16 h/8h light/dark regime. Disease progression was scored at four days post inoculation.

Inoculation with *P. syringae* pv. *tomato* DC3000 was performed by spray inoculation of a bacterial suspension of 5 × 108 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 days post inoculation.

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 *C. fulvum* was performed as previously described (de Wit 1977). Briefly, five–week–old soil–grown tomato plants were inoculated by spraying 5 ml of conidial suspension (1×10^6 conidia/ml) onto the lower surface of the leaves. Subsequently, plants were kept at 100 % RH for 48 h under a transparent plastic cover after which they we incubated at standard greenhouse conditions of 16 h/8h light/dark regime and 70% RH. Disease progression was monitored for 20 days post inoculation.

For inoculation with *F. oxysporum* f. sp. *lycopersici*, three–week–old soil–grown tomato plants were up–rooted and inoculated by dipping the roots in a conidial suspension (1×10^7 conidia/ml) in 12 g/l potato dextrose broth (Oxoid, Basingstoke, England). After re–planting in soil, plants were incubated at standard greenhouse conditions of 16 h/8 h light/dark regime and 70% RH. Disease progression was monitored for 20 days post inoculation.

Microarray sample preparation and analyses

For each experiment, independently repeated three times, three transgenic tomato plants and three MM–*Cf–0* tomato plants were grown for four weeks under standard greenhouse conditions. For each plant set, all third and fourth compound leaves containing five–seven leaflets were harvested, pooled and flash–frozen in liquid nitrogen. For RNA extraction, the frozen leaves were crushed using a spoon and approximately 100 mg of the crushed material was homogenized in Trizol® reagent (Invitrogen, Carlsbad, CA, USA). After 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, from plants grown in three independent experiments total RNA was obtained that was used for hybridization onto six individual custom designed Affymetrix tomato GeneChip arrays (made available through Syngenta Biotechnology, Inc., Research Triangle Park, NC, USA) which contain probe sets to interrogate 22,721 genes. Probe preparations and GeneChip hybridizations were carried out at ServiceXS (Leiden, The Netherlands). Microarray data sets were equilibrated and investigated with Rosetta Resolver Software (Rosetta Biosoftware, Seattle, WA, USA), which uses a proprietary algorithm error model to calculate significantly up or down regulated sequences (Weng et al., 2006). We only considered differential signals with P values below 0.01 and ratios larger than two as significant.

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

Generation of transgenic Avr4- and Avr9-producing Arabidopsis plants

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

Table 2. Primers used in this study.

Primer name	Sequence (5'-3')[1]	Description
oligo–dT	TTGGATCCTCGAGTTTTTTTTTTTTTTTT	Poly–T (*Nco*I and *Sac*I)
Nco–Avr4–F	TTCCATGGATGCACTACACAACCCTCCT	*Avr4* inverted repeat (*Nco*I)
Avr4–EcoRI–R2	GAATTCATAGCCAGGATGTCCAACCGAC	*Avr4* inverted repeat (*Eco*RI)
Avr4–NotI–R2	GCGGCCGCATAGCCAGGATGTCCAACCGAC	*Avr4* inverted repeat (*Not*I)
Avr4–(RT)–F	ACTCTCCTAGTCGGCACAG	*C. fulvum Avr4*
Avr4–(RT)–R	CAATAGCCAGGATGTCCAAC	*C. fulvum Avr4*
CF–GAPDH–F	GGAAACCGGAACCGTTCAG	*C. fulvum* actin
CF–GADPH–R	TGTTAGTGATCCCTTGTGATCCAA	*C. fulvum* actin
CF–Act	CATCGGCAACGAGCGATT	Tomato actin
CF–Act	TGGTACCACCAGACATGACAATG	Tomato actin
Array1–F	GCATGCCAGGATACTTTCCGTGGTAGAG	Vacuolar sorting protein
Array1–R	CATCGCAATGCTCCAGATGGTTCAC	Vacuolar sorting protein
Array2–F	ACCTGATCTCAAATCTTGTGGTGTTGCC	Polyphenol oxidase
Array2–R	CGGAGTTTCGTCATAGGAGGGAACTTGTAG	Polyphenol oxidase
Array3–F	CAGATGAGCTTGATAAGGCGGAGTTTGC	Polyphenol oxidase
Array3–R	CCTCCAACAGTTCAGTTATCGCCAGC	Polyphenol oxidase
Array4–F	TGAAGAATCGAAACGATGATAGATGTCCTG	Chitinase
Array4–R	CGCGCAGTATCATCACCGGTAGTAC	Chitinase
Array5–F	ATGTCAGAGGAGATTGTTCAGGTTGTTGG	RNA–binding protein–like
Array5–R	GGAGAAACCGTTCAGGAGAATGTGGC	RNA–binding protein–like
Array6–F	GGTATCCAAATACATCGTGGTCCTGATGG	Fruit set–induced
Array6–R	GGTTCTGCTCCTGGGTTGGCATTTC	Fruit set–induced
Array7–F	CTTTGGACTAACGGAACATTTATGTCAGCG	Hypothetical protein
Array7–R	GCGCAGTAGCCAACCAACCTGAC	Hypothetical protein

[1] Restriction sites are indicated in bold.

Western blot analyses

Total leaf extracts were prepared by homogenizing leaf material in demineralised water, and subsequently denatured by boiling for 5 min in an equal volume of denaturing solution (6.25 µM Tris–HCl, pH 6.8; 2% (w/v) SDS; 10% (w/v) glycerol; 5% (v/v) β–mercaptoethanol; and 0.001% (w/v) bromophenol blue). Protein samples were separated on 16 % Tricine gels and subsequently transferred to polyvinylidene difluoride (PVDF) membrane (Bio–Rad, Hercules, CA, USA) by electroblotting. The resulting blots were incubated overnight at 4 °C in blocking buffer (1 × phosphate–buffered saline (PBS), pH 7.3; 3% (w/v) BSA and 0.1% (w/v) Tween 20). Subsequently blots were incubated for 2 h at room temperature with 10 µl primary (rabbit) antibody diluted in 10 ml antibody buffer (1 × PBS, pH 7.3; 0.3% (w/v) BSA and 0.1% (v/v) Tween 20). After washing in antibody buffer, blots were incubated for 2 h at room temperature with horseradish peroxidase (HRP)–conjugated antibody. After washing, the HRP–conjugate was activated (SuperSignal, Pierce, Rockford, IL, USA) and detected on film (X–OMAT™ LS, Eastman Kodak Company, Rochester, NY, USA). Polyclonal antibodies raised against Avr4 were produced by immunization of rabbits according to standard procedures (Eurogentec, Seraing, Belgium; van Esse et al., 2006). For immunization, enterokinase–treated (for affinity–tag removal), *P. pastoris*–produced His_6–FLAG–tagged Avr4 (van Esse et al., 2006) was used.

Isolation of intracellular chitinases from tomato

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

***In vitro* fungal growth assays**

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

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

A fragment of the *Avr4*–coding sequence was amplified using cDNA from a compatible interaction between *C. fulvum* and tomato as template. Primer sequences are shown in Table 2. Construction of the binary vector containing an inverted repeat fragment of the *Avr4* gene was performed as described (Bolton et al., 2007). *A. tumefaciens*–mediated transformation of *C. fulvum* was performed as described (Bolton et al., 2007).

Quantification of Avr4 expression levels and fungal biomass

C. fulvum was inoculated onto five–week–old MM–*Cf–0* tomato plants as described above. In each of three independently repeated experiments, leaf material was harvested from inoculated MM–*Cf–0* plants at 0, 3, 7, 11 and 16 days post inoculation, flash–frozen in liquid nitrogen and stored at –80°C until used for RNA analysis. Leaf samples consisted of three leaflets obtained from the second, third, and fourth compound leaves of two tomato plants. Total RNA was isolated from infected leaf material using the RNeasy kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Total RNA was used for cDNA synthesis using an oligo(dT) primer and the SuperScript II reverse transcriptase kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Quantitative real–time PCR was conducted with primers given in Table 2, and using an ABI7300 PCR machine (Applied Biosystems, Foster City, CA) 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 10 min followed by denaturation for 15s at 95°C, annealing for 30s at 60°C, and extension for 30s at 72°C for 40 cycles and analyzed on the 7300 System SDS software (Applied Biosystems, Foster City, CA). To check for

contamination with genomic DNA, real–time PCR was also carried out on RNA without the addition of reverse transcriptase.

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Chapter 4:
The *Cladosporium fulvum* virulence protein Avr2 inhibits host proteases required for basal defense

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Abstract

Cladosporium fulvum (syn. *Passalora fulva*) is a biotrophic fungal pathogen that causes leaf mold of tomato. During growth in the apoplast, the fungus secretes effector proteins enabling it to establish disease, ten of which have been characterized. For most of these effectors, cognate *C. fulvum* (*Cf*) resistance loci have been identified that monitor the presence or (the activity of) the effector protein and mediate an immune response in tomato. 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 leads to enhanced susceptibility towards a number of extracellular fungal pathogens that include *Botrytis cinerea* and *Verticillium dahliae*, and microarray analysis of unchallenged plants showed that *Avr2* expression triggers a global transcription pattern reflecting pathogen challenge. Cysteine protease activity profiling showed that Avr2 inhibits multiple extracellular Arabidopsis cysteine proteases. In tomato, *Avr2* expression resulted in enhanced susceptibility not only towards natural Avr2–defective *C. fulvum* strains, but also towards *Botrytis cinerea* and *Verticillium dahliae*. Cysteine protease activity profiling in tomato revealed that also in this plant Avr2 inhibits multiple extracellular cysteine proteases, including Rcr3 and its close relative Pip1. Finally, silencing of the *Avr2* gene in *C. fulvum* significantly compromised its 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 in tomato. Moreover, heterologous expression in Arabidopsis and tomato enhances virulence of additional pathogens.

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 haustoria formation (Bond 1938; Lazarovits and Higgins 1976; de Wit 1977; Thomma et al., 2005). As a consequence, all molecular components secreted by the fungus can be found in the apoplast. Ten *C. fulvum* effector proteins secreted during host colonization have been characterized so far, 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 for all 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). Remarkably, although all *C. fulvum* effector proteins share some common features such as their small size and even number of cysteine residues, they display no significant sequence similarity to each other or to protein sequences deposited in public databases. The only exception is the lysine motif–

carrying Ecp6 protein for which homologues are present in various fungal species (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). According to the current view, evolution of *C. fulvum* effector molecules occurred to suppress or avoid basal host defense responses of tomato (de Wit, 2007). In turn, tomato has evolved Cf proteins to monitor the presence or activity of race–specific pathogen effector molecules (Joosten and de Wit, 1999; Rivas and Thomas, 2005; Thomma et al., 2005). Indeed, tomato genotypes carrying cognate *Cf* genes have been identified for all *C. fulvum* effector molecules tested (Jones et al., 1994; Dixon et al., 1996; 1998; Thomas et al., 1997; Laugé et al., 1998; Takken et al., 1998; de Kock et al., 2005).

Large effector repertoires have been identified from bacterial pathogens, which are typically delivered into host cells by the type III secretion system (Tang et al., 2006). Although it is often difficult to demonstrate a significant contribution to pathogen virulence due to functional redundancy, an intrinsic biological function has been uncovered for a growing number of these effectors (Grant et al., 2006; Buttner and Bonas, 2006). Besides bacterial pathogens, the number of (potential) effector molecules of filamentous fungal and oomycete plant pathogens is also growing (Xu et al., 2006). Based on the presence of the RxLR host targeting motif, it is currently predicted that the genomes of several oomycete plant pathogens encode hundreds of effectors (Whisson et al., 2007; Jiang et al., 2008). Nevertheless, the intrinsic biological function of only a few filamentous pathogen effector molecules has been identified so far (Kamoun, 2007; van der Does and Rep, 2007). 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 that Avr2 contributes to *C. fulvum* virulence with RNAi–mediated gene silencing. 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 are essential for basal host defense.

Results

Heterologous expression of *C. fulvum Avr2* in Arabidopsis enhances susceptibility towards distinct fungal pathogens

The model plant Arabidopsis is a well–characterized plant with respect to basal defense against microbial pathogens (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; 1999). 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 (data not shown). 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 (Suppl. Fig. 1A, B). 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 aploplastic fluid from progenitor wild–type Col–0 plants showed only slight non–specific chlorosis (Suppl. Fig. 1C). 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 fungal pathogens *B. cinerea* or *P. cucumerina* showed a clear enhancement of disease progression when compared to the inoculated control plants (Fig. 1). On the *At–Avr2* lines necrotic lesions developed faster and continued to enlarge compared to inoculated control plants on which lesions remained small (Fig. 1). Upon inoculation with the necrotrophic fungus *A. brassicicola*, the bacterial pathogen *P. syringae* pv. *tomato* strain DC3000, or the oomycete pathogens *P. brassicae* and *H. parasitica* strains Waco9 (virulent) and Cala2 (avirulent), no increased susceptibility of *At–Avr2* lines was observed (data not shown). Taken together, expression and extracellular targeting of Avr2 in Arabidopsis promotes the virulence of some, but not all, pathogens.

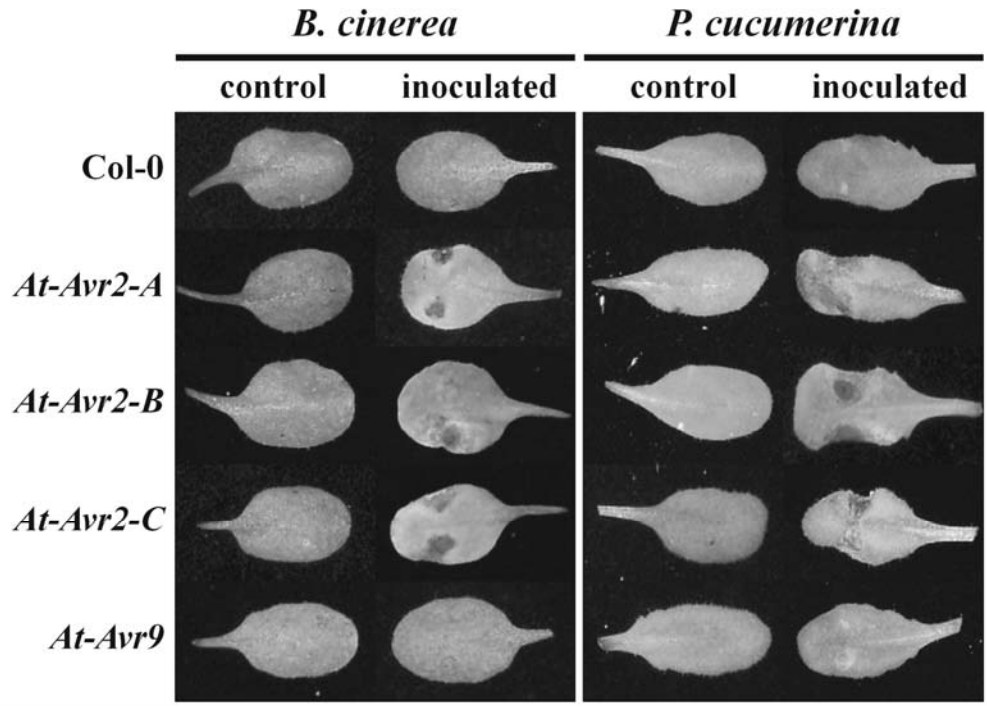


Figure 1. ***Avr2*–expressing Arabidopsis is more susceptible to the fungal pathogens *Botrytis cinerea* and *Plectosphaerella cucumerina* (see page 203 for full color version).** Typical symptoms caused by *B. cinerea* and *P. cucumerina* on four–week–old plants of three independent *Avr2*–expressing Arabidopsis lines (*At–Avr2–A* to *–C*) at four days post inoculation. Typical symptoms on the parental Col–0 line and an *Avr9*–expressing transgenic line (*At–Avr9*) are shown as control.

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. Two complementary methods were applied to relate global changes in gene expression to biological processes and pathways. In one method, Gene Ontology (GO) category enrichment is calculated via the ErmineJ software program (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, the cellular components they are associated with and their molecular and biochemical functions in a species–independent manner (Ashburner et al., 2000; Harris et al., 2004). This analysis revealed that genes participating in regulation of actin cytoskeleton reorganization, photosynthesis, and

biosynthesis of nitrogen compounds like amino acids and glucosinolates were differentially regulated, in addition to pathways related to wounding, oxidative stress, and jasmonic acid/ethylene/salicylic acid–signaling (data not shown). 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 (data not shown). This is also reflected by the molecular function of the products of the differentially regulated genes (data not shown).

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). Although this method is widely used to analyze human and murine transcriptome data, it has not yet been exploited for plants (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 our gene set was enriched for genes that are similarly found in Arabidopsis challenged with *P. syringae* or *Escherichia coli*, treatment with bacterial effectors, and treatment 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.

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]	P–value[b]	Reference
Type III–induced genes of *Pseudomonas syringae* coronatine mutant	264	0	Thilmony et al., 2006
P. syringae type III–induced genes	263	0	Thilmony et al., 2006
SNARE interactions in vesicular transport	46	0	KEGG pathway
Ndr1–specific upon challenge with *P. syringae* expressing *AvrRpt2*	50	0	Sato et al., 2007
Alternaria (AAL) toxin–induced programmed cell death at 72 hr	92	0	Gechev et al., 2004
Alternaria (AAL) toxin–induced programmed cell death at 48 hr	92	0	Gechev et al., 2004
P. syringae coronatine–regulated genes	323	0	Thilmony et al., 2006
Alternaria (AAL) toxin–induced programmed cell death at 24 hr	87	0.003	Gechev et al., 2004
N–glycan biosynthesis	20	0.003	KEGG pathway
Ribosome	180	0.008	KEGG pathway
PAMP–repressed genes	115	0.019	Thilmony et al., 2006
Nucleotide sugars metabolism	15	0.029	KEGG pathway
Salicylic acid–repressed auxin signalling pathway	20	0.036	Wang et al., 2007
E. coli strain O157:H7–induced genes	220	0.042	Thilmony et al., 2006
Fusarium oxysporum Nep1 toxin–induced death	432	0.047	Bae et al., 2006

[a]Number of genes present in the gene set or KEGG pathway
[b]Nominal p–value

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, and that can be fully competed by pretreatment with an excess of E–64 prior to labeling (Fig. 2). Interestingly, the biotinylated signals could also largely be competed with an excess of Avr2, demonstrating that Avr2 is able to inhibit cysteine proteases in Arabidopsis (Fig. 2). When compared to the progenitor Col–0 plants, a slightly different pattern of active cysteine proteases was observed in western blots of *At–Avr2–A* plants. Strikingly, although these signals could be competed with an excess of E–64, they could not be competed with an excess of Avr2 (Fig. 2), suggesting that the active cysteine proteases in *AtAvr2–A* plants are different from those in Col–0 plants and belong to a subset that cannot be inhibited by Avr2.

Subsequently, biotinylated proteins present in DCG–04 labeled protein extracts were purified on streptavidin magnetic beads and identified using LC/MS[2]. 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). Upon pre–incubation of the Col–0 extract with an excess of Avr2, subsequent DCG–04 labeling and streptavidin pull–down, only cathepsin B was detected (Table 2). In *At–Avr2–A* plants, three cysteine proteases were detected: aleurain, RD21a, and cathepsin B (Table 2). However, in this case the signals could not be competed by pre–treatment of the extract with an excess of Avr2, suggesting that in addition to cathepsin B the affinity of Avr2 for aleurain and RD21a is rather low. Since the genes encoding these three proteases were not differentially regulated in the transcriptome analysis of *At–Avr2–A* plants and progenitor Col–0 plants, it is concluded that the release and activation, rather than the production, of these proteases is enhanced in *At–Avr2–A* plants.

Table 2. Active cysteine proteases identified in total extracts of Arabidopsis plants.

		Col–0			At–Avr2–A	
Protease	Accession number	No competitor	Excess of E–64	Excess of Avr2	No competitor	Excess of E–64
XCP1	O65493	4 (2–0–1–1–0)	–	–	–	–
XCP2	Q9LM66	11 (6–3–1–1–0)	–	–	–	–
Cathepsin B	Q9ZSI0[1]	12 (10–1–1–0–0)	–	10 (6–1–1–1–1)	7 (4–3–0–0–0)	–
RD21A	P43297	13 (12–1–0–0–0)	–	–	6 (5–1–0–0–0)	–
CPR1	Q9LT77[2]	5 (2–1–1–1–0)	–	–	–	–
Aleurain	Q8H166	9 (7–2–0–0–0)	–	–	5 (4–1–0–0–0)	–
Aleurain – like	Q8RWQ9	7 (5–1–0–0–1)	–	–	–	–

[1] The same peptides were identified for Cathepsin B (Q9ZS10)
[2] The same peptides were identified for pseudotzain (Q3EB42)

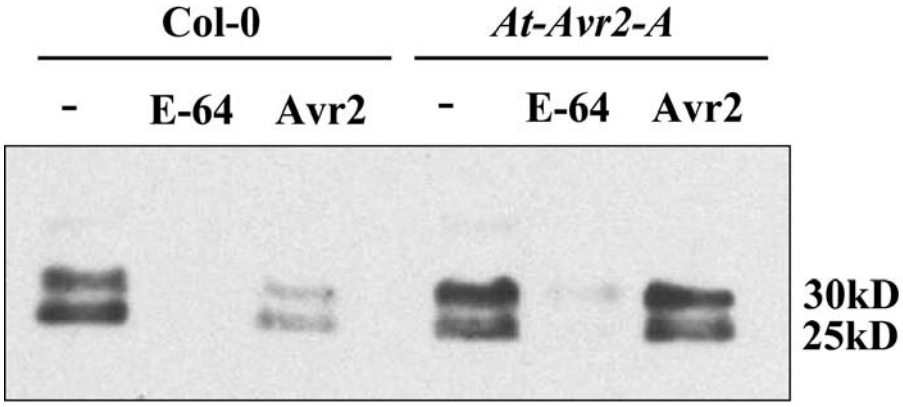


Figure 2. Avr2 inhibits cysteine proteases in Arabidopsis. Western blot of total protein extracts of Arabidopsis transformants expressing *Cladosporium fulvum* Avr2 (*At–Avr2–A*) and corresponding progenitor Col–0 plants, upon treatment with the biotinylated cysteine protease inhibitor DCG–04 and purification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

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 as for Arabidopsis, no macroscopically visible phenotypic anomalies were observed in these lines when grown under standard greenhouse conditions (Suppl. Fig. 2A). 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 (Suppl. Fig. 2C). In addition, injection of apoplastic fluids harvested from MM–*Avr2* plants, but not from the progenitor MM–*Cf–0* line, into leaves of MM–*Cf–2* plants resulted in a clearly visible HR four days post injection, confirming the presence of biologically active Avr2 in the apoplast of MM–*Avr2* lines (Suppl. Fig. 2B).

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 natural strain of *C. fulvum* lacking functional Avr2. Four–week–old MM–*Avr2* 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 (Fig. 3A). At 14 DPI, conidiophores of *C. fulvum* started to emerge from the *Avr2*–expressing plants, while conidiophores were not yet observed on MM–*Cf–0* leaves (Fig. 3A). The enhanced colonization of MM–*Avr2* plants was confirmed by real–time PCR at 11 DPI (Fig. 3B). Similar experiments with another natural *C. fulvum* strain that lacked a functional *Avr2* gene provided similar results (data not shown), strongly suggesting that Avr2 is a virulence factor of *C. fulvum*.

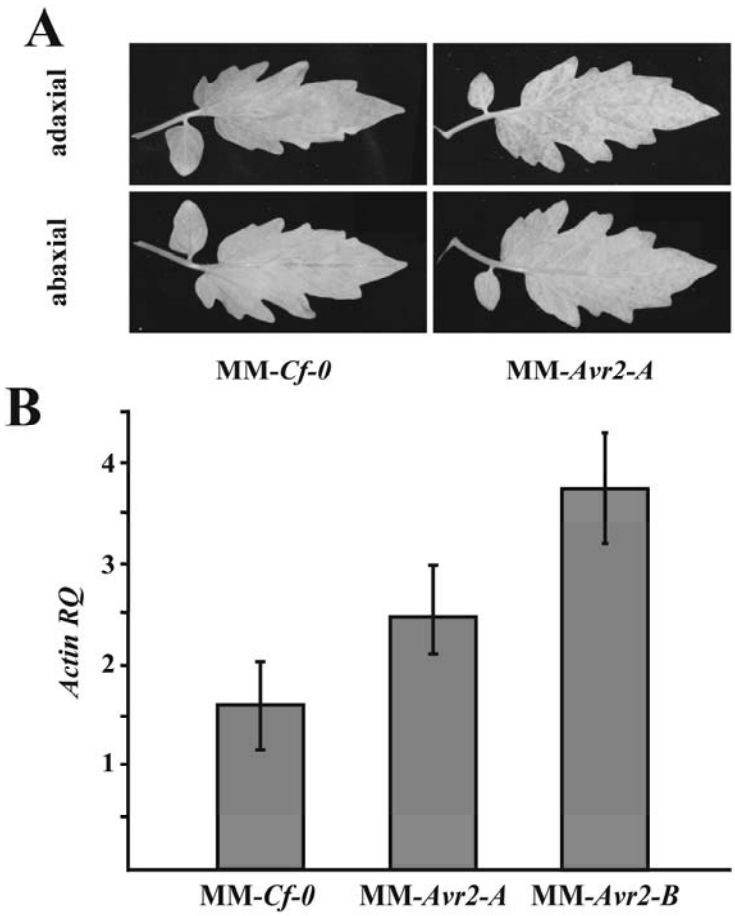


Figure 3. *Avr2*–expressing tomato is more susceptible to Race 2 *Cladosporium fulvum* (see page 204 for full color version). (A) Typical disease symptoms developed on the adaxial and abaxial leaf sides after inoculation with a *C. fulvum* race 2 strain of *Avr2*–expressing tomato (MM–*Avr2–A*), when compared to the progenitor line (MM–*Cf–0*) at 11 days post inoculation. (B) Quantitative real–time PCR of fungal colonization by comparing *C. fulvum* actin transcript levels (measure for fungal biomass) relative to tomato actin transcript levels (for equilibration) on two independent *Avr2*–expressing tomato transformants (MM–*Avr2–A* and MM–*Avr2–B*) when compared to the parental line (MM–*Cf–0*) at 11 days post inoculation.

Silencing of *Avr2* in *C. fulvum* compromises virulence on tomato

Previously, gene silencing has been used successfully to target the expression of *C. fulvum* effector genes (van Esse et al., 2007; Bolton et al., 2008). To corroborate whether Avr2 is a genuine virulence factor, *Avr2*–specific 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 *Avr2* inverted–repeat (*Avr2*–IR) transformants were obtained, three of which were used for further analysis. Growth of these transformants *in vitro* was indistinguishable from that of the progenitor strain (data not shown). 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 at 14 DPI using real–time PCR, showing a 60–70% reduction of *Avr2*–expression in each of the transformants when compared to the progenitor *C. fulvum* strain (Fig. 4A). Nevertheless, these levels were still sufficient to trigger *Cf–2*–mediated resistance in MM–*Cf–2* plants, although the response to *Avr2*–IR transformants was less vigorous than to the progenitor *C. fulvum* strain (Suppl. Fig. 3). 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 (Fig. 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 (Fig. 4C), demonstrating that Avr2 is a genuine virulence factor.

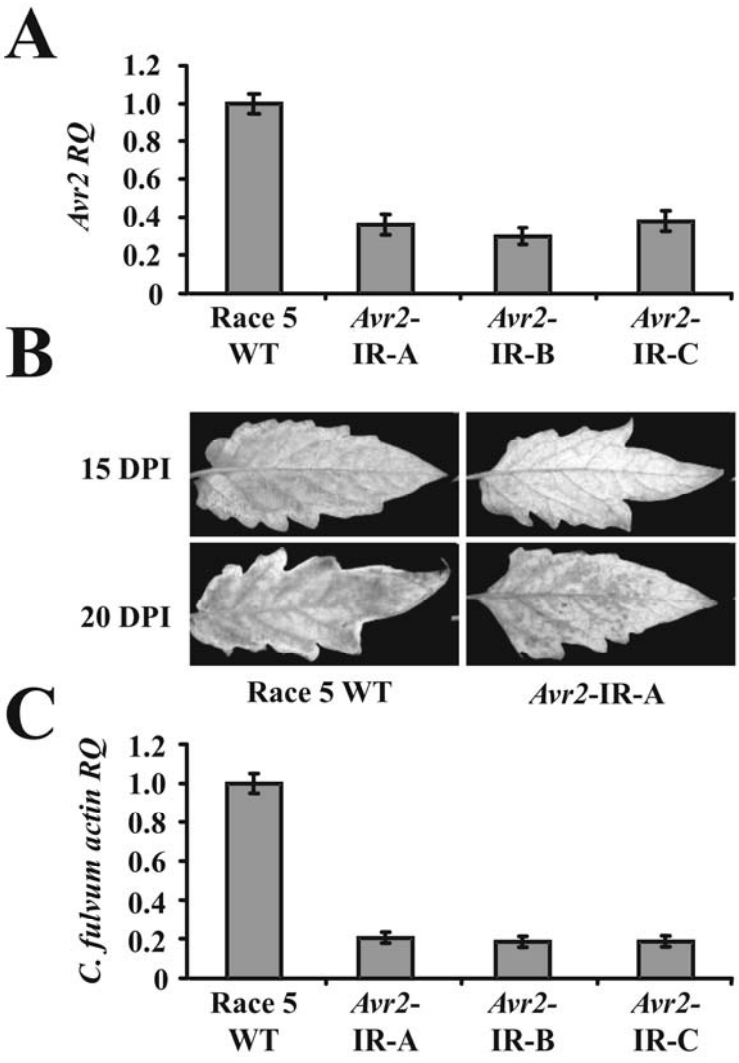


Figure 4. Silencing of *Avr2*–expression in *Cladosporium fulvum* decreases virulence on tomato (see page 205 for full color version). (A) Quantitative real–time PCR of *Avr2* transcript levels during a compatible interaction with MM–Cf–0 tomato. *Avr2* transcript levels are shown in three independent *Avr2*–silenced *C. fulvum* transformants (*Avr2*–IR–A to –C) when compared to the parental strain (Race 5 WT) at 11 days post inoculation. (B) Typical disease symptoms developed after inoculation of MM–*Cf–0* tomato plants with the *Avr2*–silenced *C. fulvum* transformant Avr2–IR–A, as typical example, when compared to the parental strain (Race 5 WT), monitored at 15 and 20 days post inoculation. (C) Quantitative real–time PCR of fungal colonization by comparing *C. fulvum* actin transcript levels (as a measure for fungal biomass) relative to tomato actin transcript levels (for equilibration) for three independent *Avr2*–silenced *C. fulvum* transformants (*Avr2*–IR–A to –C) when compared to the parental strain (Race 5 WT) at 11 days post inoculation.

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 (data not shown). However, significantly more necrosis developed upon *B. cinerea* inoculation on MM–*Avr2* lines than on the progenitor MM–*Cf–0* line (Fig. 5A), which correlated with enhanced fungal colonization as confirmed by microscopic analysis (Fig. 5B). 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, as *Avr2*–transgenic lines showed stronger stunting and more wilting (Fig. 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 (Fig. 5D).

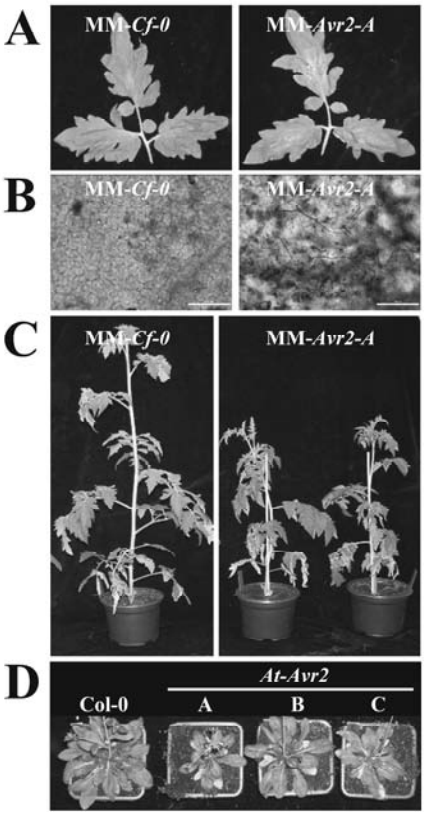


Figure 5. *Avr2*–expressing plants are more susceptible to *Verticillium dahliae* and *Botrytis cinerea* (see page 206 for full color version). (A) Typical appearance of *Avr2*–expressing tomato leaves (MM–*Avr2–A*) when compared to the parental line (MM–*Cf–0*) upon inoculation with *B. cinerea* at 60 hours post inoculation. (B) Microscopic observation of *Avr2*–expressing tomato leaves (MM–*Avr2–A*) when compared to the parental line (MM–*Cf–0*) upon inoculation with *B. cinerea* at 48 hours post inoculation after staining of fungal hyphae and death plant cells with trypan blue. (C) Typical appearance of *Avr2*–expressing tomato plants (MM–*Avr2–A*) when compared to the parental 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 parental line (Col–0) at two weeks post inoculation.

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 (race 2.4; Boukema, 1981) were assessed for the presence of active cysteine proteases with biotinylated DCG–04. A western blot, using streptavidin coupled to horseradish peroxidase (HRP) for detection, demonstrated that inoculation of tomato with a race 2 strain of *C. fulvum* that does not produce functional Avr2 results in the induction of several active apoplastic cysteine proteases (Fig. 6). Compared to 0 DPI, plants at five and seven DPI gained additional bands, including an increase in band intensities, resulting in three major signals at 25, 30 and 37 kDa at 7 DPI. At five and seven DPI, more bands appeared while also the intensities of the bands 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 (Fig. 6).

To identify 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).

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. The Rcr3, Pip1 and glycinain proteases were no longer biotinylated by DCG–04 in the presence of an excess of Avr2, suggesting that Avr2 has the highest affinity for these tomato proteases in competition with E–64 (Table 3). To directly assess Avr2 interaction targets rather than assessing competitive ability, 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. With 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, except for 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.

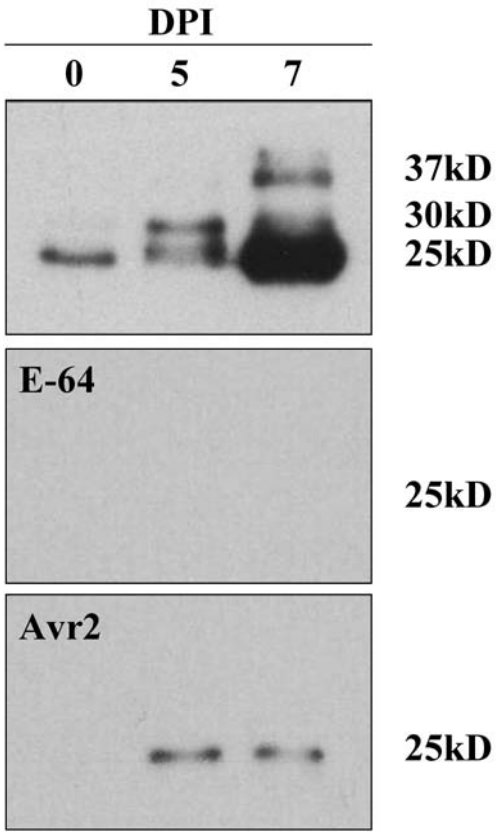


Figure 6. Active cysteine proteases in tomato apoplastic fluid upon inoculation with *Cladosporium fulvum*. Western blot of apoplastic fluids from tomato plants upon inoculation with *C. fulvum* at 0, 5 and 7 days post inoculation (DPI) upon treatment with the biotinylated cysteine protease inhibitor DCG–04 and purification using streptavidin–coated beads. Active cysteine proteases are detected with streptavidin coupled horseradish peroxidase (HRP). Prior to labeling with DCG–04, the extracts received no pre–treatment (upper panel), or were treated with E–64 (middle panel) or Avr2 (lower panel).

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 2.4**	**Non–inoculated**
Plant genotype		**MM–*Cf–0***		**MM–*Avr2–A***	**MM–*Cf–0***	**MM–*Cf–0***
Competitor		**No competitor**	**Excess of Avr2**	**No competitor**	**No competitor**	**No competitor**
Protease	Rcr3 (TC128871)	5 (4–1–0–0–0)	–	–	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 (0–1–0–0–0)	–	–

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 multiple 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), CPR1 and glycinain, 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 occur 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. This confirms that Avr2 targets multiple host cysteine proteases of the C1 class, and that so far no additional targets could be found in the tomato apoplast fluids besides these cysteine proteases.

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 our assay only detected qualitative but not quantitative differences between samples, so even a large reduction in binding of DCG–04 to TDI65 and aleurain remains 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).

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). In compliance 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 (data not shown) were overrepresented in the expression profiles. Furthermore genes associated with the secretory pathway and the exterior of the cell (apoplast) were over–represented.

The inhibition of several extracellular host proteases by Avr2 is likely to cause the enhanced susceptibility 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 did not show enhanced susceptibility towards race 2 *C. fulvum* strains that lack functional Avr2 when compared to *Cf–2/Rcr3* plants (data not shown), suggesting that loss of Rcr3 function alone is not sufficient for the enhanced disease susceptibility. It is likely that the simultaneous inhibition of several host proteases by Avr2 causes the observed enhanced disease susceptibility phenotypes. Remarkably, a similar increase in susceptibility towards *B. cinerea* and *V. dahliae* was observed on tomato and Arabidopsis. This may suggest that the same proteases in both hosts are responsible for the enhanced disease susceptibility phenotype, at least for these two pathogens.

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 partially 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).

Material and methods

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.

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

For *in planta* production of *C. fulvum* effectors, the sequence encoding each of the mature proteins was amplified and ligated into the binary pGREEN vector (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 (incubation for 1 minute in 70% EtOH, 25 minutes in 10% commercial bleach, rinsed three times in sterilized water) and sown on MS agar supplemented with sucrose (30 g/l) and incubated incubated in the dark in a growth chamber at 25°C for two days and were 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. Subsequently, the explants were 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 then transfer 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.

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.

P. infestans inoculations on tomato were performed on detached leaves similar 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 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. 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 Trizol® reagent (Invitrogen, Carlsbad, CA, USA). After 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 positives) errors. For plants, an FDR < 0.05 cut–off is a suitable global value when arrays are of high quality (DeCook et al., 2006). Since FDR values were larger than 0.05, conclusions were only based on the global ErmineJ and GSEA algorithms that are not sensitive to individual FDR values. 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 Table 4. 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

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 (Table 4) 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 Table 4, 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.

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 2.4 (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.

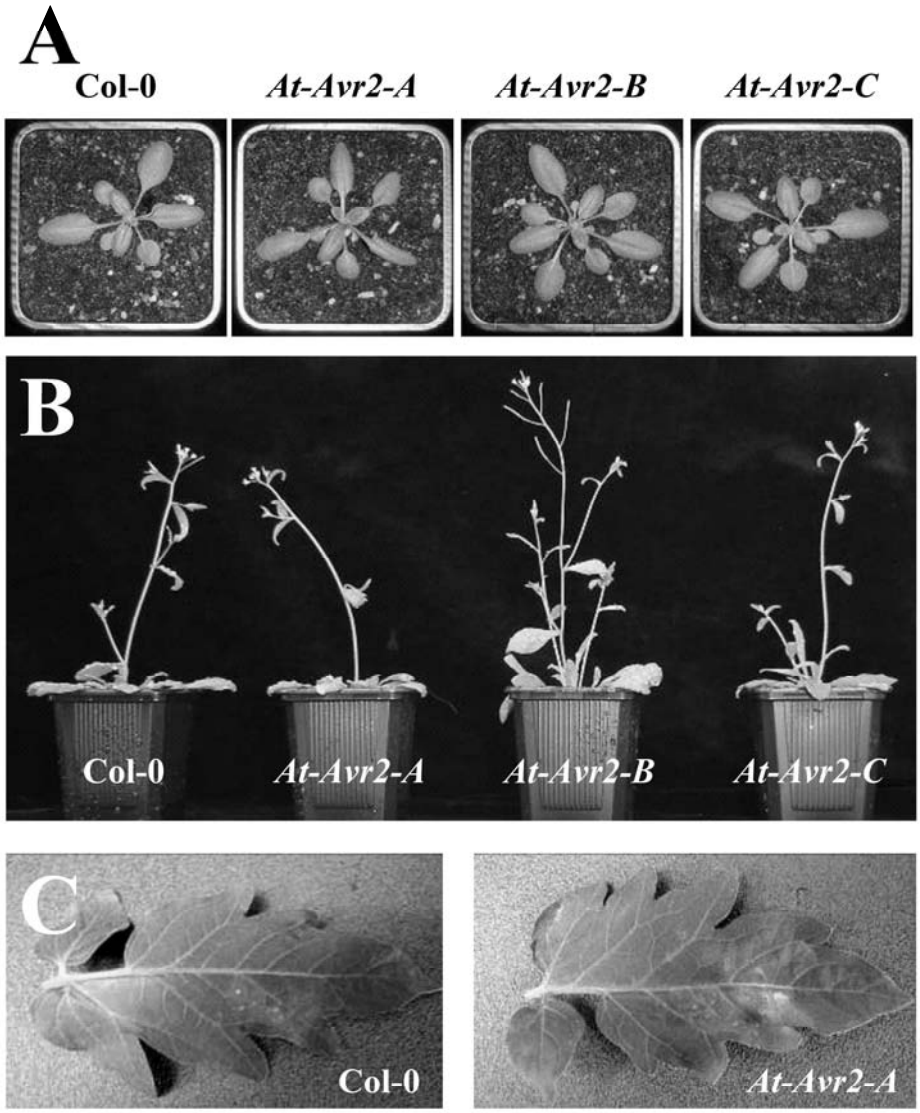
The protein samples were analyzed with LC/MS^2 by injecting 18 or 20 µl of sample on a 0.10 x 32 mm Prontosil 300–3–C18H pre–concentration column (Bischoff, Leonberg, Germany) at a flow of 3 or 6 µl/min for 10 minutes. Peptides were eluted from the pre–concentration column onto a 0.10 x 200 mm Prontosil 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.

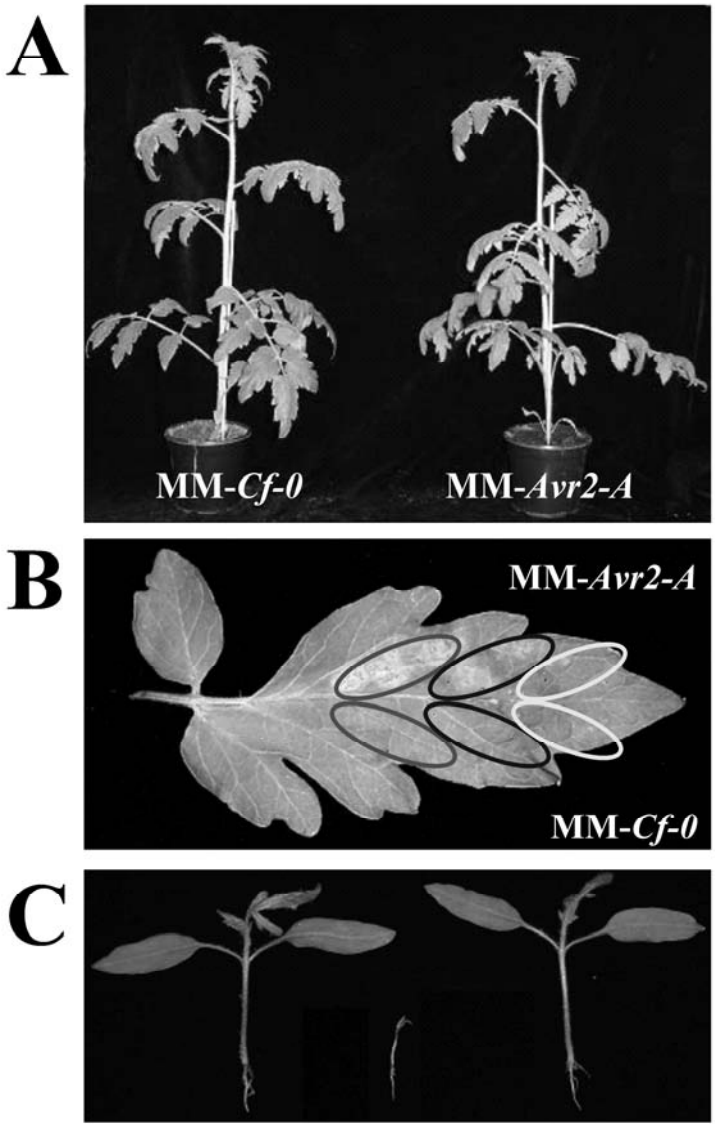
All MS^2 spectra were analyzed with Bioworks 3.2 or 3.3.1 software (Thermo Fisher Scientific, San Jose, CA, USA). A maximum of 3 differential modifications was set for oxidation of methionines and de–amidation of N and Q. Carboxamidomethylation of cysteines was set as a fixed modification. An *Arabidopsis 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).

Table 4. Primers used in this study.
[1] Restriction sites are indicated in bold.

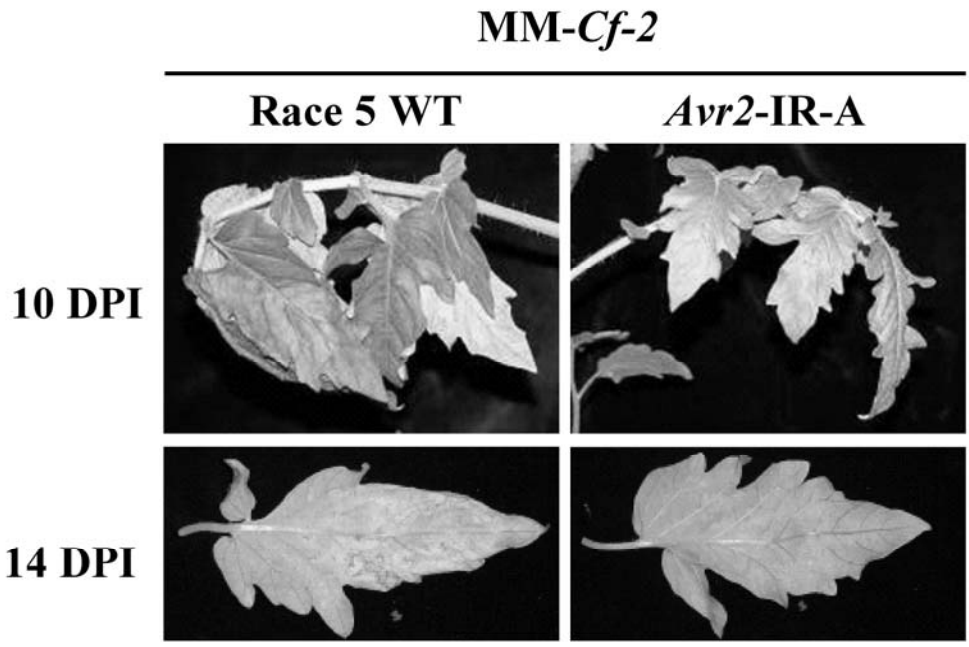
Primer name	Sequence (5'–3')[1]	Description
oligo–dT	TTGGATCCTCGAGTTTTTTTTTTTTTTTT	Poly–T (*Nco*I and *Sac*I)
Nco–Avr2–F	TTTTTTCCATGGATGAAGCTCTTCATACTG	*Avr2* inverted repeat (*Nco*I)
Avr2–EcoRI–R2	GAATTCACCGCAAAGACCAAAACAG	*Avr2* inverted repeat (*Eco*RI)
Avr2–NotI–R2	GCGGCCGCACCGCAAAGACCAAAACAG	*Avr2* 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



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 parental line (Col–0). (C) Production of Avr2 in transgenic Arabidopsis plants (line *At–Avr2–A* as example, right panel) when compared to the parental line (Col–0, left panel) demonstrated by injection of apoplastic fluid (total protein content of 1.3 µg/µl) into a leaf of MM–*Cf–2* tomato. The picture was taken four days after infiltration.



Supplemental figure 2. Characterization of *Avr2*–expressing tomato plants. (A) Typical appearance of four–week–old plant from an *Avr2*–expressing tomato lines (MM–*Avr2*) when compared to the parental line (MM–*Cf–0*). (B) Production of Avr2 in transgenic tomato (MM–*Avr2–A*), but not in the parental line (MM–*Cf–0*) demonstrated by injection of apoplastic fluid in a leaf of MM–*Cf–2* tomato. The total protein concentration was 0.6 (red), 0.2 (blue), or 0.07 (yellow) µg/µl, and the picture was taken after four days. (C) Production of Avr2 in transgenic tomato (MM–*Avr2–A*) demonstrated by crossing to MM–Cf–2 tomato. The cross results in viable seeds, but seedlings die after germination. Seedlings from left to right; MM–*Avr2–A*, progeny of cross, MM–*Cf–2*.



Supplemental figure 3. Silencing of *Avr2*–expression in *Cladosporium fulvum* attenuates Cf–2–mediated immunity. Typical appearance of MM–*Cf–2* upon inoculation with the *Avr2*–silenced *C. fulvum* transformant Avr2–IR–A, as typical example, when compared to the parental strain (Race 5 WT), monitored at 10 and 14 days post inoculation.

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Chapter 5:
The novel *Cladosporium fulvum* lysine motif effector Ecp6 is a virulence factor with orthologs in other fungal species

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Abstract

During tomato leaf colonization, the biotrophic fungus *Cladosporium fulvum* secretes several effector proteins into the apoplast. Eight effectors have previously been characterized and show no significant homology to each other or to other fungal genes. To discover novel *C. fulvum* effectors that might play a role in virulence, we utilized two–dimensional polyacrylamide gel electrophoresis (2D–PAGE) to visualize proteins secreted during *C. fulvum*–tomato interactions. Three novel *C. fulvum* proteins were identified; CfPhiA, Ecp6, and Ecp7. CfPhiA shows homology to proteins found on fungal sporogenous cells called phialides. Ecp6 contains lysine motifs (LysM domains) that are recognized as carbohydrate–binding modules. Ecp7 encodes a small, cysteine–rich protein with no homology to known proteins. Heterologous expression of *Ecp6* significantly increased the virulence of the vascular pathogen *Fusarium oxysporum* on tomato. Furthermore, by RNAi–mediated gene silencing we demonstrate that Ecp6 is instrumental for *C. fulvum* virulence on tomato. Hardly any allelic variation was observed in the *Ecp6* coding region of a worldwide collection of *C. fulvum* strains. Although none of the *C. fulvum* effectors identified so far have obvious orthologs in other organisms, conserved Ecp6 orthologs were identified in various fungal species. Homology based modelling suggests that the LysM domains of *C. fulvum* Ecp6 may be involved in chitin binding.

Introduction

Cladosporium fulvum (syn. *Passalora fulva*) is a biotrophic pathogen that causes leaf mold of tomato (*Solanum esculentum*Mill. syn. *Solanum esculentum*) (Thomma et al., 2005). After germination of conidia, the fungus produces runner hyphae that penetrate stomata predominantly on the lower side of the leaf. Once inside the apoplast, *C. fulvum* does not penetrate host cells or develop haustoria but remains confined to the intercellular space between plant mesophyll cells (de Wit 1977). Despite much research on the *C. fulvum*–tomato interaction, the molecular components that *C. fulvum* utilizes for infection and colonization are largely unknown (Thomma et al., 2005).

Plant pathogens secrete molecules called effectors that contribute to the establishment of disease to their hosts. Since the complete set of effectors of a potential pathogen determines the outcome of the interaction with a possible host, it is important to make an inventory of this effector catalogue. Many plant pathogenic bacteria inject effector proteins into the cytoplasm of host cells by means of the type III secretion system (TTSS) to subvert host cellular physiology to the bacterium's advantage (Grant et al., 2006; Tang et al., 2006). This process is orchestrated by specific *cis*–elements in the promoters of genes encoding type III effector proteins, a feature which has been exploited to identify such effectors in genome–wide functional screens (Guttman et al., 2002; Chang et al., 2005). In a similar way, the discovery that several oomycete effector molecules enter the host cytoplasm through a specific host targeting RXLR–DEER motif (Whisson et al., 2007) has been exploited to identify oomycete effector catalogues. It is currently predicted that the genomes of

oomycete plant pathogens contain hundreds of such effectors (Whisson et al., 2007; Jiang et al., 2008).

The effectors of extracellularly growing plant pathogenic fungi are usually very rich in cysteine residues involved in disulphide bridges, thereby protecting them against proteinases that occur frequently in apoplastic spaces of their host plants (Joosten and de Wit, 1999; Rep, 2005; Thomma et al., 2005; Kamoun 2006). At present, relatively few whole–genome sequences of plant pathogenic fungi are available when compared to bacteria (Xu et al., 2006). Since most effector proteins from extracellular pathogenic fungi are secreted, apoplastic extract from colonized plants is an important resource for the discovery of molecular factors important in several plant diseases (Joosten and de Wit 1999; Rep, 2005; Thomma et al., 2005; Kamoun 2006).

Since *C. fulvum* is restricted to the tomato apoplast during colonization, all communication and exchange of molecular components between *C. fulvum* and its host occurs in the apoplastic space. So far, analysis of the protein composition of the apoplastic space of *C. fulvum*– infected tomato leaves has mainly been focused on identification of race–specific avirulence proteins (Avrs) that are secreted by the fungus during infection and invoke a resistance response in tomato genotypes carrying cognate *C. fulvum* resistance (*Cf*) genes (van Kan et al., 1991; Joosten et al., 1994; Luderer et al., 2002b; Westerink et al., 2004). In addition, a number of extracellular proteins (Ecps) secreted during infection by all strains of *C. fulvum* have been identified (Joosten and de Wit 1988; Wubben et al., 1994; Laugé et al., 1998a; 2000; Haanstra et al., 1999; 2000). Like Avrs, Ecps induce a resistance response in tomato accessions carrying not yet identified *Cf–Ecp* resistance genes. Collectively, the Avrs and Ecps are the secreted effecor proteins. In total, eight *C. fulvum* secreted effector proteins have been characterized in detail and their 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., 2002b; Westerink et al., 2004; Thomma et al., 2005). All these secreted effector proteins are relatively small (ranging from 3 to 15 kDa) and contain a high and even number of cysteine residues that appear to be involved in disulphide bridge formation (Kooman–Gersmann et al., 1997; van den Burg et al., 2003). These bridges provide a compact tertiary structure that contributes to stability and activity of the secreted effector proteins in the protease–rich tomato apoplast (Joosten et al., 1997; Tornero et al., 1997; Jorda et al., 1999; Krüger et al., 2002; van Esse et al., 2006). Although all of these effector proteins elicit a defence response in plants carrying the cognate *Cf* genes in a 'gene–for–gene' manner (Kruijt et al., 2005), the observation that these proteins are maintained within the population together with their abundance and specific accumulation during pathogenesis suggest that these proteins play an important role in fungal virulence (Thomma et al., 2005). Indeed, transformants containing gene knock–outs of either *Ecp1* or *Ecp2* were shown to have impaired aggressiveness in mature tomato plants (Laugé et al., 1997). Recent data show that also Avr2 is a genuine virulence factor of *C. fulvum* (van Esse et al., 2008). It has previously been shown that Avr2 interacts with, and inhibits, the tomato cysteine protease Rcr3 which, in compliance with the guard hypothesis,

is required for Cf–2–mediated immunity (Rooney et al., 2005). In compatible interactions, however, Avr2 inhibits several additional extracellular host cysteine proteases that are required for host basal defense (van Esse et al., 2008). Protection of chitin, a major constituent of fungal cell walls, against plant chitinases by the chitin–binding Avr4 effector protein (van den Burg et al., 2006) was recently shown to contribute to *C. fulvum* virulence (van Esse et al., 2007).

In addition to the secreted effectors, *Nrf1* and *Aox* have been identified as virulence factors of *C. fulvum* (Segers et al., 2001; Thomma et al., 2006). The nitrogen response regulator Nrf1 was found to control expression of *Avr9* but no other known *Avr* or *Ecp* genes *in planta* (Pérez–García et al., 2001; Thomma et al., 2006). Interestingly, disruption of the *Nrf1* gene reduces *C. fulvum* virulence significantly (Thomma et al., 2006). Similarly, targeted disruption of *Aox1*, a starvation–induced acetaldehyde dehydrogenase, caused decreased colonization of the host plant (Segers et al., 2001).

To visualize extracellular proteins present in compatible and incompatible *C. fulvum*–tomato interactions, the apoplastic proteome of *C. fulvum*–infected tomato was analyzed using two–dimensional polyacrylamide gel electrophoresis (2D–PAGE). Several proteins that are produced during infection were identified by mass spectrometry (MS) and coding sequences for three novel *C. fulvum* proteins were obtained by reverse genetics employing PCR with degenerate primers based on MS/MS sequence tags and N–terminal sequencing. We used RNA interference (RNAi) for functional analysis in *C. fulvum* and demonstrate that one of the identified secreted effectors is crucial for *C. fulvum* virulence.

Results

Quantification of *Cladosporium fulvum* biomass in infected tomato leaves

In a compatible interaction involving the susceptible MoneyMaker *Cf–0* (MM–Cf–0) tomato cultivar which lacks resistance genes against this pathogen, the fungus colonizes the apoplast around leaf mesophyll cells. Conidiophores emerge from stomata seven days post inoculation to produce conidia (Fig. 1A). Using real–time PCR to quantify fungal biomass in the plant tissue it is evident that fungal biomass gradually increases until the fungus is extensively sporulating (Fig. 1B). In the incompatible interaction, such as with resistant MoneyMaker *Cf–4* (MM–*Cf–4*) tomato plants that recognize *C. fulvum* strains expressing wild–type *Avr4* (Joosten et al., 2004), no disease symptoms are visible (not shown). Real–time PCR confirms that in such an incompatible interaction no significant increase in fungal biomass occurs when compared to the compatible interaction (Fig. 1B).

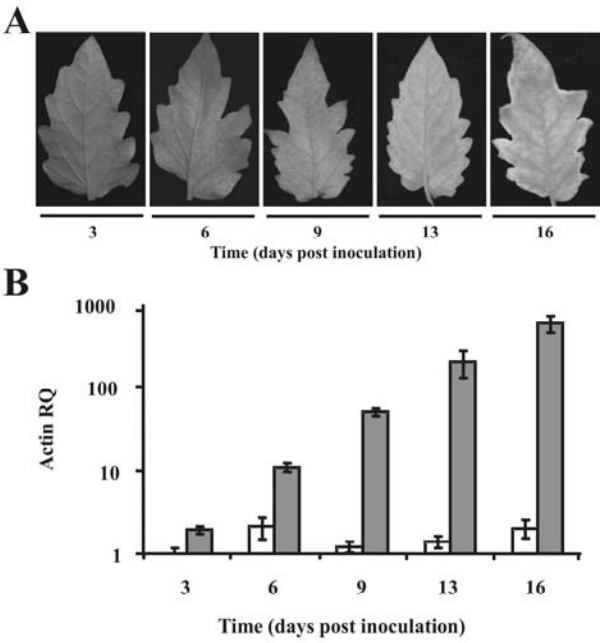

Figure 1. Disease progression of *Cladosporium fulvum* on tomato. (A) Typical symptoms caused by *C. fulvum* on susceptible MM–*Cf–0* tomato plants at 3, 6, 9, 13, and 16 days post inoculation (dpi). The fungus is not visible at early stages of infection (3 dpi) but develops white patches of conidiophores (6 dpi) that expand and cover almost the whole leaf (9 dpi). Subsequently, the conidiophores start to produce conidia (13 dpi) which give the leaf a green–brownish velvet–like appearance (16 dpi). (B) Quantitative real–time reverse transcription PCR to measure *C. fulvum* growth on resistant MM–*Cf–4* tomato plants (white) and on susceptible MM–*Cf–0* tomato plants (grey) at 3, 6, 9, 13, and 16 dpi. The extent of colonization is determined by the relative quantification (RQ) of transcript levels of the constitutively expressed *C. fulvum* actin gene (measure for fungal biomass) to the constitutively expressed tomato glyceraldehyde–3–phosphate dehydrogenase gene (measure for plant biomass) shown on a logarithmic scale. Bars represent mean values and standard errors of three leaflets taken from two plants at each time–point analyzed. The experiment was repeated twice with similar results.

Characterization of the *Cladosporium fulvum*-infected tomato apoplast proteome

In previous analyses, the protein composition of the apoplastic space of *C. fulvum*– infected tomato leaves has mainly focused on identification of effectors that are secreted by the fungus during infection and that invoke a resistance response in tomato. For an inventory of the apoplast proteome of *C. fulvum*–infected tomato and to identify secreted fungal proteins that might play a role in virulence, two–dimensional polyacrylamide gel electrophoresis (2D–PAGE) was utilized that allowed the comparison of MM–*Cf–0* and MM–*Cf–4* plants infected by a race 5 *C. fulvum* strain (compatible and incompatible interaction, respectively). At two weeks post inoculation of susceptible MM–*Cf–0* plants, the fungus has generated considerable biomass and has extensively colonized the host tissue (Fig. 1), likely resulting in a large quantity of fungal proteins in the apoplast as compared to resistant MM–*Cf–4* plants. Therefore, this time point was chosen for detailed analysis of fungal proteins (Fig. 2). Proteins present in 2 ml of apoplastic fluid isolated from the two different

interactions were analyzed with 2D–PAGE. Separation of the proteins in the first dimension was carried out on Immobiline DryStrips (pH 4–7) and for the second dimension 12.5% polyacrylamide gels were used. After Coomassie Brilliant Blue–staining, 16 protein spots specific for, or highly induced during, the compatible interaction were excised from the gel (Fig. 2). Subsequently, the proteins were digested with trypsin and the generated peptides were analyzed with matrix–assisted laser desorption ionization time–of–flight (MALDI–TOF) mass spectrometry (MS) and peptide fragment spectra were obtained with liquid chromatography (LC) MS/MS. Peptide mass fingerprints and peptide sequence information were used to search for protein identity in databases. This resulted in the identification of a tomato endochitinase and the *C. fulvum* proteins Ecp1, Ecp2, and Ecp5 (Table 1). Proteins present in the other spots could not be identified solely based on the data obtained in the MS analysis.

Six of these non–identified protein spots (5–10; Fig. 2) resulted in a comparable peptide mass fingerprint and are therefore likely to be derived from the same protein. One of the protein spots (5; Fig. 2) was subsequently subjected to N–terminal sequencing, resulting in a 46 amino acid sequence that was found to harbour the previously identified MS/MS tags. Since the obtained sequence showed homology to a structural *A. nidulans* phialide protein, this protein was designated CfPhiA (Table 1). This is a typical protein that occurs on phialides, which are sporogenous cells that release conidia from their apex by budding (Melin et al., 2003).

Three other protein spots (11 to 13; Fig. 2) also generated a comparable mass fingerprint, implicating that also these spots may be derived from the same protein. N–terminal sequencing of spot 12 resulted in a 26 amino acid sequence harbouring the corresponding MS/MS sequence tags and the corresponding protein was designated Ecp6 (Table 1).

The remaining protein spots (14, 15; Fig. 2), of which we obtained peptide mass fingerprints as well as peptide fragment spectra, were also subjected to N–terminal sequencing. For protein spot 14, the 25 amino acid sequence that was obtained matched the corresponding MS/MS sequence tags and the protein was designated Ecp7 (Table 1). Although sequence information based on MS/MS was available for protein spot 15, this protein was not considered for further study because N–terminal sequence failed repeatedly.

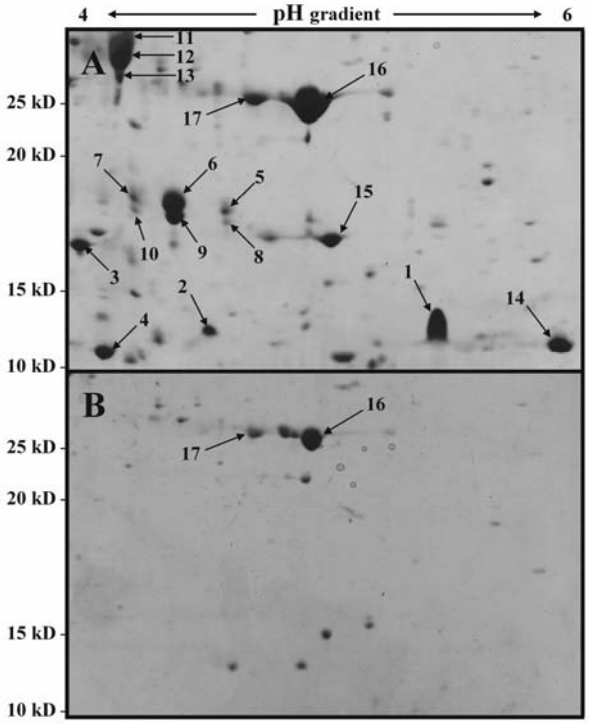

Figure 2. The apoplast proteome of *Cladosporium fulvum*-infected tomato analyzed with two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Coomassie Brilliant Blue–stained 2D–PAGE gels obtained after electrophoresis of soluble proteins present in apoplastic fluid collected from a compatible (A; race 5 *C. fulvum* strain inoculated onto MM–*Cf–0* plants), and an incompatible (B; race 5 *C. fulvum* strain inoculated onto MM–*Cf–4* plants) interaction at 14 days post inoculation. The proteins were focused over a nonlinear gradient of pH 4–7. Molecular weight markers for the second dimension are indicated on the left. The part of the gel showing the *C. fulvum*–derived differentially accumulated proteins is shown. Protein spots for which identification was pursued are numbered (see also Table 1).

Table 1. Apoplast proteins identified with mass spectrometry.

Protein	Spot numbers	Peptides confirmed with MS/MS	N-terminal amino acids (aa)	Reference
Ecp1	1,2	5	–	Joosten and de Wit 1988
Ecp2	3	7	–	Wubben et al., 1994
Ecp5	4	4	–	Laugé et al., 2000
CfPhiA	5–10	6	46 aa	This study
Ecp6	11–13	4	26 aa	This study
Ecp7	14	5	25 aa	This study
endochitinase	16, 17	PMF[1]	–	Joosten et al., 1989

[1]= Determined with MALDI–TOF generated peptide mass fingerprints (PMF)

Cloning of extracellular protein genes

Degenerate primers were designed based on the N–terminal protein sequences of CfPhiA and Ecp6 and were used in combination with an oligo–dT primer to amplify the coding regions of the corresponding genes using a cDNA library from *C. fulvum*–infected tomato leaves as template. For Ecp7, a degenerate primer based on an MS sequence tag was used because the N–terminal sequence was not yet available when the cloning was initiated. In all cases, a cDNA sequence was successfully amplified which corresponded to MS/MS and N–terminal peptide sequences. For *CfPhiA*, a 720 bp fragment encoding the mature protein and part of the 3'UTR was cloned (Suppl. Fig. 1). The predicted mature CfPhiA protein contains 175 amino acids and has a predicted molecular mass of about 19 kDa and a pI of 5.0. BlastP analysis (Altschul et al., 1997; Schäffer et al., 2001) of the amino acid sequence showed that this protein shares similarity to putative proteins of several fungal species including *Aspergillus nidulans*, *A. fumigatus* and *Neurospora crassa*. Of these orthologs, the PhiA protein from *A. nidulans* has been functionally characterized (Melin et al., 2003), and was found to be essential for growth and sporulation of the fungus as *phiA* mutants were found to be impaired in phialide development. Therefore, it is likely that the *C. fulvum* putative ortholog CfPhiA has a similar function.

A 742 bp fragment with the coding region for the mature Ecp6 protein and the 3'UTR was cloned (Suppl. Fig. 1). *Ecp6* encodes a mature protein of 199 amino acids, including eight cysteines, and has a predicted molecular mass of 21 kDa and a pI of 4.6. Furthermore, Ecp6 contains five predicted N–glycosylation sites, explaining the location of the Ecp6 protein spots on the 2D–gel. Based on BlastP analysis, Ecp6 was found to share significant homology to the glycoprotein CHI1 identified in the plant pathogenic fungus *Colletotrichum lindemuthianum* (Perfect et al., 1998). Although the contribution of CIH1 to pathogenicity is unknown, it has been shown to accumulate during infection on bean in the walls of intracellular hyphae and the interfacial matrix which separates the hyphae from the invaginated host plasma membrane (Perfect et al., 1998).

For *Ecp7*, a 464 bp cDNA fragment was cloned containing the coding region for 84 amino acids of the mature Ecp7 protein. N–terminal sequencing of Ecp7 revealed that a stretch of 16 amino acids precedes the peptide that was identified as an MS tag, and based on which the degenerate primer for cloning the cDNA was designed (Suppl. Fig. 1). Therefore it should be concluded that *Ecp7* encodes a mature protein of 100 amino acids which includes 6 cysteines and has a predicted molecular mass of 11 kDa and a pI of 6.0. BlastP analysis of the amino acid sequence revealed no significant homology of Ecp7 to other protein sequences deposited in public databases.

***CfPhiA*, *Ecp6*, and *Ecp7* are expressed during infection**

With real–time PCR assays using genomic DNA from *C. fulvum* as a template and *Avr2* as a single copy reference gene (Luderer et al., 2002b), it was determined that the *C. fulvum* genome contains only one copy of the *CfPhiA*, *Ecp6*, and *Ecp7* genes (results not shown). Furthermore, real–time PCR analysis of *CfPhiA*, *Ecp6*, and *Ecp7* transcripts, using the

constitutively–expressed *C. fulvum* actin gene as an endogenous control, revealed that all genes are expressed in both compatible and incompatible interactions (Fig. 3). *CfPhiA* expression is induced already early in the compatible interaction, at six days post inoculation (dpi), and maintains this level of expression for all time points analyzed. In the incompatible interaction, *CfPhiA* is also induced, although its expression level is approximately half of that found in the compatible interaction (Fig. 3). Both *Ecp6* and *Ecp7* show a low but steady level of expression in the incompatible interaction when compared to that of the *C. fulvum* actin gene, while the genes are clearly induced in the compatible interaction. While *Ecp7* peaks at nine dpi (Fig. 3), *Ecp6* is maximally expressed at 13 dpi (Fig. 3). In contrast to the expression pattern of the *CfPhiA* gene, the patterns of *Ecp6* and *Ecp7* typically resemble those of other genes encoding secreted *C. fulvum* effectors. For example, *C. fulvum Avr9* is highly expressed throughout the compatible interaction, with maximum expression at 9 dpi, whereas its expression in the incompatible interaction remains low (Fig. 3). Nevertheless, the expression level of the *Avr9* gene is much higher than those of *Ecp6* and *Ecp7* (Fig. 3).

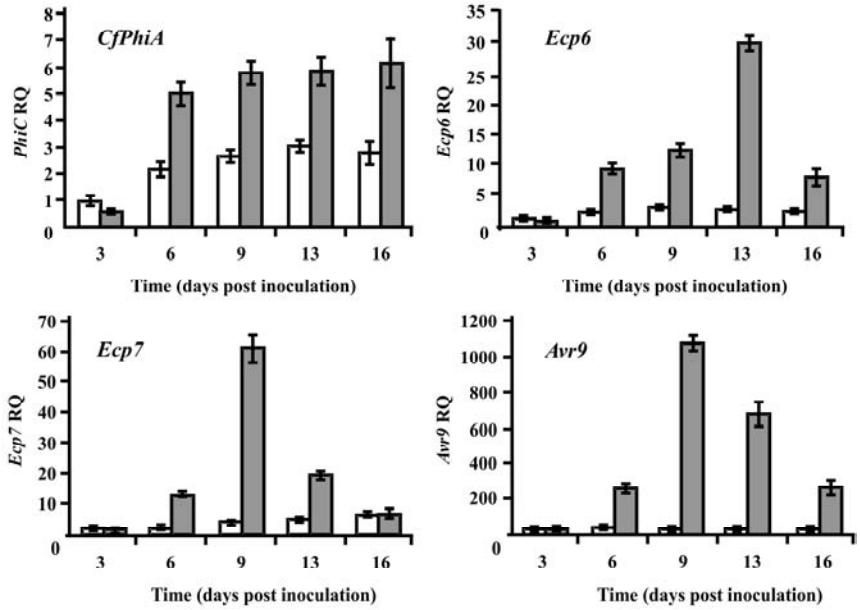

Figure 3. Expression analysis of the newly identified *Cladosporium fulvum* extracellular proteins. The expression of *CfPhiA*, *Ecp6*, *Ecp7*, and *Avr9* genes was monitored during the interaction of *C. fulvum* with MM–*Cf–4* tomato (incompatible; white bars) and MM–*Cf–0* tomato (compatible; grey bars) at 3, 6, 9, 13, and 16 days post inoculation (dpi). Real–time reverse transcriptase PCR was used for the quantification (RQ) of transcript levels of the *C. fulvum CfPhiA*, *Ecp6*, and *Ecp7* genes relative to the constitutively expressed *C. fulvum* actin gene as an endogenous control. The RQ of the *Avr9* gene is shown as an example of the expression profile of a typical *C. fulvum* effector gene. The mean and standard error of the results obtained from three leaflets taken from two plants at each time–point assayed are shown. The experiment was repeated twice with similar results.

Heterologous expression of *Ecp6* in *F. oxysporum* f. sp. *lycopersici* enhances virulence on tomato

In contrast to *C. fulvum*, *F. oxysporum* may easily be transformed using *Agrobacterium*–mediated transformation, generally resulting in large numbers of transformants (Mullins et al., 2001). To investigate whether *C. fulvum* Ecp6 and Ecp7 may act as fungal virulence factors, we over–expressed these Ecps in *F. oxysporum* f. sp. *lycopersici.* To this end, the sequences encoding the mature proteins were fused in frame with the sequence encoding the *C. fulvum* Avr4 signal peptide for extracellular targeting (Joosten et al., 1997) into a binary vector under control of the fungal constitutive *ToxA* promoter (Ciuffetti et al., 1997). Using *Agrobacterium*–mediated transformation a large number of transformants were obtained, and presence of the transgene was confirmed by PCR (data not shown). Four transformants were randomly picked for each of the *C. fulvum* Ecps and tested in an inoculation assay on tomato. Upon inoculation of tomato plants with transformants that overexpress *Ecp7*, disease development was indistinguishable from disease caused by the non–transformed progenitor strain (data not shown). In contrast, on tomato plants that were inoculated with each of the four transformants that over–express *Ecp6*, disease symptoms developed earlier and were more severe compared to the inoculation with the non–transformed progenitor *F. oxysporum* f. sp. *lycopersici* strain (Fig. 4A, B) or the transformants that overexpress *Ecp7* (data not shown). With reverse transcription PCR it was confirmed that in each of the transformants, but not in the progenitor *F. oxysporum* f. sp. *lycopersici* strain, *Ecp6* was expressed (Fig. 4C).

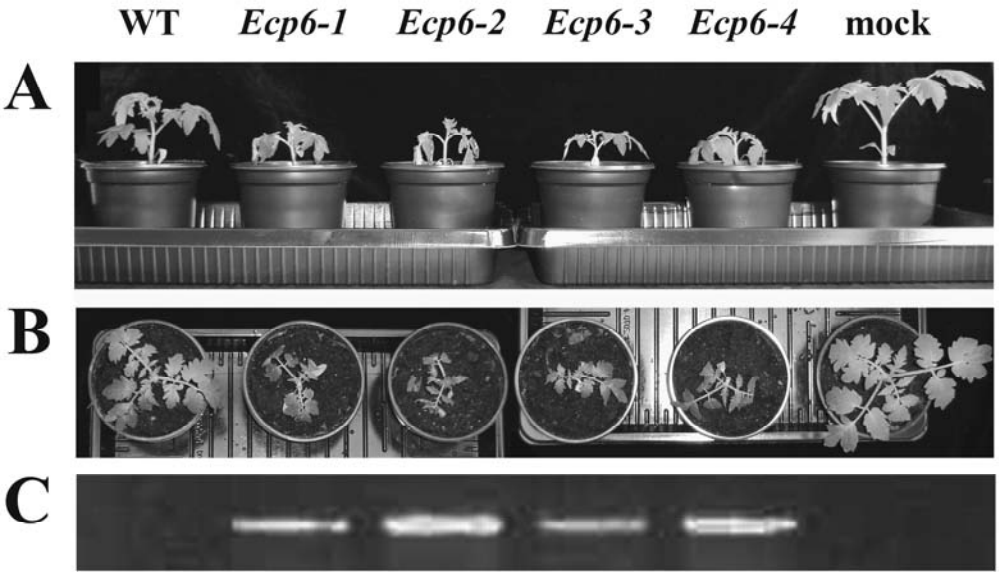

Figure 4 (previous page). Symptoms caused by wild-type *Fusarium oxysporum* f. sp. *lycopersici* and heterologous *Ecp6* over-expression transformants on susceptible tomato (see page 207 for full color version). (A) B, Side view (A) and top view (B) of the disease phenotype caused by *Fusarium oxysporum* f. sp. *lycopersici* wild–type (WT) and four independent heterologous *Ecp6* over–expression transformants (*Ecp6–1* to *Ecp6–4*) on susceptible tomato MoneyMaker plants when compared to mock–inoculated tomato (mock) at 14 days post inoculation. (C) RT–PCR to detect *in planta* transcription of heterologously expressed *C. fulvum Ecp6* in *Fusarium oxysporum* f. sp. *lycopersici* wild–type (WT) and four independent heterologous *Ecp6* over–expression transformants (*Ecp6–1* to *Ecp6–4*) on susceptible tomato MoneyMaker plants when compared to mock–inoculated tomato (mock) at 14 days post inoculation.

RNAi–mediated silencing of *Ecp6* compromises *C. fulvum* virulence on tomato

RNA–mediated gene silencing or RNA interference (RNAi) has been successfully employed for gene functional analysis in filamentous fungi (Nakayashiki et al., 2005). This is particularly relevant for fungal genomes, like that of *C. fulvum*, for which homologous recombination is not straightforward. Recent evidence has shown that PEG–mediated transformation may generate somaclonal variation that may be circumvented by *Agrobacterium*–mediated transformation which is, however, significantly less efficient (van Esse et al., 2007). Therefore, RNAi was recently successfully implemented to silence the expression of *C. fulvum* effector genes (van Esse et al., 2007; 2008).

Based on the results obtained with heterologous expression of *C. fulvum Ecp6* in *F. oxysporum* f. sp. *lycopersici*, we applied RNAi–mediated silencing for functional analysis of the *C. fulvum Ecp6* gene using *Agrobacterium*–mediated transformation with constructs aimed at generating double–stranded RNA that targets these genes (RNAi). A pGREEN–based binary vector, carrying transfer DNA (T–DNA) that contains either a nourseothricin resistance cassette or a hygromycin resistance cassette, and an inverted repeat fragment of the target gene under control of the fungal constitutive *ToxA* promoter (Ciuffetti et al., 1997), was used to provoke RNAi–mediated gene silencing. To target the expression of the *Ecp6* gene, two RNAi constructs were generated based on different sections of the *Ecp6* coding region. *Agrobacterium*–mediated transformation of the RNAi constructs generated several antibiotic–resistant transformants for each construct. Analysis of the transformants indicated that their growth *in vitro* was indistinguishable from that of the progenitor race 5 isolate (data not shown). Since *C. fulvum* effector genes show variable expression when cultured *in vitro* (Thomma et al., 2006), four–week–old MM–*Cf–0* tomato plants were inoculated with three transgenic *C. fulvum* strains to determine whether the introduction of the inverted–repeat construct resulted in *Ecp6* silencing. Utilizing real–time PCR, a strong reduction in transcription of the target gene was found when compared to the progenitor isolate in several transformed isolates using expression of the *C. fulvum* actin gene as a reference (Fig. 5A). At 10 days post inoculation, transformants *Ecp6i–1* and *Ecp6i–4* of the first construct, and *Ecp6i2–1* of the second construct, showed a reduction to 36%, 27% and 48% of the wild–type *Ecp6* expression level, respectively (Fig. 5A). At later time points, the level of *Ecp6* reduction increased for the *Ecp6i2–1* transformant, while the reduction in the *Ecp6i–1* and *Ecp6i–4* remained rather consistent, which may possibly be attributed to different regions of the transcript that are targeted for gene silencing (data not shown).

Visual inspection of the inoculated MM–*Cf–0* tomato plants showed a clearly delayed progression of disease for the *Ecp6* RNAi transformants (Fig. 6). While conidiophores were emerging from the stomata on the lower surface of tomato leaves inoculated with the wild–type progenitor strain at 10 dpi, the leaves inoculated with transformant *Ecp6i–4* were devoid of these structures (Fig. 6). Although leaves inoculated with transformants *Ecp6i–1* (Fig. 6) and *Ecp6i2–1* (data not shown) showed some fungal growth, the extent of leaf colonization appeared significantly less than that observed for the wild–type strain. To measure the extent of fungal growth of RNAi transformants compared to the parental wild–type strain, the constitutively expressed *C. fulvum* actin gene was used as a marker in real–time PCR analyses (Fig. 5B). The constitutively expressed tomato chloroplast glyceraldehyde–3–phosphate dehydrogenase gene was used as a reference for the ratio of fungal biomass to plant biomass to determine the degree of colonization. After inoculation of MM–*Cf–0* tomato lines, all *Ecp6* RNAi transformants showed significant reduction in growth compared to the parental race 5 isolate (Fig. 5B).

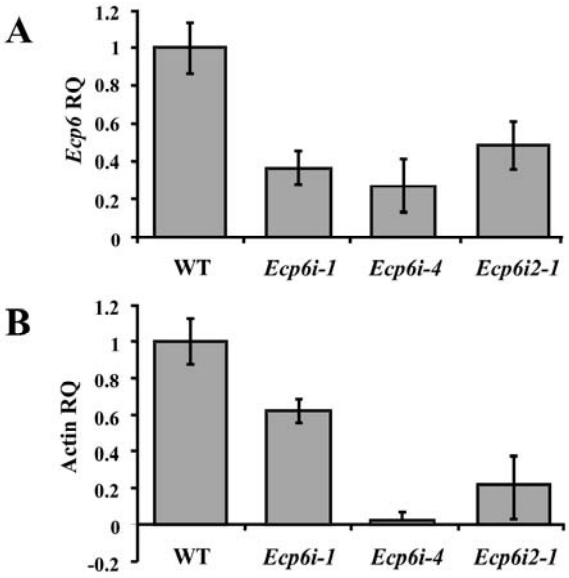

Figure 5. Expression analysis and quantification of growth of *Cladosporium fulvum* RNAi transformants silenced for *Ecp6* and *Ecp7*. (A) The expression of *Ecp6* and *Ecp7* is monitored during a compatible interaction between *C. fulvum* and MM–*Cf–0* tomato involving the wild–type (WT) *C. fulvum* and RNAi transformants at 10 days post inoculation. Real–time PCR was used to measure the relative quantity (RQ) of transcript levels of the *Ecp6* and *Ecp7* genes, as compared to the constitutively expressed *C. fulvum* actin gene as an endogenous control. Bars represent mean values and standard error of the results obtained from three leaflets taken from two infected plants. (B) Growth of WT *C. fulvum* and RNAi transformants was quantified on MM–*Cf–0* tomato plants. The transcript levels of the constitutively expressed *C. fulvum* actin gene (measure for fungal biomass) relative to the levels of the constitutively expressed tomato glyceraldehyde–3–phosphate dehydrogenase gene (measure for plant biomass) are shown to determine the degree of fungal colonization of the MM–*Cf–0* tomato leaves. Bars represent mean values and standard error of the results obtained from three infected leaflets taken from two plants.

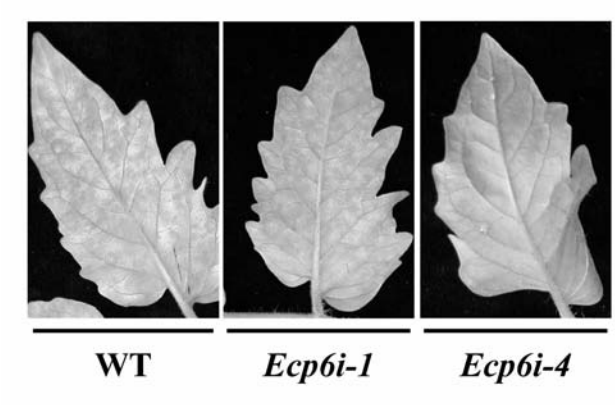

Figure 6. Typical symptoms caused by *C. fulvum* wild-type (WT) and RNAi transformants silenced for Ecp6 at 10 days post inoculation onto susceptible tomato plants (MM-*Cf-0*).

Ecp6 sequence analysis from a world-wide collection of *C. fulvum* strains

Since our results showed that Ecp6 is a virulence factor of *C. fulvum*, we assessed sequence variation of *Ecp6* in a worldwide collection of strains (Stergiopoulos et al., 2007a; b). We first obtained 691 bp of genomic sequence upstream of the region that encodes the mature Ecp6 protein by gene walking. Sequence analysis using the gene prediction algorithm FGENESH (Salamov and Solovyev, 2000) identified a putative start codon and predicted intron/exon boundaries using the genetic codes of several fungi present as models in the database. These were confirmed by cloning the *Ecp6* cDNA from infected plant material, showing that the *Ecp6* ORF is 669 bp, interrupted by two introns of 68 and 111 bp, respectively, and encodes a protein of 222 amino acids (Fig. 7).

The full–length sequence of *Ecp6* was obtained from a collection of 50 *C. fulvum* strains (Table 2). Analysis of the sequence 62 bp upstream of the start codon to 91 bp downstream of the stop codon revealed that variation within *Ecp6* was very limited, resulting in a total of five single nucleotide polymorphisms (SNPs) within these strains (Fig. 7). One SNP (G>A at 494 bp downstream of the putative start codon) occurred inside the second intron of *Ecp6*, and was only detected in one Canadian strain (#34; Table 2). The other four SNPs all occurred in seven strains originating from North America (#31, #34, #40, #41; Table 2), and Japan (#67, #71, #74; Table 2). While one SNP (G>A at 128 bp) occurred in the first intron, two other SNPs are silent mutations (C>T at 335 bp, and G>A at 662 bp). Only one SNP (C>A at 142 bp) is predicted to result in an amino acid substitution (Thr25>Asn; Fig. 7).

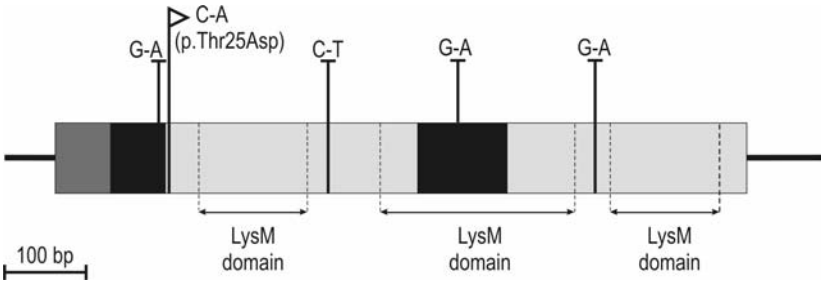

Figure 7. Allelic variation of the *Cladosporium fulvum Ecp6* gene. Open reading frames are shown as light grey boxes and introns as black boxes. The predicted signal peptide is indicated as dark grey box. The white flag indicates a single nucleotide polymorphism (SNP) that leads to an amino acid substitution in the Ecp6 protein. Silent mutations are indicated by a T. The figure is drawn at scale.

Table 2. *C. fulvum* isolates used in this study.

Strain	Code	Origin	Code	Accession	Origin
1	0	Netherlands	42	Can 84	Canada
2	2	Netherlands	52	IMI Day5 054977	UK
3	4E	Netherlands	57	IPO 2459 (30787)	Netherlands
4	2 4	Netherlands	58	IPO 2459 (50381)	Netherlands
5	2 4 11	Poland	59	IPO 2459 (60787)	Netherlands
6	2 4 5	Netherlands	60	IPO 248911 Polen	Poland
7	2 4 5 11	Netherlands	61	IPO 249 France	France
8	2 4 5 7	Netherlands	62	IPO 2679 SECRET	New Zealand
10	2 4 5 9 11 IPO	Netherlands	65	IPO 5 (15104)	Netherlands
11	2 4 8 11	Netherlands	66	IPO 80379	Netherlands
12	2 4 9 11	Poland	67	Jap 12	Japan
15	2 5 9	France	69	Jap 15	Japan
16	4	Netherlands	71	Jap Cf32	Japan
17	4 (2)	Netherlands	73	Jap Cf5	Japan
19	5 Kim	France	74	Jap Cf56	Japan
20	5 Marmeisee	France	75	Jap Cf9	Japan
22	Alenya B	France	78	MUCL723	Belgium
24	Brest 84	France	80	MUCL725	Belgium
25	Brest Rianto 85	France	82	Nantes 89	France
26	Bul 20	Bulgaria	84	Pons 89	Netherlands
31	Can USA	USA	87	T Hijwegen	Netherlands
34	Can 38	USA	111	VKM 1437	Former USSR
35	Can 43	Canada	112	Z. Am 1	South America
40	Can 62	Canada	117	Turk 1a	Turkey
41	Can 69	Canada	122	Turk 3c	Turkey

Orthologs of *Ecp6* are found in several fungal species

Interrogation of the *C. fulvum* Ecp6 protein sequence using BlastP (Altschul et al., 1997; Schäffer et al., 2001) and Pfam analysis (Finn et al., 2008) indicates that the Ecp6 protein contains three lysine motif (LysM) domains. These domains are widespread protein modules of approximately 40 amino acids, originally identified in a bacterial autolysin that degrades bacterial cell walls (Joris et al., 1992). LysM domains are also found in eukaryotic proteins, and presently LysM domains are implicated in binding of diverse carbohydrates that occur in bacterial peptidoglycan, fungal chitin, and Nod–factor signals that are produced by *Rhizobium* bacteria during the initiation of root nodules on legumes (Bateman and Bycroft, 2000; Butler et al., 1991; Amon et al., 1998; Ponting et al., 1999). We queried all available fungal genome sequences and EST libraries (Table 3) for the presence of Ecp6–like proteins using BlastP or tBlastN, respectively. The retrieved sequences were subsequently analyzed for predicted protein domains using HMMER (http://hmmer.janelia.org/) loaded with the current Pfam HMM library (http://pfam.sanger.ac.uk). Prediction of significant LysM domains (E–value cut–off 0.001) was used as a selection criterion for further analysis. Subsequently, all sequences containing predicted LysM domains were aligned, permitting for the selection of fungal proteins with high overall similarity to *C. fulvum* Ecp6. In this way, a list of 16 putative *C. fulvum* Ecp6–like proteins was generated, containing five *Aspergillus niger* proteins, two *Magnaporthe grisae* proteins, and 1 from each *Mycosphaerella fijiensis*, *M. graminicola, Botrytis cinerea, Sclerotinia sclerotiorum, A. nidulans, A. oryzae, A. flavus, C. lindemuthianum* and *Leptosphaeria maculans*. For these 17 proteins, using ClustalW (Chenna et al., 2003) a multiple sequence alignment analysis was performed (Suppl. Fig. 2). In addition to the LysM domains, the positions of the cysteine residues that flank the LysM domains, and the high abundance of proline, serine and threonine residues in the LysM linker regions appear to be conserved (Suppl. Fig. 2). Subsequently a neighbour–joining tree (Saitou and Nei, 1987) was constructed to reveal evolutionary relationships (Fig. 8). Based on this tree, the 16 Ecp6–like proteins can be divided into three groups. *C. fulvum* Ecp6 clusters with three Ecp6–like proteins of *M. graminicola*, *M. fijiensis* and *L. maculans* that all contain three LysM domains (Group 1, Fig. 8). The second group of Ecp6–like proteins encompasses the two *M. grisea* Ecp6–like proteins and CIH1 from *C. lindemuthianum* that are shorter than other Ecp6–like proteins and have only two LysM domains (Group 2, Fig. 8). The largest group of Ecp6–like proteins, encompassing the five *A. niger* proteins in addition to those of *A. nidulans*, *A. oryzae*, *S. sclerotiorum* and *B. cinerea*, contain two LysM domains and a weak, but not significant, signature of a third LysM domain (Group 3, Fig. 8).

Table 3. Fungal whole genome and EST sequence libraries screened for Ecp6-like sequences.

Species[1]	# hits[2]	LysM[3]
Cladosporium fulvum (Ecp6)	1	Yes
Colletotrichum lindemuthianum *(CIH1)*	1	Yes
EST sequences		
Alternaria brassicicola	0	N.a.
Blumeria graminis	0	N.a.
Colletotrichum gloeosporioides f	0	N.a.
Colletotrichum trifolii	0	N.a.
Fusarium sporotrichioides	0	N.a.
Leptosphaeria maculans	1	Yes
Ophiostoma novo–ulmi	0	N.a.
Phycomyces blakesleeanus	0	N.a.
Whole genome sequences		
Aspergillus flavus	2	Yes (1)
Aspergillus nidulans	1	Yes
Aspergillus niger	5	Yes (5)
Aspergillus oryzae	1	Yes
*Batrachochytrium dendrobatidis**	1	Yes
Botrytis cinerea	1	Yes
Candida sp.	0	N.a.
*Chaetomium globosum**	3	Yes
*Cryphonectria parasitica**	1	Yes
*Cryptococcus neoformans**	1	Yes
Fusarium graminearum	0	N.a.
Fusarium oxysporum	1	No
Fusarium verticilliodes	0	N.a.
*Histoplasma capsulatum**	1	Yes
Laccaria bicolor	0	N.a.
Lodderomyces elongisporus	0	N.a.
Magnaporthe grisae	2	Yes (2)
Mycosphaerella fijensis	1	Yes
Mycosphaerella graminicola	1	Yes
Nectria haematococca	0	N.a.
Neurospora crassa	1	No
Phanerochaete chrysosporium	0	N.a.
Pichia stipitis	0	N.a.
Podospora anserina	1	No
Postia placenta	0	N.a.
Sclerotinia sclerotiorum	1	Yes
*Stagonospora nodorum**	1	Yes
Sporobolomyces roseus	0	N.a.
Ustilago maydis	1	

[1] LysM containing Ecp6–like proteins of the species indicated in bold are included in the alignment shown in supplemental figure 2. The asterisks indicate species for which a LysM containing Ecp6–like protein is identified, but since the overall homology of these proteins to *C. fulvum* Ecp6 and *C. lindemuthianum* CIH1 is low they are not included in the alignment shown in supplemental figure 2.

[2] BlastP and tBlastN searches (E–value < 0.001) were performed Using *Cladosporium fulvum* Ecp6 and *Colletotrichum lindemuthianum* CIH1 as queries.

[3] Sequences were analyzed using HMMER (http://hmmer.janelia.org) loaded with the current Pfam HMM library (http://pfam.sanger.ac.uk) for the presence of LysM domains (E–value < 0.001). Numbers between brackets indicate how many of the hits contain a predicted LysM domain. N.a. is not applicable.

Homology modelling of Ecp6 LysM domains

Although LysM domains have been identified in over 1500 proteins, the three–dimensional (3D) structure of only three LysM domains has been reported. Two of these are of bacterial origin, the 3D structure of a LysM domain of the *E. coli* membrane–bound lytic murein transglycosylase D (MltD; PDB code: 1EOG; Bateman and Bycroft 2000) and the LysM domain of the *Bacillus subtilis* spore protein ykuD of unknown function (PDB code: 1Y7M; Bielnicki et al., 2006). Recently, the 3D structure of the LysM domain of the human hypothetical protein SB145 was determined using nuclear magnetic resonance (NMR) imaging (PDB code: 2DJP). The structural organization of the three LysM domains from these different proteins is highly similar, and characterized by a βααβ fold, with the two helices stacking on one side of the plate generated by a double–stranded anti–parallel β–sheet.

Recently, the first characterization of an interaction of a LysM domain with its ligand was reported (Ohnuma et al., 2007). Binding of oligomers of *N*–acetylglucosamine ($(GlcNAc)_n$), a monosaccharide derivative of glucose that is a building block for bacterial peptidoglycan and fungal chitin, to the LysM domains of a chitinase from *Pteris ryukyuensis* was monitored with NMR spectroscopy. The stoichiometry of $(GlcNAc)_n$/LysM binding was found to occur in a 1:1 ratio. Furthermore, using $(GlcNAc)_5$ it was shown that binding of this oligomer to the LysM domain occurs at a shallow groove formed by the N–terminal part of helix 1, the loop between strand 1 and helix 1, the C–terminal part of helix 2, and the loop between helix 2 and strand 2.

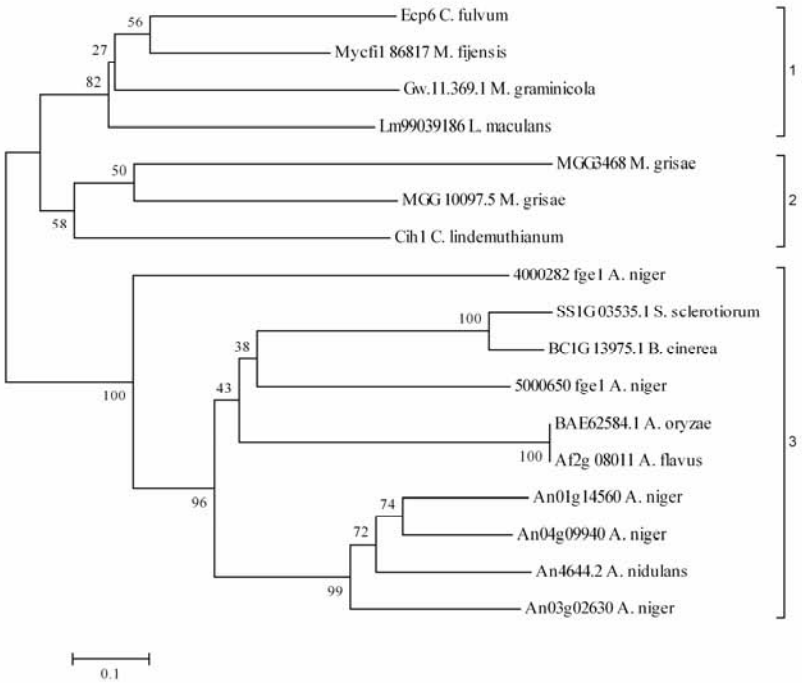

Figure 8. Homologs of Ecp6 in other fungal species. Neighbor–Joining tree of 17 Ecp6–like sequences from different fungal species. The evolutionary history of Ecp6–like protein sequences was inferred by Neighbor–Joining analysis (Saitou and Nei, 1987) and bootstrap values (%) are indicated at the nodes. The tree is drawn to scale, with branch lengths representing evolutionary distances. The positions containing alignment gaps were eliminated in pairwise sequence comparisons. A total of 220 positions were calculated in the final dataset.

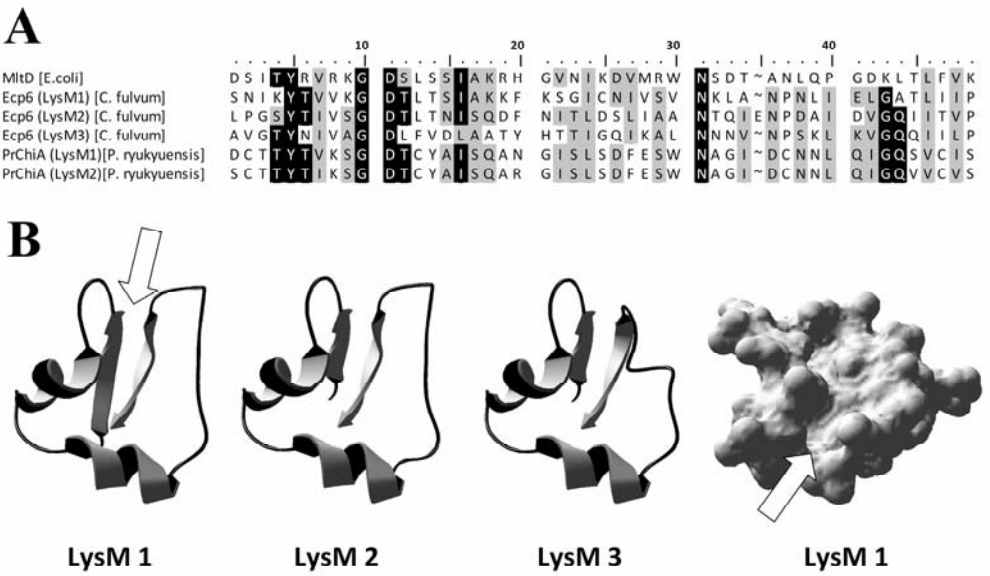

Figure 9. Homology models for the LysM domains of *Cladosporium fulvum* Ecp6. (A) Aligment of the individual LysM domains of *Cladosporium fulvum* Ecp6 (this study), *Escherichia coli* MltD (Bateman and Bycroft 2000), and *Pteris ryukyuensis* PrChi–A (Ohnuma et al., 2007). Identical amino acid residues are shaded in black and similar residues (75% treshold according to Blosum62 score) are shaded in grey. (B) LysM domains modelled based on the MltD LysM solution structure (Bateman and Bycroft 2000). Panels 1, 2, and 3 display the three–dimensional ribbon structures of the Ecp6 LysM domains 1, 2 and 3, respectively. Panel 4 shows the computed molecular surface of Ecp6 LysM domain 1. The arrow indicated in panel 1 indicates the direction of looking to obtain the view in panel 4. The arrow in panel 4 indicates the shallow groove described as the site of interaction of PrChi–A with chitin oligomers (Ohnuma et al., 2007).

To predict the ligand binding site with corresponding binding specificities of the *C. fulvum* Ecp6 LysM domains, homology–based modelling based on the three–dimensional (3D) structure of the LysM domain of the MltD structure was performed. The MltD and Ecp6 LysM domains show moderate but significant overall sequence similarity (53%, 47% and 33%, respectively, for LysM domains 1 to 3; Fig 9A). Moreover, by assessing local Kyte–Doolittle (KD)–hydrophobicity values (Kyte and Doolittle, 1982), the conserved secondary structure could reliably be predicted, which was subsequently used to predict the 3D structure. The predicted 3D structure of the three individual Ecp6 LysM domains is highly similar, with small changes in the position of the second loop of the third LysM domain (Fig. 9B). Moreover, due to sufficient similarity (52%) of LysM domain 1 of *C. fulvum* Ecp6 to LysM domains 1 and 2 of *P. ryukyuensis* PrChi–A (Fig. 9A), ligand binding can be modelled according to the interaction between chitin oligomers and PrChi–A LysM domains. The molecular surface of the first LysM domain of Ecp6 (Fig. 9B, panel 1) was computed and is shown in panel 4 of Fig. 9B. In the surface of this LysM domain, a cavity is observed that fulfils the requirements to act as binding site of chitin oligomers, based on the structural homology with PrChi–A.

Discussion

In this study, we employed a combined 2D–PAGE and proteomics approach to identify *C. fulvum* proteins produced and accumulating in compatible as compared to incompatible interactions. It was anticipated that proteins that accumulated exclusively in the compatible interaction were expected to be predominantly of pathogen origin. Indeed in this way, three novel extracellular *C. fulvum* proteins (CfPhiA, Ecp6, and Ecp7) could be identified in addition to the previously described effector proteins Ecp1, Ecp2, and Ecp5 (Table 1). CfPhiA was found to have homology to the PhiA protein from *A. nidulans* which is important for phialide and conidium development (Melin et al., 2003). Several attempts to generate RNAi transformants for *CfPhiA* have failed (data not shown), which suggests that silencing of *CfPhiA* might be detrimental or even lethal as it is required for *C. fulvum* growth and development. Furthermore, the expression pattern of *CfPhiA* compared to that of *Avr9* suggests that CfPhiA is likely not a genuine effector of the fungus.

Of the newly identified fungal extracellular proteins, Ecp7 especially resembles the previously identified Avrs and Ecps. It is relatively small (the mature protein contains 100 amino acid residues of which six are cysteines) with a calculated molecular mass of approximately 11 kDa. The even number of cysteine residues suggests their involvement in disulphide bridges that aid in their stability and activity in the harsh protease–rich apoplast (Joosten et al., 1997; Kooman–Gersmann et al., 1997; Thomma et al., 2005). In addition, the *Ecp7* expression profile during infection of tomato resembles that of other effector genes (Fig. 5). However, like for most of the previously identified Avrs and Ecps, the Ecp7 amino acid sequence did not show significant homology to sequences present in public databases. Despite the use of multiple transformants generated with two different RNAi constructs to target *Ecp7* expression, we have not been able to obtain unambiguous evidence showing that Ecp7 is a virulence factor of *C. fulvum* (data not shown). This is in contrast to the findings for *C. fulvum* Ecp6.

The mature Ecp6 protein contains 199 amino acids and has an estimated molecular mass of 21 kDa, making it the largest of the abundantly secreted effector proteins of *C. fulvum* identified so far. Previous studies on the genes encoding secreted *C. fulvum* effectors have shown that *Avr* genes accumulated considerably more polymorphisms than *Ecp* genes (Stergiopoulos et al., 2007a). This was suggested to be due to the lack of selection pressure imposed on the pathogen to overcome resistance mediated by R proteins that recognize Ecps, as these have not been deployed yet in commercial tomato lines (Stergiopoulos et al., 2007a). In line with these findings, polymorphisms in *Ecp6* were only rarely observed. Of the 50 *C. fulvum* strains, only seven strains contained allelic variants of *Ecp6*. All seven of these strains, which have previously been shown to be related (Steriopoulos et al., 2007b), contained the same four SNPs, while one strain contained an additional fifth SNP. Of these five SNPs, only one resulted in an amino acid change, while the four others concerned silent or intron mutations. The occurrence of mostly synonymous modifications in *Ecp* genes was hypothesized to imply selective constraints for maintaining Ecp protein sequences or, alternatively, a recent common ancestor gene (Stergiopoulos et al., 2007a).

However, our finding that Ecp6 markedly contributes to *C. fulvum* virulence, and that *Ecp6* has orthologs in other fungal species, favours the second hypothesis.

The Ecp6 protein contains three lysine motifs (LysM domains) that were originally found in a variety of enzymes that bind to and hydrolyze peptidoglycans present in bacterial cell walls, of which lysozyme is the best known example (Joris et al., 1992; Kariyama et al., 1992; Ruhland et al., 1993; Birkeland 1994; Longchamp et al., 1994). More recently, LysM motifs have been found to occur in plant plasma membrane receptors (Zhang et al., 2007), where they have so far been implicated in two different types of interactions with microbes (Knogge and Scheel, 2006). LysM receptor kinases are involved in the perception of oligosaccharide nodulation (Nod) factors secreted by *Rhizobium* bacteria to establish a symbiosis with their legume hosts (Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; 2007; Arrighi et al., 2006; Smit et al., 2007). LysM receptors also function in chitin signalling in plant innate immune responses against fungal pathogens (Kaku et al., 2006; Miya et al., 2007; Wan et al., 2008). For example, an insertion in the LysM containing receptor–like kinase gene CERK1 (also known as *LysM RLK1*) resulted in loss of ability to respond to the chitin elicitor $(GlcNAc)_8$ or crab shell chitin, as measured by production of reactive oxygen species, MAP kinase signalling, and induction of chitooligosaccharide–responsive genes. Moreover, enhanced susceptibility towards the fungal pathogens *Alternaria brassicicola* and *Erysiphe cichoracearum* was observed for these mutants, showing that this LysM containing receptor–like kinase is required for chitin signalling in plant innate immune responses (Miya et al., 2007; Wan et al., 2008).

LysM domains are also found in different chitinases from various organisms (Amon et al., 2000; Ponting et al., 1999). The involvement of LysM proteins in perception of chitin (β–1,4–linked poly–N–acetyl–D–glucosamine), peptidoglycan (a heteropolymer with alternating units of of acetyl–D–glucosamine and acetyl–muramic acids), and the acetyl–D–glucosamine backbone of Nod factors supports a role for LysM domains in binding of the acetyl–D–glucosamine oligosaccharide. Using domain swaps between Nod–factor receptors, it was demonstrated that these receptors mediate specific perception of Nod–factors from different *Rhizobium* bacteria and that this recognition depends on the structure of the Nod–factor (Radutoiu et al., 2007). Moreover, a single amino acid change in one of the LysM domains resulted in altered Nod–factor recognition, strongly suggesting that the LysM domains constitute the binding domains for the lipochitinoligosaccharide Nod–factors (Radutoiu et al., 2007). A high–affinity chitin–binding protein was isolated from the plasma membrane of suspension–cultured rice cells. This extracellular membrane–anchored protein, CEBiP, contains two LysM domains. Knockdown of *CEBiP* expression diminished the elicitor–induced oxidative burst as well as expression of chitin–induced genes. Moreover, binding assays as well as affinity labelling showed that the plasma membrane of knockdown lines for *CEBiP* carried less elicitor binding sites (Kaku et al., 2006).

Chitin binding has also been demonstrated for the *C. fulvum* effector protein Avr4 which contains an invertebrate chitin–binding domain (van den Burg et al., 2003). Through this chitin–binding activity, Avr4 was found to protect *C. fulvum* hyphae from hydrolysis by

plant chitinases (van den Burg et al., 2006; van Esse et al., 2007). It is tempting to speculate that *C. fulvum* Ecp6 is a chitin–binding protein too. To that end it is interesting to note that the Ecp6 homolog CIH1 of the plant pathogenic fungus *C. lindemuthianum* is found to be present at the surface of intracellularly growing fungal structures present in infected plant tissue (Perfect et al., 1998). Ecp6 may potentially act as a functional homolog of Avr4 through the ability to bind to chitin. Such functional redundancy might explain why the *C. fulvum* strain Can38, which harbours a frame–shift mutation in the *Avr4* gene and as a consequence does not produce Avr4, is still able to infect tomato (Joosten et al., 1997). Alternatively, Ecp6 may act as a "stealth factor" by shielding fungal hyphae in a similar fashion as has been suggested for hydrophobins (Whiteford and Spanu, 2002). Furthermore, the fungus may avoid recognition by the plant by sequestering chitin mono– or oligomers that act as elicitors of defense responses once they are released by the activity of plant chitinases.

Using homology modelling, docking sites for the interaction between LysM domains and their ligands have been predicted (Mulder et al., 2006; Radutoiu et al., 2007). However, only recently the first experimentally defined characterization of an interaction of a LysM domain with its ligand was reported (Ohnuma et al., 2007). Using $(GlcNAc)_5$ it was shown that binding to the LysM domain of a chitinase (PrChi–A) from *Pteris ryukyuensis* occurs at a long continuous shallow groove formed by the N–terminal part of helix 1, the loop between strand 1 and helix 1, the C–terminal part of helix 2, and the loop between helix 2 and strand 2 (Ohnuma et al., 2008). To predict if the same could be true for Ecp6 LysM domains, we used homology modelling to calculate whether GlcNAc oligomers can act as ligands for Ecp6. This analysis has shown that the Ecp6 LysM domains is likely to structurally resemble previously characterized LysM domains, and that based on structural calculations GlcNAc oligomers may indeed dock to the LysM domains of Ecp6 in a similar fashion as to the LysM domains of PrChi–A (Ohnuma et al., 2008). Future experiments will reveal whether the LysM domains of Ecp6 are able to bind chitin and, moreover, how Ecp6 contributes to fungal virulence.

Material and methods

Fungal and plant materials, and infection assays

The wild–type race 5 strain of *C. fulvum* was stored in 50% glycerol at –80°C until revitalized on potato dextrose agar (PDA; Oxoid Ltd., Hampshire, England) and was grown at room temperature in the dark. Two–week–old *C. fulvum* PDA plate cultures were used to harvest conidia by adding sterile water to the plates and rubbing the surface with a sterile glass rod to release the conidia. Conidial suspensions were filtered through Miracloth (Calbiochem–Behring, La Jolla, CA), centrifuged at 4000 rpm and washed two times with sterile water after which the conidial concentration was determined. Subsequently, the conidia were used for plant inoculations or *Agrobacterium tumefaciens*–mediated transformation.

All tomato plants were grown under standard greenhouse conditions: 21°C during the 16 hour day period, 19°C at night, 70% relative humidity (RH) and 100 W/m^2 supplemental light when the sunlight influx intensity was below 150 W/m^2. The tomato (*Solanum esculentum*) cultivar MoneyMaker, containing no resistance genes against *C. fulvum* (MM–*Cf–0*), and a MoneyMaker near isogenic line containing the *Cf–4* locus (MM–*Cf–4*) were used for all inoculations. *C. fulvum* was inoculated as described previously (de Wit, 1977). Per five–week–old tomato plant, 5 ml of conidial suspension (1 x 10^6 conidia per ml) was used for spray–inoculation on the lower surface of the leaves until drop off. Plants were kept at 100% RH under a plastic cover for 48 h after inoculation. All experiments, starting from plant inoculations, were repeated at least twice.

Preparation of protein samples and two-dimensional polyacrylamide gel electrophoresis

Leaves were harvested from *Cladosporium fulvum*–infected MM–*Cf–0* and MM–*Cf–4* lines at 14 days post inoculation and apoplastic fluid (AF) was isolated by vacuum infiltration (van Esse et al., 2006) using de–mineralized water followed by centrifugation for 5 min and stored at –20°C until further analysis. AF from both interactions was freeze–dried and the residue was re–suspended in 3.5 ml water. After centrifugation (10 min at 4000 g) samples were desalted using a PD–10 desalting column (GE Healthcare, UK), freeze–dried again, and stored at –20°C. Freeze–dried protein samples were dissolved in 340 µl of Rehydration Buffer (7 M urea, 2 M thiourea, 4% CHAPS, 60 mM DTT, 0.002% (w/v) bromophenol blue) along with 3.4 µl of IPG buffer pH 4–7 (GE Healthcare). The samples were vortexed briefly and centrifuged (10 min at 4000 g). The protein samples were applied to Immobiline DryStrips of 18 cm with a non–linear pH 4–7 gradient (GE Healthcare), covered with paraffin oil, and allowed to re–hydrate overnight at room temperature. Isoelectric focusing was performed using the Ettan IPGphor electrophoresis apparatus (GE Healthcare) at 20°C maintaining 50 µA per strip. A total focusing of 70 k Vh was achieved by following a running protocol using a step–n–hold gradient (1.5 hr 0 to 3,500 V, 6 hr 3,500 V). After first dimensional isoelectric focusing, the strips were stored at -20°C.

Subsequently, strips were placed in equilibration buffer (EB; 50 mM Tris, pH 8.8, 6 M urea, 30% (v/v) glycerol, and 2% (w/v) SDS) supplemented with 65 mM DTT. After 15 min, the buffer was replaced by EB supplemented with 135 mM iodoacetamide, and the strips were incubated for another 15 min. The proteins were subsequently separated on 12.5% polyacrylamide gels; the gels were run at 70 volts for the first 30 minutes and subsequently at 200 volts until the bromophenol blue reached the bottom of the gels. Gels were stained with Coomassie Brilliant Blue overnight and de–stained with 10% ethanol and 7.5% HAc in water.

Mass spectrometry

Protein spots were excised from the gel and digested with trypsin with an in–gel method (Shevchenko et al., 1996). The collected extracts of the resulting tryptic peptides were freeze–dried and stored at –20°C. The peptides were re–dissolved in 8 µl of 50% acetonitrile, 5% formic acid. MS and MS/MS information was acquired with a Q–Tof1 (Waters, Manchester, UK) coupled with a nano–LC Ultimate system (LC Packings Dionex, Sunnyvale, CA). After the dilution of 1–2 µl of sample 12 times with water, peptides were separated on a nano–analytical column (75 µm i.d. X 15 cm C18 PepMap, LC Packings, Dionex) using a gradient of 2–50% acetonitrile, 0.1% formic acid in 20 minutes. The flow of 300 nl min^{-1} was directly infused into the Q–Tof1, operating in data–dependent MS and MS/MS modes. The resulting MS/MS spectra were processed with Masslynx software (Waters, Manchester,UK) and used to search in MASCOT using the MSDB database. Since sequence data of both *C.*

fulvum and tomato are far from complete, MS/MS data from un–assigned spectra were analyzed by using the Masslynx Pepseq software for *de novo* sequence information. Both Blast (www.expasy.org/tools/blast) and MSBLAST were used to search for possible homologous proteins with the generated sequence information. For MALDI–TOF analysis, a 1 µl volume was spotted on a target plate after mixing the samples 1:1 (v/v) with a solution of 10 mg ml $^{-1}$ α–Cyano–4–hydroxycinnamic acid in 50% ethanol/50% acetonitrile/0.1% TFA. Reflectron MALDI–TOF spectra were acquired on a TofSpec 2E (Waters, Manchester, UK). For peptide mass fingerprinting the resulting peptide mass lists were used to search in MASCOT using the same MSDB database.

Cloning of *CfPhiA*, *Ecp6*, and *Ecp7*

Based on the N–terminal CfPhiA sequence MDPIDVVWK, the forward degenerate primer Deg–PhiA along with an oligo–dT primer (Table 4) was used to isolate the *CfPhiA* coding sequence. Likewise, degenerate forward primers (Table 4) were designed matching the ETKATDCG and QITTQDFG sequences from the N–terminal sequences of Ecp6 and Ecp7, respectively. Using the degenerate primers and a poly–T primer PCR products were amplified from a cDNA library derived from a compatible interaction between *C. fulvum* and tomato using the high fidelity polymerase ExTaq (Takara, Shiga, Japan). Products were cloned into the pGEM–T Easy vector (Promega, Madison, WI), and sequenced.

Construction of plasmids for RNAi in *C. fulvum*

Two constructs for over–expression of inverted repeat constructs for RNAi based on two different parts of the *Ecp6* coding sequence were generated. For the first RNAi construct targeting the 3'end of *Ecp6*, 218 bp of *Ecp6* was PCR–amplified from cDNA using a forward primer that added an *Nco*I restriction site to the 5' end (Ecp6i–F) and a reverse primer that added *Eco*RI and *Not*I restriction sites to the 3' end (Ecp6i–R; Table 4). PCR reactions were carried out under the following conditions: an initial denaturation step for 2 min followed by denaturation for 15 s at 94°C, annealing for 30 s at 55°C, and extension for 1 min at 72°C for 30 cycles, followed by a final elongation step at 72°C for 5 min. PCR products were separated on 1% agarose gels and were purified using the DNeasy kit (Qiagen, Valencia, CA). Subsequently, PCR products were cloned into the pGEMT–Easy vector. Vectors were digested with *Nco*I and *Not*I or with *Nco*I and *Eco*RI. Both digested inserts were cleaned from gel using the QIAquick gel extraction kit (Qiagen) and subsequently ligated with a *Not*I– and *Eco*RI–digested 129 bp spacer segment from the *Pichia pastoris Aox–1* gene into the *Nco*I–digested plasmid pFBB302 (Dr. Brandwagt, Wageningen University). The plasmid pFBB302 is constructed in the backbone of the pGreenII binary vector (Hellens et al., 2000) and contains a nourseothricin resistance cassette (Malonek et al., 2004) to select for fungal transformants, and the *Uid*A reporter gene flanked by the constitutive *ToxA* fungal promoter (Ciuffetti et al., 1997) and *trpC* terminator (Punt et al., 1987). Digestion with *Nco*I releases the *Uid*A coding sequence and allows ligation of the inverted repeat RNAi sequence.

For the second RNAi construct targeting the 5' end of *Ecp6*, two *Ecp6* PCR products were generated of 250 and 318 bp with the same forward primer that added an *Eco*RI restriction site to the 5' end (Ecp6i2–F) and two different reverse primers that added a *Not*I restriction sites to the 3' end (Ecp6i2k–R and Ecp6i2l–R, respectively; Table 4). PCR reactions and gel cleaning was performed similar as for the first RNAi construct. Subsequently, PCR products were cloned into the pGemT–Easy vector, digested with *Not*I and *Eco*RI, cleaned from gel, and ligated into the *Eco*RI–digested plasmid pFBT004. The plasmid pFBT004 is a modified version of pFBB302, in which the nourseothricin resistance cassette is replaced by a hygromycin resistance cassette (Punt et al., 1987).

Agrobacterium tumefaciens*-mediated transformation of *C. fulvum

RNAi plasmids were transformed into *Agrobacterium tumefaciens* strain LBA1100 (containing the binary vector pSoup (Hellens et al., 2000) by electroporation. A 3 ml culture of *A. tumefaciens* was grown overnight in 1xYT (Sambrook and Russell, 2001) supplemented with kanamycin (25 µg/ml). The following day, the culture was centrifuged and resuspended in 50 ml fresh minimal medium (MM) (Hooykaas et al., 1979) supplemented with kanamycin (25 µg/ml) and grown overnight. The following day, the culture was centrifuged and resuspended in 10 ml fresh MM. One ml of resuspended bacteria was used to inoculate 50 ml of induction medium (IM; MM salts plus 40 mM 2–(*N*–morpholino)ethanesulphonic acid (MES), pH 5.3, 10 mM glucose, and 0.5% (w/v) glycerol) supplemented with 200 µM acetosyringone (AS) and was grown for an additional 4 to 5 hrs until the culture reached an optical density (OD_{600}) of 0.25. At that point, the *A. tumefaciens* culture was centrifuged and

resuspended in 10 ml sterile water. In addition, while *A. tumefaciens* cultures were growing in IM+AS medium, *C. fulvum* conidia were harvested and subsequently suspended in 50 ml B5 medium (Duchefa Biochemie BV, Haarlem, the Netherlands) at a concentration of approximately $1x10^6$ conidia/ml and placed in a rotary shaker (125 rpm) at room temperature to induce germination of conidia. After 4–5 hrs, germinated conidia were centrifuged twice at 4000 rpm and re–suspended in sterile water to a final volume of $1x10^7$ conidia/ml.

500 µl from the induced *A. tumefaciens* cell suspension was mixed with 10 ml germinated conidia and plated (200 µl per plate) on a 0.45–µm pore, 45–mm diameter nitrocellulose filter (Whatman, Hillsboro, OR) and placed on co–cultivation medium (IM + 200 µM AS and 5 mM glucose and 1.5% technical agar). The co–cultivation mixture was incubated at 22°C for two days. Following incubation, the filter was transferred to PDA supplemented with 50 µg/ml nourseothricin (Werner BioAgents, Jena, Germany) or with 100 µg/ml hygromycin B (Duchefa Biochemie BV, Haarlem, The Netherlands) as a selection agent for transformants and 200 µg/ml cefotaxime (Duchefa Biochemie BV, Haarlem, The Netherlands) to kill *A. tumefaciens* cells. Individual transformants were transferred to new selection plates and incubated until conidiogenesis under normal growth conditions. Conidia from these plates were stored in 50% glycerol at –80°C until further analysis.

Real-time PCR analyses

Three leaflets were harvested from inoculated MM–*Cf–0* and MM–*Cf–4* plants at 3, 6, 9, 13, and 16 days post inoculation. Leaf samples were composed of three leaflets from the 2nd, 3rd, and 4th tomato leaves of two tomato plants taken at each time point, immediately frozen in liquid nitrogen, and stored at –80°C until used for RNA analysis. A similar procedure was used for RNAi transformant analysis. *Ecp6* RNAi transformants Ecp6i–1 and Ecp6i–4 along with *Ecp7* RNAi transformants Ecp7i–1, Ecp7i–3, and Ecp7i–7 were randomly chosen for inoculation and analysis with the progenitor race 5 wild–type strain inoculated on MM–*Cf–0* plants. Leaf samples were taken at 10 days post inoculation, immediately frozen in liquid nitrogen, and stored at –80°C until used for RNA analysis.

Total RNA was isolated from infected leaf material using the RNeasy kit (Qiagen, Valencia, CA), including an in–column DNAse treatment (Qiagen) according to manufacturer's instruction. Total RNA was used for cDNA synthesis using an oligo–(dT) primer and the SuperScript II reverse transcriptase kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Quantitative real–time PCR was conducted using an ABI7300 PCR machine (Applied Biosystems, Foster City, CA) with the GoldStar SYBR green PCR kit (Eurogentec, Seraing, Belgium). All primer sequences are shown in Table 4. Expression primers were designed so that the reverse primer was not included in the RNAi construct to prevent detection of the constitutively expressed RNAi construct. For the first RNAi construct, primer pair Ecp6–RNAi–RQ–F and Ecp6–RNAi–RQ–R was used, and for the second RNAi construct primer pair Ecp6–RNAi2–RQ–F and Ecp6–RNAi2–RQ–R. Real–time PCR conditions were as follows: an initial 95°C denaturation step for 10 min followed by denaturation for 15s at 95°C, annealing for 30s at 60°C, and extension for 30s at 72°C for 40 cycles and analyzed on the 7300 System SDS software (Applied Biosystems, Foster City, CA). To ensure no genomic DNA contaminated RNA samples, real–time PCR was also carried out on RNA without the addition of reverse transcriptase. All experiments, including leaf inoculations, were repeated twice.

Heterologous expression of *C. fulvum Ecp6* in *F. oxysporum* f. sp. *lycopersici*

For *C. fulvum Ecp6*, the cDNA corresponding to the mature protein was amplified using primer Ecp6OE–F that also contained the sequence encoding the the *C. fulvum* Avr4 signal peptide for extracellular targeting (Table 4). For *C. fulvum Ecp7*, the cDNA corresponding to the mature protein was amplified in two steps. Since the 5' coding sequence was lacking from our cDNA clone, a primer was designed to add a 5' codon–optimized sequence stretch based on the N–terminal protein sequence (Ecp7NtermF) and used in combination with the reverse primer Ecp7OE–R (Table 4). The resulting PCR product was used as template for a second PCR with primer Ecp7OE–F that also contained the sequence encoding the the *C. fulvum* Avr4 signal peptide for extracellular targeting and a *Hin*dIII restriction site in combination with the reverse primer Ecp7OE–R that contained a *Xma*I restriction site (Table 4). All PCR reactions were carried out under the following conditions: an initial denaturation step for 2 min followed by denaturation for 15 s at 94°C, annealing for 30 s at 56°C, and extension for 1 min at 72°C for 30 cycles, followed by a final elongation step at 72°C for 5 min. PCR products were separated on 1% agarose gels and purified using the DNeasy kit (Qiagen, Valencia, CA). Subsequently, PCR products were cloned into the

pGemT–Easy vector and sequenced. A correct clone was digested with *Eco*RI (for *Ecp6*) or *Hin*dIII and *Xma*I (for *Ecp7*), cleaned from gel, and ligated into the *Eco*RI– (for *Ecp6*) or *Hin*dIII– and *Xma*I– (for *Ecp7*) digested plasmid pFBT004. The constructs were transformed into *A. tumefaciens* strain LBA1100 (containing the binary vector pSoup (Hellens et al., 2000)) by electroporation essentially as described by Mersereau et al., 1990). *Agrobacterium*–mediated transformation of *F. oxysporum* f. sp. *lycopersici* was performed as described (Mullins et al., 2001).

***Ecp6* gene walking**

Three primers designed on the region encoding the mature Ecp6 protein (TSP1, TSP2 and TSP3; Table 4) were used to amplify the genomic DNA sequence upstream of the region that encodes the mature Ecp6 protein using the DNA Walking SpeedUp™ Premix Kit (Seegene Inc., Rockville, Maryland) according to the manufacturer's instructions. Amplified products were cloned in the pGEM–T Easy vector (Promega, Madison, WI) and sequenced. Putative open reading frames (ORFs) were predicted using the FGENESH program (Salamov and Solovyev, 2000) of the MOLQUEST software package (available at http://sun1.softberry.com/berry.phtml; Softberry Inc. NY, USA) using the genetic codes of several fungi present in the database as models. ORFs were verified by cloning *Ecp6* cDNA. For this purpose, total RNA was isolated from leaves of MM–*Cf–0* plants inoculated with a race 5 strain of *C. fulvum* at 11 days post inoculation and used for cDNA synthesis using an oligo–(dT) primer (Table 4) and the SuperScript II reverse transcriptase kit (Invitrogen, Carsbad, CA) as described previously (van Esse et al., 2007). The generated cDNA was used as template for the primers Ecp6_ChrWal_F1 and Ecp6_R (Table 4) to amplify the predicted *Ecp6* ORF. The primers Ecp6_F3, Ecp6_F2, Ecp6_R3, Ecp6_R2 (Table 4) that hybridized outside the predicted *Ecp6* ORF were used as negative controls. The 50 µl PCR–reaction mixes contained 5.0 µl of 10x SuperTaq PCR–reaction buffer, 10 mM of each dNTP (Promega Benelux bv, Leiden, The Netherlands), 20 µM of each primer, 1 Unit of SuperTaq DNA polymerase (HT Biotechnology Ltd., Cambridge, UK) and approximately 100 ng of cDNA as template. The PCR program consisted of an initial 5 min denaturation step at 94 °C, followed by 35 cycles of denaturation at 94 °C (30 s), annealing at 55 °C (30 s) and extension at 72 °C (60 s). A final extension step at 72 °C (7 min) concluded the reaction. Amplified products were cloned in the pGEM–T Easy vector (Promega, Madison, WI) and sequenced.

Ecp6 allelic variation

Allelic variation in *Ecp6* was determined for 50 *C. fulvum* strains (Table 2) that are part of a previously described collection (Stergiopoulos et al., 2007a; b). Strains were cultured on half–strength PDA (Oxoid Ltd., Hampshire, England) at 22°C. Conidia were harvested from 15–day–old cultures and freeze–dried prior to DNA extraction. Genomic DNA isolations were performed using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The forward primer Ecp6_F3, located 424 bp upstream of the *Ecp6* translation start codon, and the reverse primer Ecp6_R3, located 99 bp downstream of the *Ecp6* stop codon, were used to amplify *Ecp6* (Table 4). The 50 µl PCR–reaction mixes contained 5.0 µl of 10x SuperTaq PCR–reaction buffer, 10 mM of each dNTP (Promega Benelux bv, Leiden, The Netherlands), 20 µM of each primer, 1 unit of SuperTaq DNA polymerase (HT Biotechnology Ltd., Cambridge, UK) and approximately 100 ng of genomic DNA as template. The PCR program consisted of an initial 5 min denaturation step at 94 °C, followed by 35 cycles of denaturation at 94 °C (30 s), annealing at 55 °C (30 s) and extension at 72 °C (60 s). A final extension step at 72 °C (7 min) concluded the reaction. Amplified PCR products were excised from 0.8% agarose gels, purified using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences UK limited, Buckinghamshire, England), and sequenced using the forward primers Ecp6_F2 and Ecp6_F in combination with the reverse primer Ecp6_R3 (Table 4).

Bioinformatical analysis of Ecp6-like proteins

EST sequences from various fungal pathogens were downloaded from the COGEME Phytopathogenic Fungi and Oomycete EST Database version 1.6 (http://cogeme.ex.ac.uk) (Soanes and Talbot, 2006). The genome sequences of various fungi listed in Table 3 were consulted at the website of Fungal Genome Initiative of the Broad Institute of MIT and Harvard (http://www.broad.mit.edu/annotation/fgi/) or at the website of the USA Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/genomes). The mining of Ecp6–like proteins was performed using NCBI BLAST, and the Standalone–BLAST version 2.2.3 (Altschul et al., 1997; Schäffer et al.,

2001). HMMpfam analysis of each identified candidate was performed by running a customized Perl script for Pfam HMM detection, available at ftp://ftp.sanger.ac.uk/pub/databases/Pfam, using Bioperl version 1.4 (http://bioperl.org) and HMMER version 2.3.2 (http://hmmer.janelia.org), which was loaded with the current Pfam *ls* and *fs* models (02.10.2007), for whole domain and fragment models respectively. An E–value of 0.001 was used as cut–off. The retained sequences were analyzed in BioEdit version 7.0.5.3 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Multiple sequence alignment was performed by ClustalW version 1.83 and for phylogenetic tree construction Molecular Evolutionary Genetic Analysis 4.0 (MEGA) was used (Kumar et al., 2001; Tamura et al., 2007). Phylogeny construction of fungal Ecp6–like proteins was performed by Neighbour–Joining analysis. We used p–distance as the distance parameter as specified in the program MEGA. The inferred phylogeny was tested by 500 bootstrap replicates (Felsenstein et al., 1985).

Three–dimensional modelling was performed using the Protein Homology/analogY Recognition Engine (Phyre), a protein fold recognition server (http://www.sbg.bio.ic.ac.uk/~phyre/; Bennet–Lovsey et al., 2008; Kelley et al., 2000). Estimated precision of generated models was used as an indication of significance. Subsequent analyses, visualization and preparation of 3D figures were performed in the Swiss–PdbViewer version 3.7 (http://www.expasy.org/spdbv).

Table 4. Primers used in this study.

Primer name	Sequence (5'-3')	Description
Deg–PhiA	ATGGAYCCNATHGAYGTNGTNTGGAA	Degenerate primer for *CfPhiA* cloning
Deg–Ecp6	GARACNAARGCNACNGAYTGYGG	Degenerate primer for *Ecp6* cloning
Deg–Ecp7	CARATHACNACNCARGAYTTYGG	Degenerate primer for *Ecp7* cloning
oligo–dT	TTGGATCCTCGAGTTTTTTTTTTTTTTTT	Poly–T primer with *Nco*I (bold) and *Sac*I (underlined)
Avr2RQ–F	ACCGCATCCGAAGTAATAGCA	*Avr2* qRT–PCR expression forward
Avr2RQ–R	CCAGACTTCTCCTTCACTTTGCA	*Avr2* qRT–PCR expression reverse
Avr9RQ–F	GAGCTTGCTCTCCTAATTGCTACTACT	*Avr9* qRT–PCR expression forward
Avr9RQ–R	GTAGTCTAGCCCGACTCCCAATC	*Avr9* qRT–PCR expression reverse
CfPhiARQ–F	TGAGGACCAGAAGTGGACTCTTTC	*CfPhiA* qRT–PCR expression forward
CfPhiARQ–R	ATCTCGCACAAATGCCTTGAG	*CfPhiA* qRT–PCR expression reverse
Ecp6RQ–F	GCTCAAGGTTGGTCAGCAGAT	*Ecp6* qRT–PCR expression forward
Ecp6RQ–R	TTCACACCTGACAGATCACTTATGC	*Ecp6* qRT–PCR expression reverse
Ecp7RQ–F	TGGTTTTTCTTCTTTCTATAGTCGAGTCTA	*Ecp7* qRT–PCR expression forward
Ecp7RQ–R	TTCTTAGCCCCTGCGTTCTGT	*Ecp7* qRT–PCR expression reverse
CfPhiAi–F	**CCATGG**AGCACCCAAGGTCGGCGACA	*CfPhiA* RNAi forward with *Nco*I (bold)
CfPhiAi–R	**GAATTC**GCGGCCGCACACTGCAGTATCTCGCACA	*CfPhiA* RNAi reverse with *Eco*RI (bold) and *Not*I (underlined)
Ecp6i–F	**CCATGG**AGATCGAGAACCCAGATGCC	*Ecp6* RNAi forward with *Nco*I

		(bold)
Ecp6i–R	**GAATTC**GCGGCCGCCCCGACCATCTTCACACCTG	*Ecp6* RNAi reverse with *Eco*RI (bold) and *Not*I (underlined)
Ecp6i2–F	**GAATTC**GAAGGCGACGGATTGCGGTT	*Ecp6* RNAi2 forward with *Eco*RI (bold)
Ecp6i2k–R	**GCGGCCGC**TGGAAGACCTGGCACGCAAG	*Ecp6* RNAi2 reverse with *Not*I (bold)
Ecp6i2l–R	**GCGGCCGC**TCGAGCGTGATGTTGAAGTC	*Ecp6* RNAi2 reverse with *Not*I (bold)
Ecp6–RNAi–RQ–F	GTCAGATTAAGGCTCTCAAC	*Ecp6* qRT–PCR RNAi expression forward
Ecp6–RNAi–RQ–R	GTTTAAGTACAAGACCATTC	*Ecp6* qRT–PCR RNAi expression reverse
Ecp6–RNAi2–RQ–F	GTCAGATTAAGGCTCTCAAC	*Ecp6* qRT–PCR RNAi expression forward
Ecp6–RNAi2–RQ–R	GTTTAAGTACAAGACCATTC	*Ecp6* qRT–PCR RNAi expression reverse
Ecp6OE–F	AAGCTT**ATGGGATTTGTTCTCTTTTCACAATTGCCTTCATTTCTTCTTGTCTCTACACTTCTCTTATTCCTAGTAATATCCCACTCTTGCCGTGCCCAAAAT**GAAACCAAAGCG ACGGAC	*Ecp6* over–expression with coding sequence for *C. fulvum Avr4* signal peptide (bold) and *Hin*dIII restriction site (underlined)
Ecp6OE–R	TTATGCCACAGCAGTAGTGA	*Ecp6* over–expression
Ecp7NtermF	CACTACTTGACCATCTACAGCAACATCGGCTGCCGCAAG GGCAGCCAGATTACGACGCAGGATTTTGGTCACGAG	*Ecp7* over–expression primer to obtain coding sequence for mature protein (bold)
Ecp7OE–F	AAGCTT**ATGGGATTTGTTCTCTTTTCACAATTGCCTTCTTTCTTCTTGTCTCTACACTTCTCTTATTCCTAGTAATATCCCACTCTTGCCGTGCCCAAAAT**CACTACTTGACCATCT AC	*Ecp7* over–expression with coding sequence for *C. fulvum Avr4* signal peptide (bold) and *Hin*dIII restriction site (underlined)
Ecp7OE–R	**CCCGGG**AATTCTTAACAATCAACTCTG	*Ecp7* over–expression with *Xma*I site (bold)
TSP1	TTGACGGATACGATGTTG	Gene walking
TSP2	TTGGCAATGGAGGTGAGG	Gene walking
TSP3	CCTTGACGACAGTGTATTTGATG	Gene walking
Ecp6_ChromWal_F1	CCATGCAGTCG**ATG**ATTC	cDNA cloning, start codon in bold
Ecp6_R	ACAGCAGTAGTGACGTTCTTG	cDNA cloning
Ecp6_F2	ACTCTCGTTAGATTGCATTC	Allelic variation
Ecp6_R2	GTTACTCTCAACACGCTG	Allelic variation
Ecp6_F3	CCTCGCTGCTATCACATC	Allelic variation
Ecp6_R3	GTTGTCGAATAGCTGATG	Allelic variation
Ecp6_F1	AAATACACTGTCGTCAAGGG	Allelic variation

CfPhiA

```
1     ATGGACCCGATHGATGTBGTTTGGAAAGCACCCAAGGTCGGCGACAAATTCGGCATCGCA
      M  D  P  I  D  V  V  W  K  A  P  K  V  G  D  K  F  G  I  A

61    GCCACAGGCGAAGGCATCTTCAACAAGGGCCTCACTGCAACGATGGGGGGCATCTTCGTT
      A  T  G  E  G  I  F  N  K  G  L  T  A  T  M  G  G  I  F  V

121   GGAGGCAAGCAAAGTCCATCTTGCGACAGAGGCGCGAGGCAAGACTTTGCGAATTTCTGG
      G  G  K  Q  S  P  S  C  D  R  G  A  R  Q  D  F  A  N  F  W

181   CTCAAGGAGGACACCAGCATCAGTCTGTACAAGACCGACAACCCTCCACAAGACCTCTGG
      L  K  E  D  T  S  I  S  L  Y  K  T  D  N  P  P  Q  D  L  W

241   GTTGACGCGTCGGACATGGGCGGAGGTCTTGTTGGATACACTACTGGAGTCTTTGAGCAG
      V  D  A  S  D  M  G  G  G  L  V  G  Y  T  T  G  V  F  E  Q

301   CTACCAAAGAGCGCGGCAAGAACTGGATTCGCGGTCGATCCTGACACGAGAGTTCTCACC
      L  P  K  S  A  A  R  T  G  F  A  V  D  P  D  T  R  V  L  T

361   TTCAACGGTGTTGGCGGCAAGGCGTGCCCGACTGGTGAGGACCAGAAGTGGACTCTTTCG
      F  N  G  V  G  G  K  A  C  P  T  G  E  D  Q  K  W  T  L  S

421   TTCACCGACAGCGAGAGGCCTCGCAACCAGCATGGCTGCGTCACCGTGGAGCTCAAGGCA
      F  T  D  S  E  R  P  R  N  Q  H  G  C  V  T  V  E  L  K  A

481   TTTGTGCGAGATACTGCAGTGTCGTGCTGGTACTCGGACTCGTCATAGGTGTCACATCAG
      F  V  R  D  T  A  V  S  C  W  Y  S  D  S  S  *

541   AGGGAGTTGCGGAGGCCATGGCGACGGTGTCGATCAGCATGGGAAGCATAAGGTGGCATA
601   GTATAGAGCATGGGAGAGCATGGTGTAGCATGGGGGATTTGTCGGGTCGACAATCATGGT
661   CTAATAGTTGTATAGGCGTTTCCTTACAGGACATTTCTTTCCCCGGGAAAAAAAAGAAAA
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Ecp6

```
1     GAAACCAAAGCGACGGACTGCGGTTCGACCAGCAACATCAAATACACTGTCGTCAAGGGT
      E  T  K  A  T  D  C  G  S  T  S  N  I  K  Y  T  V  V  K  G

61    GACACCCTCACCTCCATTGCCAAGAAATTCAAGTCCGGCATTTGCAACATCGTATCCGTC
      D  T  L  T  S  I  A  K  K  F  K  S  G  I  C  N  I  V  S  V

121   AACAAACTCGCCAACCCCAACCTCATCGAGCTCGGCGCAACCCTCATCATCCCAGAGAAC
      N  K  L  A  N  P  N  L  I  E  L  G  A  T  L  I  I  P  E  N

181   TGTTCTAACCCCGACAACAAGTCCTGCGTGTCGACACCGGCCGAGCCCACCGAGACTTGC
      C  S  N  P  D  N  K  S  C  V  S  T  P  A  E  P  T  E  T  C

241   GTGCCAGGTCTTCCAGGCAGCTACACCATCGTCAGCGGCGACACTCTCACCAACATCTCC
      V  P  G  L  P  G  S  Y  T  I  V  S  G  D  T  L  T  N  I  S

301   CAGGACTTCAACATCACGCTCGACTCCCTCATCGCTGCCAACACTCAGATCGAGAACCCA
      Q  D  F  N  I  T  L  D  S  L  I  A  A  N  T  Q  I  E  N  P

361   GATGCCATCGATGTTGGCCAGATCATCACCGTCCCAGTCTGCCCATCGTCCCAGTGCGAG
      D  A  I  D  V  G  Q  I  I  T  V  P  V  C  P  S  S  Q  C  E

421   GCTGTCGGTACTTACAACATTGTGGCCGGTGACCTTTTCGTCGATTTGGCCGCTACCTAC
      A  V  G  T  Y  N  I  V  A  G  D  L  F  V  D  L  A  A  T  Y

481   CACACCACTATCGGTCAGATTAAGGCTCTCAACAACAACGTTAACCCATCTAAGCTCAAG
      H  T  T  I  G  Q  I  K  A  L  N  N  N  V  N  P  S  K  L  K

540   GTTGGTCAGCAGATCATTCTGCCACAGGACTGCAAGAACGTCACTACTGCTGTGGCATAA
      V  G  Q  Q  I  I  L  P  Q  D  C  K  N  V  T  T  A  V  A  *

601   GTGATCTGTCAGGTGTGAAGATGGTCGGGACAGAATGGTCTTGTACTTAAACTCAGCGTG
661   TTGAGAGTAACTGTGTGTATACATCAGCTATTCGACAACCAAAAAAAAAAAAAAAAAACT
721   CGAGGATCCAAAATCGAATTC
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Ecp7

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                                                                ATTACGACGCAG
      H  Y  L  T  I  Y  S  N  I  G  C  R  K  G  S  Q  I  T  T  Q

13    GATTTTGGTCACGAGCGACCCGGCTGGACTTCCGGTTGCAAGTCCATCAGTCCAAGTGGT
      D  F  G  H  E  R  P  G  W  T  S  G  C  K  S  I  S  P  S  G

73    CGGTCAATCGACATGCACTTCGACAACCGAGGTACGGACTGCACGGCAAAAATCTATGAC
      R  S  I  D  M  H  F  D  N  R  G  T  D  C  T  A  K  I  Y  D

133   GACAATGCATGCCAGCACGAACTGATTGGAATGTCTGGGTGGGTTGACCCACACACCTAC
      D  N  A  C  Q  H  E  L  I  G  M  S  G  W  V  D  P  H  T  Y

193   GGCTGCCTTGCTCATGATGATTGGGGCAAGGGGTATGTCAATTCATTCAGAGTTGATTGT
      G  C  L  A  H  D  D  W  G  K  G  Y  V  N  S  F  R  V  D  C

253   TAAGAATTCACCAAGCTCTGAATGTACAATGCGTGCCGGAGGATACCT
      *

313   ATATGTTAGATAGTGGTTTTTCTTCTTTCTATAGTCGAGTCTACATGGCTGTCACGGTCT
373   CTTCCCAGAAGGCACAGAACGCAGGGGCTAAGAAGCCTTCTGCGCCGAGGGAGGCCCGAA
433   GTGAATGTCAAGACAAAAAAAAAAAAAAAAAA
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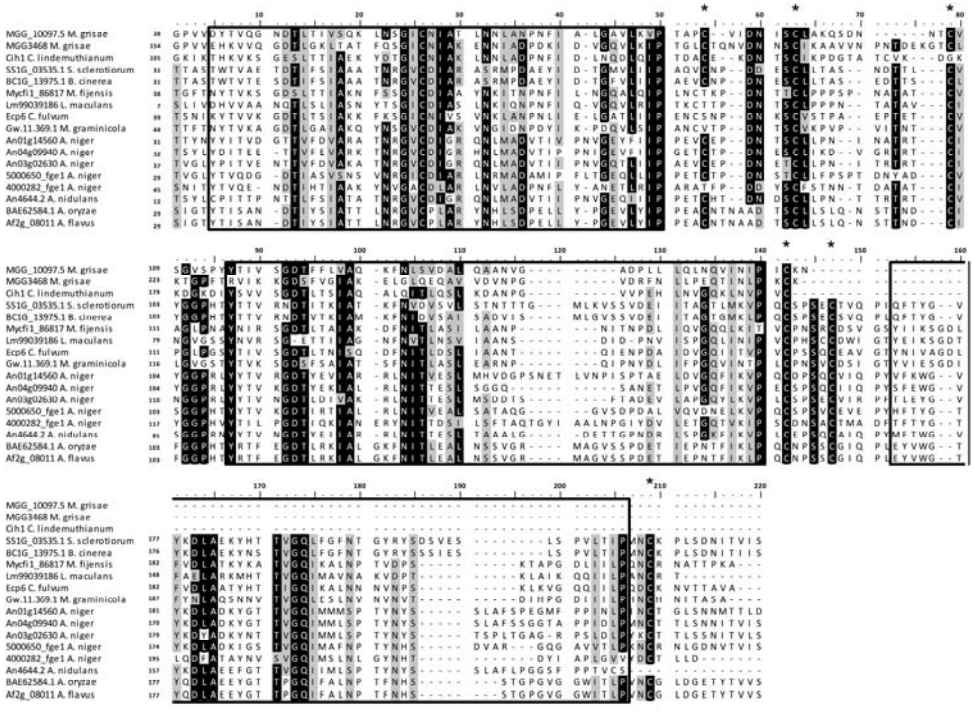
Supplemental Figure 1 (above and previous page). Nucleotide and deduced amino acid sequences of *CfPhiA*, *Ecp6*, and *Ecp7* from *Cladosporium fulvum*. The predicted amino acid sequence of each protein is shown below the nucleotide sequence. The first 16 N–terminal amino acids of Ecp7 were obtained by N–terminal sequencing of protein spot 14 (Fig. 2). Sequence information obtained by MS/MS peptide sequencing is indicated in bold. The underlined nucleotide sequences of *Ecp6* correspond to encoded LysM signatures. Cysteine residues are shaded.



Supplemental Figure 2 (previous page). Multiple sequence alignment of Ecp6–like proteins from various fungal species. Included in the alignment are *Cladosporium fulvum* Ecp6 (Ecp 6) and 16 Ecp6–like fungal protein sequences from *Aspergillus flavus* (Af2g_08011), *A. nidulans* (An 4644.2), *A. niger* (An 01g14560; An 04g09940; An 03g02630; 5000650_fge; 4000282_fge), *A. oryzae* (BAE62584.1), *Botrytis cinerea* (BC1G_13975.1), *Colletotrichum lindemuthianum* (Cih1), *Leptosphaeria maculans* (Lm99039186), *Magnaporthe grisea* (MGG_10097.5; MGG3468), *Mycosphaerella graminicola* (Gw.11.369.1), *M. fijensis* (Mycfi1_86817) and *Sclerotinia sclerotiorum* (SS1g_03535.1). Identical amino acid residues are shaded in black and similar residues (70% treshold according to Blosum62 score) are shaded in grey, while cysteine residues are indicated by asterisks. LysM domain signatures are indicated by the boxed areas. The third LysM domain is only identified with sufficient statistical support (E–value <0.001) for the Ecp6–like proteins of *C. fulvum*, *M. fijiensis*, *M. graminicola* and *L. maculans* (Ecp6, Mf_86817, Mycfi1_86817, Gw.11.369.1 and Lm99039186, respectively). (B)

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Chapter 6:
The tomato transcriptomes upon infection with a foliar and a vascular fungal pathogen show little overlap

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(*Submitted*)

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Abstract

Plants have evolved various defense mechanisms to defend themselves against attack by microbial pathogens, which activation often requires significant host transcriptional reprogramming. Microarrays provide a powerful tool to monitor these transcriptional changes. In this study, we performed global transcriptional profiling to compare transcriptional changes in tomato during compatible and incompatible interactions with the foliar pathogenic fungus *Cladosporium fulvum* and the soil–borne vascular pathogenic fungus *Verticillium dahliae*. Although both pathogens colonize different host tissues, they display significant commonalities in their infection strategies as they both penetrate natural openings and grow strictly extracellular without the formation of haustoria. Furthermore, in incompatible interactions with both pathogens resistance is conveyed by extracellular transmembrane receptors that belong to the class of receptor–like proteins. For each of the two pathogens, the transcriptomes of the compatible and incompatible interaction largely overlap. However, the *C. fulvum*–induced transcriptomes shows little overlap with the *V. dahliae*–induced transcriptomes, as most genes are uniquely regulated by one of the two pathogens. This also applies to both incompatible interactions, despite defense activation by the same type of resistance protein. Remarkably, of the relatively small subset of genes that is regulated by both pathogens a large portion shows an inverse regulation; induced by one pathogen and repressed by the other. With pathway reconstruction, interacting networks of tomato genes implicated in photorespiration, hypoxia and glycoxylate metabolism were identified that are repressed upon infection with *C. fulvum* and induced by *V. dahliae*. Similarly, auxin signaling seems to be differentially affected by the two pathogens. To our knowledge, this is the first microarray study to compare the defense transcriptome of tomato upon infection by two fungal pathogens.

Introduction

Plants are continuously exposed to microbial pathogens that aim to parasitize their potential hosts. In response to this threat, they have evolved various defense mechanisms to protect themselves against microbial attack. These comprise constitutive defense barriers, and defenses that are activated upon detection of a potential invader. Plants may activate basal defense responses upon detection of microbe–associated molecular patterns (MAMPs); non–self molecular components that are common to specific groups of microbes, such as fungal chitin or ergosterol (Felix et al., 1993; Granado et al., 1995), and bacterial flagellin, elongation factor Tu, or lipopolysaccharides (Felix et al., 1999; Dow et al., 2000; Kunze et al., 2004). Such MAMPs are generally detected through transmembrane pattern recognition receptors that activate basal defense (Altenbach and Robatzek, 2007). Successful pathogens are able to overcome MAMP–triggered basal defense through the activity of pathogen effector molecules that are delivered inside the host (Chisholm et al., 2006). In turn, plants have evolved the means to recognize pathogen presence based on these effector molecules through resistance (R) proteins (Chisholm et al., 2006; Jones and Dangl, 2006). Upon

recognition of (the activities of) pathogen effectors by R proteins, often a hypersensitive response (HR) is activated in which plant cells in close proximity of the site of pathogen ingress undergo cell death.

Whether a pathogen is arrested in growth on the host or not, and whether growth arrest is established by MAMP–triggered immunity or effector–triggered immunity, microbial attack generally leads to significant transcriptional changes of the host. A few decades ago, the first pathogenesis–related (*PR*) genes have been identified that are induced by pathogen attack, and of which the corresponding gene products have been associated with pathogen defense (van Loon et al., 1994; 2006; van Loon and van Strien, 1999). PR proteins play a role not only in basal defense, but also in effector–triggered immunity (Thomma et al., 2001; Thordal–Christensen 2003), demonstrating that both types of defense responses at least partly employ the same defense genes. Interestingly, upon pathogen attack similar defense genes are activated in different plant species, but the effect on a given pathogen might be completely different. For instance, basal defense in Arabidopsis and tobacco against *Botrytis cinerea* is mainly conveyed by jasmonate– and ethylene–induced defense genes, while in tomato *B. cinerea* resistance is achieved by salicylic acid–dependent defense genes (Thomma et al., 1998; 1999; 2001; Audenaert et al., 2002; Geraats et al., 2003; Achuo et al., 2004). More recently, transcriptional reprogramming can be monitored at a genome–wide scale with the use of microarrays in a growing number of plant species (Quirino and Bent 2003; Wise et al., 2007; van Baarlen et al., 2008). Plant microarray data demonstrate that in the interactions of plant pathogens with their hosts hundreds of genes are activated and repressed, irrespective whether the interaction is compatible or incompatible. Currently, most microarray analyses are initiated for gene discovery, in order to associate novel genes with effective host defense responses as a lead to select appropriate candidate genes for functional analysis (Wan et al., 2002; AbuQamar, 2006; Wise et al., 2007). Alternatively, microarray analyses may also be used to profile the transcriptional activity of a plant tissue, providing global insight into the cell biology of the host (van Baarlen et al., 2008; van Esse et al., 2008). Such approach accelerates the identification of cellular pathways or processes that are modulated, and facilitates a clear overview of cellular processes that are differentially regulated under a certain condition, which helps to understand the underlying biological processes (van Baarlen et al., 2008).

It has been suggested that a plant is resistant or susceptible to a specific pathogen depending on the speed and rate at which the same host defense molecules are produced, implicating that resistance is based on quantitative rather than qualitative differences in host defense (Tao et al., 2003; Navarro et al., 2004; Eulgem et al., 2004; Thilmony et al., 2006; Jones and Dangl, 2006). However, others have shown significantly divergent gene expression not only between compatible and incompatible interactions (Caldo et al., 2004), but even between incompatible interactions of the same pathogen–host combination that are mediated by different R proteins (Adams–Philips et al., 2008). By querying microarray databases such as Genevestigator, the response of a single host to various pathogens can be compared *in silico* (Zimmermann et al., 2004). However, only few studies have

investigated transcriptional changes in one host upon challenge by different pathogens. In Arabidopsis, transcriptional responses were profiled upon challenge with a host (*Erysiphe cichoracearum*) and a non–host (*Blumeria graminis hordei*) powdery mildew isolate. This analysis showed that barley powdery mildew elicited a stronger response in Arabidopsis than Arabidopsis powdery mildew (Zimmerli et al., 2004; Stein et al., 2006). Two–thirds of the differentially regulated genes, mainly involved in photosynthesis and general metabolism, appeared to be repressed, while induced transcripts primarily included defense–related transcripts (Zimmerli et al., 2004).

Until recently, the vast amount of microarray data was produced for the model plant species Arabidopsis, and most transcriptomics studies upon pathogen attack relied on inoculations with the bacterium *Pseudomonas syringae* (Maleck et al., 2000; Scheideler et al., 2002), the fungus *Alternaria brassicicola* (Schenk et al., 2000; van Wees et al., 2003), and the oomycete pathogen *Hyaloperonospora parasitica* (Maleck et al., 2000; Eulgem et al., 2004). Currently, however, high–density microarrays are available for a growing set of crop species (Wise et al., 2007) including the Solanaceous crop plant tomato (van Esse et al., 2007).

In this study, we performed global transcriptional profiling to compare transcriptional changes in tomato during compatible and incompatible interactions with the two fungal pathogens *Cladosporium fulvum* and *Verticillium dahliae*. Both pathogens have fundamentally different infection strategies as *C. fulvum* is a foliar pathogen that causes leaf mold on its sole host tomato (Thomma et al., 2005), while *V. dahliae* is a soil–borne vascular pathogen of over 200 host plants (Fradin et al., 2006). Despite these different infection strategies, both pathogens share a number of characteristics as they invade their host through natural openings, and grow strictly extracellular without the formation of feeding structures such as haustoria (Thomma et al., 2005; Fradin et al., 2006). Furthermore, in incompatible interactions with tomato, recognition of these pathogens is mediated by plasmamembrane–anchored extracellular receptor proteins that belong to the receptor–like protein class of resistance proteins (Jones et al., 1994; Kawchuk et al., 2001; Thomma et al., 2005; Fritz–Laylin et al., 2005; Wang et al., 2008). The aim of this study was to compare global transcriptional profiles in response to host attack by *C. fulvum* and *V. dahliae* in order to identify differences and similarities in compatible and incompatible interactions with a foliar and a vascular fungal pathogen.

Results

The transcriptome of tomato upon *C. fulvum* inoculation

Tomato genes that were differentially regulated (≥ 2–fold change with a P–value ≤ 0.001) upon inoculation with *C. fulvum* were identified at 3, 7 and 10 DPI using the samples harvested at 0 DPI as a common reference (Fig. 1A). In the compatible interaction, the number of differentially induced genes gradually increased from 144 genes via 1,093 to 3,360 genes at 3, 7 and 10 DPI, respectively (Table 1). A small subset of only 32 genes was found to be differentially regulated throughout the three time points. In the incompatible interaction, 118, 1,227 and 3,318 genes were differentially regulated at 3, 7 and 10 DPI, respectively (Table 1). Thus, although in the incompatible interaction the fungus is arrested soon after tissue penetration, the number of differentially regulated genes is comparable to that in the compatible interaction. Moreover, a similarly low number of 31 genes was found to be differentially regulated at the three time points throughout the incompatible interaction. Thus, the tomato transcriptome upon *C. fulvum* inoculation is highly dynamic and strictly based on the number of differentially regulated genes, the incompatible and compatible interaction of *C. fulvum* with tomato cannot be discriminated.

Often similar gene sets are induced during compatible and incompatible plant–pathogen interactions, albeit that the regulation occurs with different rates and amplitudes (Tao et al., 2003; Navarro et al., 2004; Eulgem et al., 2004; Thilmony et al., 2006). To account for temporal variation, the differentially regulated genes were pooled to one gene set of 3,500 genes for the compatible, and one set of 3,573 genes for the incompatible interaction. Roughly, two thirds of the regulated genes appeared to overlap between both interactions (Fig. 1A). Subsequently, hierarchical clustering (HCL, Eisen et al., 1998) was performed to cluster genes based on similarity in expression patterns, showing that most overlapping genes displayed similar expression patterns over time in the compatible and incompatible interaction (Fig. 1B).

We were able to subsequently assign Gene Ontology (GO) annotations (The Gene Ontology Consortium, 2000) to 6,730 of the 22,721 probed gene transcripts by BLASTing the probed EST sequences to the Pfam database. These GO annotations were used to identify the major differentially regulated biological processes (Table 2), showing that most differentially regulated genes belong to the categories transport (27%), metabolism (25%), and phosphorylation (11%). Interestingly, despite the observation that only two thirds of the regulated genes appeared to overlap for both interactions, no significant differences were observed in differentially regulated biological processes between the compatible and the incompatible interaction.

The transcriptome of tomato upon *V. dahliae* inoculation

Similar as for the foliar pathogen *C. fulvum*, transcriptional changes were monitored in tomato upon inoculation with the vascular pathogen *V. dahliae*. Since *V. dahliae* is a soil–borne pathogen, samples were taken of foliage (leaves and stems) and of roots at 3 and 7 DPI. *V. dahliae* inoculation requires uprooting of the plants followed by root–dip inoculation. Since this method is likely to introduce transcriptional changes that are not related to pathogen attack, in this case mock–inoculated plants harvested at 3 DPI were taken as common reference. In the compatible interaction, 0 and 2 genes were found to be differentially regulated at 3 DPI in foliage and roots, respectively, while at 7 DPI 518 and 1,188 genes were differentially regulated in those tissues (Table 1). In the incompatible interaction, 3 and 22 genes were differentially regulated at 3 DPI, while 389 and 130 genes were differentially regulated at 7 DPI in the foliage and roots, respectively (Table 1). Thus, in contrast to the *C. fulvum*–induced transcriptome, the *V. dahliae*–induced transcriptome of the compatible interaction was significantly larger than that of the incompatible interaction. In roots, the number of differentially expressed genes in the compatible interaction was almost ten–fold the number of differentials in the incompatible interaction.

Similar as for the *C. fulvum*–tomato transcriptome, temporal variation was countered by pooling the transcriptomes of the samples harvested at 3 DPI and 7 DPI for each interaction. Overall, in the foliage 280 genes were identified in the overlap between the in total 518 and 389 differentially regulated genes in the compatible and incompatible interaction, respectively (Fig. 1A). In roots, 94 genes were identified in the overlap between the 1,188 differentially regulated genes of the compatible and the 147 of the incompatible interaction (Fig. 1A). Like for *C. fulvum*, HCL showed that most overlapping genes displayed similar expression patterns over time in the compatible and incompatible interaction (Fig. 1B).

Interestingly, the assessment of GO categories for the *V. dahliae* regulated genes showed that phosphorylation, photosynthesis, transcription, and stress responses are slightly over–represented in the compatible interaction in foliage, while proteolysis and transport were slightly over–represented in foliage in the incompatible interaction (Table 2).

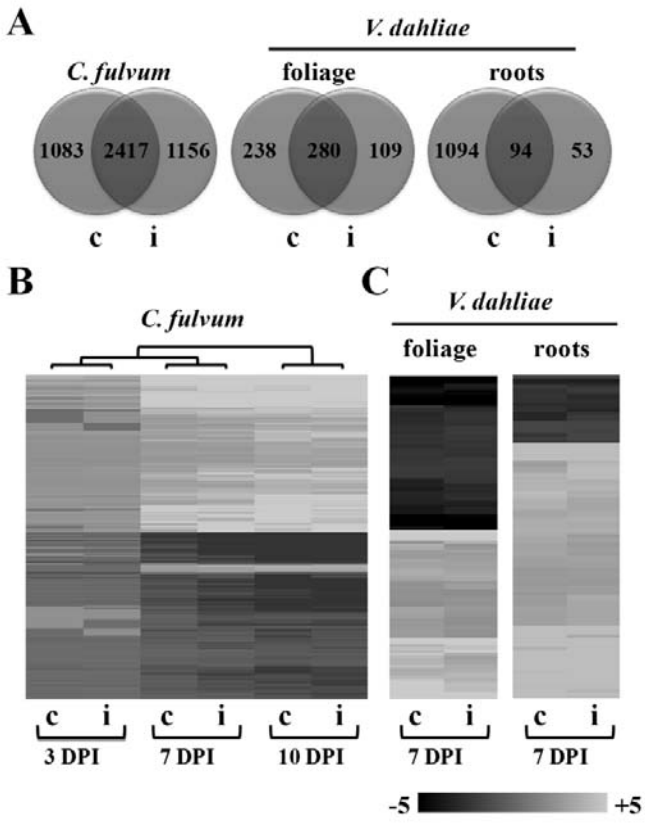

Figure 1. Differentially regulated tomato gene sets during compatible and incompatible interactions with *Cladosporium fulvum* and *Verticillium dahliae* (see page 208 for full color version). (A) Venn diagrams displaying specificity and overlap in differentially regulated gene sets between compatible (c) and incompatible (i) interactions with tomato. (B) Expression profiles of differentially regulated genes in the compatible (c) and incompatible (i) *C. fulvum*–tomato interaction at 3, 7 and 10 days post inoculation (DPI), respectively. (C) Expression profiles of differentially regulated genes in foliage and roots in the compatible (c) and incompatible (i) *V. dahliae*–tomato interaction at 7 DPI in foliage and roots.

Table 1. Number of differentially regulated genes identified in the diverse interactions of tomato with *Cladosporium fulvum* and *Verticillium dahliae* at 3, 7 and 10 days post inoculation (DPI).

Pathogen	Interaction	Tissue	Orientation	3 DPI	7 DPI	10 DPI
C. fulvum	Compatible	Foliage	Induced	46	592	1655
C. fulvum	Compatible	Foliage	Repressed	98	501	1705
C. fulvum	Incompatible	Foliage	Induced	45	763	1774
C. fulvum	Incompatible	Foliage	Repressed	73	464	1544
V. dahliae	Compatible	Foliage	Induced	0	255	n.d.[1]
V. dahliae	Compatible	Foliage	Repressed	0	263	n.d.
V. dahliae	Compatible	Roots	Induced	2	486	n.d.
V. dahliae	Compatible	Roots	Repressed	0	702	n.d.
V. dahliae	Incompatible	Foliage	Induced	2	218	n.d.
V. dahliae	Incompatible	Foliage	Repressed	1	171	n.d.
V. dahliae	Incompatible	Roots	Induced	22	102	n.d.
V. dahliae	Incompatible	Roots	Repressed	0	28	n.d.

[1] n.d. is = not determined.

Table 2. Differentially regulated biological processes (GO process) identified in the diverse interactions of tomato with *Cladosporium fulvum* and *Verticillium dahliae*.

GO process	*C. fulvum* Compatible Foliage	*C. fulvum* Incompatible Foliage	*V. dahliae* Compatible Foliage	*V. dahliae* Incompatible Foliage	*V. dahliae* Compatible Roots	*V. dahliae* Incompatible Roots
biosynthesis	11%	11%	9%	10%	9%	36%
metabolism	29%	29%	23%	24%	22%	21%
phosphorylation	13%	12%	7%	5%	11%	7%
photosynthesis	4%	3%	6%	4%	2%	–
proteolysis	7%	8%	7%	9%	10%	–
response to stress	4%	4%	9%	7%	3%	2%
transcription	4%	5%	5%	2%	4%	–
transport	26%	25%	29%	33%	35%	25%
signal transduct..	1%	1%	2%	2%	2%	2%
cell wall	2%	2%	3%	4%	2%	7%

Transcriptome comparison of foliage and roots upon *V. dahliae* inoculation

V. dahliae is a soil–borne pathogen that penetrates the roots and travels through the vascular system towards the green tissues. When comparing the transcriptomes of roots and foliage, a rather small overlap was observed (Fig. 2A). For the compatible interaction 59 genes overlapped, while for the incompatible interaction only 16 genes overlapped between the differentially regulated gene sets for roots and foliage (Fig. 2A). In the overlapping gene set of the compatible interaction, the *PR*–genes encoding P69B, PR–5 and hevein–like protein, a homolog of the Arabidopsis monosaccharide symporter *STP6* gene (Scholz–Starke et al., 2003), and several aquaporin δ–TIP genes that are implied in osmotic housekeeping (Daniels et al., 1996) were induced in both tissue types, while the TAS14 dehydrin gene was strongly repressed. Of the 59 genes, 13 genes displayed an inverse expression pattern (Fig. 2B), among which was a homologue of the tobacco nitrate reductase *nir–3* gene (Kronenberger et al., 1993) that was induced in foliage and repressed in roots. Highly induced foliage–specific genes upon *V. dahliae* inoculation included photosynthesis genes, aquaporin δ–TIP genes (Daniels et al., 1996), genes implicated in salt tolerance (Nagaoka and Takano, 2003), an ammonium transporter gene and a nitrate transporter gene. Interestingly, root–specific induced genes included the *PR* genes *PR1a*, *PR–5x* and *P96F*, but also the *Pip1* and *Rcr3* genes that are induced upon infection with *C. fulvum* and *Phytophthora infestans*, respectively (Krüger et al., 2002; Tian et al., 2007). Other root–specific induced genes were the inorganic phosphate transporter gene *LePT2*, the iron transporter gene *LeIRT1* (Eckhardt et al., 2001) and an Arabidopsis copper transporter gene homolog. In the incompatible interaction, the overlap between the transcriptomes of the roots and foliage consisted of only 16 genes with similar expression patterns (Fig. 2A, B) that included the *PR* genes *PR–5x*, and chitinase genes. Genes involved in proteolysis and transcription were induced in foliage, but not in roots, as was the case for the pathogen–inducible *Rcr3*, *Pip1* and *P69B* genes. In roots, the iron transporter gene *LeIRT1* (Eckhardt et al., 2001) was induced. Overall, it can be concluded

that the root and foliage transcriptomes of tomato upon *V. dahliae* inoculation are highly divergent.

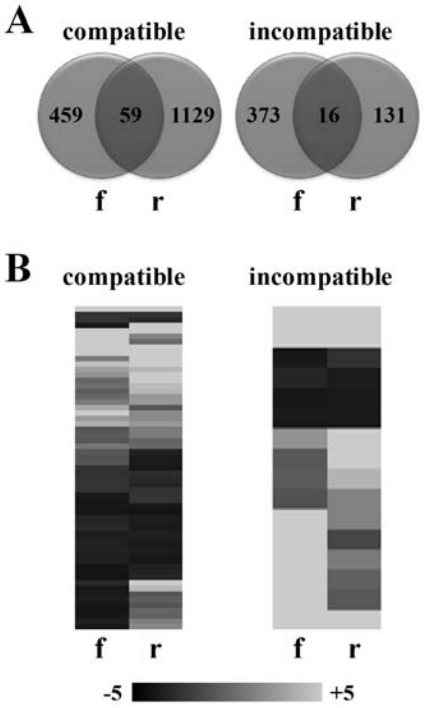

Figure 2. Differentially regulated tomato gene sets in foliar and root tissues during a compatible and incompatible interaction with *Verticillium dahliae* (see page 209 for full color version). (A) Venn diagrams displaying specificity and overlap in differentially regulated gene sets the compatible and an incompatible interaction between foliar tissues (f) and roots (r). (B) Expression profiles of differentially regulated genes in the compatible and incompatible interaction between foliar tissues (f) and roots (r).

Inoculation with *C. fulvum* and *V. dahliae* results in largely different transcriptomes

Although *C. fulvum* and *V. dahliae* colonize different host tissues, both pathogens have significant commonalities in their infection strategies as they only penetrate natural openings, and grow strictly extracellular without the formation of haustoria. However, during compatible interactions with tomato, the major differentially regulated biological processes by these pathogens differed (Table 2). Interestingly, the *C. fulvum*–induced foliar transcriptome more resembled the *V. dahliae*–induced root transcriptome than it resembled the *V. dahliae*–induced foliar transcriptome (Table 2). Nevertheless, the categories transport–related and proteolysis (the class that also contains many *PR* genes) were stronger represented in roots during the compatible interaction with *V. dahliae*, than with *C. fulvum*, while metabolism was stronger represented in the compatible interaction with *C. fulvum* than in *V. dahliae*–infected roots.

For both *C. fulvum* and *V. dahliae*, resistance in the incompatible interaction with resistant tomato genotypes is conveyed by extracellular transmembrane receptors that belong to the class of receptor–like proteins (Thomma et al., 2005; Fradin et al., 2006). It may be anticipated that pathogen receptors that belong to the same class converge into the

same signal transduction cascade that mediates a defense response. In such case, the transcriptomes are expected to largely overlap. In contrast to the compatible interaction, the *C. fulvum*–induced foliar transcriptome of the incompatible interaction most resembled the foliar response to *V. dahliae*. Nevertheless, overall significant differences are observed in the major regulated biological processes in both incompatible interactions (Table 2).

To study the *C. fulvum*– and *V. dahliae*–induced transcriptomes in more detail, we compared the 3500 *C. fulvum*–regulated genes to the 1647 *V. dahliae*–regulated genes in the roots and foliage for both compatible interactions, showing that the overlap contained only 454 genes (Fig. 3A). Similarly, both incompatible interactions were compared, showing only 172 overlapping genes (Fig. 3A). The *C. fulvum*–specific genes in both the compatible and incompatible interaction encompassed glutathione S–transferase genes and serine–type protease inhibitor genes. Both types of proteins have been implicated in attenuation of the oxidative burst (Lamb and Dixon, 1997; Guo et al., 1998; Chen and Sing, 1999). Furthermore, many WRKY transcription factor genes were specifically regulated in the *C. fulvum*–tomato interaction (12 in the compatible and 18 in the incompatible interaction), while no regulation of WRKY transcription factor genes was monitored in the interactions with *V. dahliae*. Similarly, NAM–like (no apical meristem) proteins were found to be specifically induced in the C. *fulvum*–tomato interaction (6 in the compatible and 10 in the incompatible interaction). Unique *V. dahliae*–regulated genes in both the compatible and incompatible interaction included genes that encode osmotic housekeeping factors such as; δ–tonoplast intrinsic aquaporins (Kjellbom et al., 1999), salt tolerance–like proteins, RD22–like dehydration–induced protein (Yamaguchi–Shinozaki et al., 1992), a homologue of the SOS2 salt tolerance protein (Liu et al., 2000), and an RD–28 water channel homologue (Daniels et al., 1994). In addition, in the compatible interaction an inorganic phosphate transporter (Daram et al., 1998) and iron–regulated transporter 1 (Eckhardt et al., 2001) were highly induced in root tissue.

K–Means clustering (Soukas et al., 2000) was performed on the overlapping genes (displayed in Fig. 3A) to group genes with similar expression pattern. Between the *C. fulvum*– and *V. dahliae*–induced transcriptomes, we identified 51 induced and 17 repressed genes in both compatible interactions. Similarly, 35 and 2 genes were induced and repressed, respectively, in both incompatible interactions. As may be expected, *PR* genes were induced in all interactions. HCL on the subsets of overlapping genes for the compatible, but also for the incompatible interaction, showed that most of the differentially regulated genes in *V. dahliae*–infected roots tissues displayed a similar regulation as in *C. fulvum*–infected leaves, while the same genes showed an inverse regulation in the *V. dahliae*–infected foliage (Fig. 3B).

Overall, our results show that the transcriptional response of tomato towards both pathogens is completely different. Within the 454 differentially regulated genes that were shared between both compatible pathogen interactions, 164 genes displayed an inverse expression pattern, consisting of 62 genes that were induced by *C. fulvum* and repressed by *V. dahliae*, and 102 genes that were repressed by *C. fulvum* and induced by *V. dahliae* (Fig.

3A, B). A similar situation was true for the incompatible interactions for which 72 of the 172 shared differentials displayed inverse regulation (Fig. 3A, B). Of the 102 inversely regulated genes in the compatible interactions, 16 genes could be implied in photosynthesis. To further investigate the effect of both pathogens on photosynthesis, HCL was performed on the 41 photosynthesis genes represented on the GeneChip by plotting the response of these 41 genes to *C. fulvum* and *V. dahliae* infection at the various time points (Fig. 4). Strikingly, most of these 41 genes were inversely regulated upon inoculation with the two pathogens. This strongly suggests that upon *C. fulvum* infection photosynthesis is repressed, while *V. dahliae* infection induces photosynthesis genes both in the compatible and the incompatible interaction (Fig. 4).

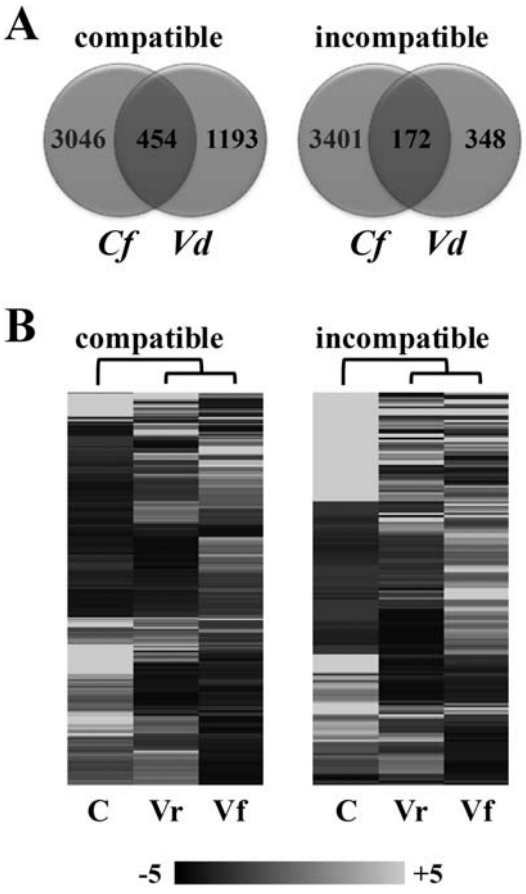

Figure 3. *Cladosporium fulvum* and *Verticillium dahliae* induce largely different transcriptomes (see page 210 for full color version). (A) Venn diagrams displaying specificity and overlap in compatible and incompatible tomato interactions with *C. fulvum* (*Cf*) and *V. dahliae* (*Vd*). (B) Expression profiles of differentially regulated genes in compatible and incompatible tomato interactions with *C. fulvum*-infected tomato (C), *V. dahliae*-infected foliar (Vf) and root (Vr) tissues at 7 DPI.

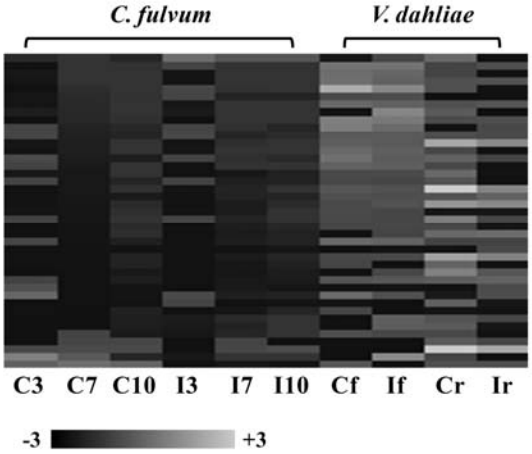

Figure 4. *Cladosporium fulvum* and *Verticillium dahliae* have inverse effects on photosynthesis in tomato (see page 211 for full color version). Transcriptional regulation of the 41 photosynthesis genes that are represented on the tomato GeneChip in the interactions of tomato with *C. fulvum* and *V. dahliae* at various time points. The different lanes represent the compatible tomato interaction with *C. fulvum* at 3, 7 and 10 DPI (C3, C7 and C10, respectively), the incompatible interaction with *C. fulvum* at the same time points (I3, I7 and I10, respectively), the compatible and incompatible tomato interaction with *V. dahliae* in foliage at 7 DPI (Cf and If, respectively), and the compatible and incompatible tomato interaction with *V. dahliae* in roots at 7 DPI (Cr and Ir, respectively).

Pathway reconstruction reveals inversely regulated processes by *C. fulvum* and *V. dahliae*

Pathway reconstruction is a valuable tool to identify cellular processes that might otherwise be obscured by the large amount of primary transcription data. Although this tool is widely used for analysis of human and murine transcriptome data (van Baarlen et al., 2008), it has hardly been used to analyze plant transcriptome data (van Baarlen et al., 2008; van Esse et al., 2008). Despite the currently available tools that facilitate cellular pathway reconstruction from plant gene expression data pathway reconstruction in plants remains a challenge, especially in non–model plants such as tomato (van Baarlen et al., 2008). For tomato, a whole–genome sequence is not yet available and gene annotation and associated information is still limited. Therefore, many genes still have to be screened manually to obtain the most relevant information. Here, we performed pathway reconstruction on the subset of genes that was regulated by both pathogens in the compatible or in the incompatible interactions, but displayed inverse regulation. This set of 164 inversely regulated genes between the compatible, and 72 between the incompatible interactions (Fig. 3), may reveal biological processes that are activated by one pathogen and repressed by the other.

As a first step, these genes were Blasted against the Reference Sequence (RefSeq) collection (RefSeq_protein NCBI database; http://www.ncbi.nlm.nih.gov/blast/) that provides a comprehensive, integrated and non–redundant set of sequences that can be used as a stable reference for gene identification. In this way, the RefSeq accessions of the closest Arabidopsis homologue were obtained. These RefSeq accessions were loaded into the BioNetBuilder plugin (http://err.bio.nyu.edu/cytoscape/bionetbuilder/; Avila–Campillo et al., 2007), an open–source tool that enables to generate biological networks based on integrated information from several databases that include the Biomolecular Interaction Network Database (BIND; Gilberd. 2005), Prolinks (Bowers et al., 2004) and the Kyoto

Encyclopedia of Genes and Genomes database (KEGG; Kanehisa et al., 2002). Cytoscape vizualizes the biological networks that are identified by BioNetBuilder as network graphs by representing molecular components (such as proteins) as nodes, and intermolecular interactions as links (edges) between nodes (Shannon et al., 2003). With the first "neighbor of nodes" option in BioNetBuilder, a pathway was identified with 14 interacting gene products that can be implicated in photorespiration, hypoxia and glycoxylate metabolism (Fig. 5). We subsequently grafted the expression data of the corresponding genes ($P < 0.05$) onto the identified pathway, showing a differential regulation of this pathway by both pathogens in the compatible interaction (Fig. 5), and similarly also in the incompatible interaction (data not shown). While most components of this pathway are repressed upon *C. fulvum* inoculation (Fig. 5A), they are induced by *V. dahliae* in roots (Fig. 5B), and even stronger in the foliage (Fig. 5C). In a similar way, a small interaction cascade with two genes that are involved in auxin signaling was identified. Both genes, *AtAux2–11* (Wyatt et al., 1993) and the *IAA16* transcription factor gene (Kim et al., 1997), were repressed by *C. fulvum* and induced by *V. dahliae*. BioNetBuilder allows expanding gene sets to include neighboring nodes in iterative steps to find new interactions with molecular components identified in the previous step (Avila–Campillo et al., 2007). In this way, after three iterative steps a predicted network containing 21 proteins was identified based on the two auxin signaling genes (Fig. 6). For 12 of these 21 proteins, probe sets were present on the tomato GeneChip. Remarkably, nine of those genes were differentially regulated ($P < 0.05$) upon inoculation with least one of the pathogens. Similar to *AtAux–11* and *IAA16*, the genes encoding the negative auxin signaling regulator AXR3 (Leyser et al., 1996) and the Skp1–CUL1–F–box protein ASK2 (Arabidopsis Skp–1–like 2; Park et al., 1993) were inversely regulated. Also present in the pathway is the Tir1 (transport inhibitor response) protein, an orthologue of human Skp2, that is able to bind to ASK2 (Ruegger et al., 1998). Interestingly, in addition to Tir1, also ASK2 is known to bind to the negative regulator of jasmonate signaling COI1 (Devoto et al., 2002). The gene encoding AXR2 (Nagpal et al., 2000) was specifically induced in the *V. dahliae*–tomato interaction (Fig. 6). Recently, it has been demonstrated that Arabidopsis mutants in *AXR2* display 10–fold reduction of *P. syringae* pv. *maculicola* colonization (Wang et al., 2007). Finally, also the auxin–inducible *IAA18* gene (Reed, 2001) was induced by *C. fulvum* and repressed by *V. dahliae* (Fig. 6).

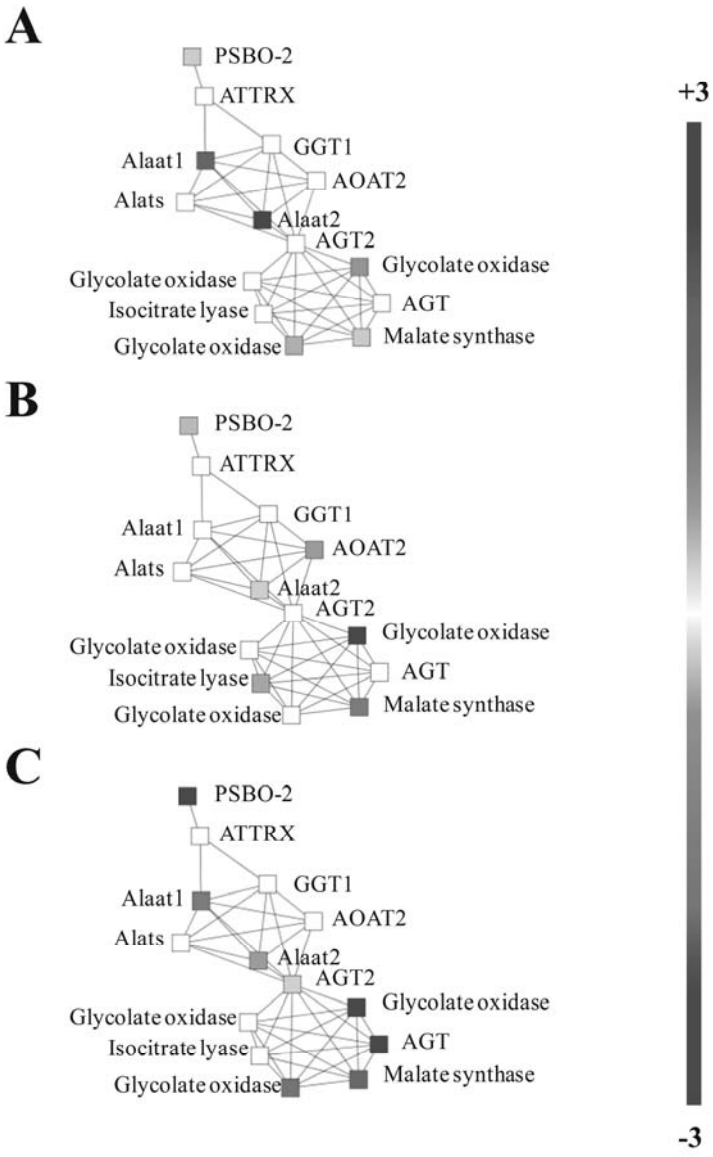

Figure 5. Pathway reconstruction reveals protein interaction networks (see page 212 for full color version). Responses of susceptible tomato upon inoculation of *Cladosporium fulvum* were compared those of tomato inoculated with *Verticillium dahliae* using the BioNetBuilder plug-in. A protein interaction network implied in both interactions was retrieved and visualized in Cytoscape. Subsequently, expression data monitored with the tomato GeneChip were grafted onto the network. (A) Network response upon inoculation with *C. fulvum*. (B) Network response in foliar tissues upon inoculation with *V. dahliae*. (C) Network response in root tissues upon inoculation with *V. dahliae*.

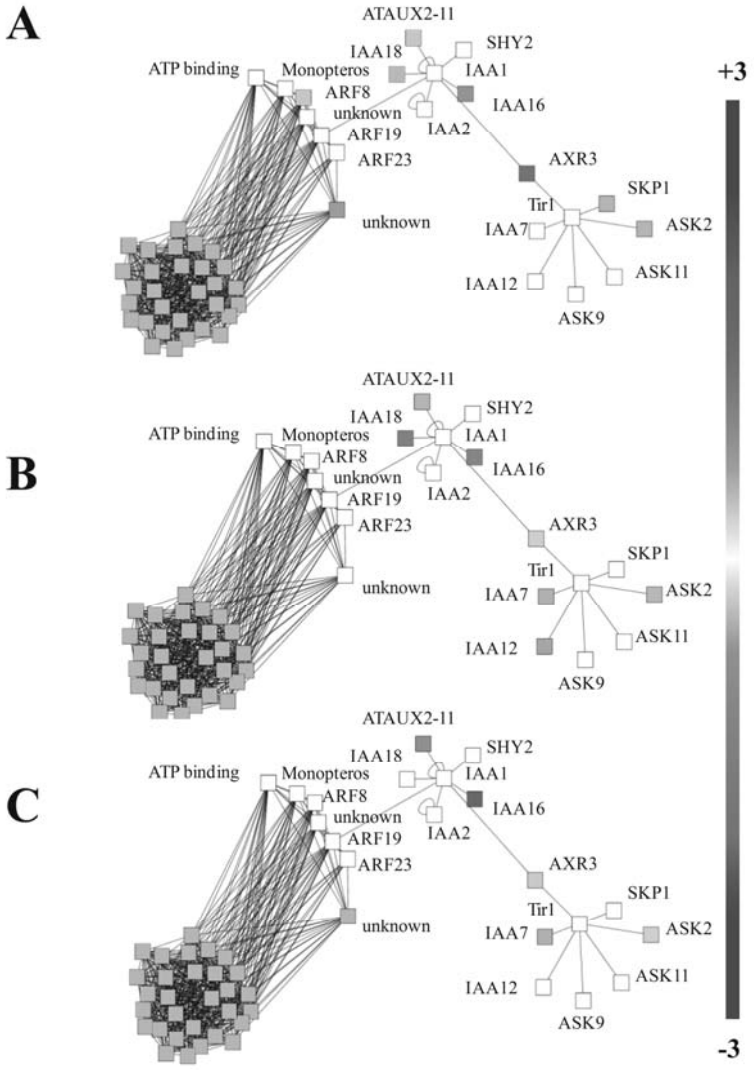

Figure 6. Tomato auxin signaling cascade in response to *Cladosporium fulvum* and *Verticillium dahliae* inoculation (see page 213 for full color version). Responses of susceptible tomato upon inoculation of *C. fulvum* were compared those of tomato inoculated with *V. dahliae* using the BioNetBuilder plug–in. A small protein interaction network implied in auxin signalling was retrieved. After 3 iterative steps in the BioNetBuilder, a protein interaction network was obtained that is visualized in Cytoscape. Subsequently, expression data monitored with the tomato GeneChip were grafted onto the network. Grey nodes indicate proteins for which expression was not considered. (A) Network response upon inoculation with *C. fulvum*. (B) Network response in foliar tissues upon inoculation with *V. dahliae*. (C) Network response in root tissues upon inoculation with *V. dahliae*.

Discussion

In this study, we have used microarrays to monitor global transcriptional responses of tomato upon inoculation with the foliar pathogen *C. fulvum* and the vascular pathogen *V. dahliae*, both in a compatible and an incompatible interaction. When examining the response to each of the two pathogens separately a significant overlap was observed in the response of tomato when comparing the compatible and the incompatible interaction. These observations are similar to other transcriptomics studies that compared compatible and incompatible plant–pathogen interactions (Tao et al., 2003; Navarro et al., 2004; Eulgem et al., 2004; Thilmony et al., 2006). For both pathogens, approximately two thirds of the genes that are differentially regulated in the incompatible interaction are also differentially regulated in the compatible interaction (Fig. 1). This complies with the currently held hypothesis that effector triggered immunity (ETI) can largely be seen as an accelerated and amplified PAMP–triggered immunity (PTI) response which culminates into an HR, a response which leads to disease resistance (Jones and Dangl, 2006; Shen et al., 2007).

In incompatible interactions with resistant tomato genotypes, resistance against *C. fulvum* and *V. dahliae* is conveyed by extracellular transmembrane receptors that belong to the class of receptor–like proteins (RLPs; Kruijt et al., 2005; Fritz–Laylin et al., 2005; Wang et al., 2008). Resistance against *C. fulvum* is governed by *Cf* genes (Thomma et al., 2005), of which we employed the *Cf–9* gene in this study (Jones et al., 1994), while in all currently used *V. dahliae*–resistant tomato cultivars resistance is governed by a single locus that contains two resistance genes, *Ve1* and *Ve2* (Kawchuk et al., 2001). Since RLPs lack obvious cytoplasmic signaling domains, it has been proposed that they associate with receptor–like kinases (RLKs) to relay signals across the plasmamembrane, as has been demonstrated for the RLP CLV2 that associates with the RLK CLV1 to initiate meristem maintenance upon perception of the peptide ligand CLV3 (Ogawa et al., 2008; Jeong et al., 1999; Joosten and de Wit, 1999). It may be anticipated that different RLPs associate with the same RLK to activate a general defense signaling cascade. However, our study suggests that the signaling cascade that is activated by *Cf–9* is different from the cascade that is activated by *Ve1* and *Ve2*, since both pathogens regulate highly divergent gene sets in the incompatible interactions (Fig. 3).

Both *C. fulvum* and *V. dahliae* display significant commonalities in their infection strategies as they penetrate natural openings and grow strictly extracellular without the formation of haustoria. Nevertheless, the tomato transcriptomes induced by the two pathogens in their compatible interactions hardly overlap. Previously, the transcriptional response of Arabidopsis upon challenge with a host (*E. cichoracearum*) and a non–host (*B. graminis* f.sp. *hordei*) powdery mildew isolate revealed that *B. g. hordei* elicited a more dramatic response than *E. cichoracearum*. It was suggested plants respond more powerful to *B. g. hordei* because it cannot evade or suppress basal defenses as efficiently as the host powdery mildew, *E. cichoracearum* (Zimmerli et al., 2004; Stein et al., 2006). Moreover, attacker–specific transcriptomes have also been observed in Arabidopsis upon challenge with different microbial pathogens and feeding insects (de Vos et al., 2005).

However, apart from the commonalities in the colonization strategies of *C. fulvum* and *V. dahliae*, there are also obvious differences. The most significant difference is the tissue that is colonized; the leaf apoplast by *C. fulvum* and the xylem by *V. dahliae*. Whereas *C. fulvum* is in close contact with mesophyll cells, *V. dahliae* resides in dead tracheids and may not have direct physical contact with living plant cells. Furthermore, during colonization *V. dahliae* has been reported to secrete potent phytotoxins into the xylem that are dispersed throughout the plant with the sap stream, while *C. fulvum* is not known to utilize toxin activity (Thomma et al., 2005; Fradin et al., 2006). Since *V. dahliae* is a vascular wilt pathogen, the more prominent expression of genes that are associated with water stress and dehydration such as several δ–TIP aquaporin–like genes, and transporters may be expected. A more remarkable difference between the tomato transcriptomes induced by both pathogens is the prominent occurrence of WRKY transcription factors in the *C. fulvum* interaction, while in the interaction with *V. dahliae* the genes coding for these transcription factors are not induced. Upon *C. fulvum* infection, the tomato homologs of the Arabidopsis WRKY transcription factors 2, 3, 4, 6, 7, 23, 51, 53 and 71 are differentially regulated. Of these, WRKY 4, 6, 7, 51 and 53 have previously been implicated in plant defense and senescence responses (Eulgem et al., 2000; Robatzek and Somssich, 2001; Kalde et al., 2003; Dong et al., 2003; Eulgem and Somssich, 2007). Furthermore, induction of glutathione S–transferases and serine–type protease inhibitors is specifically observed in the *C. fulvum*–tomato interaction. Both types of proteins may attenuate the oxidative burst (Lamb and Dixon, 1997) that plays a role in tomato defense against *C. fulvum* (Hammond–Kosack et al., 1996). Other genes that are specifically induced by *C. fulvum* in tomato are those that code for NAM–like proteins (Souer et al., 1996) that, together with ATAF, and CUP–SHAPED COTYLEDON (CUC) are part of a larger NAC (for NAM, ATAF, and CUC) protein family of transcription factors (Aida et al., 1997). Some of *NAC* genes, such as the *ATAF1* and *ATAF2* genes from *Arabidopsis* and the *StNAC* gene from potato, are induced by pathogen attack and wounding (Aida et al., 1997; Collinge and Boller, 2001). Furthermore, many *NAC* genes were differentially regulated in the interaction between the nematode *Heterodera glycines* and soybean (Klink et al., 2007). Recently, the barley *NAC* gene *HvNAC6* was implicated in basal defense against the barley powdery mildew pathogen *B. graminis* f.sp. *hordei* (Jensen et al., 2007). Furthermore, Arabidopsis ATAF2 is known to repress *PR* genes, and *ATAF2* overexpressing plants showed a higher susceptibility to the soil–borne vascular fungal pathogen *Fusarium oxysporum* (Delessert et al., 2005). Other Arabidopsis *NAC* members are involved in secondary cell wall thickening (Zhong and Ye, 2007), and in auxin and ethylene signalling (Guo et al., 2005; He et al., 2005). Also in a cDNA–AFLP analysis on tomato transgenes that undergo a controlled HR due to heterologous expression of *C. fulvum* Avr4 in *Cf–4* plants, transcripts for NAM–like proteins and a WRKY transcription factor were identified (Gabriëls et al., 2006). Another surprising observation is the inverse regulation of photosynthesis in tomato upon challenge by *C. fulvum* and *V. dahliae*. Strong repression of photosynthesis, like in *C. fulvum*–challenged tomato, is a typical plant response to pathogen attack (Scheideler et al., 2002;

Dowd et al., 2004; Zimmerli et al., 2004; Berger et al., 2007). However, local stimulation of photosynthesis has been noted too, as *A. candida* infection of Arabidopsis and *B. cinerea* infection of tomato results in decreased photosynthesis at the infection site, surrounded by a zone of enhanced photosynthesis (Chou et al., 2000; Berger et al., 2004). In a recent study, transcription profiles were determined for stem tissue of tomato plants infected with two different *V. dahliae* isolates using a cDNA array to interrogate expression of approximately 8,600 tomato genes (Robb et al., 2007). Infection with isolate Vd1 resulted in a compatible interaction, while infection with isolate E6 resulted in a tolerant interaction in which tomato develops few symptoms despite substantial fungal colonization. Similar to our findings, also in this study stimulation of photosynthesis by *V. dahliae* was observed in both the tolerant and compatible interaction (Robb et al., 2007). Moreover, Robb et al. (2007) noted induction of a gene encoding a 14–3–3 protein in the compatible interaction with *V. dahliae*, while this gene was repressed in the tolerant interaction, suggesting that this gene may be elementary for the tolerance phenotype. However, in a tolerant cotton genotype, a gene encoding a 14–3–3 protein was found to be activated upon *V. dahliae* challenge (Hill et al., 1999). In our study, genes encoding 14–3–3 proteins were found to be induced both in the compatible and incompatible interaction with *V. dahliae* in leaves and roots, but also in the interactions with *C. fulvum*.

Few microarray studies have been performed to monitor the transcriptome of different plant tissues upon pathogen infection. One example is the profiling in cotton roots and hypocotyls in response to infection with *Fusarium oxysporum* (Dowd et al., 2004). Also in this study it appeared that tissues responded quite differently to infection. Substantially more induced plant genes were identified in infected cotton hypocotyl tissues than in root tissues, suggesting that the fungus may be suppressing plant defense responses in the root tissue (Dowd et al., 2004). Also our transcriptome profiling of roots and foliage of *V. dahliae*–infected tomato revealed significant differences in expression profiles between the two tissues. In contrast to the *Fusarium oxysporum*–infected cotton, in our study more genes were induced in the roots than in the foliage. Perhaps most striking is the relatively low number of differentially regulated genes in roots in the incompatible interaction at 7 DPI, which possibly reflects that the fungus has largely been arrested in growth as has previously been observed (Heinz et al., 1998). Cell wall–regulated transcripts are among the differentially regulated genes, which is likely to reflect that resistance against *V. dahliae* largely depends on the isolation of the fungus in xylem vessels through cell wall fortifications (Fradin et al., 2006).

With pathway reconstruction, it was attempted to uncover biological processes in the differentially regulated gene sets. Among the inversely regulated gene sets by the two pathogens is a pathway that can be implicated in photorespiration, hypoxia and glycoxylate metabolism. It has previously been shown that these processes are repressed upon pathogen attack (Scheideler et al., 2002; Zimmerli et al., 2004; Berger et al., 2007). GGT1 or glutamate:glyoxylate aminotransferase is a peroxisomal enzyme that plays a central role in the photorespiratory pathway (Liepman & Olsen 2003). Peroxisomal alanine:glyoxylate

aminotransferases AGT1 and –2 and alanine:2–oxoglutarate aminotransferase AOAT2 have also been implicated in photorespiration, although the exact metabolic function of AGT2 and AOAT2 remain unclear (Igarashi et al., 2002; Liepman & Olsen 2003). ALaAT1 and ALaAT2 have recently been shown to be induced by oxygen deprivation (Miyashita et al., 2007). Interestingly, malate synthase is induced during the *V. dahliae*–tomato interaction and repressed during the *C. fulvum*–tomato interaction. Malate syntase is part of the glyoxylate cycle and expression of this gene is repressed when sucrose is being synthesized (e.g. in photosynthetic tissues), although induction has also been observed in senescent organs (Graham et al., 1992). Peroxisomal glycolate oxidases have a clear role in plant defense and PCD (Hammond–Kosack and Jones, 1996; Grant and Loake., 2000) but also play a role in photoresperation (Fukao et al., 2002). Another gene in the pathway, *PSBO–2*, is part of the photosystem II, and clearly links this pathway to the differential regulation of photosynthetic genes. Altogether, this pathway analysis shows that, apart from photosynthesis, also the photoresperatory system of tomato is differentially regulated upon infection by the two pathogens.

A similar situation occurs for an auxin signaling pathway. Previously, auxin signaling has been implicated in the response of cotton to *F. oxysporum*–infection (Dowd et al., 2004). With respect to the role of auxin in plant defenses, it has been shown that an Arabidopsis miRNA contributes to resistance against *P. syringae* by repressing auxin signaling (Navarro et al., 2006), and salicylic acid was found to inhibit the growth of pathogens via repression of the auxin signaling pathway (Wang et al., 2007). Recently, the *Pseudomonas syringae* type III effector AvrRpt2 was found to alter auxin physiology in Arabidopsis (Chen et al., 2007a), suggesting that modulation of auxin signaling contributes to host susceptibility. Furthermore, some strains of the bacterial pathogen *Ralstonia solanacearum* are known to produce auxin, the production of which is controlled by a *Hrp* master regulatory gene whose activity is induced in presence of plant cells (Valls et al., 2006). Finally, auxin produced by *Pseudomonas syringae* pv. *savastanoi* appears to be required for the inhibition of plant defences (Robinette and Matthysse, 1990).

Overall, it can be concluded that global transcriptional profiling of transcriptional changes in tomato during compatible and incompatible interactions with the foliar pathogenic fungus *C.fulvum* and the vascular pathogenic fungus *V. dahliae* revealed only limited overlap, suggesting that host defense signaling is highly sophisticated and pathogen–specific.

Materials and methods

Cultivation of micro-organisms and plant inoculations

C. fulvum and *V. dahliae* were cultured at room temperature on half–strength potato dextrose broth (Oxoid, Basingstoke, England) supplemented with 7 g/l technical agar (Oxoid, Basingstoke, England). 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.

Inoculation of tomato with *C. fulvum* was performed as previously described (de Wit, 1977). Briefly, four–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.

For inoculation with *V. dahliae*, ten–day–old soil–grown tomato plants were up–rooted and inoculated by dipping the roots for two 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.

Microarray sample preparation and data analyses

All samples were collected in three independently repeated experiments. A race 5 strain of *C. fulvum* was used for inoculation. MoneyMaker *Cf–0* was used as a *C. fulvum*–susceptible genotype as it is devoid of functional *Cf* resistance genes, while the isogenic MoneyMaker *Cf–9* line was used as a resistant genotype as it recognizes the Avr9 elicitor that is produced by the race 5 *C. fulvum* strain. At 0, 3, 7 and 10 days post inoculation, leaf material was harvested from the secondary and tertiary leaves of three plants, pooled and flash frozen in liquid nitrogen. Material harvested at 0 DPI was used as common reference.

For *V. dahliae,* a race 1 strain was used for inoculation on MoneyMaker and Motelle tomato plants. MoneyMaker was used as a susceptible genotype because it lacks *Verticillium* resistance, while Motelle contains the *Ve* locus that provides resistance against race 1 *Verticillium* strains (Schaible et al., 1951; Kawchuk et al., 2001). At 3 and 7 days post inoculation (DPI), foliar and root material was harvested from nine plants, pooled and flash frozen in liquid nitrogen. As common reference foliar and root material of mock–inoculated plants at 3 DPI was used.

For RNA extraction, the frozen plant material was grinded using a mortar and pestle, and approximately 100 mg of the material was homogenized in Trizol® reagent (Invitrogen, Carlsbad, CA, USA). After 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 ATH1 Affymetrix Arabidopsis whole–genome arrays. Probe preparations and GeneChip hybridizations were carried out at ServiceXS (Leiden, The Netherlands).

Microarray data analyses

Bioconductor packages (www.bioconductor.org; Gentleman et al., 2004) were used to analyse 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). Venn diagrams and basic comparisons were performed in Microsoft Excel. HCL and K–Means clustering were performed using the TM4 microarray software suite (http://www.tm4.org/mev.html) and data were imported as Tab Delimited, Multiple Sample Files (TDMS). The TM4 software suite runs in a Java (http://www.java.com/en/) environment (Sun Microsystems, Inc. Santa Clara, U.S.A.).

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Chapter 7:
General discussion

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Impact of phytopathology

In agricultural practice worldwide, plant diseases regularly cause severe crop losses that may devastate the staple food of millions of people, thus causing famines, and collectively result in economic damage of billions of euros (Agrios, 2005). Famous examples from the past are the Irish potato famine (1845–1847), when the oomycete pathogen *Phytophthora infestans* destroyed most of the potato harvest (Large, 1940), and the great Bengal famine (1942–1943), when the rice pathogen *Helrninthosporium oryzae* caused a food shortage that resulted in the death of two million people (Padmanab, 1973). Furthermore, many fungal pathogens affect food and feed quality, not only by inflicting damage resulting in reduced quality of the produce, but also through contamination by mycotoxins (Yoshizaw and Morooka, 1973; Abbas et al., 2006; Magan and Aldred, 2007), low–molecular weight secondary metabolites that are toxic to humans and animals. Ingestion of low levels of mycotoxins may already lead to chronic effects such as reduced growth and development, immuno–supression and even cancer development (Bryden, 2007). According to recent estimations, 50% of the animal feed may be contaminated by mycotoxins in Europe (Binder et al., 2007). Finally, the use of fungicides to combat plant diseases may pose significant environmental and/or human health risks (Henriques et al., 1997; Garry et al., 2002), which has resulted in the pressure to reduce the use of these fungicides and provide more environmental–friendly alternatives. As a consequence of this all, efforts to unravel infection strategies of fungal pathogens have never been more important as now.

The role of secreted effectors in *C. fulvum* pathogenicity

The work presented in this thesis for the first time clearly links the intrinsic biological functions of two fungal effector genes, *C. fulvum Avr2* and *Avr4*, to pathogen virulence (chapters 3 and 4). Furthermore, for the LysM effector gene *Ecp6* an unambiguous role in fungal virulence has been established (Chapter 5). Using similar strategies as employed for *Avr2* and *Avr4*, we expect that the current leads towards a biological function will result in the identification of the intrinsic role for this effector in the near future. Previously, also the secreted *C. fulvum* effectors Ecp1 and Ecp2 were shown to be virulence factors (Laugé et al., 1997). The measurable contribution of all these individual effectors to fungal virulence upon targeted deletion (for *Ecp1* and *Ecp2*) or RNAi mediated gene silencing (for *Avr2*, *Avr4* and *Ecp6*) is remarkable, since similar strategies to target genes that encode secreted effectors in other fungal pathogens often not results in reduced virulence of the pathogen. For example, absence of several effectors in the flax rust pathogen *Melampsora lini* (Dodds et al., 2004; Dodds et al., 2006) and in the barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (formerly *Erysiphe graminis*; Bronson and Ellingboe, 1986; Brown and Wolfe, 1990) did not result in significant loss of virulence. Only in a handful of cases a marked role for a secreted effector in fungal virulence was recorded; two avirulence proteins from the barley powdery mildew fungus *B. 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., 2005a; Ridout et al., 2006).

So far, the only secreted C. *fulvum* effector for which a knockout of the corresponding gene did not result in a virulence penalty is Avr9 (Marmeisse et al., 1993; Thomma et al., 2006), suggesting that Avr9 function is redundant or dispensable for virulence. Indeed, also transgenic Arabidopsis plants that express *Avr9* did not show enhanced susceptibility towards *B. cinerea*, *P. cucumerina*, *H. parasitica*, *P. brassicae* and *P. syringae* (Chapters 3 and 4). Nevertheless, preliminary data suggest that heterologous expression of *C. fulvum Avr9* in Arabidopsis and tomato does result in enhanced susceptibility to the vascular pathogen *Verticillium dahliae*, indicating that Avr9 may still be a virulence factor of *C. fulvum* after all. Presently, we know that PEG transformation may generate significant variation in *C. fulvum* virulence that is independent of the transgene itself, but may be caused by the protoplasting and regeneration procedure. This does not obscure (qualitative) experiments to assess loss of recognition when targeting avirulence factors, such as the observation that targeted deletion of *C. fulvum Avr9* results in loss of recognition in *Cf–9* tomato (Marmeisse et al., 1993), but it may obscure (quantitative) experiments that are aimed at assessing different degrees of virulence. To avoid these complications, currently *Agrobacterium tumefaciens*–mediated transformation of *C. fuvum* is implemented (van Esse et al., 2007; 2008; Bolton et al., 2008).

Interestingly, *C .fulvum* no longer poses a serious threat to commercial tomato cultivation since the introduction of the *Cf–9* gene cluster in tomato cultivars in the late 1970s. Introgressed from *Solanum pimpinellifolium*, the *Cf–9* the locus contains five homologues, *Hcr9–9A* to *Hcr9–9E*, of which *Cf–9* (*Hcr9–9C*) is able to recognize Avr9 (van der Hoorn et al., 2001). The only mechanism described so far to overcome *Cf–9*–mediated recognition is the loss of *Avr9* from the genome of *C. fulvum* (van Kan et al., 1991). This may be explained in three ways, namely that Avr9 is indispensable for *C. fulvum* virulence, Avr9 is indispensable for survival outside the host, or the *Cf–9* locus recognizes an additional, yet unidentified *C. fulvum* effector. The observation that *Avr9* is highly induced in the plant once the fungus penetrates an open stoma (van den Ackerveken et al., 1994), argues against a crucial role for *Avr9* outside, but not in, the host. Although unambiguous proof for dispensability of *Avr9* for *C. fulvum* virulence remains to be demonstrated, there is evidence for an additional *C. fulvum* component that is recognized via the *Cf–9* gene cluster. It has been shown that *Hcr9–9B* functions as a genuine resistance gene in mature tomato plants, and recognizes a different, yet unidentified, elicitor than *Cf–9* (Panter et al., 2002). Although the resistance is weaker than that provided by *Cf–9*, *Hcr9–9B* and possibly other members of the cluster that may still act as resistance genes, which might explain the durability of the resistance provided by the gene cluster. Similarly, two of the five resistance gene homologs present in the the *Cf–4* gene cluster, *Hcr9–4D* and *Hcr9–4E*, mediate recognition of the two sequence–unrelated *C. fulvum* effectors Avr4 and Avr4E, respectively (Takken et al., 1999).

Due to the increased availability of bacterial genomes, one can now predict complete effector catalogs of bacteria that, in most bacteria studied so far, are predicted encompass between 20 and 50 effectors. *P. syringae* pv. *tomato* DC 3000 is estimated to employ approximately 33 effectors that are injected by the T3SS into the host cell (Schechter et al., 2006). The number of T3SS effectors secreted by *P. syringae* pv. *phaseolicola* 1448A is estimated to be 27 (Vencato et al., 2006), while 22 T3SS effectors are secreted by the pathogen *P. syringae* pv. *syringae* B728a (Vinatzer et al., 2006). Similar numbers are found for T3SS effectors of other species. For example, *Xanthomonas campestris* pv. *vesicatoria* has 29 predicted secreted effectors (Thieme et al., 2005), and *Ralstonia solanacearum* is predicted to secrete 48 effectors (Cunnac et al., 2004). For bacterial effectors, it is often difficult to demonstrate a significant contribution to pathogen virulence due to functional redundancy. Interestingly, the *P. syringae* pv. *tomato* DC 3000 effectors AvrRpm1, AvrB, and AvrRpt2 all target the Arabidopsis protein RIN4 (Mackey et al., 2002; Kim et al., 2005). Nevertheless, AvrRpm1and AvrRpt2 are still required for full pathogen virulence (Chen et al., 2000; Ritter and Dangl, 1995), because they have other targets in addition to RIN4 (Belkhadir et al., 2004; Lim and Kunkel, 2004; Chisholm et al., 2005). Similarly, both *P. syringae* pv. *tomato* effectors AvrPto and AvrPtoB target the Pto kinase (Pedley and Martin, 2003) and are required for full pathogen virulence (Lin and Martin, 2005). Nevertheless, often deletion of effectors does not result in an attenuated virulence (Grant et al., 2006).

The number of effectors in pathogenic bacteria is dwarfed by the number of predicted effector proteins present in the genomes of the oomycetous plant pathogens. Based on the presence of the RxLR host targeting motif, it is currently predicted that the genomes of these pathogens encode hundreds of effectors (Tyler et al., 2006; Whisson et al., 2007; Jiang et al., 2008). The predicted number of secreted effectors encoded in the genomes of fungal pathogens is quite variable. The *Magnaporthe grisea* genome revealed 739 putatively secreted proteins (Dean et al., 2005), while for *Ustilago maydis* 426 putatively secreted proteins were identified (Kämper et al., 2006), and the secretome of *Fusarium graminearum* is predicted to consist of 127 secreted proteins (Cuomo et al., 2007). *U. maydis* is a haustorial pathogen that furthermore induces tumor formation via fungal–induced alterations in plant growth (Kämper et al., 2006). These processes require an intimate relationship with the host. Therefore, non–haustorial pathogens like *F. graminearum* and *C. fulvum* might require less effector proteins to establish the interaction with their hosts, which is less intimate in nature. In a recent analyses of the *F. graminearum*, 120 *in planta* secreted proteins were identified, of which 77 may have a role in pathogen virulence (Paper et al., 2007). This set comprised 26 cell wall–degrading enzymes, 11 proteases and 10 oxidoreductases (Paper et al., 2007). Furthermore, 29 proteins of unknown function were identified, of which only 11 proteins with a molecular mass <17 kDa , reminiscent of *C. fulvum* effectors (Paper et al., 2007).

As discussed in the introduction of this thesis, *C. fulvum* may be considered as a "baseline pathogen" that employs a rather simple infection strategy and only has the minimal

requirements to invade a host plant. The currently known effector arsenal of *C. fulvum* consists of 10 proteins, of which five have been demonstrated to contribute markedly to pathogen virulence. Since there is no evidence that *C. fulvum* effectors have virulence targets in the interior of host cells, and it can be anticipated that the number of virulence targets in the host apoplast is limited, *C. fulvum* may only require a limited number of effectors to colonize on tomato. The availability of the *C. fulvum* genome sequence in the near future will reveal whether this hypothesis is indeed true

Arabidopsis as a heterologous system to study pathogen effectors

Notable obstacles may be encountered when studying novel pathogen effectors. In addition to the earlier discussed functional redundancy, many plant pathogens can not readily be transformed. This is especially a problem for obligate biotrophic pathogens such as *Hyaloperonospora parasitica* (Slusarenko and Schlaich, 2003), *M. lini* (Ellis et al., 2007) and *B. graminis hordei* (Ellis et al., 2007). To overcome such problems, heterologous systems may be employed to study the role of individual effector proteins. An early example of the use of a heterologous organism to study a putative virulence determinant is the *B. graminis PKA–c* gene that was used to complement a *CPKA* deletion mutant of *M. grisea* to restore appressorium development (Bindslev et al., 2001). However, based on sequence homology, in this case a function for the *B. graminis* gene was already envisaged, and this approach is not feasible for unique effectors for which no homologs in other species have been identified, which is true for most *C. fulvum* effectors (Thomma et al., 2005). In this thesis, we have used heterologous expression of *C. fulvum* effectors in the host plant tomato, and the non–host plant Arabidopsis to unravel biological functions (Chapters 3 and 4). In a similar approach, the *B. graminis* f. sp. *hordei* effectors AVRa10 and AVRk1 were recently demonstrated to contribute to successful infection by transient expression in the host plant barley (Ridout et al., 2006). Finally, in a novel approach the *H. parasitica* effectors ATR1 and ATR13 were found to promote disease susceptibility in Arabidopsis by fusing the coding sequences to the N terminus of the *P. syringae* type III effector protein AvrRPS4, resulting in type III mediated cytoplasmic delivery of the effectors which, in turn, resulted in enhanced bacterial colonization (Sohn et al., 2007).

In this thesis, we used Arabidopsis to study the roles of *C. fulvum* effectors, which has several advantages. Arabidopsis may readily be screened with a wide variety of pathogens (Thomma et al., 2001). This is useful, since specific effectors may not contribute to the virulence of all pathogens. For example, Arabidopsis plants that produce Avr2 only displayed enhanced susceptibility towards *V. dahliae*, *Botrytis cinerea*, and *Plectosphaerella cucumerina* while no enhanced susceptibly was observed upon challenge with *Ps. syringae*, *Ph. brassicae* and *H. parasitica*. Furthermore, as noted earlier, transgenic Arabidopsis plant expressing *Avr9* did not show enhanced susceptibility towards *B. cinerea*, *Pl. cucumerina*, *H. parasitica*, *Ph. brassicae* and *Ps. syringae* (Chapter 3 and

4), but preliminary data suggest that heterologous expression of *C. fulvum Avr9* results in enhanced susceptibility to the vascular pathogen *V. dahliae*. The screening of Arabidopsis transgenes that produce other *C. fulvum* effectors with pathogens that have not yet been tested may still reveal effectors that play contribute to pathogen virulence. Another benefit of the use of Arabidopsis as tool to study fungal effectors is the availability of genomic tools such as a genome sequence, mutant libraries and whole genome microarrays (van Baarlen et al., 2007b). In this study, microarrays for Arabidopsis, and also tomato, have been used to analyze transgenic plants that express individual *C. fulvum* effectors, and thus investigate the effect they have on the host.

Challenges in plant cellular pathway reconstruction based on gene expression profiling

Microarrays are used to profile transcriptional activity, providing global cell biology insight. Particularly for plants, interpretation of transcriptional profiles is challenging because many genes have unknown functions. Furthermore, many plant gene sequences do not have clear homologs in other model organisms. Over the past five years, various tools that assist plant scientists have been developed. The remainder of the discussion will be an evaluation of the currently available *in silico* tools for reconstruction of cellular (metabolic, biochemical and signal transduction) pathways based on plant gene expression datasets. Furthermore, it is shown how expression–profile comparison at the level of these various cellular pathways contributes to the postulation of novel hypotheses which, after experimental verification, can provide further insight into decisive elements that have roles in cellular processes.

Maximizing information retrieval from plant microarray datasets

A microarray, or gene chip, is an assembly of microscopic DNA spots arrayed on a solid surface that is commonly used for expression profiling (transcriptional analysis) in which the expression levels of thousands of genes are simultaneously monitored. The use of microarrays has made it possible to profile changes in transcriptional activity to specific stimuli at a genome–wide level. However, to link expression profiles to biological pathways as they occur in the cell remains a challenge. When compared with several other models, such as human, mouse, the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*, the biological function(s) of many genes and their encoded products is still obscure in plants. Furthermore, single genes can have roles in multiple, diverse biological processes (Kemmerling et al., 2007; Swindell et al., 2007)

Several tools, such as databases and software packages, that facilitate the analyses and exploration of plant microarray data, are now available (Fig. 1, Box 1). Still, researchers often find that many genes with unknown function remain in the final results of the array analysis. Furthermore, it is technically challenging and laborious to retrieve all, or even most, relevant information from *in silico* analysis of plant microarray datasets. For human

datasets, excellent software tools are available for *in silico* reconstruction of metabolic, biochemical and signal transduction pathways at the cellular level. This facilitates the prediction of processes in which unknown genes are involved and can even predict the function of unknown genes. Here, we will discuss the possibilities for cellular–pathway reconstruction in plants and suggest how to select, from the many tools that have been developed for microarray analysis so far, the right tools to maximize information retrieval from microarray datasets and to assign gene function whenever possible (Fig. 1, Box 1). A first step to assign gene function is to identify orthologous genes.

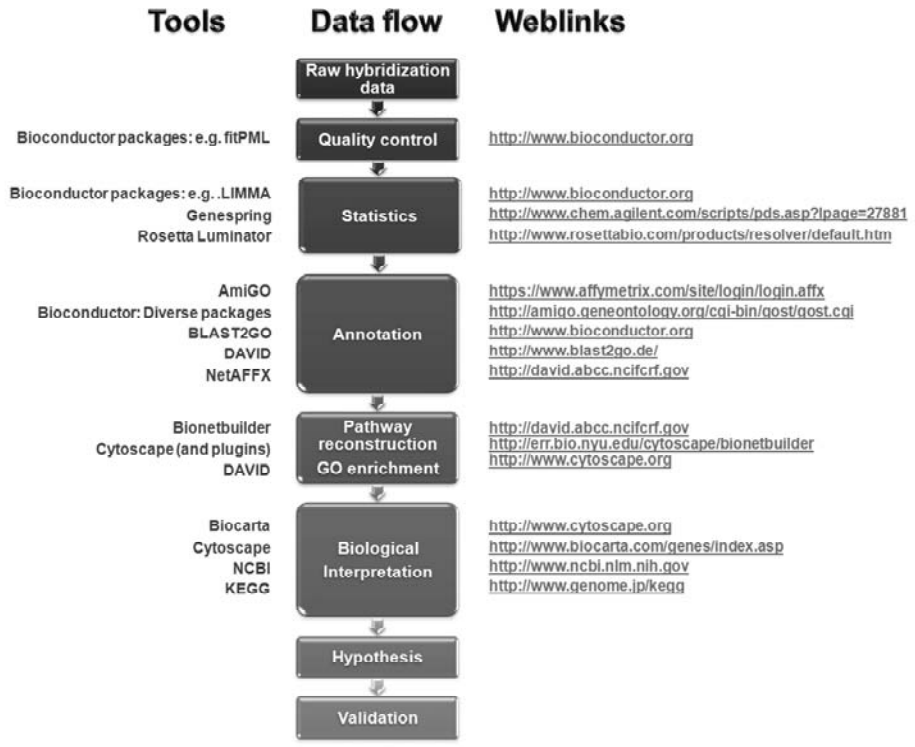

Figure 1. Flow chart for microarray data analysis. Microarray data analysis requires hybridization quality control to detect technical anomalies that affect subsequent statistical analyses. Subsequent statistical analyses to monitor gene transcript levels can be performed using open–source packages provided by bioconductor or the commercial packages GeneSpring or Rosetta Resolver. After functional annotation, pathway reconstruction can be conducted. To have a good graphical representation of genes that function in a single pathway and of modulated cellular processes the Cytoscape and the Biocarta websites provide valuable software tools, whereas the NCBI database and the KEGG pathway databases provide more information on specific genes. Finally, novel hypotheses can be made, that can be validated by biological experimentation.

Box 1. Assigning functions to genes; a general flow of microarray data analysis As a first step in microarray data analysis (see flow diagram in Fig. 1 in main text), a hybridization quality control should be performed to validate the technical accuracy of sample hybridization to the array (Alison et al., 2006; Heber and Sick, 2006) Databases and software packages are available to facilitate subsequent data interpretation and integration with the literature (Hoffmann and Valencia, 2004; Bajic et al., 2005; Hoffmann and Valencia, 2005; Hoffmann et al., 2005; Dennis Jr et al., 2007) (Fig. 1). Furthermore, tools are available for integration of the dataset with available biochemical and cell biological data (Mueller et al., 2003; Thimm et al., 2004; Zhang et al., 2005; Benedict et al., 2006; Wei et al., 2006; Dennis Jr et al., 2007; Poultney et al., 2007; Urbanczyk–Wochniak and Sumner, 2007). If using the above–mentioned tools cannot assign a biological function to a given sequence, additional analyses can be performed to identify possible gene functions.

Gene orthologies are often based on known orthologs. Several methods to identify orthologs (Page and Charleston, 1997; Remm et al., 2001; Chen et al., 2007b) or orthologous domains (Storm and Sonnhammer, 2003), or to retrieve orthologs from online resources (Penkett et al., 2006; von Mering et al., 2007; Spannagl et al., 2007) are available. A starting point to explore poorly characterized genes and predict plant protein function is the use of Gene Ontology (GO) annotation (Thomas et al., 2007), a controlled formal vocabulary that consists of general terms for gene and protein annotation in any organism. Consequently, comparisons across diverged taxa, such as plants and animals, become possible (Ashburner et al., 2001; Thomas et al., 2007). GO annotation for sequence data can be obtained through the use of the 'BLAST Search' option of AmiGO (http://amigo.geneontology.org/cgi–bin/amigo/go.cgi) or the BLAST2GO tool (http://www.blast2 go.de/); existing GO annotations can also be retrieved via the AmiGO browser.

To avoid the need to access multiple individual databases manually, software packages that enable integration of data from transcriptomics, proteomics and metabolomics databases have been developed. For instance, the DAVID (http://david.abcc.ncifcrf.gov/) and NetAffx (http://www.affymetrix.com/analysis/index.affx) databases can be used for data analysis and exploration because large gene lists can be uploaded and queried. DAVID also provides functional clustering of genes and can thereby give important hints of the major processes and pathways that are modulated by the differentials.

Based on the research question, for instance the cellular responses to pathogen infection or cell metabolism during drought stress, other software tools can be used to extract maximum information. For pathogen infection, pathway reconstruction using the Cytoscape Bionetbuilder plug–in can, together with the co–expression data from the DAVID output, give insight into regulated pathways. Metabolic pathways can be investigated using the MapMan software tool.

Orthology prediction aids the identification of gene functions

Orthology prediction is an important tool that evaluates relationships of genes with those of model organisms to predict (plant) protein function (Bowers et al., 2004; Chen et al., 2006). Orthologous genes, or orthologs, are genes in different species that originated from a common ancestor and were separated by a speciation event. Nevertheless, they might have retained their original function in both organisms (Koonin et al., 2004; Koonin et al., 2005); thus, the identification of an ortholog (e.g. from human) with a described function is often indicative of the function of a plant gene that has not been functionally characterized.

Several methods to identify orthologs (Page and Charleston, 1997; Remm et al., 2001; Chen et al., 2007b) or orthologous domains (Storm and Sonnhammer, 2003), or to retrieve orthologs from online resources (Penkett et al., 2006; von Mering et al., 2007; Spannagl et al., 2007) are available. Orthologous proteins can be retrieved via the KOG (clusters of euKaryotic Orthologous Groups) section of NCBI's COG database via the 'Clusters of Orthologous Groups' Hot Spot at the NCBI homepage (http://www.ncbi.nlm.nih.gov.ezproxy.library.wur.nl). Online retrieval of orthologous sequences for a given protein sequence can be performed via the WU–BLAST2 server (http://dove.embl–heidelberg.de/Blast2/), and the output can be used as input in the GeneTree software tool, an experimental program for comparing gene and species trees (Page, 1998). By combining GO annotation (Gene Ontology; Box 1) and orthology inference, for instance, via ProLinks or YOGY, the comparison and interpretation of the biological functions of proteins can be more reliably extrapolated to less well–studied taxa (Bowers et al., 2004). However, because GO annotations can be based on orthology inference or predictions from other species, care should be taken when making statements based on a combination of GO annotation and orthology.

Using orthology, when annotating gene function, is worthwhile. For instance, when assessing the response of Arabidopsis to treatment with *Escherichia coli* (Thilmony et al., 2006) 350 significantly regulated Affymetrix probe sets (the set of probes to monitor expression of a single gene) can be translated into 305 Arabidopsis proteins using Cytoscape's Bionetbuilder plug–in (Box 1). For these proteins, a total of 223 interactions ('edges') can be found when searching in different databases; 0 edges in Biogrid, four in BIND, 14 in KEGG and 205 in the ProLinks database, which incorporates GO annotations and orthology predictions. This example illustrates that databases differ in the amount of information they provide and that orthology prediction results in a greater number of *in silico* protein interactions (physical as well as indirect). A next step to assign gene function is to identify co–regulated known and unknown genes.

Putative biological functions inferred from co-expression

Shared expression patterns of genes with unknown functions together with characterised plant genes and genes that have eukaryotic orthologs can hint towards biological functions of the 'unknown' genes. In this way, unknown genes can be associated with a specific stimulus or condition. This can be used to formulate novel hypotheses to test specifically

the role of a given gene in a given process and can give important clues to the function of unknown genes that are consistently found to be co–expressed with other genes in known pathways (Wille et al., 2004; Persson et al., 2005; Hirai et al., 2007). This will be exemplified with an evaluation of microarray studies on early and late *Alternaria alternata* f. sp. *lycopersici* AAL toxin–induced responses in Arabidopsis that links unknown genes to oxidative stress.

Produced by the fungus *A. alternata* f. sp. *lycopersic*i, the AAL toxin induces programmed cell death (PCD) in Arabidopsis and tomato (Gechev et al., 2004; Wang et al., 2006). PCD is a process that can be executed through diverse mechanisms (Doorn and Woltering, 2005). It can occur as a regular feature of the plant's life cycle, which is executed by a range of chloroplast, cytoplasmic and mitochondrial proteins (Doukhanina et al., 2006; Eckhard et al., 2006; Ichimura et al., 2006; Kim et al., 2006; Yao and Greenberg, 2006). However, plant cell death can also be induced by toxins of plant pathogens, as in the case of the AAL toxin. Microarray analysis of early and late AAL toxin–induced Arabidopsis responses displayed a complex up– and downregulation of genes involved in the production of reactive oxygen species, ethylene biosynthesis, ethylene signaling, cell wall modification, photosynthesis, growth and development (Gechev et al., 2004). In addition to the genes with known functions, nearly 30 genes with unknown function were discovered that were co–activated in response to cell death–associated oxidative stress. Similar stress–induced genes are also regulated during the response to menadione, a commonly used oxidative–stress inducer (Baxter et al., 2007). By clustering genes with known and unknown function with attention to the orientation of their regulation (induction or repression), genes with unknown functions can be classified together with previously annotated genes to specific processes, in this case, oxidative stress.

Determining the specificity of a given gene for a given process is not straightforward. However, the translation of transcriptional profiles into more standard signal transduction and other, easier to compare cellular pathways facilitates the assignment of genes to processes. Several *in silico* tools are available to pursue the conversion of transcriptional data into cellular pathways.

Accelerated identification of biological roles of unknown genes via functional clustering of cotranscribed genes

Co–expressed genes can be grouped based on functional annotation, such as GO annotation, by performing a gene–set enrichment analysis (GSEA) (Subramanian et al., 2005) or through the online tools offered by the DAVID (Database for Annotation, Visualization and Integrated Discovery) database (http://david.abcc.ncifcrf.gov/) (Dennis Jr et al., 2007). These tools enable researchers to convert microarray expression datasets into clusters of genes that participate in a similar biological process or pathway, supported by statistical tests. In this way, large datasets that consist of thousands of genes are reduced to dozens of biological processes (e.g. ‘response to oxidative stress’). Based on functional annotation, genes that regulate these processes can also be clustered. These lists of

biological processes are subsequently ranked in order of statistical significance. Interacting processes or pathways can be combined further, which results in a better understanding of the cellular context at the time of RNA extraction. Thus, functional clustering reveals activated pathways, and the integration of data that are available on interactions of the corresponding gene products (the proteins) can provide further leads towards biological functions.

Integration of protein–protein interaction networks with gene expression data identifies regulated cellular pathways

Several databases that aid *in silico* data analysis are available for plant research, such as STRING, BIND and ProLinks. Although some of these databases are user–friendly, intuitive to use and require only little input from the user (e.g. STRING), other databases (including DAVID and BIND) require more experience or input from the user (Box 2). Many databases provide data on protein–DNA and protein–protein interactions, including protein–complex formation, phosphorylation and ubiquitylation. These interactions can be based on different types of evidence: (i) biological assays, such as co–immunoprecipitations, (ii) high–throughput experiments, such as yeast two–hybrid analyses, (iii) *in silico*–predicted interactions based on orthology or sequence similarity and (iv) information extracted from literature databases (Hoffmann and Valencia, 2005; Jensen et al., 2006).

Protein interactions can be visualized in a network structure (Box 1), where the nodes represent proteins and the edges represent interactions. Color intensity of the network nodes reflects expression levels of the encoding gene. This way, in one view, gene–expression levels can be visualized in addition to protein interactions. Some software tools provide clickable nodes and edges that link out to database resources (e.g. Entrez Gene or PubMed), which enables the researcher to explore conveniently these interactions and the resulting networks. Although these tools already greatly assist plant scientists, next–generation commercial software tools, such as Ingenuity Pathway Analysis (IPA; Box 3), which are developed to analyze human and murine microarray datasets, take *in silico* analysis several steps further.

Software tools such as IPA are able to generate protein networks according to biological and functional categories, such as ‘cancer’, ‘cell death’ or ‘cell cycle’, and to make use of advanced network visualization to provide considerable amounts of information in an accessible and synoptic manner. This accelerates the identification of modulated cellular pathways or processes. Further integration of interacting networks makes possible the reconstruction of pathways that belong to a specific cellular process (e.g. ‘immune response’ or ‘cell death’). A clear overview of cellular processes that are differentially regulated under certain conditions helps us to understand the underlying biological changes and, thus, helps to assign genes to biological processes.

Limited pathway reconstruction possibilities for plants

To compare pathway reconstruction from plant and human datasets directly, two studies that investigated similar processes in these organisms were selected. The innate immune systems of plants and vertebrates share important features and function in similar ways to halt potential microbial pathogens (Ausubel, 2005; van Baarlen et al., 2007a; van Baarlen et al., 2007b). These similarities include molecular structures that are as diverse as the receptors involved in pathogen recognition, mitogen–associated protein kinase (MAPK)–based downstream signaling pathways, use of a respiratory burst and the production of antimicrobial peptides. Several microbial pathogens even have the capability to infect hosts as diverse as plants and humans (van Baarlen et al., 2007a; van Baarlen et al., 2007b). Analyzing plant and human transcriptional profiles upon challenge by the same microbial pathogen enables the comparison of tools for maximum data retrieval in plant and human biology. When comparing the information that can be obtained from published microarray studies that investigate the interaction of the pathogenic enterohaemorrhagic *E. coli* (EHEC) strain O157:H7 with Arabidopsis (Thilmony et al., 2006) and humans (Nau et al., 2002), it is evident that much more information can be retrieved from the human dataset (Box 3). Functional annotation is unavailable for many plant genes and knowledge on the participation of the gene products in co–regulated pathways or cellular functions is often absent, which limits understanding of the biology. The software tool IPA (www.ingenuity.com), which is developed to analyze human and murine Affymetrix genechips, enables the reconstruction and visualization of cellular pathways and functions, leading to models of a total cellular ‘context’ that can be captured into a cellular ‘snapshot’ to accelerate the interpretation and understanding of the underlying biology (Fig. 2).

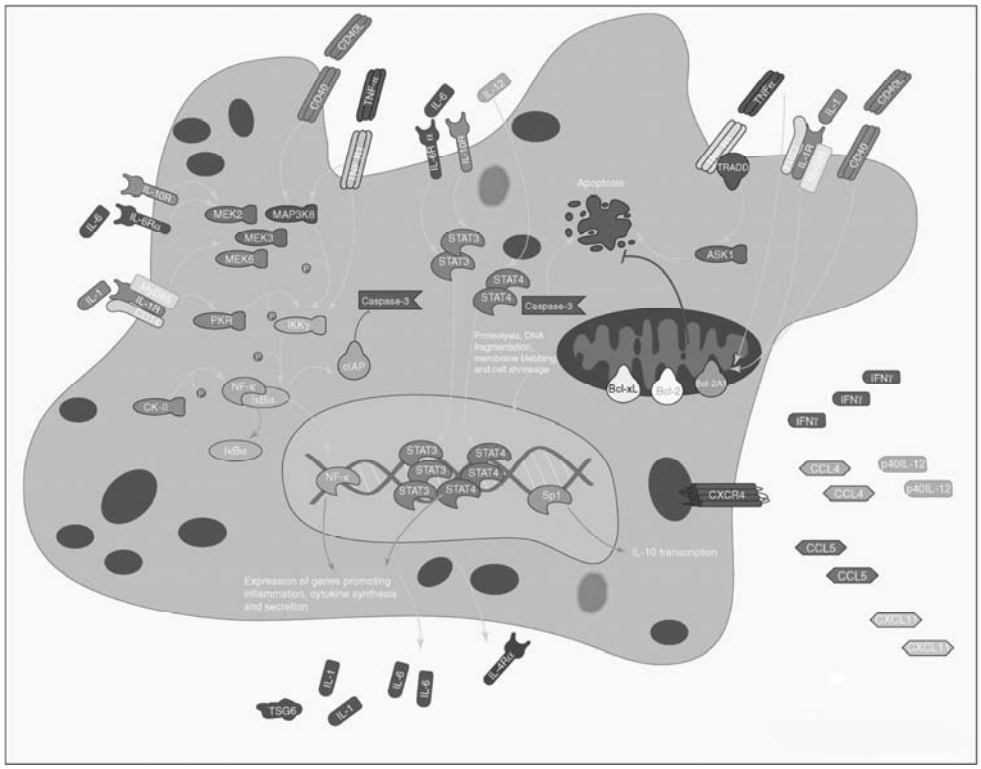

Figure 2. Human macrophage responses to EHEC *E. coli* O157:H7 in a cellular environment. The darker pink ovals indicate macrophage granules (lysosomes) (see page 214 for full color version). Proteins with a colored icon are upregulated with fold–changes over 1.5. The proteins depicted as an icon with a white background (Bcl–2 family) have an altered transcription but with a fold–change lower than 1.5; the biological relevance of this smaller transcriptional change is unclear. Proteins depicted as a 'transparent' icon are downregulated. Major extracellular cytokines with transcriptional fold–changes over 1.5, such as the interleukins and interferon–gamma, are also depicted. Visualization of individual proteins and cellular structures uses standard icons that can be downloaded from the BioCarta website (www.biocarta.com).

Box 2. Hands-on guide for array data handling. Not all databases and online tools are intuitive or easy to use for inexperienced users. Here, we provide general tips to make these tools accessible for novel users. Some databases allow the use of Affymetrix probe set IDs as input. Differentially expressed Affymetrix IDs can be uploaded into the NetAffx database (free registration) with the option 'Retrieve annotations for a probe list [Batch Query]' from the 'Expression' category. On the next page, the GeneChip array type should be indicated (for instance, ATH1–121501 for Arabidopsis) and the ID type that is used as input (the default Probe Set ID in this example). The input text file should be in ASCII format, listing the IDs in a single column (typically, a precompiled list from a spreadsheet program saved as ASCII or MS–DOS text). When choosing 'Annotation List' as view option, a table with general annotations (including GO) for each expressed gene is provided. Choosing 'Public Database References' as view option provides, among others, RefSeq Transcript IDs that can be used as input in the Bionetbuilder plug–in. Annotation lists and public database references can be exported as spreadsheet–compatible files by choosing the option TSV (Tab Separated Values) under 'Define your own format'. It is advisable to keep one spreadsheet with all different IDs together with the corresponding Affymetrix probe set IDs and fold–changes (or some other measure of differential gene expression) for use in other resources and software tools.

Databases such as STRING and DAVID contain (often precompiled) information regarding genes and their encoded proteins, including protein–protein interactions. STRING provides additional information of the representation of a given protein and its homologs and interacting partners across different species, incorporating information from scientific literature. Many conventional gene IDs or names are automatically recognized by STRING, which makes it user–friendly. DAVID computes a measure of enrichment; it computes whether the co–expressed genes participate more often than expected by mere chance in a given biological process or pathway, providing an ideal starting point for further expression data analysis. DAVID also provides information of gene participation (and enrichment) in known cellular pathways, and links out to KEGG and BioCarta pathway databases. It is somewhat less easy to use than STRING because the user has to select the ID type used as input, although DAVID is usually able to 'guess'.

Box 3. Comparison of pathway reconstruction in plant and human. Plant and vertebrate innate immunity shares important features and functions similarly to halt microbial pathogens, some of which are able to infect species of these unrelated hosts (Ausubel, 2005; van Baarlen et al., 2007a; van Baarlen et al., 2007b). To compare pathway reconstruction possibilities, the maximum of information retrieved from published microarray analyses of Arabidopsis (Thilmony et al., 2006) and human (Nau et al., 2002) responses to enterohaemorrhagic *E. coli* (EHEC) strain O157:H7 is compared.

Using whole–genome Affymetrix arrays, 350 differentially regulated genes were scored in EHEC–inoculated Arabidopsis (Thilmony et al., 2006). Using the Bionetbuilder plugin (http://err.bio.nyu.edu/cytoscape/bionetbuilder/) (Avila–Campillo et al., 2007), 305 proteins were retrieved yielding 223 interactions including protein–protein interactions and shared compounds. This is visualized in Cytoscape (www.cytoscape.org) (Shannon et al., 2003). A MAPK cascade that is also associated with oxidative stress (Gechev et al., 2004; Bae et al., 2006) is upregulated (data not shown). Unfortunately, primary gene and protein annotation cannot be directly retrieved within Bionetbuilder and expression data need to be manually integrated with protein information. Moreover, the nature of interactions between components is unclear. Additional plug–ins, such as APID2NET (http://bioinfow.dep.usal.es/apid/apid2net.html) and GOlorize (http://www.pasteur.fr/recherche/unites/Biolsys/GOlorize/index.htm) can be used to address these interactions. Published evidence for interactions can be searched via the Agilent Literature Search plugin (http://www.agilent.com/laboratories/research/mtl/projects/sysbio/sysinformatics/litsearch.html) for Cytoscape, although not all published literature is covered in this database. Using Affymetrix Hu6800 GeneChips, nearly a thousand differentially regulated genes were identified in EHEC–induced human macrophages (Nau et al., 2002). IPA (www.ingenuity.com) enables the reconstruction and visualization of cellular pathways and functions, and a cellular 'context' model can be constructed by listing significantly altered cellular functions and pathways in clickable diagrams that can be plotted in maps that depict direct and indirect interactions and protein associations. In the dataset prostaglandin synthesis (PTGS2; fatty acid–derived lipids that induce inflammatory responses) is upregulated (in red), whereas expression of central transcription factors, such as tumor protein 53 (TP53) and FBJ murine osteosarcoma viral oncogene homolog (FOS, involved in regulation of cell cycle), is downregulated (in green). Cellular reconstruction suggests that the cells actively regulate their cell cycle and synthesize immune–response–related factors as part of an antimicrobial response. Pro–inflammatory IL–1 (Interleukin 1) and NF–κB (Nuclear Factor–kappa B) are perceived with extracellular receptors and signaling passes the cytosol to the nucleus where secreted molecules such as interleukins are produced, showing regulation of genes encoding proteins that belong to different cellular locations. By further combining multiple regulated cellular pathways into a graphical cellular environment (www.biocarta.com), overall cellular activity and responses can be compiled (Fig. 2 in main text).

Pathway reconstruction in plants to define novel hypotheses

The fungus *C. fulvum* is the causal agent of tomato leaf mould (Thomma et al., 2005). During colonization of the host, *C. fulvum* secretes proteinaceous effector molecules that are thought to promote pathogen virulence (Thomma et al., 2005; van Esse et al., 2007), although the exact roles of these effectors are largely unknown. The effector molecule Avr2 is a protease inhibitor with an undefined role in pathogenesis (Rooney et al., 2005). Transgenic Arabidopsis plants that constitutively produce Avr2 were the subject of a microarray study. With the use of the tools described in this manuscript we found that Avr2 expression leads to modulation of genes mediating chloroplast and vacuolar function including photosynthesis and iron metabolism, genes regulating the biosynthesis of stilbenes, coumarines and lignin, activation of ubiquitylation processes, stimulation of transcriptional activity, regulation of genes involved in responses to biotic stimuli (hormones and endogenous), and genes participating in Ras GTPase signaling and leucine–rich–repeat (LRR) receptor protein signaling. This outcome is currently verified in biological experiments.

Conclusions

The available databases and software packages that are dedicated to plant science research maximize the amount of information that can be retrieved from DNA microarray datasets. Furthermore, tools are currently available that enable cellular pathway reconstruction from plant–gene expression data. As a result, cellular processes that might otherwise be obscured by the large amount of primary data can now be revealed.

However, with the current state of the art, a researcher requires substantial knowledge of the available software tools to retrieve the maximum amount of information. Moreover, the current integration of plant gene expression profiles and protein functional data and *in silico* possibilities for their visualization are not as advanced as in human biology. The various ways by which information is integrated, made available and visualized by tools such as IPA (Box 3) is superior to what can currently be achieved by software tools and databases dedicated to plant sciences. Two recent studies clearly show how pathway reconstruction using IPA contributes to the identification of altered pathways in mouse cells that respond to food–borne microbes and lead to the identification of pathways that are differentially activated in different mouse cell types (Giannakis et al., 2006; Lecuit et al., 2007). Such tools greatly facilitate the discovery of novel signaling and other cellular pathways.

However, limitations in software packages do not pose the largest obstacle for advanced microarray data analysis in plant sciences. Rather, the largest difference between what can be achieved *in silico* in plant and human biology is due to the lower amount of known general biological function(s) of plant genes and proteins. Remarkably, the information that can be extracted automatically from the major public databases is less than what is actually published in plant scientific literature. One way to improve plant microarray data analysis is by the further development of software tools that reach the level of data retrieval and integration that is achieved with a tool such as Ingenuity PA. For companies such as

Ingenuity, the incorporation of a plant model in their knowledge base requires more functional information on plant genes and proteins. Even then, addition of plant pathways to the Ingenuity tool might not be commercially viable as the marketplace might not support the investment required to incorporate plant pathways.

More comprehensive information per gene and protein is not only valuable for plant sciences, but also for comparative studies between plants and humans. Ideally, in future research, it should be possible to find and compare plant and human cellular pathways directly based on microarray data. The wealth of knowledge of human biology can then be better exploited by plant biologists, and full use can be made of the power of comparative biology.

Summary

Cladosporium fulvum (syn. *Passalora fulva*) is a biotrophic fungal pathogen that causes leaf mould of tomato (*Solanum esculentum*). **Chapter 1** is a "pathogen profile" describing the biology of the pathogen. During growth in the leaf apoplast, the intercellular space surrounding the mesophyll cells, the fungus secretes effector proteins that are thought to play a role in disease establishment. Eight of these effectors have been characterized in detail. For most of these effectors, cognate *C. fulvum* (*Cf*) resistance loci have been identified in tomato that mediate an immune response upon recognition of (the activity of) the cognate effector.

In **chapter 2**, a targeted proteomics approach to investigate the role of these effector proteins and to identify possible *in planta* targets is described. *C. fulvum* proteins were expressed as recombinant fusion proteins carrying various affinity–tags at either their C– or N–terminus. Although these fusion proteins were correctly expressed and secreted into the leaf apoplast, detection of affinity–tagged C. *fulvum* proteins failed and affinity–purification did not result in the recovery of these proteins. However, when using *C. fulvum* effector protein–specific antibodies, specific signals were obtained for the different proteins. It was therefore concluded that the stability of the *in planta* expressed recombinant fusion proteins is insufficient, which resulted in removal of the affinity–tag from the fusion proteins, irrespective of C– or N–terminal fusion or the nature of the affinity–tag. Similar observations were made when the fusion proteins were expressed in other Solanaceous species, but not when expressed in *Arabidopsis thaliana*.

Previous studies have demonstrated that Avr4 binds to chitin present in fungal cell walls, and that this binding by Avr4 can protect these cell walls against hydrolysis by plant chitinases. In **chapter 3** it is described that *Avr4*–expression in Arabidopsis results in increased virulence of several fungal pathogens with exposed chitin in their cell walls, whereas the virulence of a bacterium and an oomycete remained unaltered. Heterologous expression of *Avr4* in tomato increased the virulence of *Fusarium oxysporum* f. sp. *lycopersici*. Tomato GeneChip analysis was used to demonstrate that *Avr4*–expression in tomato results in the induced expression of only a handful of genes. Finally, silencing of the *Avr4* gene in *C. fulvum* decreased fungal virulence on tomato. In conclusion, **chapter 3** is the first report on the intrinsic function of a fungal avirulence protein that displays self-defense activity which is required for full pathogen virulence.

In **chapter 4**, a study on the intrinsic biological function of Avr2 is presented. The Avr2 effector interacts with the apoplastic tomato cysteine protease Rcr3, which is required for Cf–2–mediated immunity. In this chapter it is demonstrated that Avr2 is a genuine virulence factor of *C. fulvum*. Heterologous expression of *Avr2* in Arabidopsis resulted in enhanced susceptibility towards a number of extracellular fungal pathogens that include *Botrytis cinerea* and *Verticillium dahliae*, and microarray analysis of unchallenged Arabidopsis plants showed that *Avr2* expression triggered a global transcription profile that is reminiscent of pathogen challenge. Cysteine protease activity profiling revealed that

Avr2 inhibits multiple extracellular Arabidopsis cysteine proteases. In tomato, *Avr2* expression resulted in enhanced susceptibility not only towards natural Avr2–defective *C. fulvum* strains, but also towards *Botrytis cinerea* and *Verticillium dahliae*. Cysteine protease activity profiling in tomato revealed that Avr2 inhibits multiple extracellular cysteine proteases including Rcr3 and its close relative PIP1. Finally, silencing of the *Avr2* gene in *C. fulvum* significantly compromised fungal virulence on tomato. This all shows that Avr2 is a genuine virulence factor of *C. fulvum* that inhibits several cysteine proteases required for plant basal defense in tomato.

Chapter 5 describes the discovery and characterization of a novel effector protein of *C. fulvum*, Ecp6. To discover novel *C. fulvum* effectors that might play a role in virulence, two–dimensional polyacrylamide gel electrophoresis (2D–PAGE) was used to visualize proteins secreted during *C. fulvum*–tomato interactions. Three novel *C. fulvum* proteins were identified; CfPhiA, Ecp6, and Ecp7. CfPhiA shows homology to proteins found on fungal sporogenous cells called phialides, while Ecp6 contains lysine motifs (LysM domains), which are recognized as carbohydrate–binding modules. Finally, Ecp7 encodes a small, cysteine–rich protein with no homology to known proteins. Heterologous expression of *Ecp6* significantly increased the virulence of the vascular pathogen *Fusarium oxysporum* on tomato. Furthermore, by RNAi–mediated gene silencing it was demonstrated that Ecp6 is instrumental for *C. fulvum* virulence on tomato. Hardly any allelic variation was observed in the *Ecp6* coding region of a worldwide collection of *C. fulvum* strains. Although none of the *C. fulvum* effectors identified so far have obvious orthologs in other organisms, conserved Ecp6 orthologs were identified in various fungal species. Homology based modelling suggests that the LysM domains of *C. fulvum* Ecp6 may be involved in chitin binding.

Chapter 6 presents global transcriptional profiling study to compare transcriptional changes in tomato during compatible and incompatible interactions with the foliar pathogenic fungus *Cladosporium fulvum* and the soil–borne vascular pathogenic fungus *Verticillium dahliae*. Although both pathogens colonize different host tissues, they display significant commonalities in their infection strategies as they both penetrate natural openings and grow strictly extracellular without the formation of haustoria. Furthermore, in incompatible interactions with both pathogens resistance is conveyed by extracellular transmembrane receptors that belong to the class of receptor–like proteins. For each of the two pathogens, the transcriptomes of the compatible and incompatible interaction largely overlapped. However, the *C. fulvum*–induced transcriptomes showed little overlap with the *V. dahliae*–induced transcriptomes, as most genes were uniquely regulated by one of the two pathogens. This also applied to both incompatible interactions, despite defense activation by the same type of resistance protein. Remarkably, of the relatively small subset of genes that was regulated by both pathogens a large portion showed an inverse regulation; induced by one pathogen and repressed by the other. With pathway reconstruction, interacting networks of tomato genes implicated in photorespiration, hypoxia and glycoxylate metabolism were identified that were repressed upon infection with *C. fulvum*

and induced by *V. dahliae*. Similarly, auxin signaling was differentially affected by the two pathogens.

In **chapter 7**, the general discussion, the implications are of the data that are presented in this thesis are discussed for the use of *C. fulvum* as a model, and for fungal pathogens in general. Furthermore, the use of heterologous expression systems to study fungal effectors is briefly discussed. In several of the chapters presented in this thesis, the use of microarrays has been instrumental to investigate the biology of *C. fulvum* and the role of specific effectors secreted by the pathogen. Therefore, an overview of the currently available *in silico* tools for reconstruction of cellular pathways based on plant gene expression datasets is presented.

Samenvatting

Cladosporium fulvum (syn. *Passolora fulva*) is een biotrofe schimmel die de bladvlekkenziekte van tomaat (*Solanum esculentum*) veroorzaakt. **Hoofdstuk 1** is een literatuurstudie die de biologie van deze schimmel beschrijft. Tomatenplanten raken besmet met *C. fulvum* via luchtstromen of spatwater dat conidia van deze schimmel bevat. Wanneer een conidium aan de onderkant van het blad terecht komt kiemt deze en vormt loophyfen. Wanneer een loophyfe vervolgens een openstaand huidmondje tegenkomt dringt deze de plant binnen. Tijdens groei in de apoplast, de intracellulaire ruimte die de mesofylcellen omgeeft, scheidt *C. fulvum* zogenaamde effectoren (eiwitten) uit die het tot stand komen van een succesvolle infectie faciliteren. Tot nu toe zijn acht van deze effectoren gekarakteriseerd, en voor de meesten zijn *C. fulvum* (*Cf*) resistentie-loci geïdentificeerd in tomaat waarvan de produkten, ook wel receptor-like proteins (RLPs) genoemd, de bijbehorende effectoren direct of indirect herkennen. Na (in)directe herkenning van een effector door een Cf eiwit activeert de tomatenplant zijn afweer wat resulteert in een overgevoeligheidsreactie (in het Engels hypersensitive response of afgekort HR) waarbij lokale geprogrammeerde celdood de infectie een halt toeroept.

In **hoofdstuk 2** staan biochemische methoden bescheven om de doelwitten van de acht gekarakteriseerde *C. fulvum* effectoren in de plant te kunnen identificeren. *C. fulvum* effectoren zijn tot expressie gebracht als fusie-eiwitten met verscheidene affiniteits-merkers aan de C- of N- terminus. Deze merkers faciliteren de detectie en opzuivering van de eiwitten waaraan zij gefuseerd zijn. Ondanks het feit dat de fusie-eiwitten wel tot expressie kwamen en uitgescheiden werden in de apoplast, lukte het niet de fusie-eiwitten op basis van hun affiniteits-merkers op te zuiveren of te detecteren. Detectie was echter wel mogelijk met specifieke antilichamen tegen de *C. fulvum* effectoreiwitten zelf. Om de stabiliteit van de fusie-eiwitten te testen zijn deze *in vitro* geïncubeerd in apoplastvloeistof, waaruit bleek dat de merkerfusie niet stabiel was. De fusie-eiwitten bleken ook niet stabiel in andere Solanaceae soorten, maar wel in *Arabidopsis thaliana*.

Eerdere studies hebben aangetoond dat het effectoreiwit Avr4 bindt aan chitine in de schimmelcelwand en zo bescherming biedt tegen chitinases van tomaat. In **hoofdstuk 3** is aangetoond dat Avr4 een virulentie factor van *C. fulvum* is op grond van zijn beschermende werking tegen plant chitinases. *Avr4*-expressie in *A. thaliana* resulteerde in verhoogde virulentie van verschillende schimmels. In tegenstelling tot deze schimmels bleef de virulentie van bacterieën en een oömyceet, die geen chitine in hun celwand hebben, ongewijzigd. Heterologe expressie van *Avr4* in tomaat resulteerde in een verhoogde virulentie van de vaatparasiet *Fusarium oxysporum* f. sp. *lycopersici* die gevoelig is voor chitinases. Daarnaast is met behulp van micro-arrays bepaald dat *Avr4*-expressie in tomaat resulteert in de inductie van slechts enkele genen. Tenslotte is aangetoond dat silencing van *Avr4* in *C. fulvum* de virulentie van deze schimmel vermindert.

Van de *C. fulvum* effector Avr2 was bekend dat hij een interactie aangaat met het apoplastische cysteine-protease Rcr3, en dat deze interactie vereist is voor *Cf-2*-

gemedieerde resistentie. In **hoofdstuk 4** wordt aangetoond dat Avr2 bijdraagt aan virulentie van *C. fulvum* door de remming van meerdere cysteine-proteases die vereist zijn voor de basale afweer van tomaat tegen verscheidene schimmels. Heterologe expressie van *Avr2* in *A. thaliana* resulteerde in verhoogde vatbaarheid voor een aantal extracellulaire schimmelpathogenen, waaronder *Botrytis cinerea* en *Verticillium dahliae.* Uit een micro-array-analyse is gebleken dat het transcriptie-profiel van Avr2-producerende *A. thaliana* lijkt op dat van *A. thaliana* planten die door een pathogeen aangevallen worden. Avr2 verstoort dus niet een aantal algemene huishoudelijke processen in de plant, hetgeen tot een verhoogde vatbaarheid voor pathogenen zou kunnen leiden, maar specifiek de basale afweer. Via een biochemische analyse van de protease-activiteit is aangetoond dat Avr2 verschillende extracellulaire cysteine-proteases van *A. thaliana* remt. In tomaat resulteerde *Avr2*-expressie in een verhoogde vatbaarheid voor *C. fulvum, B. cinerea* en *V.dahliae*. Biochemische analyse van de cysteine-protease-activiteit toonde aan dat Avr2 verschillende cysteine-proteases remt waaronder het eerder genoemde Rcr3. Tenslotte is aangetoond dat silencing van het *Avr2* gen in *C. fulvum* resulteert in verminderde virulentie.

Hoofdstuk 5 beschrijft de identificatie van drie nieuwe eiwitten van *C. fulvum*, waarvan er één, Ecp6, in detail gekarakteriseerd is. Twee-dimensionale polyacrylamidegelelectroforese (2D-PAGE) is gebruikt om eiwitten te visualiseren die *C. fulvum* tijdens infectie van tomaat uitscheidt. Met behulp van deze techniek zijn de drie nieuwe *C. fulvum* eiwitten geïdentificeerd, CfPhiA, Ecp6, en Ecp7. CfPhiA vertoont homologie met structurele eiwitten die voorkomen op conidioforen van schimmels, terwijl Ecp6 lysine domeinen (LysM domeinen) bevat die betrokken zijn in de binding van koolhydraatmoleculen. Ecp7, tenslotte, is een klein cysteine-rijk eiwit met onbekende functie. Heterologe expressie van *Ecp6* verhoogde de virulentie van de vaatparasiet *F. oxysporum* f. sp. *lycopercici*. Verder is met behulp van silencing aangetoond dat *Ecp6* een bijdrage levert aan de virulentie van *C. fulvum* op tomaat. In een wereldwijde collectie van *C. fulvum* isolaten komt amper allelische variatie voor in de coderende regio van *Ecp6*. Geconserveerde Ecp6 orthologen zijn gevonden in verschillende schimmelsoorten. Dit is opmerkelijk omdat geen van de andere, tot nu toe bekende, *C. fulvum* effectoren duidelijke orthologen heeft in andere organismen. Modellering op basis van homologie met chitine-bindende LysM domeinen in andere organismen suggereert dat ook de LysM domeinen van Ecp6 chitine binden. Om de precieze functie van Ecp6 te achterhalen is echter meer onderzoek nodig.

In **hoofdstuk 6** wordt een studie van genoomwijde transcriptieprofielen in tomaat tijdens infectie door *C. fulvum* en *V. dahliae* gepresenteerd. Hierbij is gekeken naar veranderingen in de gen-expressie van tomaat tijdens een compatibele en incompatibele interactie met de bladschimmel *C. fulvum* en de vaatparasiet *V. dahliae.* Ondanks het feit dat deze schimmels andere weefsels van hun waardplant koloniseren vertonen ze een aantal overeenkomsten. Beide schimmels dringen de plant binnen via natuurlijke openingen en groeien stikt extracellulair zonder vorming van haustoria (voedingsstructuren). Verder worden beide

schimmels tijdens incompatibele interacties herkend door resistentie-eiwitten die behoren tot de klasse van RLPs.

Bij zowel *C. fulvum* als *V. dahliae* geïnduceerde transcriptieprofielen was een grote overlap waar te nemen tussen compatibele en incompatibele interacties. Echter, de *C. fulvum*-geïnduceerde transcriptieprofielen vertoonden weinig overlap met de *V. dahliae*-geïnduceerde profielen. Opmerkelijk is dat binnen de kleine subset van genen die in beide interacties differentiëel gereguleerd werd, veel genen een reciprook expressie patroon vertoonden; de genen die geïnduceerd worden door de ene schimmel, worden onderdrukt door de andere. Op deze set van genen is een netwerk-analyse uitgevoerd die genen betrokken in eenzelfde biologisch proces groepeert, en de onderlinge relatie van genen binnen een groep laat zien. Zo zijn groepen genen geïdentificeerd die zijn betrokken bij fotorespiratie, hypoxia, glycoxylaat metabolisme en auxine signaaltransductie.

In **hoofdstuk 7**, de algemene discussie, wordt kort ingegaan op het belang van de fytopathologie voor de hedendaagse landbouw. Verder worden de implicaties van de bevindingen die gepresenteerd zijn in dit proefschift besproken in de context van het gebruik van *C. fulvum* als een modelpathogeen. Ook wordt het gebruik van heterologe expressiesystemen om effectoren van pathogenen te bestuderen besproken. In een aantal hoofdstukken van dit proefschrift is het gebruik van micro-arrays essentiëel gebleken om de functie van bepaalde effectoren in de biologie van *C. fulvum* te achterhalen. Daarom is in dit hoofdstuk een overzicht gepresenteerd van de beschikbare *in silico*-programma's om cellulaire netwerken te reconstrueren, gebaseerd op beschikbare genoomwijde expressiedata van planten.

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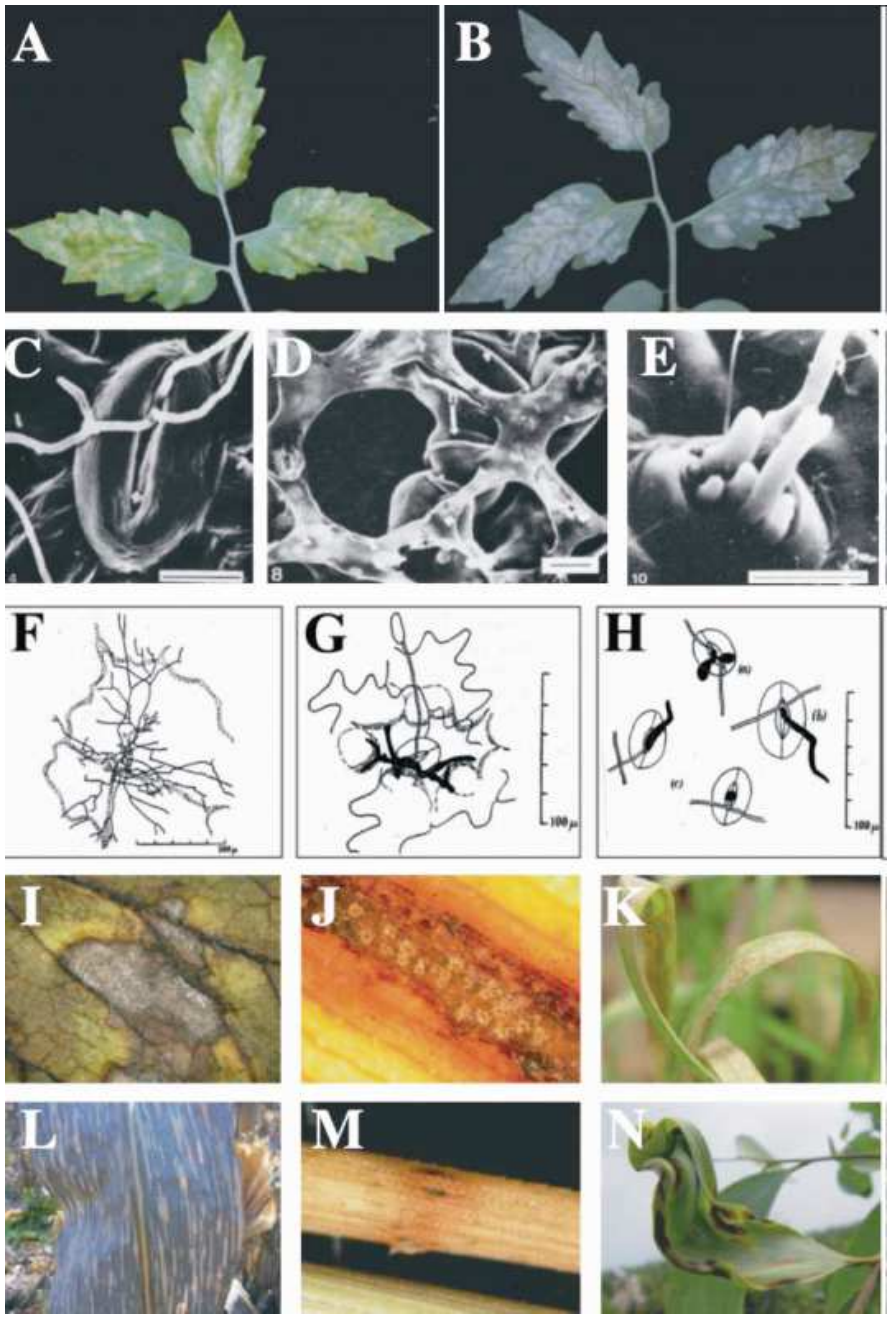
Curriculum Vitae

Hendrikus Pieter (Peter) van Esse werd geboren op 14 Juli 1980 te Elst (Utr). Na het behalen van zijn HAVO diploma in 1997 begon hij aan de HLO opleiding Medische Biotechnologie aan de Hogeschool van Utrecht. Tijdens zijn afstudeeronderzoek bij Plant Research International heeft hij gewerkt aan de flavonoïd biosynthese in tomaat onder begeleiding van Elio Schijlen en dr. ir. Arnaud Bovy. Na het succesvol afronden van zijn HLO opleiding begon hij in 2001 aan de studie Biotechnologie aan de Wageningen universiteit. Tijdens zijn afstudeeronderzoek heeft hij gewerkt aan tomato spotted wilt virus bij de leerstoelgroep Virologie onder begeleiding van Christiano Lacorte en dr. ir. Marcel Prins. Na het voltooien van zijn M. Sc. opleiding in januari 2003 heeft hij als assistent in opleiding onderzoek gedaan aan de *C. fulvum*-tomaat interactie bij de leerstoelgroep Fytopathologie aan de Wageningen Universiteit, onder begeleiding van dr. ir. Bart Thomma en prof. dr. ir. Pierre de Wit. In maart 2008 werd hij aangesteld als postdoctoraal onderzoeker bij de vakgroep Fytopathologie aan de Wageningen Universiteit om binnen de onderzoeksgroep van dr. ir. Bart Thomma onderzoek te verrichten aan de biologie van de vaatparasiet *Verticillium dahliae*.

Full color figures

Full color figures chapter 1:



Full color figures **. 198**

Figure 1 (previous page): Physiology of the *Cladosporium fulvum* infection on host and non-host plants (A–H) and typical symptoms on host plants caused by other plant pathogenic Mycosphaerellaceae as found in nature (I–N). (A) Adaxial side of a tomato leaf (MoneyMaker *Cf–0*) 18 days after inoculation with a compatible race of *C. fulvum.* Distinctive yellow spots can be seen as a result of dead palisade parenchyma cells. (B) Abaxial side of a tomato leaf *(*MoneyMaker *Cf–0)* 18 days after inoculation with a compatible race of *C. fulvum*. White mold can be seen developing into light brown patches where sporulation takes place. (C–E) SEM images from *C. fulvum*–infected tomato leaves in a compatible interaction at different timepoints after inoculation (pictures are taken from: de Wit, P.J.G.M. Light and scanning–electron microscopic study of infection of tomato plants by virulent and avirulent races of *Cladosporium fulvum* Neth. J. Plant Pathol. (1977) 83, 109–122, with permission). (C) *C. fulvum*–infected tomato leaf in a compatible interaction 2 days post inoculation with fungal hyphae entering a stoma. (D) *C. fulvum*–infected tomato leaf in a compatible interaction 7 days post inoculation. In the spongy mesophyll hyphae (h) grow in close contact with the plant cells. (E) *C. fulvum*–infected tomato leaf in a compatible interaction 12 days post inoculation. Young conidiophores emerging from the stomata are observed. (F–H) Drawings upon microscopic analysis of lactophenol–stained leaf material of several plant species upon inoculation with *C. fulvum* (drawings are reproduced from: Bond, T.E.T. Infection experiments with *Cladosporium fulvum* Cooke and related species.Ann. Appl. Biol. (1938) 25, 277–307, by permission of Oxford University Press). (F) Growth of *C. fulvum* mycelium in the tomato cultivar 'Giant red' 7 days after inoculation. The growth is characterized by long runner hyphae that pass between spongy mesophyll cells to send out ascending branches. (G) Limited growth of mycelium in *Hyoscyamus niger* (Solanaceae) 6 days after inoculation. Fungal growth does not go further than the substomatal cavity and a ring of discolored cells is observed. (H) Penetration of *C. fulvum* in so–called inappropriate hosts (or non–hosts) 6 days after inoculation: *Anthirrhinum majus* (a), *Bryonia dioica* (b) and *Callistephus* sp. (c). Mycelium is confined to single peg–like branches. (I) *Cercospora beticola* sporulating on sugarbeet leaves (*Beta vulgaris*). (J) Fasciculate conidiophores of *Pseudocercospora fijiensis* on banana (*Musa*) leaves. (K) Pycnidia of *Mycosphaerella graminicola* on wheat. (L) Angular leaf spots of *Cercospora zeae–maydis* on maize (*Zea mays*). (M) Conidiomata of *Dothistroma septospora*, causing red band needle disease of *Pinus* sp.(N) *Passalora perplexa* causing Crassicarpa leaf blight on *Acacia crassicarpa*.

Full color figures chapter 2:

Figure 2. PVX-mediated expression of affinity-tagged *Cladosporium fulvum* effector protein leads to production of biologically active proteins. His_6–FLAG–tagged Ecp2 is expressed and targeted towards the apoplast of tomato leaves by making use of a binary potato virus X (PVX)–based expression system. (A) Typical spreading necrosis phenotype in a tomato plant carrying the corresponding *Cf–Ecp2* resistance gene 14 days post inoculation(DPI). (B) Phenotype of a tomato *Cf–0* plant, not carrying *Cf*–resistance genes, 14 days post PVX–inoculation. (C) Injection of AF isolated from a tomato *Cf–0* plant inoculated with a binary PVX vector encoding His_6–FLAG–tagged Ecp2 in a leaf of a *Cf–Ecp2* tomato plant. (D) Injection of AF solated from a tomato *Cf–0* plant inoculated with a binary PVX vector encoding His_6–FLAG–tagged Ecp2 in a leaf of a tomato *Cf–0* plant.

Full color figures chapter 3:

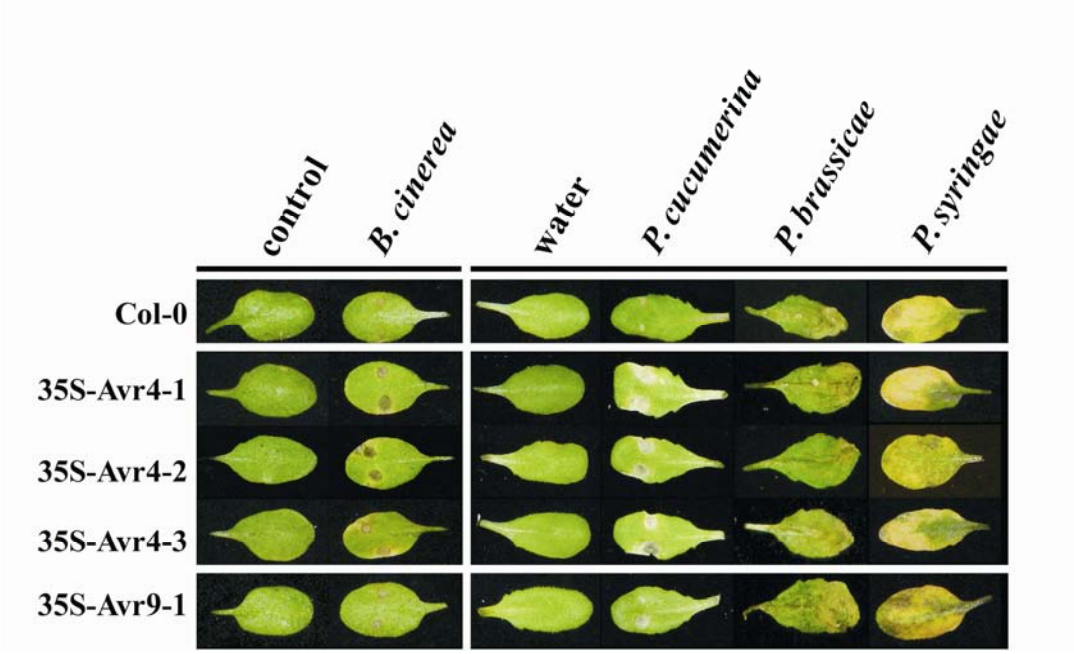


Figure 2. Avr4-producing Arabidopsis is more susceptible to several fungal pathogens. Typical symptoms caused by *Botrytis cinerea, Plectosphaerella cucumerina, Phytophthora brassicae* and *Pseudomonas syringae* on four–week–old plants of three independent Avr4–producing Arabidopsis lines at four days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

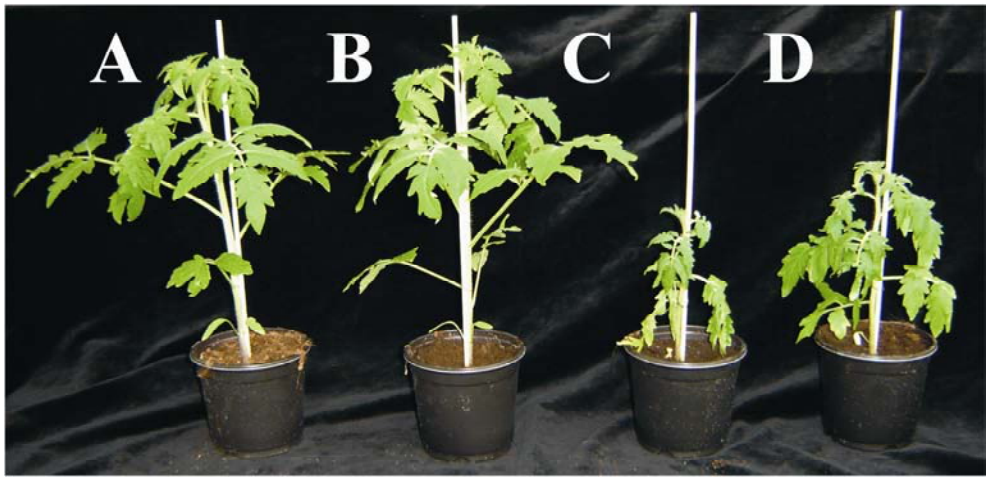


Figure 4. Heterologous expression of *Avr4* in tomato results in increased susceptibility to *Fusarium oxysporum* f. sp. *lycopersici*. Typical symptoms of disease after inoculation of four–week–old Avr4–producing tomato plants with *Fusarium oxysporum* f. sp. *lycopersici* at 14 days post inoculation.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

Full color figures **. 202**

Full color figures chapter 4:

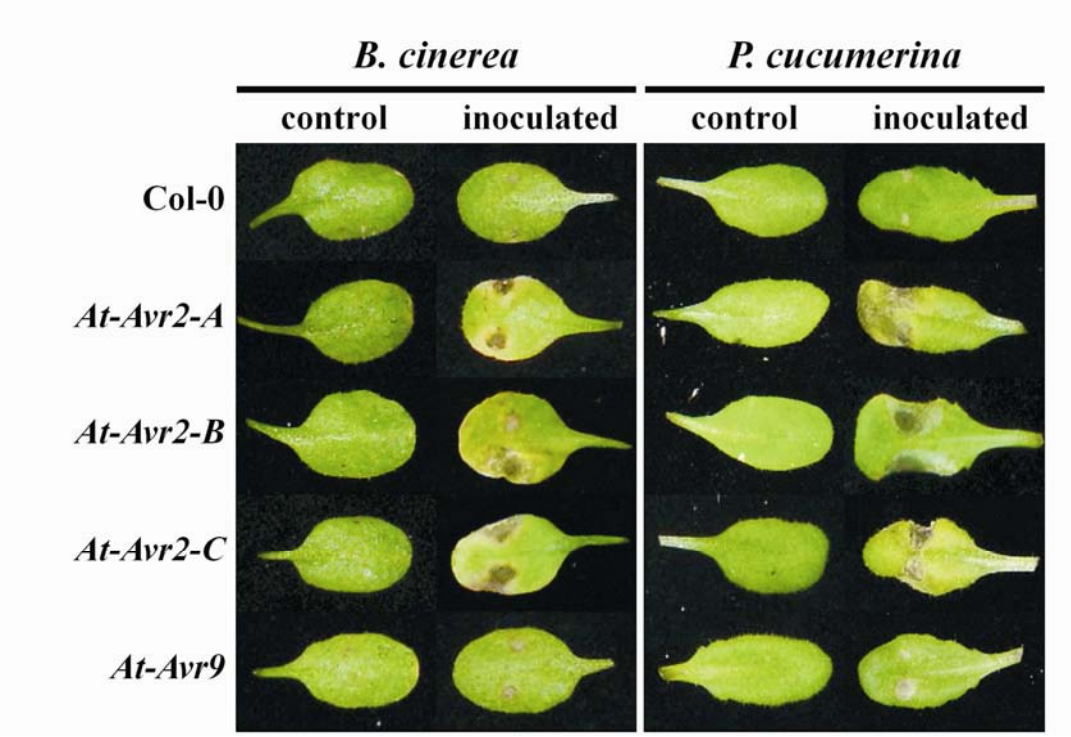


Figure 1. *Avr2*–expressing Arabidopsis is more susceptible to the fungal pathogens *Botrytis cinerea* and *Plectosphaerella cucumerina*. Typical symptoms caused by *B. cinerea* and *P. cucumerina* on four–week–old plants of three independent *Avr2*–expressing Arabidopsis lines (*At–Avr2–A* to *–C*) at four days post inoculation. Typical symptoms on the parental Col–0 line and an *Avr9*–expressing transgenic line (*At–Avr9*) are shown as control.

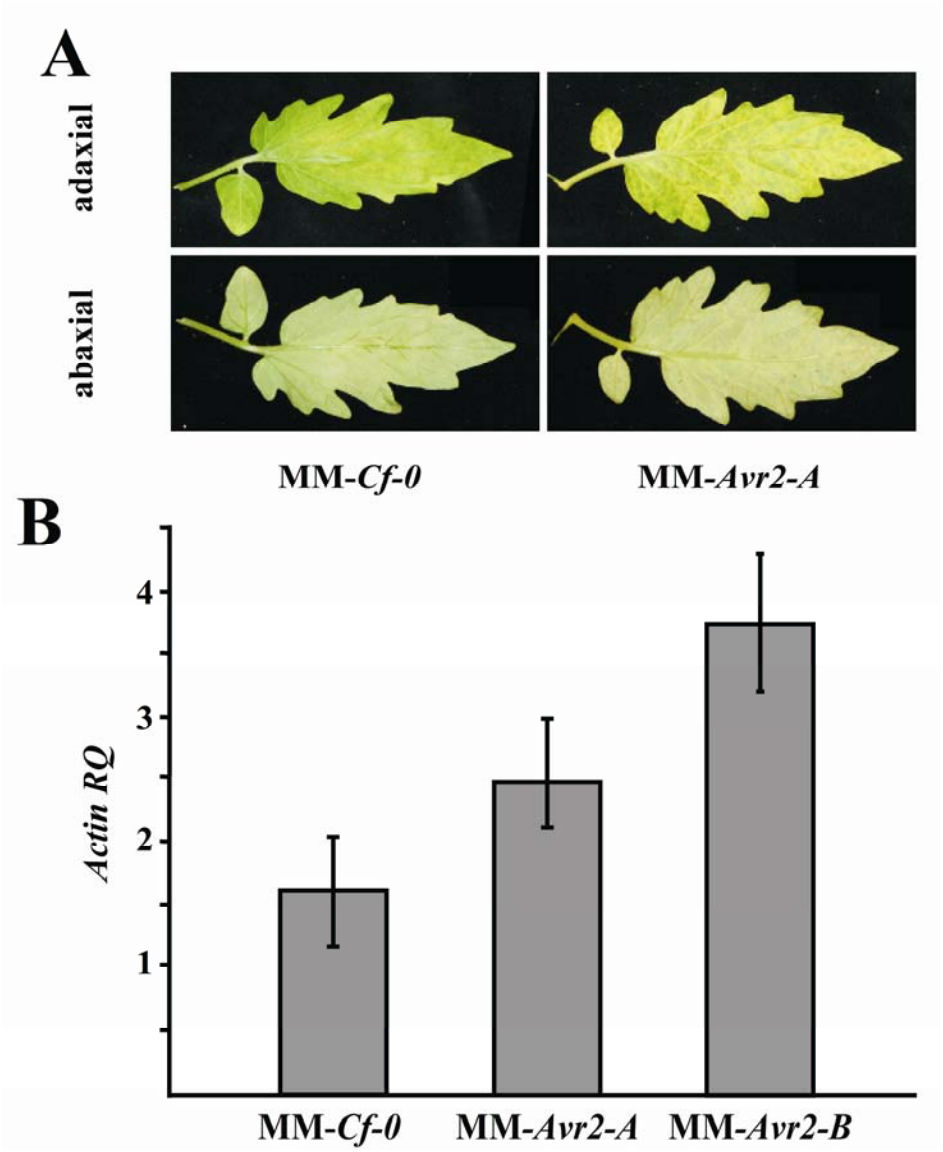


Figure 3. *Avr2*–expressing tomato is more susceptible to Race 2 *Cladosporium fulvum*. (A) Typical disease symptoms developed on the adaxial and abaxial leaf sides after inoculation with a *C. fulvum* race 2 strain of *Avr2*–expressing tomato (MM–*Avr2–A*), when compared to the progenitor line (MM–*Cf–0*) at 11 days post inoculation. (B) Quantitative real–time PCR of fungal colonization by comparing *C. fulvum* actin transcript levels (measure for fungal biomass) relative to tomato actin transcript levels (for equilibration) on two independent *Avr2*–expressing tomato transformants (MM–*Avr2–A* and MM–*Avr2–B*) when compared to the parental line (MM–*Cf–0*) at 11 days post inoculation.

Full color figures **. 204**

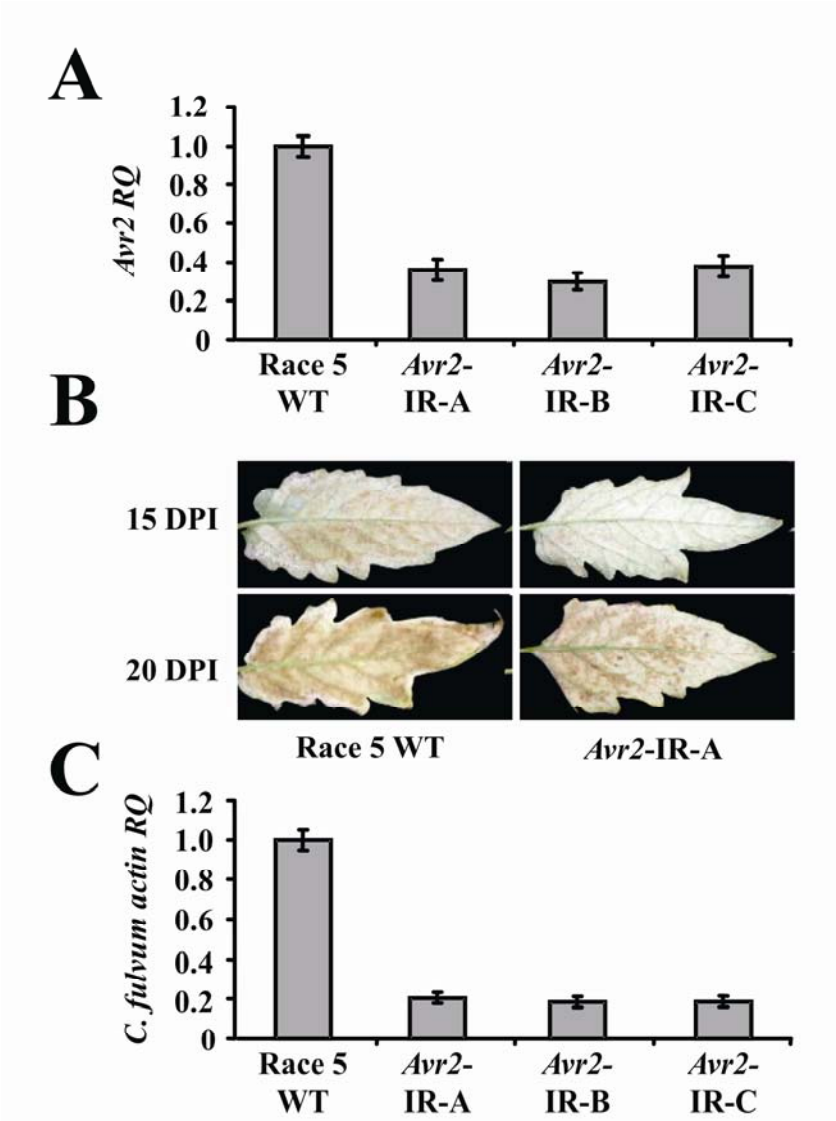


Figure 4. Silencing of *Avr2*–expression in *Cladosporium fulvum* decreases virulence on tomato. (A) Quantitative real–time PCR of *Avr2* transcript levels during a compatible interaction with MM–Cf–0 tomato. *Avr2* transcript levels are shown in three independent *Avr2*–silenced *C. fulvum* transformants (*Avr2*–IR–A to –C) when compared to the parental strain (Race 5 WT) at 11 days post inoculation. (B) Typical disease symptoms developed after inoculation of MM–*Cf–0* tomato plants with the *Avr2*–silenced *C. fulvum* transformant Avr2–IR–A, as typical example, when compared to the parental strain (Race 5 WT), monitored at 15 and 20 days post inoculation. (C) Quantitative real–time PCR of fungal colonization by comparing *C. fulvum* actin transcript levels (as a measure for fungal biomass) relative to tomato actin transcript levels (for equilibration) for three independent *Avr2*–silenced *C. fulvum* transformants (*Avr2*–IR–A to –C) when compared to the parental strain (Race 5 WT) at 11 days post inoculation.

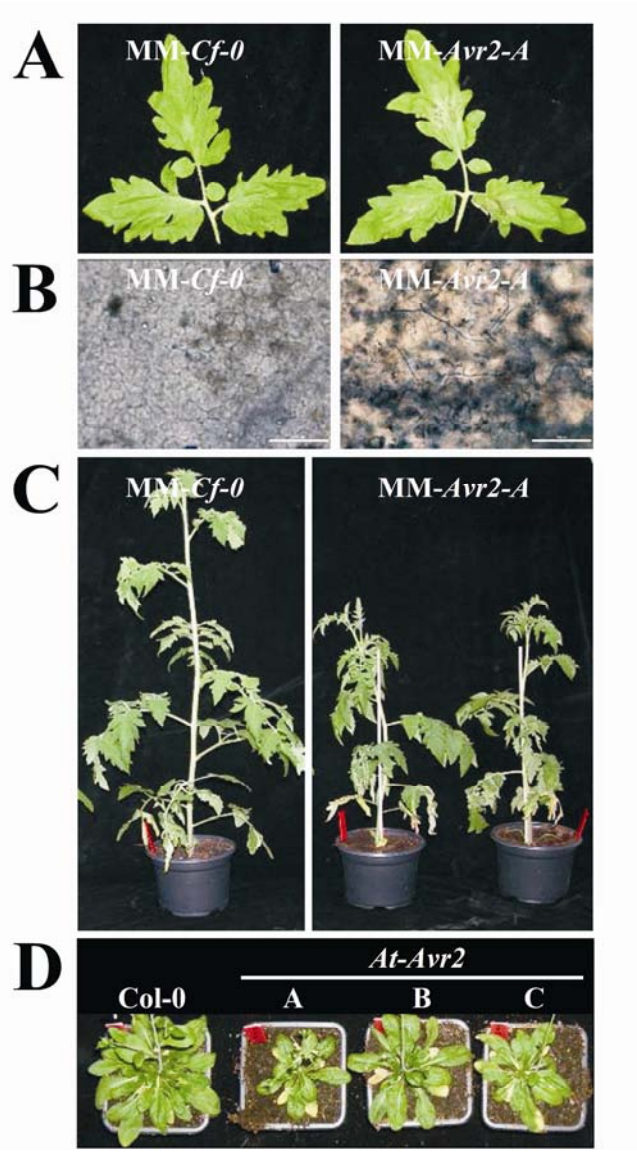


Figure 5. *Avr2*–expressing plants are more susceptible to *Verticillium dahliae* and *Botrytis cinerea*. (A) Typical appearance of *Avr2*–expressing tomato leaves (MM–*Avr2–A*) when compared to the parental line (MM–*Cf–0*) upon inoculation with *B. cinerea* at 60 hours post inoculation. (B) Microscopic observation of *Avr2*–expressing tomato leaves (MM–*Avr2–A*) when compared to the parental line (MM–*Cf–0*) upon inoculation with *B. cinerea* at 48 hours post inoculation after staining of fungal hyphae and death plant cells with trypan blue. (C) Typical appearance of *Avr2*–expressing tomato plants (MM–*Avr2–A*) when compared to the parental 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 parental line (Col–0) at two weeks post inoculation.

Full color figures **. 206**

Full color figures chapter 5:

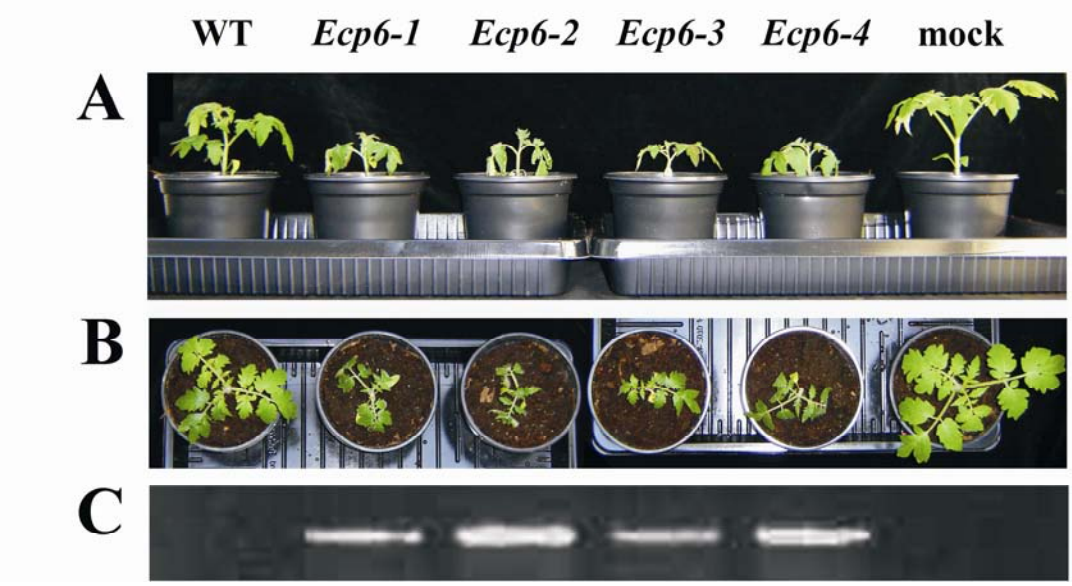


Figure 4. Symptoms caused by wild-type *Fusarium oxysporum* f. sp. *lycopersici* and heterologous *Ecp6* over-expression transformants on susceptible tomato. (A) B, Side view (A) and top view (B) of the disease phenotype caused by *Fusarium oxysporum* f. sp. *lycopersici* wild–type (WT) and four independent heterologous *Ecp6* over–expression transformants (*Ecp6–1* to *Ecp6–4*) on susceptible tomato MoneyMaker plants when compared to mock–inoculated tomato (mock) at 14 days post inoculation. (C) RT–PCR to detect *in planta* transcription of heterologously expressed *C. fulvum Ecp6* in *Fusarium oxysporum* f. sp. *lycopersici* wild–type (WT) and four independent heterologous *Ecp6* over–expression transformants (*Ecp6–1* to *Ecp6–4*) on susceptible tomato MoneyMaker plants when compared to mock–inoculated tomato (mock) at 14 days post inoculation.

Full color figures chapter 6:

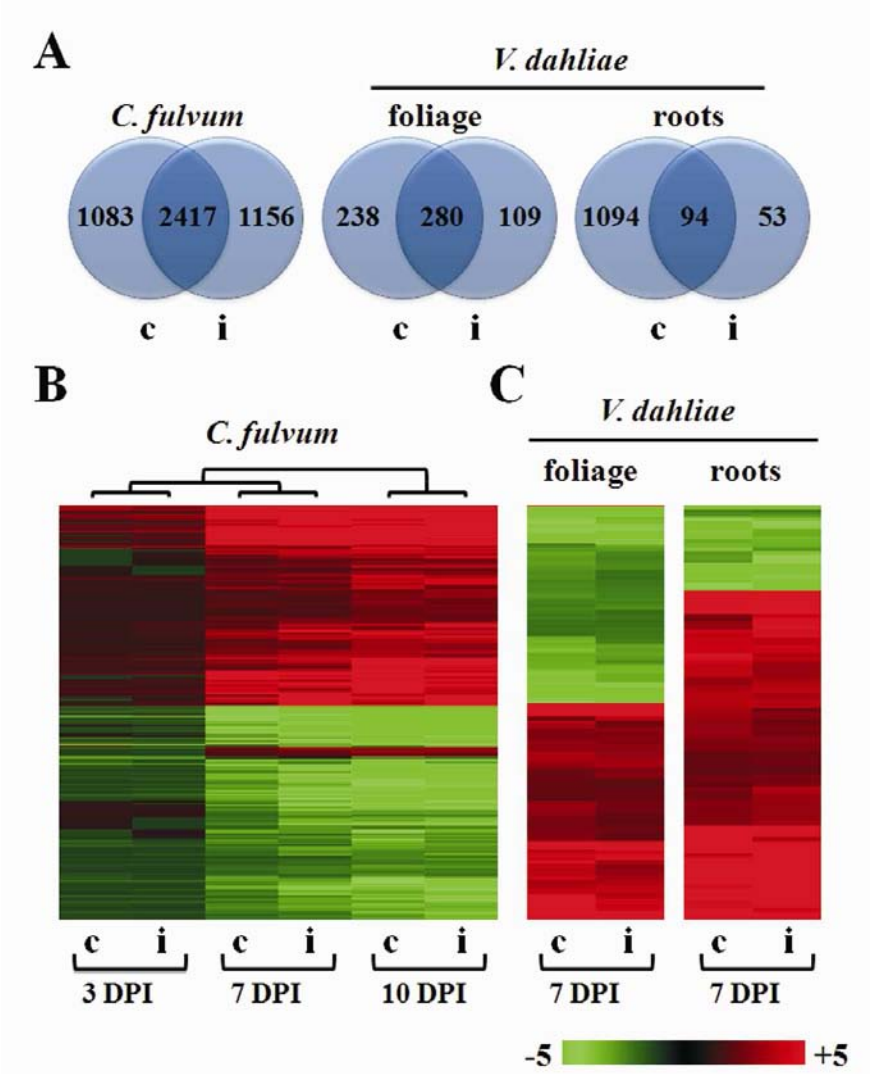


Figure 1. Differentially regulated tomato gene sets during compatible and incompatible interactions with *Cladosporium fulvum* and *Verticillium dahliae.* (A) Venn diagrams displaying specificity and overlap in differentially regulated gene sets between compatible (c) and incompatible (i) interactions with tomato. (B) Expression profiles of differentially regulated genes in the compatible (c) and incompatible (i) *C. fulvum*–tomato interaction at 3, 7 and 10 days post inoculation (DPI), respectively. (C) Expression profiles of differentially regulated genes in foliage and roots in the compatible (c) and incompatible (i) *V. dahliae*–tomato interaction at 7 DPI in foliage and roots.

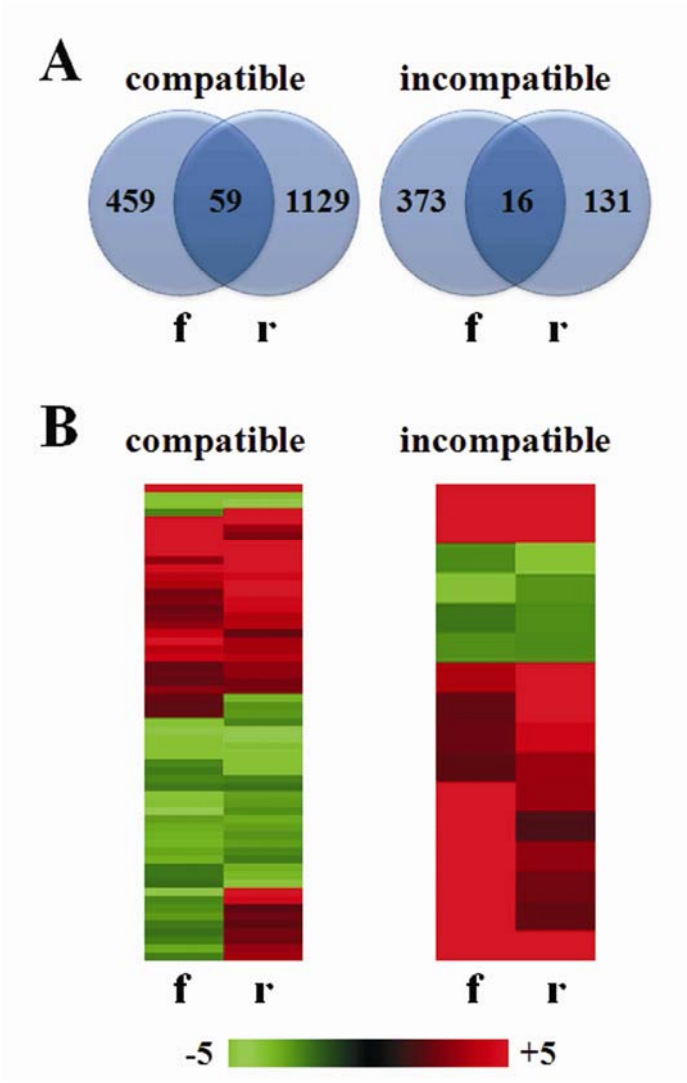


Figure 2. Differentially regulated tomato gene sets in foliar and root tissues during a compatible and incompatible interaction with *Verticillium dahliae.* (A) Venn diagrams displaying specificity and overlap in differentially regulated gene sets the compatible and an incompatible interaction between foliar tissues (f) and roots (r). (B) Expression profiles of differentially regulated genes in the compatible and incompatible interaction between foliar tissues (f) and roots (r).

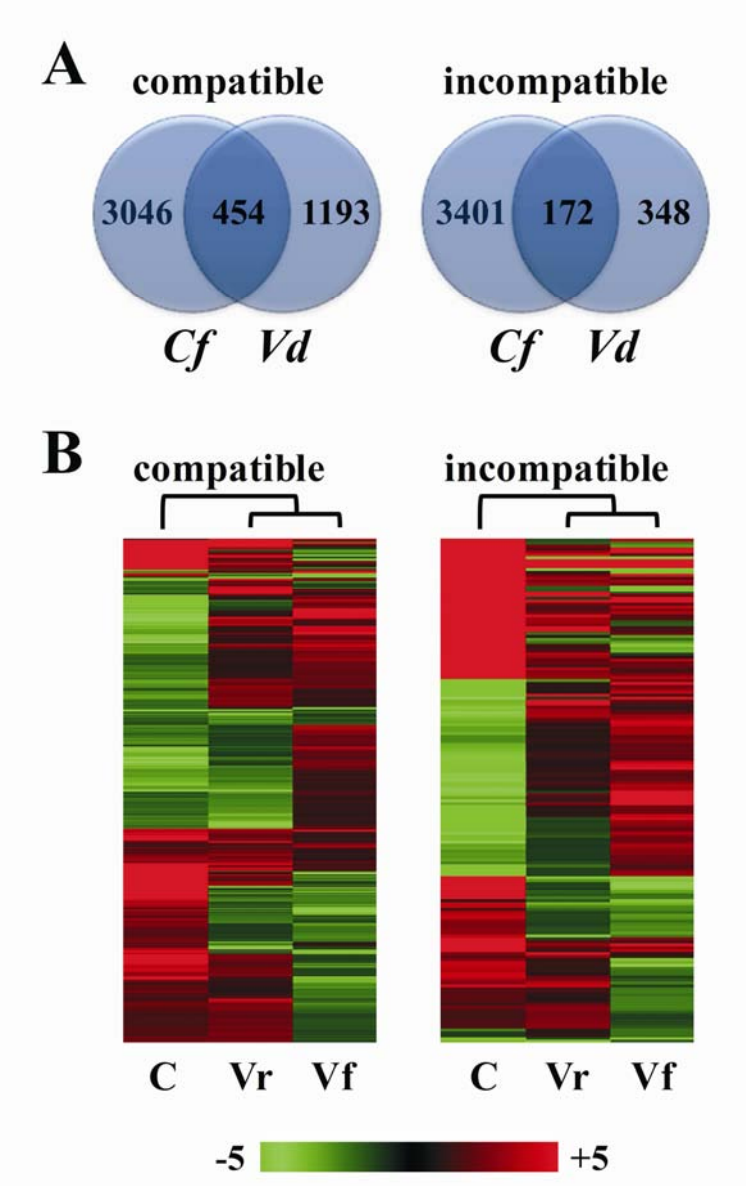


Figure 3. *Cladosporium fulvum* and *Verticillium dahliae* induce largely different transcriptomes. (A) Venn diagrams displaying specificity and overlap in compatible and incompatible tomato interactions with *C. fulvum* (*Cf*) and *V. dahliae* (*Vd*). (B) Expression profiles of differentially regulated genes in compatible and incompatible tomato interactions with *C. fulvum*-infected tomato (C), *V. dahliae*-infected foliar (Vf) and root (Vr) tissues at 7 DPI.

Full color figures **. 210**

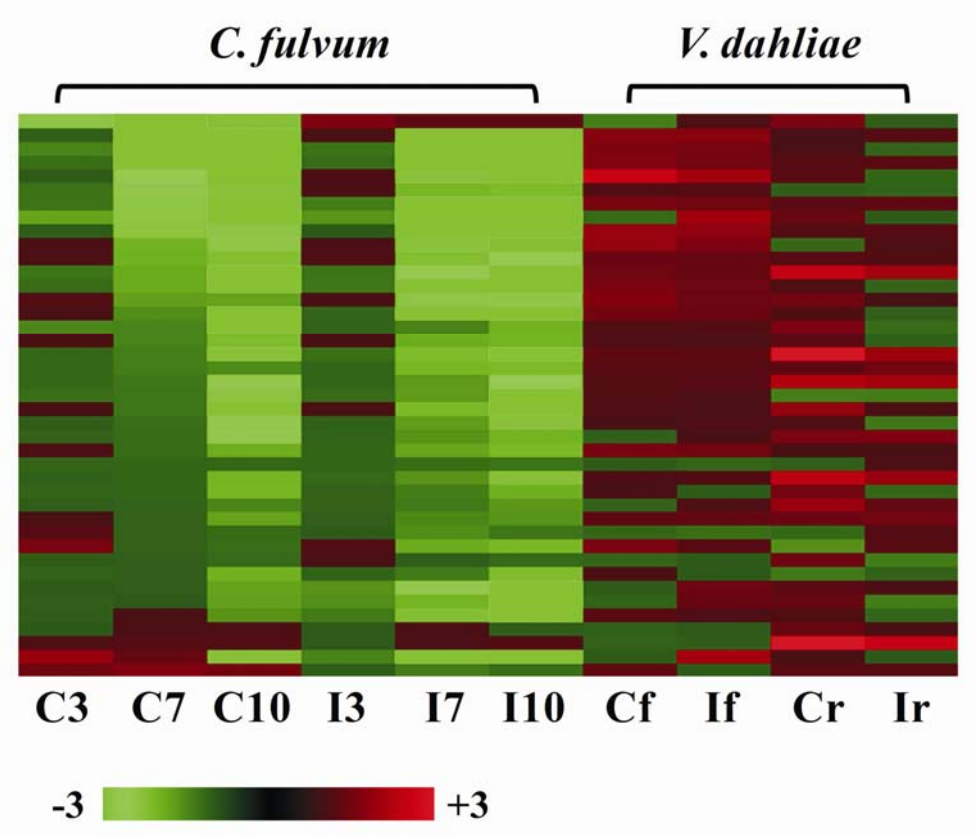


Figure 4. ***Cladosporium fulvum*** **and** ***Verticillium dahliae*** **have inverse effects on photosynthesis in tomato.** Transcriptional regulation of the 41 photosynthesis genes that are represented on the tomato GeneChip in the interactions of tomato with *C. fulvum* and *V. dahliae* at various time points. The different lanes represent the compatible tomato interaction with *C. fulvum* at 3, 7 and 10 DPI (C3, C7 and C10, respectively), the incompatible interaction with *C. fulvum* at the same time points (I3, I7 and I10, respectively), the compatible and incompatible tomato interaction with *V. dahliae* in foliage at 7 DPI (Cf and If, respectively), and the compatible and incompatible tomato interaction with *V. dahliae* in roots at 7 DPI (Cr and Ir, respectively).

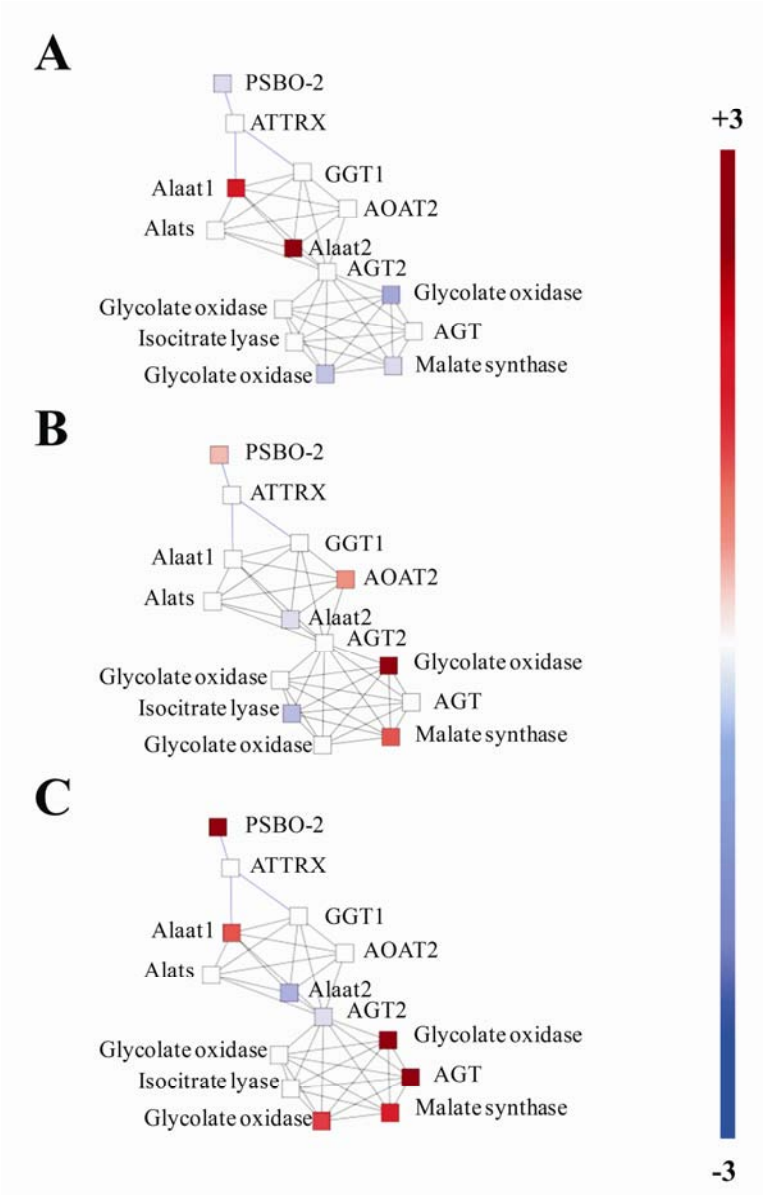


Figure 5. Pathway reconstruction reveals protein interaction networks. Responses of susceptible tomato upon inoculation of *Cladosporium fulvum* were compared those of tomato inoculated with *Verticillium dahliae* using the BioNetBuilder plug-in. A protein interaction network implied in both interactions was retrieved and visualized in Cytoscape. Subsequently, expression data monitored with the tomato GeneChip were grafted onto the network. (A) Network response upon inoculation with *C. fulvum*. (B) Network response in foliar tissues upon inoculation with *V. dahliae*. (C) Network response in root tissues upon inoculation with *V. dahliae*.

Full color figures **. 212**

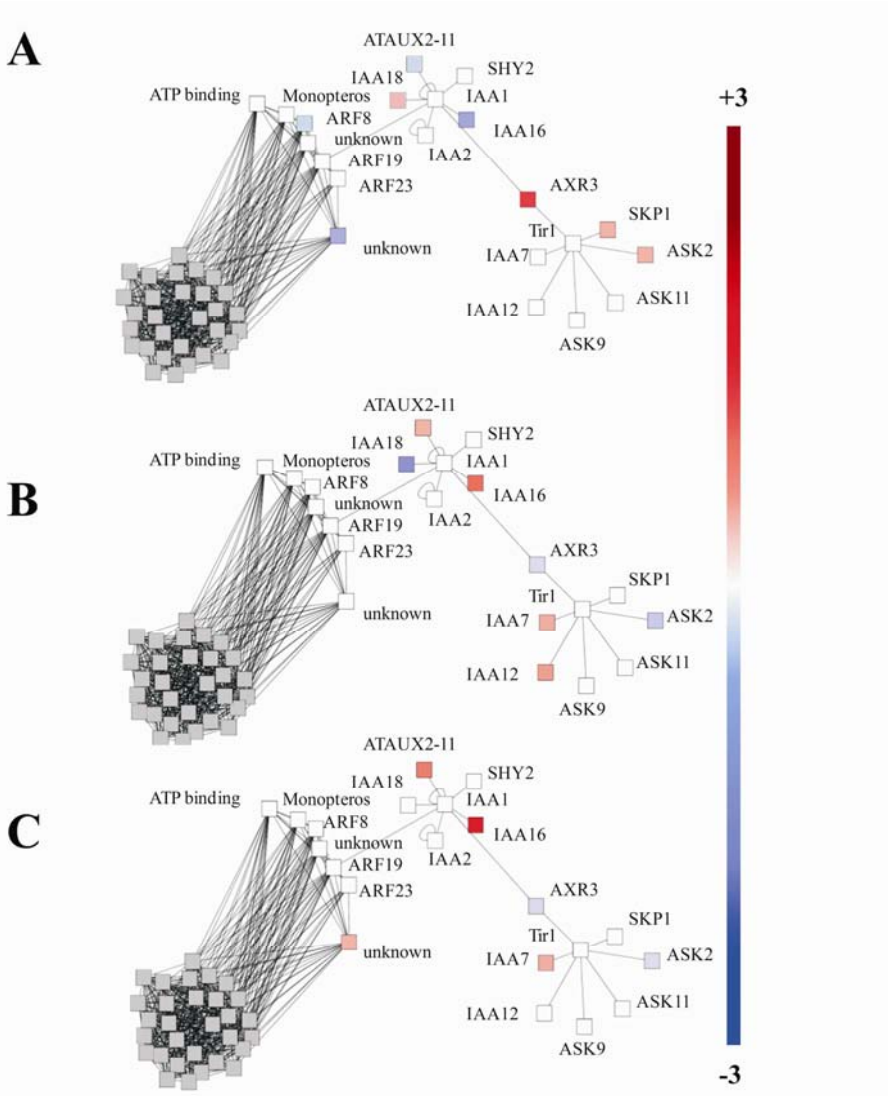


Figure 6. Tomato auxin signaling cascade in response to *Cladosporium fulvum* and *Verticillium dahliae* inoculation. Responses of susceptible tomato upon inoculation of *C. fulvum* were compared those of tomato inoculated with *V. dahliae* using the BioNetBuilder plug–in. A small protein interaction network implied in auxin signalling was retrieved. After 3 iterative steps in the BioNetBuilder, a protein interaction network was obtained that is visualized in Cytoscape. Subsequently, expression data monitored with the tomato GeneChip were grafted onto the network. Grey nodes indicate proteins for which expression was not considered. (A) Network response upon inoculation with *C. fulvum*. (B) Network response in foliar tissues upon inoculation with *V. dahliae*. (C) Network response in root tissues upon inoculation with *V. dahliae*.

Full color figures chapter 7

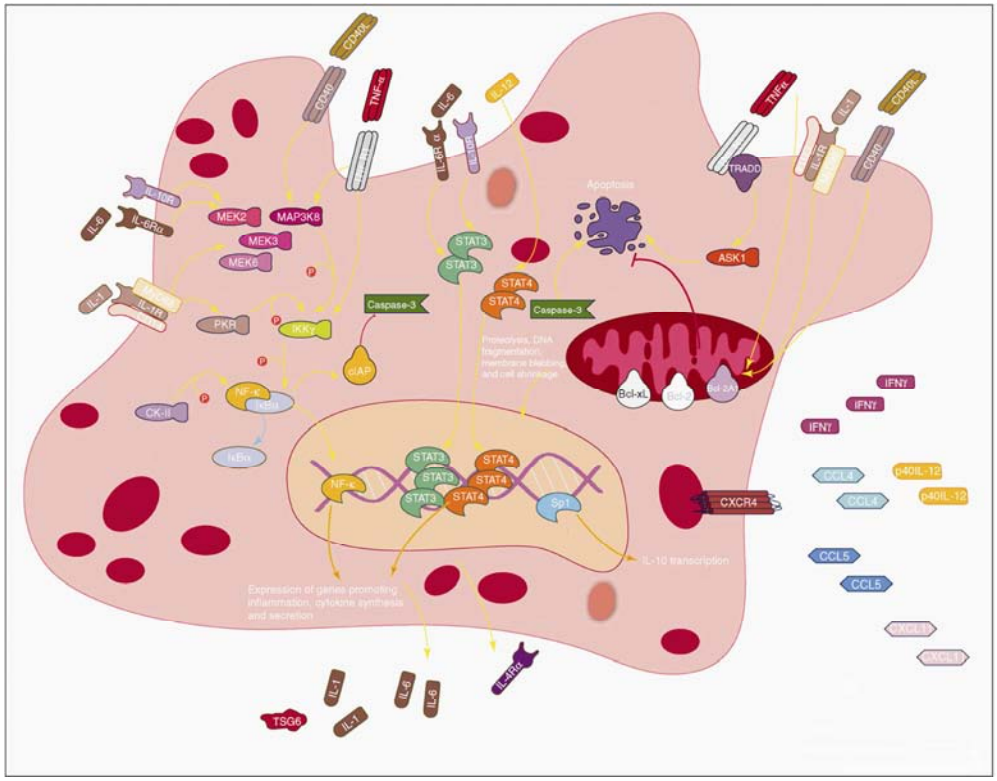


Figure 2. Human macrophage responses to EHEC *E. coli* O157:H7 in a cellular environment. The darker pink ovals indicate macrophage granules (lysosomes). Proteins with a colored icon are upregulated with fold–changes over 1.5. The proteins depicted as an icon with a white background (Bcl–2 family) have an altered transcription but with a fold–change lower than 1.5; the biological relevance of this smaller transcriptional change is unclear. Proteins depicted as a 'transparent' icon are downregulated. Major extracellular cytokines with transcriptional fold–changes over 1.5, such as the interleukins and interferon–gamma, are also depicted. Visualization of individual proteins and cellular structures uses standard icons that can be downloaded from the BioCarta website (www.biocarta.com).

Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: H. Peter van Esse
Date: June 09, 2008
Group: Wageningen University, Laboratory of Phytopathology

1) Start-up phase	date
► **First presentation of your project**	
The *Cladosporium fulvum* effector proteins and their virulence targets in tomato	Feb 17, 2003
► **Writing or rewriting a project proposal**	
The *Cladosporium fulvum* effector proteins and their virulence targets in tomato	Nov 02, 2003
► **Writing a review or book chapter**	
Cladosporium fulvum (syn. *Passalora fulva*), a highly specialized plant pathogen as a model for functional studies on plant pathogenic Mycosphaerellaceae. Molecular Plant Pathology 6, 379-393.	2005
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*8.0 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD Student Day, Vrije Universiteit	Jun 03, 2004
EPS PhD Student Day, Radboud University	Jun 02, 2005
EPS PhD Student Day, Wageningen University	Sep 19, 2006
EPS PhD Student Day, Wageningen University	Sep 13, 2007
► **EPS theme symposia**	
EPS Theme 3 symposium 'Metabolism and Adaptation', University of Amsterdam	Dec 11, 2003
EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents', Wageningen University	Dec 12, 2003
EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents', Utrecht University	Sep 17, 2004
EPS Theme 3 symposium 'Metabolism and Adaptation', Wageningen University	Oct 25, 2004
EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents', Leiden University	Jun 23, 2005
EPS Theme 3 symposium 'Metabolism and Adaptation', Utrecht University	Nov 24, 2005
EPS Theme 3 symposium 'Metabolism and Adaptation', University of Amsterdam	Nov 10, 2006
EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents', University of Amsterdam	Feb 02, 2007
► **NWO Lunteren days and other National Platforms**	
Willie Commelin Scholten day, Utrecht	Jan 22, 2004
NWO-ALW 'Experimental Plant Sciences', Lunteren	Apr 05-06, 2004
NWO-ALW 'Molecular Genetics Platform'	Oct 28-29 2004
NWO-ALW 'Experimental Plant Sciences', Lunteren	Apr 04-05, 2005
Willie Commelin Scholten day, Utrecht	Jan 19, 2006
NWO-ALW 'Experimental Plant Sciences', Lunteren	Apr 03-04, 2006
NWO-ALW 'Molecular Genetics Platform'	Oct 05-06, 2006
NWO-ALW 'Experimental Plant Sciences', Lunteren	Apr 02-03, 2007
NWO-ALW 'Molecular Genetics Platform'	Oct 27-28, 2007
► **Seminars (series), workshops and symposia**	
Seminar series 'Frontiers in Plant Biotic Interactions' (3x)	2003-2007
Seminar series 'Plant Development' (4x)	2003-2007
Seminar series 'Phytopathology' (4x)	2003-2007
Seminar series 'Molecular Biology' (4x)	2003-2007
Seminar series 'Genomics' (2x)	2003-2007
Seminar: Barbara Baker	Jun 02, 2004
Seminar: Steven Clark	Jun 28, 2004
Seminar: Nicholas Talbot	May 03, 2006
Seminar: Andrew Bent	Jun 18, 2007
Seminar: Scott Poethig	sept 24, 2007
Symposium on systems Biology 'in Honor of Prof.dr. Pierre de Wit'	Nov 04, 2004
► **Seminar plus**	
► **International symposia and congresses**	
XII Intern. Congress on Molecular Plant-Microbe Interactions, Merida (Mexico)	Dec 14-19, 2005
XIII Intern. Congress on Molecular Plant-Microbe Interactions, Sorrento (Italy)	Jul 21-27, 2007
► **Presentations**	
Oral: Presentation at the North Dakota State University, department of plant pathology (USA)	Oct 28, 2005
Molecular Plant-Microbe Interactions, Merida (Mexico)	Dec 14-19, 2005
Oral: Presentation at the Willie Commelin Scholten Day	Feb 02, 2007
XIII Intern. Congress on Molecular Plant-Microbe Interactions, Sorrento (Italy)	Jul 21-27, 2007
► **IAB interview**	Sep 18, 2006
► **Excursions**	
Subtotal Scientific Exposure	*18.0 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
Summerschool 'Signaling in Plant Development and Plant Defence'	Jun 19-21, 2006
► **Journal club**	
Member of literature discussion group 'Phytopathology'	2003-2007
► **Individual research training**	
Subtotal In-Depth Studies	*3.9 credits**

4) Personal development	date
► **Skill training courses**	
Course on intelectual property right (CBSG)	Oct 29, 2004
Scientific Writing	Apr 07-Jun 02, 2005
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Member of the EPS PhD Council	2004-2007
Wageningen PhD Council	2005-2007
Subtotal Personal Development	*4.9 credits**

TOTAL NUMBER OF CREDIT POINTS*	**34.8**

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 credits

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

The work described in this thesis was performed at the Laboratory of Phytopathology of Wageningen University with financial support of the 'Centre for Biosystems Genomics'.

The printing of this thesis was financially supported by the 'J.E. Jurriaanse Stichting'.

Front cover:
A tomato leaf infected by *C. fulvum*, at 14 days post inoculation.

Background:
Illumination of labeled RNA hybridized to an Affymetrix array. The RNA used for labeling was isolated from tomato leaves infected by *C. fulvum*, at 10 days post inoculation.

Reproduction: Ponsen & Looijen B.V., Wageningen