Identification and characterization of novel effectors of *Cladosporium fulvum*

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The *Cladosporium fulvum*-tomato pathosystem

INTRODUCTION

In their natural environment, plants are continuously exposed to a wide range of abiotic and biotic stresses. Viruses, bacteria, fungi, oomycetes, nematodes and insects are the most important biotic agents that can cause serious diseases or damages, resulting in large yield losses of economically important crops. Among these agents, fungi and oomycetes have been studied extensively to improve crop protection by a combination of strategies, including the use of major resistance genes. Efficient disease management requires an extensive knowledge of the interaction between a given pathogen and its host plant.

During co-evolution between pathogens and their host plants, different types of interactions have developed. These include interactions where pathogens have become fully dependent on their host plants, the so-called obligate biotrophic pathogens that cannot be cultured *in vitro*, such as *Blumeria graminis* f. sp. *hordei* responsible for powdery mildew on barley (Spanu *et al.*, 2010)*.* Another class of biotrophic pathogens depends on their host plants in nature but can also be cultured *in vitro*, such as *Cladosporium fulvum* responsible for leaf mould on tomato (Stergiopoulos & de Wit, 2009). Both types of biotrophic pathogens colonize the apoplast surrounding living plant tissues. Although some biotrophic pathogens produce haustoria to retrieve nutrients from the host cells (Voegele *et al.*, 2001), these feeding structures are not produced by others suggesting another still unknown mechanism for nutrient uptake. A third class of plant pathogens is represented by hemi-biotrophs, which demonstrate a short biotrophic interaction with their host plants during which no symptoms are visible. However, they gradually become necrotrophic by killing host cells, concomitant with the appearance of symptoms. These pathogens feed on nutrients that are released from the destroyed host cells to sustain their growth. Finally, necrotrophic pathogens kill host cells from the onset of the infection and feed on nutrients released from dead host cells. The latter two types of pathogens can easily be cultured *in vitro*.

During these diverse types of interactions, host plants have developed sophisticated defense strategies that enable them to recognize pathogens. All plants can recognize pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) that mediate PAMP-triggered immunity (PTI), a response that is sufficiently effective to protect plants against potential microbial pathogens (Jones & Dangl, 2006; Bent & Mackey, 2007). Generally, PAMPs induce basal structural and chemical defense responses, including callose deposition, cell wall enforcements and accumulation of pathogenesis-related (PR) proteins such as proteases, chitinases and glucanases. Although plants have developed this first layer of basal defense against microbes, successful pathogens have found ways to overcome these basal defense responses. They can suppress PTI by producing different types of effectors that target various components of PTI. By suppressing PTI, they cause effector-triggered susceptibility (ETS). Plants have evolved sophisticated ways to respond to these effectors. In addition to PRRs, plants have developed resistance proteins that recognize effectors or guard host plant targets of effectors resulting in effector-triggered immunity (ETI) (Jones & Dangl, 2006). One of the most typical characteristics of ETI is the hypersensitive response (HR), a special type of programmed cell death, where the cells surrounding the initial penetration site die quickly and prevent further growth of a biotrophic pathogen.

At the host species and cultivar level, co-evolution between pathogen and host has led to the development of numerous novel effectors and corresponding resistance proteins, which has culminated in the gene-for-gene concept, which is the outcome of continuous offenses by pathogen and counter-defenses by host (Flor, 1947; Chisholm *et al.*, 2006; Jones & Dangl, 2006). This working model of PAMP and effector recognition reflects an arms-race between pathogens and plants (Thomma *et al.*, 2011). In this chapter, the *C. fulvum*-tomato pathosystem is used as a model to discuss more recent developments in research on ETS and ETI.

History of the interaction between *Cladosporium fulvum* and tomato

C. fulvum (syn. *Passalora fulva*) is a non-obligate biotrophic fungal pathogen that causes leaf mould of tomato (Joosten & de Wit, 1999). *C. fulvum* likely originates from South and Central America, the center of origin of tomato (*Solanum lycopersicum* L.), which is the only host known so far for *C. fulvum* and is thought to be native to regions between Mexico and Peru (Bai & Lindhout, 2007). The first infection of tomato by *C. fulvum* was observed and described by Cooke on cultivated tomato in 1883 in North Carolina (Cooke, 1883). Before the 1960s, *C. fulvum* caused serious economic losses in tomato production worldwide. Since then, the introduction of several *Cf* (for *C. fulvum*) resistance genes from wild *Solanum* species into commercial tomato cultivars kept infections by *C. fulvum* under control. However, recent outbreaks have been reported in countries where tomato cultivars lacking *Cf* resistance genes are grown and in areas where intensive year-round cultivation of resistant tomato plants has led to fungal strains overcoming *Cf* resistance genes (Cooke, 1883; de Wit, 1992; Iida *et al.*, 2010). Because the genetics of tomato are well developed and many races of *C. fulvum* adapted to tomato cultivars with particular *Cf* resistance genes exist, the interaction between tomato and *C. fulvum* has become a model system to study the molecular basis of plant-pathogen interactions.

Compatible and incompatible interactions

Under high relative humidity and cool temperatures, conidia of *C. fulvum* germinate to differentiate runner hyphae on the lower side of tomato leaves (Joosten & de Wit, 1999; Thomma *et al.*, 2005). Runner hyphae enter tomato leaves through open stomata. After penetration, hyphae increase in diameter and colonize the apoplastic space around tomato mesophyll cells (Fig. 1A) (Joosten & de Wit, 1999; Thomma *et al.*, 2005). *C. fulvum* does not differentiate any feeding structure, such as haustoria, but lives on apoplastic sugars and amino acids, including saccharose and glutamine. Saccharose is hydrolyzed by *C. fulvum* invertase into glucose and fructose, which are subsequently converted by fungal mannitol dehydrogenase into mannitol,

which cannot be metabolized by the host plant (Joosten *et al.*, 1990). Approximately 10-14 days after penetration, the fungus produces large numbers of conidiophores that re-emerge from stomata present at the lower side of infected leaves. Consequently, regulation of opening and closure of stomata is obstructed causing wilting of leaves (Thomma *et al.*, 2005). However, in the incompatible interaction upon entrance of fungal runner hyphae into the apoplastic space, mesophyll cells recognize the presence of the fungus and induce defense responses, including an HR that restricts further growth of the biotrophic fungus (Fig. 1B) (Joosten & de Wit, 1999; Stergiopoulos & de Wit, 2009).
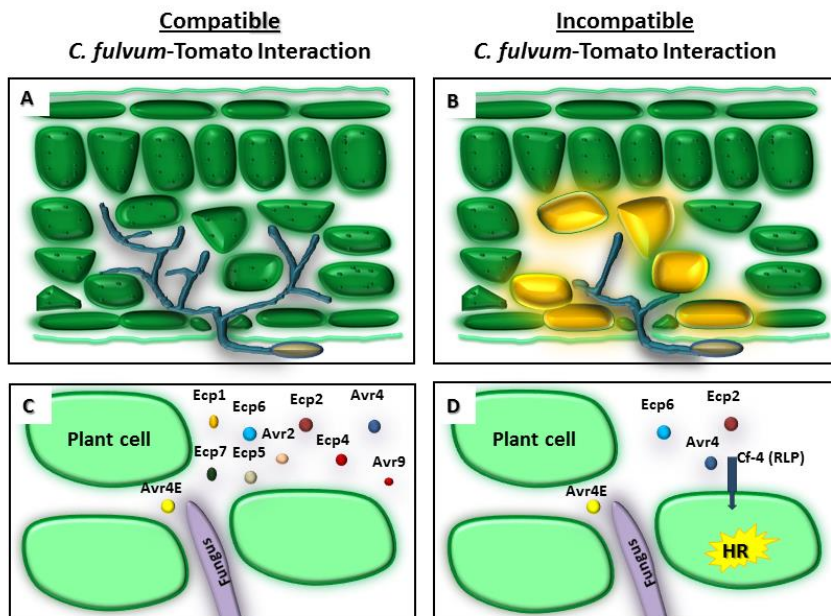
Fig. 1. Infection of tomato leaves by *Cladosporium fulvum.* **(A)** Compatible interaction between *C. fulvum* and tomato. Following germination of a conidium, a runner hypha penetrates plant tissues through open stomata and colonizes the apoplastic space of tomato leaves. **(B)** In an incompatible interaction, a tomato resistance protein recognizes the presence of the fungus, and a hypersensitive response (HR) is induced, which stops further growth of the fungus. **(C)** During infection, *C. fulvum* secretes several small cysteine-rich effector proteins (Avrs and Ecps) to manipulate host defenses and support fungal growth. **(D)** In resistant plants, effectors are recognized by corresponding receptor-like proteins (RLPs), which activate Cf-mediated defense responses, including an HR.

During infection, *C. fulvum* secretes various small (<21 kDA) proteins (effectors) into the apoplastic space of tomato leaves (Fig. 1C) (Stergiopoulos & de Wit, 2009). These effectors enable the fungus to invade and colonize the tomato plant by acting as offensive (e.g., *C. fulvum* Avr2 effector) or defensive (e.g., *C. fulvum* Avr4 and Ecp6 effectors) virulence factors (Rooney *et al.*, 2005; van den Burg *et al.*, 2006; de Jonge *et al.*, 2010). The same virulence factors can also be recognized in resistant tomato plants and trigger defense responses that compromise further growth of the pathogen (Fig. 1D). When a virulence factor is recognized by a resistant plant, it causes avirulence to the fungus, and it is called an avirulence (Avr) factor. At the molecular level, the perception of *C. fulvum* effectors by tomato is mediated by *Cf* resistance genes encoding resistance proteins that are membrane-anchored extracytoplasmic glycoproteins, which belong to the class of receptor like proteins (RLPs) (Thomas *et al.*, 1998; de Wit & Joosten, 1999; Kruijt *et al.*, 2005). Perception of Avr factors by corresponding Cf resistance proteins triggers plant defense responses, including an HR (Rivas & Thomas, 2002). Ten effector genes have been identified from *C. fulvum* so far, including four *Avr* genes (*Avr2*, *Avr4*, *Avr4E*, and *Avr9*) and six extracellular protein-encoding (*Ecp*) genes (*Ecp1*, *Ecp2*, *Ecp4*, *Ecp5*, *Ecp6*, and *Ecp7*) (Stergiopoulos & de Wit, 2009). The different *C. fulvum* effectors are supposed to have different intrinsic functions related to virulence on tomato. So far, the intrinsic functions of only three *C. fulvum* effector proteins -Avr2, Avr4, and Ecp6- have been characterized; for others, a role in virulence has been established, but their intrinsic functions remain unknown.

Avr2, an inhibitor of plant cysteine proteases

The mature Avr2 effector protein contains 58 amino acids with eight cysteine residues that are involved in formation of four disulfide bridges (Luderer *et al.*, 2002). Disulfide bridges are important for stability of proteins that are secreted in the apoplast, which is a harsh environment containing many plant hydrolytic enzymes, including proteases. The Avr2 effector is an offensive virulence factor because plant cysteine protease activity profiling showed that it can inhibit several extracellular plant

cysteine proteases, including Rcr3[pim] (required for *C. fulvum* resistance 3), Pip1 (*Phytophthora*-inhibited protease 1), aleurain and TDI65 (tomato-drought induced 65) (Kruger *et al.*, 2002; van Esse *et al.*, 2008). Mutational studies on Rcr3[pim] showed that this protein has an active role in plant defense against *Phytophthora infestans*, which secretes EPIC1 and EPIC2B proteins that also bind and inhibit Rcr3[pim] (Song *et al.*, 2009). Inhibition of an array of plant cysteine proteases contributes to virulence of different classes of pathogens. Heterologous expression of *Avr2* in tomato and *Arabidopsis thaliana* resulted in increased susceptibility to different fungal pathogens, such as *Botrytis cinerea* and *Verticillium dahliae* (van Esse et al., 2008).

Avr4, a chitin-binding protein that protects fungal hyphae against plant chitinases

The *Avr4* gene encodes a 135 amino acids pre-protein, which is processed at the N-terminus and C-terminus after secretion into the apoplast. In its mature form, Avr4 contains 86 amino acids with eight cysteine residues that are involved in disulfide bridge formation (Joosten *et al.*, 1994). Avr4 is a chitin-binding lectin that protects fungal cell walls against the deleterious effects of plant chitinases, providing a defensive role during infection. Avr4 binds to chitin in the cell wall of *Fusarium solani* and *Trichoderma viride* and protects them against plant chitinases *in vitro* (van den Burg *et al.*, 2004; van den Burg *et al.*, 2006). In addition, silencing of the *Avr4* gene in *C. fulvum* reduces virulence on tomato plants (van Esse *et al.*, 2007). Protection against plant chitinases is likely important for virulence of most plant pathogenic fungi because chitin is a basic component of fungal cell walls. It is assumed that many other fungal species could carry homologues of *Avr4*. Indeed, recently Avr4 homologues have been identified in several Dothideomycete fungi, including *Mycosphaerella fijiensis*, and these exhibit protection against plant chitinases as well (Stergiopoulos et al., 2010).

Ecp6, an effector that interferes with chitin-triggered immunity

Ecp6 encodes a protein (228 amino acids) that contains three LysM carbohydrate-binding domains (Bolton *et al.*, 2008). The intrinsic function of Ecp6 has been characterized more recently (de Jonge *et al.*, 2010). Although similar to Avr4, Ecp6 also specifically binds to chitin; this binding does not protect the fungus against basic plant chitinases. Further experiments revealed that Ecp6 is able to bind small chitin fragments that are released from the fungal cell wall *in planta*, and by doing so it prevents these fragments from being recognized by chitin receptors present at the plant plasma membrane (Fig. 2) (de Jonge *et al.*, 2010). Preventing chitin recognition is important because silencing of the *Ecp6* gene results in reduced virulence in *C. fulvum* (Bolton *et al.*, 2008). Many homologues of *Ecp6* were identified in different fungal species (de Jonge & Thomma, 2009), suggesting an important function in preventing chitin-triggered immunity in many plant-fungus interactions. Accordingly, more recent studies showed that *Zymoseptoria tritici* (syn. *Mycosphaerella graminicola*) secretes three LysM effectors called Mg1LysM, Mg3LysM and MgxLysM (Marshall *et al.*, 2011). Similar to the *C. fulvum* Ecp6 protein, both Mg1LysM and Mg3LysM are able to bind chitin. In contrast to Ecp6, both *Z. tritici* LysM effectors protect fungi against plant chitinases, whereas only Mg3LysM prevents chitin-triggered immunity. However, only the Mg3LysM deletion mutant showed significantly reduced virulence, indicating the importance of avoding chitin-triggered immunity during infection (Marshall *et al.*, 2011). Similar to Ecp6 and Mg3LysM effectors, the *Magnaporthe oryzae* LysM effector Slp1 also binds and scavenges small chitin fragments. In rice, the receptor CEBiP (chitin elicitor binding protein) that recognizes chitin and activates PTI is well characterized. Mentlak *et al.* (2012) showed that Slp1 prevents chitin-triggered immunity by competing with CEBiP for chitin binding. It is likely that Ecp6 also competes with the tomato PRR that recognizes chitin.

Other effectors secreted by *Cladosporium fulvum* with unknown function

The *Avr4E* gene encodes a cysteine-rich (6 cysteines) protein containing 101 amino acids in its mature form (Westerink *et al.*, 2004). However, the intrinsic function of Avr4E has not been identified so far.

The *Avr9* gene encodes a secreted protein of 63 amino acids, which is gradually processed into a mature peptide of 28 amino acids. The mature Avr9 peptide contains six cysteine residues that all are involved in disulfide bridge formation and form a cystine knot structure (Van Kan *et al.*, 1991; van den Hooven *et al.*, 2001). The intrinsic function of Avr9 has not been characterized yet, but Avr9 shows some similarity to carboxypeptidase inhibitors based on cysteine spacing and overall structure. However, carboxypeptidase inhibitor activity of Avr9 has not been proven experimentally so far (van den Hooven *et al.*, 2001).

Of the six Ecp effectors that were identified from apoplastic fluids of tomato leaves infected by *C. fulvum*, intrinsic functions of Ecp1, Ecp2, Ecp4, Ecp5 and Ecp7 have not been characterized yet, but the presence of their encoding genes in all *C. fulvum* races suggests that they are important virulence factors for the fungus. Indeed, deletion of *Ecp1* and *Ecp2* severely decreased virulence of *C. fulvum* during tomato infection (Lauge *et al.*, 1997). The *Ecp2* gene does occur in different Dothideomycete fungi, including *M. fijiensis*, suggesting it might be important in virulence of other fungi as well (Stergiopoulos et al., 2010).

Tomato Cf resistance proteins

Resistance proteins (R) can be categorized into two major classes based on their domain organization. The first class of R proteins is represented by nucleotide binding- leucine-rich repeat (NB-LRR) proteins that are cytoplasmic. NB-LRR proteins are the most abundant resistance proteins, and their members can be classified in two subgroups based on their N-terminal domain: coiled-coil (CC)-NB-LRR and Toll-interleukin-1 receptor (TIR)-NB-LRR (Hammond-Kosack & Jones, 1997; Pan *et al.*, 2000).

The second class of R proteins is represented by receptors containing LRRs and a transmembrane domain. These receptors can be divided into two subgroups according to whether they harbor a C-terminal kinase domain [receptor like kinases (RLKs)] or not [receptor like proteins (RLPs)].

Structure of Cf resistance proteins

Several *Cf* genes have been cloned so far, including *Cf-2* (Dixon *et al.*, 1996), *Cf-4* (Thomas *et al.*, 1997), *Cf-4E* (Takken *et al.*, 1999), *Cf-5* (Dixon *et al.*, 1998), *Cf-9* (Jones *et al.*, 1994) and, *Cf-9DC* (Van der Hoorn *et al.*, 2001). These *Cf* genes can be divided in two families: (i) *Hcr9s* (homologous of the *C. fulvum* resistance gene *Cf-9*) and (ii) *Hcr2s* (homologous of the *C. fulvum* resistance gene *Cf-2*). *Cf-4*, *Cf-4E*, *Cf-9*, and *Cf-9DC* genes are highly homologous to each other and belong to the *Hcr9* gene family that is located on the short arm of chromosome 1 at the Milky Way (MW) locus (Van der Beek *et al.*, 1992; Takken *et al.*, 1998). *Cf-2* and *Cf-5* genes are located on chromosome 6 and belong to the *Hcr2* gene family (Dixon *et al.*, 1998).

All Cf proteins characterized so far belong to the RLP subclass of R proteins. The structure of these receptors has been divided in several domains according to their putative function. The A domain is a signal peptide that is required for targeting of the R protein into the secretory pathway. Domain B is the mature N-terminal part with no predicted function, whereas domain C comprises a varying number of LRRs supposed to be involved in recognition. Domain D does not have any obvious feature, and domain E contains many acidic amino acid residues. Finally, domain F is a transmembrane domain that contains many aliphatic amino acid residues, and domain G is a short C-terminal tail that lacks obvious signalling functions (Kruijt *et al.*, 2005; van der Hoorn *et al.*, 2005). Because Cf proteins lack obvious intracellular signaling domains, it is anticipated that at least one additional interacting signaling protein is required for downstream signal transduction. Recognition capacity of RLPs resides in the LRRs of the C-domain. The number of LRRs varies among the different Cf proteins, and domain swaps between LRRs of different Cf proteins assigned LRRs and amino

acids that are crucial for recognition of corresponding Avr proteins (Van der Hoorn et al., 2001; van der Hoorn et al., 2005).

Gene-for-gene interactions: recognition of *Cladosporium fulvum* effectors by tomato;

Avr2-Cf-2 gene pair

C. fulvum strains expressing Avr2 protein trigger an HR in tomato lines that carry the corresponding *Cf-2* resistance gene (Dixon *et al.*, 1996). However, the recognition of the Avr2 is not direct and is in support of the guard hypothesis (Rooney *et al.*, 2005). The Avr2 effector targets several tomato cysteine proteases, such as Rcr3^{pim} and Pip1, to promote disease in tomato. Inhibition of Rcr3^{pim} by Avr2 is thought to cause a structural modification of Rcr3^{pim} on binding, which is monitored by the Cf-2 protein (Fig. 2) (Rooney *et al.*, 2005). By mutation analysis of Avr2, it was found that affinity of Avr2 mutants for Rcr3^{pim} was correlated with the intensity of Cf-2-mediated HR of these mutants (van't Klooster *et al.*, 2011). The higher the affinity of Avr2 for Rcr3^{pim}, the stronger Avr2-triggered Cf-2-mediated HR. It is assumed that a pathogen virulence target has an important role in plant defense, and along this line other pathogens could evolve effectors that are able to inhibit the same host target. Song *et al.* (2009) showed that EPIC1 and EPIC2B effectors in the oomycete *P. infestans* also have the ability to inhibit Rcr3^{pim} activity (Song *et al.*, 2009). In contrast to interaction with Avr2, inhibition of Rcr3^{pim} by these two *P. infestans* effectors does not trigger a Cf-2-mediated HR. It seems that the Cf-2 receptor protein cannot monitor all modifications of Rcr3 and trigger a Cf-2-mediated HR. The suggestion that structural changes of Rcr3 are sensed by the Cf-2 protein is supported by the fact that a *Solanum esculentum* homologue of Rcr3, Rcr3^{esc}, that can induce a Cf-2-mediated HR in the absence of Avr2 exists (Kruger *et al.*, 2002).

***Avr4-Cf-4* gene pair**

The *C. fulvum* chitin-binding protein Avr4 is recognized by the corresponding Cf-4 resistance protein in tomato. Heterologous expression of Avr4 in *Cf-4* tomato plants revealed that Avr4 can be recognized by Cf-4 in the absence of chitin fragments, indicating that chitin-binding is not required for recognition of Avr4 (Fig. 2) (Thomas *et al.*, 2000). Allelic variation studies in natural *C. fulvum* populations showed that *C. fulvum* strains that contain the *Avr4* gene can overcome Cf-4-mediated recognition via substitutions rather than frame shift or gene deletion mutations, suggesting that recognition of Avr4 by Cf-4 might be direct (Stergiopoulos *et al.*, 2007). Microarray analysis of *Avr4*-expressing tomato plants also supports the direct interaction between Avr4 and Cf-4 because expression of the *Avr4* gene in tomato does not induce any differential gene expression in the absence of the Cf-4 protein (van Esse *et al.*, 2008). However, so far, investigators have been unable to show physical interaction between Avr4 and Cf-4 experimentally. Avr4 homologues have been identified in other fungi, including *M. fijiensis* that can still trigger a Cf-4-mediated HR, suggesting they are structurally related to *C. fulvum* Avr4 (Stergiopoulos *et al.*, 2010).

***Avr9-Cf-9* gene pair**

The *C. fulvum* Avr9 effector protein is recognized by the corresponding Cf-9 resistance protein in tomato. Although many interaction studies have been performed for the Cf-9/Avr9 protein pair, direct interaction between these proteins has not been shown so far (Fig. 2) (Luderer *et al.*, 2002). Binding studies showed that Avr9 binds to plasma membrane of both *Cf-0* and *Cf-9* tomato and other solanaceous plant species with similar binding kinetics and capacity but not to membranes of non-solanaceous species that were analyzed (Kooman-Gersmann *et al.*, 1996). This finding suggests the presence of a high affinity binding site (HABS) for Avr9 in solanaceous plants. Heterologous expression of the *Cf-9/Avr9* gene pairs in different solanaceous plants, such as tobacco, petunia, and potato, resulted in induction of HR, but such expression did not result in HR induction in non-solanaceous plants, such as *Arabidopsis thaliana*

and lettuce, suggesting that the presence of HABS is essential for Avr9-triggered, Cf-9-mediated HR (Van der Hoorn *et al.*, 2000). Mutational analysis of Avr9 also revealed that binding affinity of Avr9 for HABS is positively correlated with the ability of Avr9 to trigger Cf-9-mediated HR (Kooman-Gersmann *et al.*, 1998). We speculate that HABS is the target of Avr9 and that modification of the HABS by Avr9 triggers Cf-9-mediated HR (Fig. 2). However, HABS has not been identified so far.

Other *effector-Cf* gene pairs

C. fulvum strains that express *Avr4E* induce an HR on tomato plants carrying the corresponding *Cf-4E* resistance gene (Westerink *et al.*, 2004), but no further studies on this gene pair have been performed. Matching resistance genes (*Cf-Ecps*) have been identified in tomato plants for Ecp1, Ecp2, Ecp4, and Ecp5 effectors; however, so far, no tomato accessions have been identified that show HR after treatment with Ecp6 or Ecp7 effectors (Stergiopoulos *et al.*, 2007; Bolton *et al.*, 2008). No detailed studies of *Cf-Ecp/Ecp* gene pairs have been performed.

Avoidance of Cf-mediated HR by allelic variation in *Cladosporium fulvum* effector genes

As discussed previously, *C. fulvum* effectors are virulence factors, but they are also recognized by the corresponding Cf resistance proteins in resistant tomato lines. However, several *C. fulvum* strains have evolved mutated versions of effectors that are no longer recognized by corresponding Cf proteins. These strains have thus become virulent on previously resistant tomato cultivars. Mutated alleles of most effector genes contain non-synonymous substitutions, whereas *Avr2* alleles contain transposon insertions (Joosten *et al.*, 1997; Luderer *et al.*, 2002; Westerink *et al.*, 2004). Other mutations in *Avr2* are insertions or deletions of nucleotides in the coding region, resulting in frame shifts that cause production of truncated and non-functional Avr2 proteins (Stergiopoulos *et al.*, 2007). A targeted mutational analysis performed on Avr2 indicated that the C-terminal tail of Avr2 most likely interacts with Rcr3 (van't Klooster

et al., 2011). Non-functional Avr2 proteins no longer interact with their target, Rcr3, and cannot trigger a Cf-2-mediated HR. Most of the polymorphisms observed in the *Avr4* gene represent point mutations mostly resulting in substitutions of cysteine residues in the encoded Avr4 protein. Cysteine substitutions cause the production of Avr4 proteins that can still bind chitin but no longer accumulate in the apoplast to trigger a Cf-4-mediated HR because they are unstable in this harsh environment (van den Burg *et al.*, 2004). Allelic variation studies in natural *C. fulvum* populations revealed that all race 9 and some race 4E strains had lost the *Avr9* and *Avr4E* gene to evade Cf-9-mediated and Cf-4E-mediated resistance, respectively (Stergiopoulos *et al.*, 2007).

In contrast to *Avr* genes, very little allelic variation has been observed for *Ecp* genes; this is likely due to the fact that *Cf-Ecp R*-traits have not yet been deployed in commercial tomato cultivars, and so no strong selection pressure has been imposed on the matching fungal *Ecp* genes (Stergiopoulos *et al.*, 2007). Introduction of these *Cf-Ecp R*-traits might provide a sustainable form of resistance to *C. fulvum* when introduced in commercial tomato cultivars. Pyramiding of several *Cf* genes can also be a good alternative to control *C. fulvum* in the future.

Cf-mediated downstream signaling

Recognition of effectors by corresponding tomato Cf receptors results in the activation of defense responses through induction of the expression of specific genes. Many genetic and biochemical approaches have been performed to identify signaling components that pass on the recognition signal from the apoplast to the host nucleus. Yeast-two-hybrid studies revealed that the C-terminal part of the Cf-4 and Cf-9 protein interacts with a tomato protein that has homology to thioredoxin [Cf-9-interacting thioredoxin (CITRX)] (Rivas *et al.*, 2004). Gene silencing of *CITRX* in both tomato and tobacco plants revealed that *CITRX* is a negative regulator of Cf-4- and Cf-9-mediated defense responses. *CITRX* gene-silenced plants showed faster Cf-4/Avr4- and Cf-9/Avr9-mediated HR induction and enhanced ROS accumulation. Although CITRX

negatively regulates Cf-4/Avr4- and Cf-9/Avr9-mediated defense responses, it does not seem to regulate Cf-2/Avr2-mediated defense responses (Rivas *et al.*, 2004). This is probably due to the fact that CITRX cannot interact with Cf-2 because its C-terminal tail is different from that of the conserved Cf-9 and Cf-4 terminal tail (Rivas *et al.*, 2004).

Transcriptome profiling of *Cf-4* tomato plants or *Cf-9* expressing transgenic tobacco cell cultures revealed several genes that are either up- or down-regulated upon matching Avr treatments (Durrant *et al.*, 2000; Gabriels *et al.*, 2006). Virus-induced gene silencing (VIGS) of up-regulated genes was performed to test their role in plant defense. Silencing of *NtCMPG* (a putative E3 ubiquitin ligase gene) in *N. benthamiana* resulted in reduction of HR for the *Cf-9/Avr9* gene pair, whereas overexpression resulted in enhancement of HR induction (Gonzalez-Lamothe *et al.*, 2006). Similarly, silencing of *Avr9/Cf-9-induced kinase1* (*ACIK1*), which encodes a Ser/Thr protein kinase, showed that it is a positive regulator of Cf-4/Avr4- and Cf-9/Avr9-mediated HR but not of HR mediated by other Cf/Avr gene pairs (Rowland *et al.*, 2005). In *Cf-9* tomato lines, silencing of *ACIK1* decreased Cf-9-mediated resistance against *C. fulvum* strains containing the *Avr9* gene. Further studies showed that ACIK1 binds and phosphorylates CITRX after treatment of *Cf-9* plants with Avr9. The ACIK1/CITRIX complex binds to the C-terminus of Cf-9 and mediates downstream signaling (Nekrasov *et al.*, 2006). In contrast to ACIK1, CITRX is a negative regulator of Cf-9/Avr9-mediated defense responses, suggesting that the ACIK1/CITRIX complex is present in non-infected plants and on Avr9 recognition ACIK1 phosphorylates CITRX, which results in destabilization and release of the complex from the Cf-9 protein that activate downstream defense signaling components.

In another approach to discover downstream defense signaling components, transgenic tomato plants expressing *Cf-4* and *Avr4* were crossed to get progeny that contained both *Cf-4* and *Avr4* genes (de Jong *et al.*, 2002). Such progeny expressing *Cf-4* and *Avr4* does not induce HR as long as it is grown at 33°C. However, synchronized HR is induced in these so-called dying seedlings as soon as the temperature is shifted from 33°C to room temperature (de Jong *et al.*, 2002). Transcriptome comparison of

dying seedlings upon HR induction and the *Cf-4* or *Avr4* parental lines revealed several differentially expressed genes upon Avr4 recognition by Cf-4. Additional VIGS studies confirmed the role of four genes that affect Cf-4/Avr4-mediated HR, including the gene NB-LRR required for HR-associated cell death 1 (NRC1) (Gabriels *et al.*, 2006). *NRC1* encodes a CC-NB-LRR type resistance (R) protein analogue that not only affects Cf-4/Avr4-induced HR but also compromises Cf-4-mediated resistance to *C. fulvum*. Upon Avr4 recognition, NRC1 induces the activation of mitogen-activated protein kinases (MAPKs), which are important for Cf-4-mediated HR. In addition, VIGS of NRC1 in *N. benthamiana* revealed that this protein is also required for HR induced by the Cf-9, LeEix, Pto, Rx and Mi R proteins (Gabriels *et al.*, 2007).

Avr4 treatment of *Cf-4*-transgenic tobacco cell cultures activates phospholipase C (PLC), which leads to formation of diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP3). DAG is converted into phosphatidic acid (PA) by diacylglycerol kinase (DGK) (de Jong *et al.*, 2004). Accumulation of PA stimulates NADPH-oxidase, which is responsible for production of reactive oxygen species (ROS). Formation of IP3 results into Ca^{2+} export from vacuole into cytoplasm activating calcium-dependent protein kinase (CDPK) by phosphorylation of its kinase domain (Romeis *et al.*, 2000; Ludwig *et al.*, 2005). Phosphorylated CDPK can activate NADPH-oxidase and increase ethylene production, which results in induction of several defense responses. VIGS studies in *N. benthamiana* showed that silencing of the *NtCDPK2* gene causes a reduced HR triggered by *Cf-4/ Avr4* and *Cf-9 /Avr9* gene pairs (Romeis *et al.*, 2001).

C. fulvum effectors in other fungi with similar infection strategies

Several approaches, including positional cloning and reverse genetics, have been used to identify effector genes from plant pathogens. However, these methods have their limitations. Positional cloning cannot be applied to fungi without known sexual cycle, such as *C. fulvum*. Identification of genes via a proteome approach

requires sufficient amounts of protein encoded by the genes that need to be cloned, which in most cases is unavailable or very difficult to obtain.
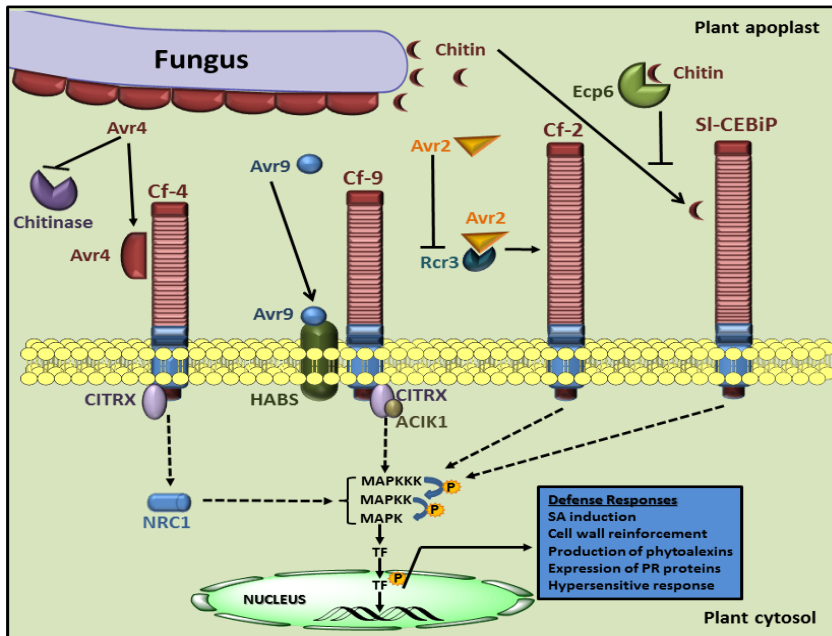

Fig. 2. Avr effector-triggered Cf-mediated defense signaling in tomato. The Avr4 effector binds chitin of fungal cell wall to protect the fungus against plant chitinases. In the presence of the Cf-4 protein, Avr4 triggers Cf-4-mediated defense responses. The NRC1 protein is required for Cf-4-mediated resistance. The Avr9 effector binds to HABS, which is located in the plant membrane. Most likely, the Avr9-HABS complex is recognized by the Cf-9 protein and triggers downstream defense signalling. In the absence of Avr9, ACIK1 and CITRIX most likely form a complex. On Avr9 recognition, ACIK1 phosphorylates CITRX, which destabilizes and releases the complex from the Cf-9 protein and results in activation of downstream defense signaling. Avr2, a cysteine protease inhibitor, binds and inhibits Rcr3. In the presence of the Cf-2 protein, the Avr2-Rcr3 complex can be recognized and induces Cf-2-mediated defense responses. Chitin fragments are PAMPs and can be recognized by Sl-CEBiP and trigger basal defense responses. However, Ecp6 effector has the ability to scavenge chitin fragments and prevents chitin fragments from being recognized. Abbreviations: Rcr3, required for *C. fulvum* resistance 3; HABS, high affinity binding site; CITRX, Cf-9-interacting thioredoxin; ACIK1, Avr9/Cf-9-induced kinase; NRC1, NB-LRR protein required for HR-associated cell death-1; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; TF, transcription factor. (Figure is modified from Stergiopoulos *et al.*, 2009).

In the era of genomics, the availability of genomes of many different fungal species provides new strategies for the identification of novel effector genes. Comparative genomics of *C. fulvum* with other fungal species enabled us to find homologous genes in related *Dothideomycete* fungi with similar infection strategies and possibly other classes of fungi as well.

Until more recently, most of the known *C. fulvum* effectors appeared to be species-specific with the exception of Ecp6, whose homologues occur in many fungal species (Bolton *et al.*, 2008; de Jonge & Thomma, 2009). More recent comparative genomics studies in Dothideomycete fungi also showed that orthologs of *Avr4* and *Ecp2* are present in closely related fungal pathogens such as, *M. fijiensis*, the causal agent of black sigatoka disease on banana (Stergiopoulos *et al.*, 2010), and *Dothistroma septosporum*, the causal agent of Dothistroma needle blight on pine (de Wit *et al.*, 2012). Comparison of the *C. fulvum* genome with other *Dothideomycetes* revealed that *D. septosporum* has the highest number of *C. fulvum* effector homologues discovered so far, including *Avr4, Ecp2, Ecp2-2, Ecp2-3, Ecp4, Ecp5*, and *Ecp6*. Avr4, Ecp2, and Ecp6 homologues in *D. septosporum* show the highest identity with those present in *C. fulvum.* Induction of Cf-4-mediated and Cf-Ecp2-mediated HR upon heterologous gene expression of *M. fijiensis* and *D. septosporum Avr4* and *Ecp2* in *Cf-4* and *Cf-Ecp2* carrying tomato plants suggests that banana and pine could also carry homologues of Cf-4 and Cf-Ecp2 to defend themselves against these pathogens (Stergiopoulos *et al.*, 2010; de Wit *et al.*, 2012). It was also shown that similar to the *C. fulvum* Avr4 protein, the *M. fijiensis* Avr4 protein binds to chitin and protects the fungal cell wall against hydrolytic activity of basic plant chitinases (Stergiopoulos *et al.*, 2010). Based on genome-wide search analysis performed so far, it is assumed that the *Avr4*, *Ecp2*, and *Ecp6* genes encode core effectors present in many fungi, whereas the *Avr2*, *Avr4E*, and *Avr9* genes are specific to *C. fulvum* (Stergiopoulos *et al.*, 2010; de Wit *et al.*, 2012). In the future, many more fungal genomes, including that of *C. fulvum* and other Dothideomycete fungi*,* will become available enabling the discovery of more core and species-specific effectors. Comparison of more related genomes will allow us

to identify infection strategies that are shared among pathogens as well as infection strategies that differentiate them.

CONCLUSION

The *C. fulvum*-tomato pathosystem is a model system to study extracellular fungal pathogens. Many effectors have been characterized, and more will be identified in the future when the *C. fulvum* genome sequence becomes available. Most of the *C. fulvum* effectors are involved in virulence; the intrinsic function of three effectors (Avr2, Avr4, and Ecp6) is known and has been studied in detail. Most of the effectors are also avirulence factors that are recognized by tomato Cf resistance proteins. However, little is known, so far, about downstream of Cf-mediated defense signalling. Availability of both the *C. fulvum* and the tomato genome sequences would speed up the discovery of new virulence factors of *C. fulvum* and enable the dissection of effector-triggered Cf-mediated plant defense signaling pathways by combined transcriptome, proteome, and functional analyses and performing gene knockout/silencing or overexpression of interesting fungal and plant genes.

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THESIS OUTLINE

Plants have an efficient surveillance system to detect and respond to invading pathogens by both constitutive and inducible defense mechanisms. Constitutive defense systems comprise biochemicals (secondary metabolites, antifungal proteins), whereas inducible defense responses comprise a two-layer-defense system of which the first responds to pathogen associated molecular patterns (PAMPs) leading to PAMP-triggered immunity (PTI). However, successful plant pathogens counterattack PTI by delivering effectors into the apoplast, cytoplasm or even nucleus of the cells of their host to avoid or suppress PTI. In the second line of defense, plants perceive these secreted effectors and induce effector-triggered immunity (ETI). There is continuous arms-race between pathogens and their hosts in which pathogens evolve new effectors that escape recognition and plants evolve resistance proteins that recognize these new effectors re-installing ETI again. Identification and characterization of the main components of this arms-race will provide a better understanding of the molecular basis of plant-pathogen interactions and thus provide knowledge to improve existing disease management strategies. Genome sequencing of fungal pathogens provides the opportunity to identify novel effectors of these pathogens, which will also provide new insight into the molecular basis of the different lifestyles of plant pathogens.

The objective of this thesis is to mine the genome of *Cladosporium fulvum* (which has recently been sequenced) with a specific focus on the discovery of new effectors involved in virulence and avirulence of this fungus.

Chapter 1 reviews the molecular basis of the *C. fulvum*-tomato interaction, which introduces the concept of effector proteins secreted by this fungal pathogen. On the one hand, molecular features of *C. fulvum* effectors that play defensive or offensive roles in a compatible interaction are discussed. On the other hand, molecular mechanisms of effector perception by resistance protein receptors and of downstream

signaling pathways that lead to activation of defense responses in incompatible interaction are briefly described.

Chapter 2 addresses the identification of novel effectors involved in virulence and avirulence of *C. fulvum*. For this purpose, the genome sequence of *C. fulvum* was mined for genes encoding putative effector proteins. A subset of effector candidates was screened for the ability to specifically elicit defense responses on tomato lines carrying different *Cf* resistance genes, of which the matching avirulence genes are still unknown. In addition, genome mining resulted in the identification of effectors that trigger non-specific necrosis, which were further characterized to understand their function in the context of *C. fulvum's* biotrophic lifestyle.

Chapter 3 describes a forward genetic approach to identify and characterize a novel enzyme effector, the tomatinase *CfTom1*, which is secreted by *C. fulvum* to detoxify the constitutively produced antifungal α-tomatine in tomato. The ability of *C. fulvum* to detoxify α-tomatine was tested both *in vitro* and during infection of tomato. In addition, *CfTom1* was deleted in *C. fulvum* to assess the role of this gene in α-tomatine detoxification and virulence of *C. fulvum*. Furthermore, suppression and toxicity assays were performed for both α-tomatine and tomatidine on tomato to assess the claimed dual role of fungal tomatinases.

Chapter 4 describes the functional characterization of CfWor1 from *C. fulvum*, a homologue of Wor1 transcriptional regulator that is conserved in a wide range of fungal species. While Wor1 homologues present in dimorphic human pathogenic yeast species is involved in the regulation of specific morphological switches, Wor1 homologues of filamentous plant pathogenic fungi regulate and affect conidiogenesis, secondary metabolite production, expression of effector genes and consequently virulence. This chapter brings new insights into the role of Wor1-like proteins in the regulation of virulence of phytopathogenic fungi through regulation of effectors, secondary metabolism and development.

Chapter 5 provides a general discussion on the different approaches and their implications employed in the last decade for identification of novel effectors involved in fungal virulence and avirulence, with particular emphasis on comparative genomics and transcriptomics.

Chapter 2

Search for novel effectors in the predicted secretome of the biotrophic fungal pathogen *Cladosporium fulvum*

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ABSTRACT

Infection of tomato by the fungus *Cladosporium fulvum*, like in other pathosystems, mostly relies on the secretion of effector proteins. Although these effectors quantitatively contribute to virulence, they can also provide an avirulence signal when specifically recognized by the corresponding receptors encoded by the *Cf* resistance genes, triggering a hypersensitive response (HR). Ten effectors from *C. fulvum* have been cloned so far, but full virulence of this fungus likely involves many more. In this study, we mined the *C. fulvum* genome for novel effectors involved in virulence and avirulence of this fungus. Sixty out of 271 secreted small cysteine-rich proteins (SSCPs) identified in the genome were tested for their ability to specifically elicit a Cf-1-, Cf-3-, Cf-5-, Cf-9B-, Cf-11- or Cf-Ecp3-mediated HR in tomato. None of them induced a specific Cf-mediated HR, but two SSCPs triggered non-specific necrosis in tomato, including a protein belonging to glycoside hydrolase family 17 and a protein that carries an MD-2-related lipid-recognition (ML)-domain. In addition, a NEP1-like protein, NLP1, was identified *in silico* and was shown to also trigger non-specific necrosis in tomato and tobacco. Quantitative PCR performed on 21 of the 60 SSCPs revealed that half of them, including one of the non-specific necrosis triggering proteins, are poorly expressed during infection compared to the known effectors of *C. fulvum*. Finally, we showed that deletion of the highly induced Ecp4 and Ecp5 effectors results in decreased virulence of *C. fulvum* on tomato. Altogether, our results indicate that the identification of virulence and avirulence factors should preferentially focus on *SSCP* genes that are highly up-regulated during infection.

INTRODUCTION

Effectors are proteins secreted by pathogens, including bacteria, fungi, oomycetes and nematodes, to manipulate host physiology and promote disease. Effectors play a particularly crucial role in attenuation or suppression of the plant immune system to avoid recognition. For example, both AvrPiz-t of *Magnaporthe oryzae* and Avr3a of *Phytophthora infestans* suppress pathogen-associated molecular

pattern (PAMP)-triggered immunity (PTI) in plants (Bos *et al.*, 2010; Park *et al.*, 2012). Some effectors have the ability to suppress effector-triggered immunity (ETI), such as Avr1 of *Fusarium oxysporum* f. sp. *lycopersici*, which suppresses I-2- and I-3-mediated resistance (Houterman *et al.*, 2008; Houterman *et al.*, 2009), and the three cytoplasmic effectors of *Phytophthora sojae*, Avr3b, Avh172 and Avh238, which suppress ETI-triggered cell death and ROS accumulation (Dong *et al.*, 2011; Wang *et al.*, 2011). Other effectors, such as Avr2 of the fungus *Cladosporium fulvum*, EPIC1 and EPIC2B of the oomycete *P. infestans*, and Gr-VAP1 of the nematode *Globodera rostochiensis* inhibit the same target, the cysteine protease Rcr3^{pim} that is involved in basal defense of tomato (Kruger *et al.*, 2002; Song *et al.*, 2009; Lozano-Torres *et al.*, 2012). Since effectors are important virulence factors, their identification and characterization might bring new insights into the understanding of plant-pathogen interactions at the molecular level. The increasing number of genome sequences from fungal and oomycete phytopathogens have allowed the identification of many more effector candidates. The selection criteria generally used for the identification of effector candidates include (i) the presence of a N-terminal signal peptide for secretion, (ii) relatively small protein size [<300 amino acids (aa) in length], (iii) the occurrence of at least four cysteine residues, (iv) the presence of pathogenicity-related motifs or domains or aa sequence repeats, (v) localization to gene-sparse or repeat-rich regions of the genome, or (vi) specific induction during plant infection (Raffaele *et al.*, 2010; Hacquard *et al.*, 2012; Saunders *et al.*, 2012). Using this kind of approach, novel effectors that suppress PTI were successfully identified in the oomycete *Hyaloperonospora arabidopsidis* (Fabro *et al.*, 2011).

       C. fulvum is a non-obligate biotrophic fungal pathogen that causes leaf mould of tomato (*Solanum lycopersicum* L.) (Thomma *et al.*, 2005). During infection this fungus remains exclusively in the apoplastic space of tomato leaves where it secretes effectors that are needed to establish the disease (Stergiopoulos & de Wit, 2009). To date, only ten effector genes from *C. fulvum* have been cloned, and the intrinsic functions of only three of them have been characterized. Avr2 and Avr4 provide

protection against host enzymes deployed as a form of basal defense (Kruger *et al.*, 2002; van den Burg *et al.*, 2006; van Esse *et al.*, 2007). Specifically, Avr2 is an inhibitor of four tomato cysteine proteases (Rcr3[pim], Pip1, aleurain and TDI-65) (Kruger *et al.*, 2002; Rooney *et al.*, 2005; van Esse *et al.*, 2008) and Avr4 binds chitin present in the fungal cell wall, protecting it from hydrolysis by host chitinases (van den Burg *et al.*, 2003; van den Burg *et al.*, 2004; van den Burg *et al.*, 2006; van Esse *et al.*, 2007). The third functionally characterized effector, Ecp6, is a LysM domain-containing protein that sequesters fungal chitin fragments and competes with plant chitin receptors involved in PTI (de Jonge *et al.*, 2010). However, some effectors might also have a larger size as exemplified by *C. fulvum* CfTom1, which is a glycosyl hydrolase family 10 (GH10) enzyme that degrades the antifungal glycoalkaloid α-tomatine to the less fungi-toxic compound tomatidine (Ökmen *et al.*, 2013). Although effectors of *C. fulvum* seem to support fungal growth during colonization of susceptible plants (compatible interaction), they are also avirulence factors because they can be recognized by receptor-like proteins (RLPs) encoded by the *Cf* resistance genes (*R*), leading to tomato resistance (incompatible interaction). Eight identified *C. fulvum* effectors (Avr2, Avr4, Avr4E, Avr9, Ecp1, Ecp2, Ecp4 and Ecp5) are recognized by the corresponding tomato RLP Cf-2, Cf-4, Cf-4E, Cf-9, Cf-Ecp1, Cf-Ecp2, Cf-Ecp4 and Cf-Ecp5, respectively (de Wit *et al.*, 2009). Additional *R*-traits, of which the matching *C. fulvum* effector genes are unknown, have been identified in different tomato lines, such as *Cf-1*, *Cf-3*, *Cf-5*, *Cf-6*, *Cf-9B*, *Cf-11* and *Cf-Ecp3* (Dixon *et al.*, 1998; Panter *et al.*, 2002; Yuan *et al.*, 2002).

Analysis of the genome sequence of *C. fulvum* identified 1,195 genes encoding secreted proteins among the 14,127 predicted gene models (de Wit *et al.*, 2012). This *in silico* secretome comprises Avr4, Avr4E, Ecp1, Ecp2, Ecp4 and Ecp6 effectors (others are not included because gene models were not predicted), but it might also contain effectors that are recognized by the *R*-traits mentioned above. In this study, we aimed at identifying such novel effectors that specifically elicit a Cf-1-, Cf-3-, Cf-5-, Cf-9B-, Cf-11- or Cf-Ecp3-mediated HR in tomato. In addition we addressed the virulence function of the newly identified NLP1 [necrosis and ethylene-inducing protein 1 (NEP1)-like

protein 1] homologue and of two previously identified effectors of *C. fulvum*, Ecp4 and Ecp5.

RESULTS

Functional annotation of the *Cladosporium fulvum* secretome

The predicted secretome of *C. fulvum* was mined for proteins with the potential to function as effectors. So far, all characterized effector proteins of *C. fulvum*, with the exception of CfTom1 and Ecp2-3 (Stergiopoulos *et al.*, 2010; Ökmen *et al.*, 2013), are shorter than 300 amino acids in length and have four or more cysteine residues. Based on these criteria, 271 out of the 1,195 secreted proteins were designated as secreted small cysteine-rich proteins (SSCPs) (de Wit *et al.*, 2012). These putative effectors were re-annotated using Interpro Scan and Gene Ontology terms to attain information about their possible role in virulence. 106 SSCPs did not have any BLASTP hit against proteins present in the NCBI database, suggesting that they are *C. fulvum*-specific, a commonly accepted characteristic of fungal effectors (Stergiopoulos & de Wit, 2009). 58 SSCPs were homologous to conserved hypothetical proteins and they might correspond to conserved effectors with yet unknown function. 20 CAZy enzymes and 10 hydrophobins were identified in that SSCP catalogue (de Wit *et al.*, 2012). In addition to Ecp6 that contains three Lys-M motifs, two other proteins that contain a single Lys-M motif (PF01476) were identified. Three homologues of the *Venturia inaequalis* candidate effector 14 contain an SCP-like protein domain (PF00188) (Bowen *et al.*, 2009). One putative effector is a member of the cerato-platanin-like protein family (PF07249) that comprises phytotoxins (Meinhardt *et al.*, 2008). Other interesting candidates have a WSC-domain (PF01822) involved in carbohydrate binding, a ribonuclease domain (PF00545) or a histone deacetylase domain (PF02146) that might be involved in transcriptional regulation. Also, a protein similar to NEP1-like proteins, which cause necrosis on plants, was found in this predicted secretome of *C. fulvum*.

Screening SSCPs for Cf-mediated hypersensitive responses

Many *Cf* genes, which provide resistance against different strains of *C. fulvum* that secrete the corresponding effectors, have been identified in different tomato cultivars. While the *R* and avirulence genes were cloned for several gene pairs, the effectors that trigger Cf-1-, Cf-3-, Cf-5-, Cf-9B-, Cf-11- or Cf-Ecp3-mediated HR have not been identified yet. In an attempt to identify one or more of these effector genes, 60 candidates were selected from the list of 271 predicted SSCP genes. The chosen candidates include 20 *C. fulvum*-specific genes, 22 genes encoding proteins with a conserved domain and 18 genes encoding proteins shared with other fungal plant pathogens (Supporting information Table S1). In addition to these criteria, preference was also given to those effector gene candidates that are located within 10 kb of repetitive genome sequence, similar to the genomic location of many identified effector genes from *C. fulvum* and other plant pathogenic fungi (Rep & Kistler, 2010; de Wit *et al.*, 2012). All 60 candidates were cloned into a binary *Potato Virus X* (PVX)-based expression vector, *pSfinx*, under the control of the *Cauliflower Mosaic Virus* (CaMV) 35S constitutive promoter for heterologous expression in tomato lines carrying the *Cf-1*, *Cf-3*, *Cf-5*, *Cf-9B*, *Cf-11* or *Cf-Ecp3* genes in order to identify the corresponding *Avr1*, *Avr3*, *Avr5*, *Avr9B, Avr11* and *Ecp3* effector genes, respectively. The *Cf-0* tomato line that does not carry any known *Cf* gene was used as a negative control. None of the candidates induced specific necrosis on given tomato lines, showing that none of them corresponds to the avirulence signals recognized by the *Cf* genes employed in this experiment. However, two effector candidates triggered non-specific chlorosis and necrosis on most of the tested tomato lines, including *Cf-0* (Fig. 1). One candidate is a member of glycoside hydrolase family 17 (GH17) (184408) and is predicted to be an exo-beta-1,3-glucanase (Supporting information Fig. S1). This protein triggered strong necrosis on all tested tomato lines, except *Cf-Ecp3* (Fig. 1B). The tomato lines consistently responded differently to the expression of this protein, with the strongest necrosis found for the *Cf-1* line (Fig. 1B). The other candidate is an ML (MD-2-related lipid-recognition; PF02221) domain-containing protein (192503), involved in lipid

recognition but its specific function remains elusive. This protein induced non-specific chlorosis and weak necrosis on all tested tomato lines after three weeks (Fig. 1A).
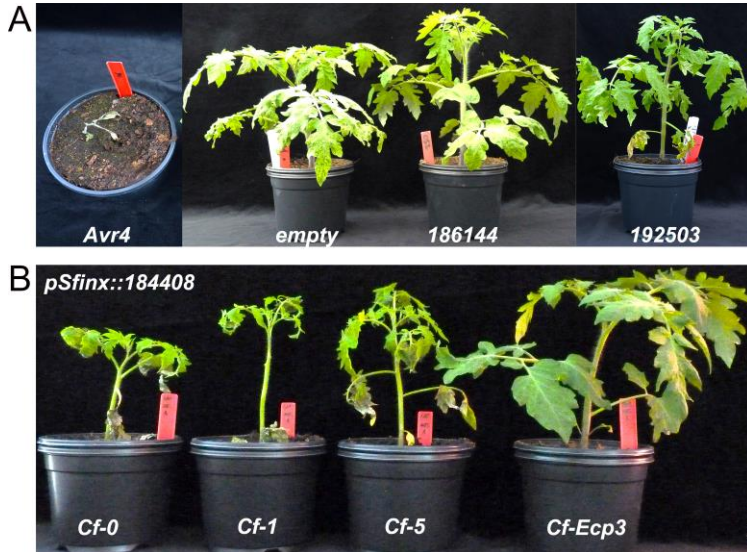

Fig. 1. Screening of *Cladosporium fulvum* effector candidates on tomato lines carrying different *Cf* resistance genes. (A) *Cf-0*, *Cf-1*, *Cf-3*, *Cf-4*, *Cf-5*, *Cf-9B*, *Cf-11* and *Cf-Ecp3* tomato lines were inoculated with *Agrobacterium tumefaciens* containing *pSfinx* plasmids that contain each one of the 60 effector candidates, *Avr4* as a positive control or empty vector as a negative control. *186144* is shown as a representative of effector candidates that did not trigger necrosis on any tested tomato line. *192503* elicited non-specific chlorosis and weak necrosis on all tested tomato lines. Necrosis induced in *Cf-5* tomato is shown as a representative response. **(B)** *184408* triggered non-specific necrosis on all tested tomato lines but *Cf-Ecp3*. Representative necrosis is shown for a few tomato lines only. Pictures were taken 3-4 weeks post inoculation.

Because none of the effector candidates triggered a specific Cf-mediated HR and two of them induced non-specific necrosis, we reasoned that many of these genes might not be expressed during infection of tomato by *C. fulvum*. Indeed, quantitative PCR showed that all characterized *C. fulvum* effector genes are not or lowly expressed *in vitro*, but they are highly up-regulated during infection of tomato (Fig. 2A). In contrast, all but two genes (187184 and 193896) show an expression level lower than

that of *Avr4E*, the *C. fulvum* effector gene with the lowest expression level (Fig. 2B). However, seven genes are significantly up-regulated *in planta* to an expression level similar or higher than that of tubulin. In particular, the candidate 184408 that causes strong non-specific necrosis is up-regulated during the late stages of infection (Fig. 2B). The other non-specific necrosis-inducing protein 192503 is expressed *in vitro* and appears to be down-regulated *in planta*.
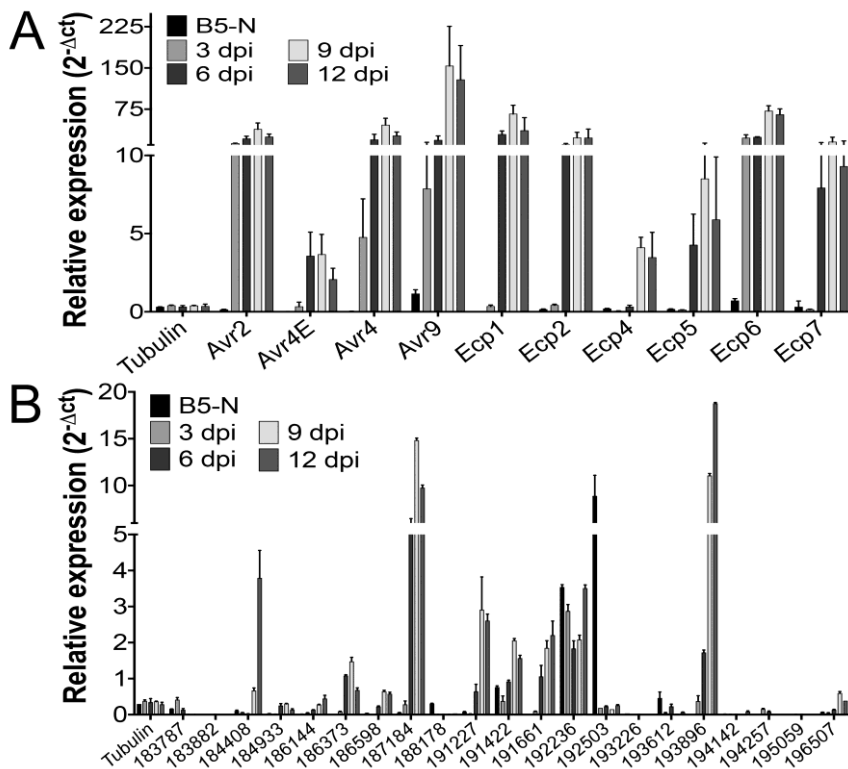

Fig. 2. Expression profile of *Cladosporium fulvum* effector genes *in vitro* and *in planta*. (A) Relative expression of characterized effector genes was assessed using quantitative PCR during *in vitro* growth in B5-N medium and from three to 12 days post inoculation (dpi) of tomato. **(B)** Relative expression of 21 effector candidates was assessed using quantitative PCR under the same conditions as in (A). Expression was normalized using the *actin* gene of *C. fulvum* using to the $2^{-\Delta Ct}$ method. The tubulin gene was used as a control for normalization. Error bars represent standard deviation of at least two biological repeats.

Identification and characterization of a NEP1-like protein

NLP1 proteins [necrosis and ethylene-inducing protein 1 (NEP1)-like protein 1] are widely distributed among prokaryotic and eukaryotic plant pathogens (Gijzen & Nuernberger, 2006). These proteins are important virulence factors in hemi-biotrophic and necrotrophic plant pathogens, such as *P. sojae* and *Verticillium dahliae* because they are up-regulated during the transition from a biotrophic to a necrotrophic stage (Qutob *et al.*, 2002; Kanneganti *et al.*, 2006; Santhanam *et al.*, 2013). A homologue of *NLP1* was found in the genome of *C. fulvum* and its role in the life cycle of *C. fulvum* is questioned because of its biotrophic lifestyle. The predicted NLP1 homologue of *C. fulvum* was designated CfNLP1 (*C. fulvum* NEP1-Like Protein 1; 194232). The CfNLP1 protein shares 46%, 54%, 43% and 47% aa identity to NLP1 from *P. infestans*, *Botrytis cinerea*, *F. oxysporum* f. sp. *lycopersici* and *Bacillus subtilis*, respectively (Bailey, 1995; Kanneganti *et al.*, 2006; Schouten *et al.*, 2008). CfNLP1 has features typical of type 1 NLP1s, including the presence of an NPP1-domain (Pfam domain PF05630), the conserved heptapeptide motif 'GHRHDWE' (GHR<u>F</u>DWE in CfNLP1), and two strictly conserved cysteine residues (Ottmann *et al.*, 2009).

To examine the necrosis-inducing activity of CfNLP1, the corresponding gene was heterologously expressed in *Nicotiana tabacum* under the control of the CaMV 35S promoter using the *Agrobacterium tumefaciens*-mediated transient gene expression assay (ATTA) (Stergiopoulos *et al.*, 2010). This assay revealed that CfNLP1 triggers strong necrosis in *N. tabacum* leaves at 3 days post inoculation (dpi) (Fig. 3A), showing that the conserved heptapeptide motif is functional. Indeed, substitution of the conserved aa histidine (H) at position two of this motif by alanine (A) (H106>A) resulted in complete loss of necrosis-inducing activity in *N. tabacum* (Fig. 3A). Since tomato (*Solanum lycopersicum* L.) is the only known host of *C. fulvum*, the necrosis-inducing activity of CfNLP1 was also tested in *S. lycopersicum* Money Maker (MM)-*Cf-4* using the *pSfinx* expression vector for heterologous expression of *CfNLP1*. Consistent with the result obtained for *N. tabacum*, CfNLP1 induced necrosis in tomato as well. This response was stronger and faster than the necrosis induced by Avr4 upon

recognition by Cf-4 in MM-*Cf-4* (*pSfinx::Avr4* as a positive control) (Fig. 3B). Finally, the expression profile of *CfNLP1 in vitro* and *in planta* was determined using quantitative PCR. This analysis revealed that *CfNLP1* is hardly expressed in both conditions (Fig. 3C).
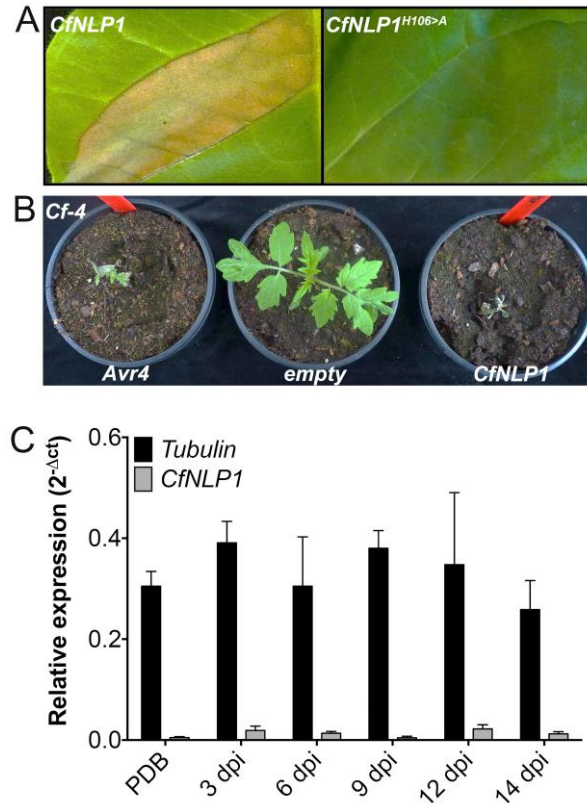

Fig.3. CfNLP1 induces non-specific necrosis in tobacco and tomato. (A) The wild-type and H106>A substituted version of *CfNLP1* of *C. fulvum* were heterologously expressed in *Nicotiana tabacum* using the *Agrobacterium tumefaciens* transient transformation assay. Photographs were taken three days post inoculation (dpi). **(B)** The necrosis-inducing activity of CfNLP1 was tested on *Cf-4* tomato plants after inoculation with *A. tumefaciens* carrying *pSfinx* plasmids that contain *CfNLP1*, *Avr4* as a positive control or empty vector as a negative control. Photographs were taken two weeks post inoculation. **(C)** Relative expression level of *CfNLP1* and *tubulin* genes was assessed by quantitative PCR during *in vitro* growth in PDB medium and during infection of tomato from three to 14 dpi. Expression was normalized using the *actin* gene of *C. fulvum* using the $2^{-\Delta Ct}$ method. Error bars represent standard deviation of three biological repeats.
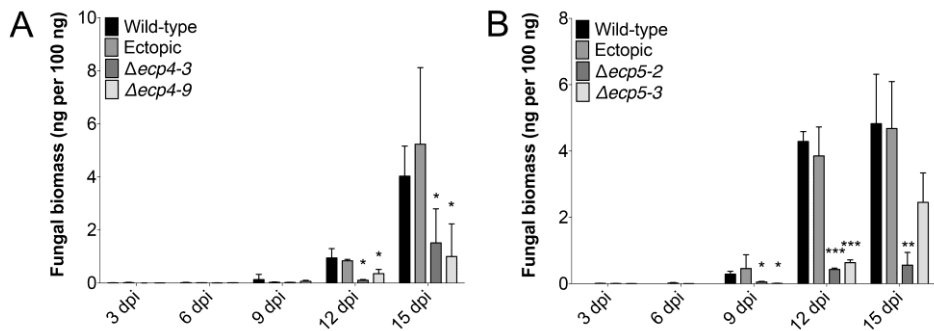
Fig. 4. Δ*ecp4* and Δ*ecp5* deletion mutants show reduced virulence on tomato. Virulence of the wild-type *Cladosporium fulvum* strain, ectopic transformants and two independent Δ*ecp4* **(A)** and two independent Δ*ecp5* **(B)** mutants was assessed by quantification of fungal biomass from three to 15 days post inoculation (dpi). Quantitative PCR was performed on genomic DNA using the *actin* gene of *C. fulvum* as a standard. The fungal biomass was deduced from a standard curve. A multiple t test analysis was performed and followed by a Holm-Sidak test. Only significant differences compared to wild type are indicated (* P<0.05; ** P<0.01; *** P<0.001). Error bars represent standard deviation of at least two biological repeats.

Ecp4 and Ecp5 are required for full virulence of *Cladosporium fulvum* on tomato

Most of the characterized effectors of *C. fulvum* reported so far were shown to be avirulence factors, but also key components of *C. fulvum* virulence on tomato. Ecp4 and Ecp5 were also shown to trigger Cf-Ecp4- and Cf-Ecp5-mediated HR, respectively (Lauge *et al.*, 2000), but it is not known whether they play a role in virulence. To address this question, targeted gene replacement of the *Ecp4* and *Ecp5* genes was carried out. Gene deletion and occurrence of a single insertion event were confirmed by PCR and quantitative PCR, respectively (Supporting information Figs. S2 and S3). Both mutants did not show any phenotype *in vitro*. Susceptible tomato plants were inoculated with the deletion mutants, ectopic transformants and wild-type strain to assess the role of Ecp4 and Ecp5 in virulence. The fungal biomass was quantified by quantitative PCR as a means to follow plant infection from three to 15 dpi. No significant differences in fungal biomass were observed between wild-type and ectopic

transformants (Fig. 4A-B). In contrast, the fungal biomass of the Δ*ecp4* and Δ*ecp5* mutants was significantly reduced compared to wild-type as early as 12 and 9 dpi, respectively (Fig. 4A-B). These results indicate that both genes are required for full virulence of *C. fulvum* on tomato.

DISCUSSION

For successful colonization and infection to occur, plant pathogenic fungi must evade or suppress the host defense system, which is achieved by secretion of a cocktail of effector proteins. Identification and functional characterization of effectors are of great interest to obtain new insights into the mechanisms that plant pathogens employ to establish disease. The *C. fulvum*-tomato pathosystem has been used for decades to study the role of effectors in fungal virulence and avirulence. Yet, only a limited number of effectors have been characterized from this fungus. The availability of the genome sequence of *C. fulvum* strain 0WU (de Wit *et al.*, 2012) allowed us to screen for novel effectors involved in avirulence and virulence. For this purpose, the SSCP catalogue of *C. fulvum* was predicted using features typical of known *C. fulvum* effectors as criteria for the search, including a protein size shorter than 300 aa and four or more cysteine residues. From this catalogue, we selected and screened 60 SSCP candidates for their ability to elicit an HR in tomato lines carrying different *Cf* genes. Tested candidates included 20 *C. fulvum*-specific proteins, 22 proteins that contain pathogenicity-related domains such as a LysM and chitin-binding domain and 18 proteins that are conserved in other plant pathogenic fungi. However, none of the effector candidates triggered a Cf-mediated HR, suggesting that the remaining 211 predicted SSCPs should be tested. But this list might not contain all effector genes of *C. fulvum* because of the bias of the criteria used in the selection. Indeed, some known fungal effectors are not cysteine rich proteins like AvrLm1 of *Leptosphaeria maculans* and Avr2 of *F. oxysporum* f. sp. *lycopersicum*, which have only one and two cysteine residues, respectively (Gout *et al.*, 2006; Houterman *et al.*, 2007). Others are longer than 300 aa such as AvrM of *Melamspora lini* that contains 314 aa, including only one

cysteine residue (Catanzariti *et al.*, 2006). Similarly, CfTom1 of *C. fulvum*, which is responsible for α-tomatine degradation during infection, is an effector required for full virulence, but it has more than 300 aa and contains only two cysteine residues (Ökmen *et al.*, 2013). Thus, candidates that do not comply with the criteria used in this study should be considered in case the list of 271 SSCPs does not contain the avirulence signals that correspond to the known *R* genes.

While no novel effectors were identified that triggered a Cf-mediated HR, we found two effectors triggering non-specific necrosis on tomato. We also identified *CfNLP1* and showed that its product induced non-specific necrosis on tomato and tobacco. In addition, other SSCPs display homology to known cell death-inducing proteins from other plant pathogenic fungi. For example, 183787 and 186485 possess a CFEM domain (Pfam domain PF05730) that also occurs in the cell death-inducing effector MoCDIP2 of the hemi-biotrophic rice blast fungus *M. oryzae* (Chen *et al.*, 2013). The CFEM domain-containing region of MoCDIP2 is sufficient for effective cell death induction (Chen *et al.*, 2013). Another *C. fulvum* SSCP, 186144, is homologous to fungal-secreted cerato-platanins (Pfam domain PF07249), a family of non-catalytic proteins that can function as virulence factors and/or elicitors of defense responses (Zaparoli *et al.*, 2009; Lombardi *et al.*, 2013). While none of these homologues were found to induce non-specific necrosis in tomato, two SSCPs of *C. fulvum*, 184408 (GH17 enzyme) and 192503 (ML domain-containing protein), caused non-specific necrosis on most of the tested tomato cultivars. Expression of 184408 at late stages of tomato infection by *C. fulvum* suggests that it might play a role in virulence when the fungal biomass dramatically increases and more nutrients are required. *C. fulvum* might secrete this enzyme for retrieval of monosaccharides from tomato cell walls for growth at this stage of infection because 184408 is predicted to have an exo-β-1,3-glucanase activity. The phenotype observed in plants constitutively expressing this enzyme is unlikely the result of plant cell wall degradation (maceration) because no necrosis is observed on *Cf-Ecp3* plants. Instead, 184408 might release significant quantities of damage-associated molecular patterns (DAMPs) from the plant cell wall, which would

induce defense responses. Lack of cell death induction in *Cf-Ecp3* tomato lines could point to lack of a DAMP recognition receptor that is present in all other tested tomato lines. The other effector candidate, 192503, is highly expressed *in vitro* but much less so during infection, indicating it is likely not involved in virulence and its role in infection and the *in planta* phenotype remains elusive. Similarly, although the predicted secretome of *C. fulvum* contains an *NLP1* homologue, this gene does not seem to be expressed in any of the conditions tested, including during tomato infection. With a few exceptions, NLPs are predominantly produced by hemi-biotrophic and necrotrophic plant pathogens, suggesting a function in the transition from a biotrophic to a necrotrophic stage concomitant with their necrosis-inducing activity, as has been shown for *P. sojae*, *P. infestans* and *Colletotrichum higginsianum* (Qutob *et al.*, 2002; Kanneganti *et al.*, 2006; Kleemann *et al.*, 2012). Although 12 NLPs have been identified in the obligate biotroph *H. arabidopsidis*, none showed necrosis-inducing activity, suggesting that they have evolved to carry out functions relevant to a biotrophic lifestyle (Cabral *et al.*, 2012). The low expression of *CfNLP1* also correlates with the biotrophic lifestyle of *C. fulvum*. *CfNLP1* likely plays a role in other parts of the lifecycle of *C. fulvum*, possibly during its saprophitic stage of which hardly anything is known. Alternatively, the *CfNLP1* might be an ancestral gene that is on its way to lose its original function.

All the characterized effectors of *C. fulvum* are highly up-regulated during infection, which seems to be the most important feature shared by all *C. fulvum* effector genes reported so far. Of the 60 effector candidates tested for recognition, only a few showed this expression pattern and most were barely expressed during infection of tomato. Thus, while mining genomes of phytopathogenic fungi is very efficient for identification of putative effector genes, such bioinformatics analysis alone does not give a true representation of the biologically relevant effector catalogue. We therefore suggest that future research to identify novel *C. fulvum* effector candidates requires large-scale gene expression profiling as a primary selection criterion. This approach has been employed successfully in the *C.*

higginsianum-Arabidopsis pathosystem to identify genes that are highly up-regulated during the infection process, including effector candidates (Kawahara *et al.*, 2012; O'Connell *et al.*, 2012).

MATERIAL AND METHODS

Fungal and plant materials

C. fulvum race 0WU [CBS131901; (de Wit *et al.*, 2012)] was grown on half-strength potato dextrose agar (19.5 g l^{-1} PDA and 15 g l^{-1} technical agar, Oxoid, Cambridge, UK) at 20°C for 2-3 weeks for conidium production and DNA isolation. For the analysis of *in vitro* gene expression, *C. fulvum* was pre-incubated in Gamborg B5 medium (Duchefa Biochemie) supplemented with 20 g l^{-1} sucrose at 22°C and incubated in an orbital shaker at 200 rpm for 6 days. *C. fulvum* mycelia were then transferred to B5 medium without nitrogen and incubated at 22°C in an orbital shaker at 200 rpm for 24 h.

Susceptible Money Maker (MM)-*Cf-0*, MM-*Cf-1*, MM-*Cf-3*, MM-*Cf-4*, MM-*Cf-5*, MM-*Cf-9B*, MM-*Cf-11* and MM-*Cf-Ecp3 Solanum lysopersicum* cultivars were used for the *pSfinx* screening experiment and MM-*Cf-0* for *C. fulvum* inoculation experiments. Tomato plants were grown in a greenhouse at 70% relative humidity, at 23-25°C during the day and at 19-21°C during the night, with a light/dark regime of 16/8 h and 100 Watt m^{-2} supplemental light when the sunlight influx intensity was less than 150 Watt m^{-2}. *Nicotina tabacum* cultivars were grown in a growth chamber at 20°C and 70% relative humidity with a photoperiod of 12 h.

Functional annotation

The Blast2GO program was used to predict multiple associated Gene Ontology categories for each protein with default parameter settings and given BLAST results against the non-redundant database of NCBI (December 2012) as input (Conesa *et al.*, 2005). Additionally, each protein sequence was scanned for the presence of

protein domains using InterproScan v4.4 including domain profiles from Pfam (December 2012).

Nucleic acid methods

Total RNA was isolated from ground mycelia or *C. fulvum*-infected tomato leaves using the combination of the trizol and RNeasy protocols described by Islas-Flores *et al.* (2006). Approximately 100 mg of the ground material was homogenized in 1 ml Trizol[®] reagent (Invitrogen, Carlsbad, CA, USA). After 5 min incubation at room temperature, 0.2 volume of chloroform was added to the sample, followed by gentle mixing. The sample mixture was incubated at room temperature for 5 min and centrifuged at maximal speed for 15 min. Subsequently, supernatant was transferred to a new tube and 0.5 volume of 80% ethanol was added to the aqueous phase. Further total RNA purification was performed with the NucleoSpin RNA Plant Kit (Macherey-Nagel GmbH, Duren, Germany) according to manufacturer's instructions. cDNA was synthesized from 5 µg of total RNA using the SuperScript II reverse transcriptase kit (Invitrogen) according to manufacturer's instructions. Quantitative PCR was performed with the 7300 System (Applied Biosystems, Foster City, CA, USA): each reaction was performed in 25 µl containing 12.5 µl Sensimix (Bioline, London, UK), 1 µl of each forward and reverse primer (5 µM), 100 ng of template cDNA and 9.5 µl of double-distilled H$_2$O. The thermal profile included an initial 95°C denaturation step for 10 min, followed by denaturation for 15 sec at 95°C and annealing/extension for 45 sec at 60°C for 40 cycles. Quantitative PCR oligonucleotide pairs were designed with Primer3 Plus (Supporting information Table S2) (Untergasser *et al.*, 2007). The efficiency and specificity of the oligonucleotide pairs were determined with a dilution series of genomic DNA before use. The *C. fulvum* actin gene was used as a reference gene for normalization, and results were analysed using the $2^{-\Delta Ct}$ method (Livak & Schmittgen, 2001). The results are the average of three biological repeats. All PCRs were performed in 25 µl reaction mixture using *GoTaq* DNA polymerase (Promega), following the manufacturer's recommendations, and using 100 ng of genomic DNA or

cDNA as template. The PCR program was initiated by a denaturation step at 94°C for 5 min, followed by 32 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 45 sec and extension at 72°C for 1 min, with a final extension step at 72°C for 7 min.

Construction of *pSfinx* expression vectors

All 60 putative effector candidates, including CfNLP1, from the *C. fulvum* secretome were amplified from cDNA by PCR using oligonucleotide sets depicted in Supporting information Table S2. All forward primers exclude the native signal peptide sequence of the genes and include a 15-nucleotide overhang sequence corresponding to the *PR1A* signal peptide sequence; all the reverse primers contain the *Not*I restriction site (Supporting information Table S2). A second PCR was performed to amplify the *PR1A* signal peptide sequence by using the PR1A_F primer containing either *Asc*I or *Cla*I restriction sites and PR1A_R primer. Overlapping PCRs were performed to fuse the *PR1A* signal peptide to the putative effector genes by using PR1A_F and appropriate R primer for each gene (Supporting information Table S2). Amplified PR1A-candidate effector fragments were purified from the agarose gel by using the Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. The purified DNA fragments were subsequently cloned into the pGEM-T Easy vector (Promega) according to the manufacturer's instructions. Recombinant pGEM-T Easy vectors were transformed into chemically competent *Escherichia coli* cells (DH5α) by standard heat shock transformation protocol. Recombinant pGEM-T Easy vectors were retrieved from *E. coli* by using a Miniprep plasmid isolation kit (Qiagen Benelux bv, Venlo, The Netherlands) and sequenced at Macrogen Inc (Amsterdam, The Netherlands). The correct inserts were obtained from recombinant pGEM-T clones using *Asc*I (New England Biolabs) or *Cla*I, and *Not*I restriction enzymes (Promega) and were subsequently directionally ligated into the binary *Potato Virus X* (PVX)-based vector, pSfinx, (Hammond-Kosack *et al.*, 1995) that was also digested with *Asc*I or *Cla*I and *Not*I restriction enzymes. Ligation reactions were transformed into *E. coli* cells in order to

isolate *pSfinx::PR1A-candidate effector* constructs. Finally the recombinant *pSfinx::PR1A-candidate effector* constructs were transformed into *A. tumefaciens* (GV3101) by electroporation. *A. tumefaciens* strain containing the *pSfinx::PR1A-candidate effector* constructs were cultured on plates containing LB medium (10 g l^{-1} bacto-peptone; 5 g l^{-1} yeast extract; 5 g l^{-1} NaCl) supplemented with 50 µg ml^{-1} kanamycin and 25 µg ml^{-1} rifampicin for 48 h at 28°C. Heterologous expression of *pSfinx::PR1A-candidate effectors* in different tomato lines was performed as described in Stergiopoulos *et al*. 2010.

Heterologous expression of *CfNLP1* in tomato and tobacco plants

The *CfNLP1* gene (194232) from *C. fulvum* was amplified from *pSfinx::CfNLP1* construct by PCR using the following oligonucleotide set: the *AttB1*_PR1A_F and the *AttB2*_CfNLP1_R primer contain Gateway cloning recombination sites (Supporting information Table S2). Purified CfNLP1 fragments were cloned into *pDONR*^{TM} *p207*, using 1 µl of BP clonase^{TM} II enzyme mix (Invitrogen^{TM}), 70 ng of insert DNA in a 5 µl reaction volume, according to the manufacturer's instructions, and introduced into *E. coli*. To construct the final destination vector, an LR reaction was performed with 70 ng of *pENTRY*^{TM} *p207_PR1A*::*CfNLP1* and 70 ng of *pK2GW7* destination vector in the presence of 1 µl of LR clonase^{TM} II enzyme mix (Invitrogen) in a total volume of 5 µl. LR reaction was introduced into *E. coli* to obtain the final *pK2GW7::CfNLP1* plasmid. Insertion of all fragments was confirmed by PCR using insert-specific primers. The following primer sets were used to substitute histidine (H) 106 to alanine (A) via PCR; *AttB1*_PR1A_F and CfNLP1_R2, and CfNLP1_F2 and *AttB2*_CfNLP1_R (Supporting information Table S2). After overlapping PCR to fuse these two PCR fragments, full length H106>A CfNLP1 was cloned in to *pK2GW7* as described above.

Construction of the *Ecp4* and *Ecp5* gene deletion plasmids

The gene replacement vectors *pR4R3Δecp4* and *pR4R3Δecp5* were constructed using the MultiSite Gateway® Three-Fragment Vector Construction Kit

(Invitrogen[TM]) according to the manufacturer's instructions. The upstream (US) and downstream (DS) flanking regions of *Ecp4* (US, 2.3 kb; DS, 2.4 kb) and *Ecp5* (US, 0.85 kb; DS, 2 kb) were amplified using primers with overhang sequences homologous to the *AttB4, AttB1r*, *AttB2r* or *AttB3* recombination sites (Supporting information Table S2). The final *pR4R3Δecp4* and *pR4R3Δecp5* plasmids were constructed as described by Ökmen *et al*. (2013).

Agrobacterium tumefaciens-mediated fungal transformation and mutant screening

The *pR4R3Δecp4* and *pR4R3Δecp5* constructs for deletion of the *Ecp4* and *Ecp5* genes in *C. fulvum,* respectively, were transformed into *A. tumefaciens* strain AGL1 by electroporation. *A. tumefaciens*-mediated transformation of *C. fulvum* was performed as described previously by Ökmen *et al*. (2013) and, the *Ecp4* and *Ecp5* genes were replaced with the *hygromycin resistance* (*HYG*) and *green fluorescent protein* (*GFP*) genes by homologous recombination. Deletion of the targeted genes was confirmed by PCR, while a single insertion event for the deletion cassettes in the genome of the mutants, reflected by the number of inserted *hygromycin* gene copies, was calculated using *actin* gene for normalization and the single copy gene *Avr4* as reference, using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Virulence assays

Wild-type *C. fulvum* (race 0WU; CBS131901), one ectopic and two independent Δ*ecp4* (Δ*ecp4-3* and Δ*ecp4-9*) and two independent Δ*ecp5* (Δ*ecp5-2* and Δ*ecp5-3*) mutants were grown on half-strength PDA plates for 2-3 weeks at 20°C. *C. fulvum* inoculations were performed on five-week-old MM-*Cf-0* tomato plants as described by Ökmen *et al.* (2013). Disease development of wild-type fungus and ectopic transformant, as well as Δ*ecp4* and Δ*ecp5* mutants was investigated at 3, 6, 9, 12 and 15 days post inoculation (dpi) with at least two biological replicates. Fungal biomass was quantified by quantitative PCR analysis as described previously, but with

genomic DNA as the template. Genomic DNA was isolated from tomato leaves infected by the wild-type fungus and ectopic transformant, as well as the Δ*ecp4* and Δ*ecp5* mutants, and diluted to a final concentration of 100 ng µl^{-1}. A standard curve was constructed using serial dilutions of *C. fulvum* genomic DNA (10 ng µl^{-1}; 1 ng µl^{-1}; 0.1 ng µl^{-1}; 0.01 ng µl^{-1}; 0.001 ng µl^{-1}) with *actin* as a reference gene. Logarithms (base 10) of DNA concentrations were plotted against the crossing point of C$_t$ (cycle threshold) values.

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SUPPORTING INFORMATION
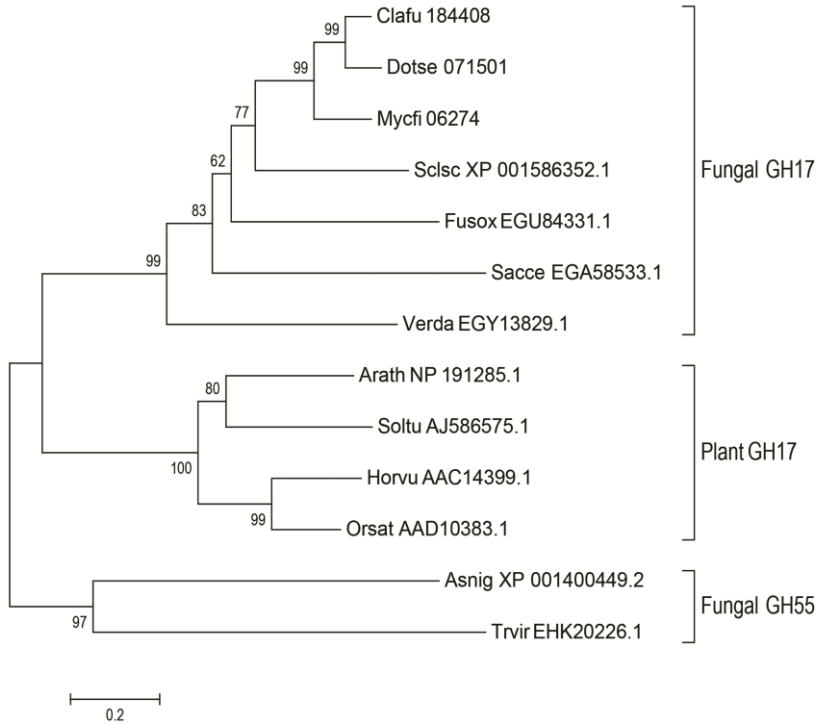
Supporting information Fig. S1. Phylogenetic tree of fungal and plant GH17 enzymes. Amino acid sequences were aligned using ClustalW2 and the phylogenetic tree was constructed with the Mega 5.1 program using the minimum evolution algorithm and performing 1,000 bootstraps. Fungal GH55 members were used as an out group. Scale bar shows the genetic distance (substitutions per site). *Clafu: Cladosporium fulvum; Dotse: Dothistroma septosporum; Mycfi: Mycosphaerella fijiensis; Sclsc: Sclerotinia sclerotiorum; Fusox: Fusarium oxysporum f. sp. lycopersici; Sacce: Saccharomyces cerevisiae; Verda: Verticillium dahliae; Arath: Arabidopsis thaliana; Soltu: Solanum tuberosum; Horvu: Hordeum vulgare; Orysa: Oryza sativa; Aspni: Aspergillus niger; Trivi: Trichoderma viride.*
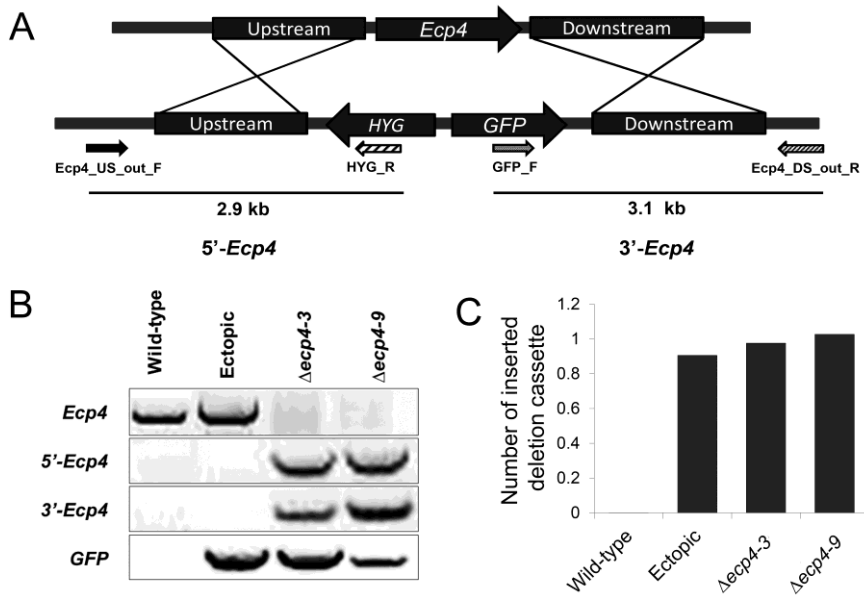
Supporting information Fig. S2. Molecular analysis of Δ*ecp4* mutants of *Cladosporium fulvum*. (A) Representation of the *Ecp4* locus in the wild-type and the Δ*ecp4* deletion mutant of *C. fulvum* after homologous recombination. The *Ecp4* gene is replaced by *hygromycin resistance* (*HYG*) and *green fluorescent protein* (*GFP*) genes. Oligonucleotides used to screen the mutants are indicated below. **(B)** Targeted gene deletion of *Ecp4* was confirmed by PCR using oligonucleotides shown in (A). **(C)** Single insertion event of the gene deletion cassette was confirmed by quantitative PCR using genomic DNA of each transgenic strain. The *HYG* gene was used as a measure for number of insertion events, together with the *actin* gene for normalization and the *Avr4* gene as a single copy reference gene, using the $2^{-\Delta Ct}$ method.
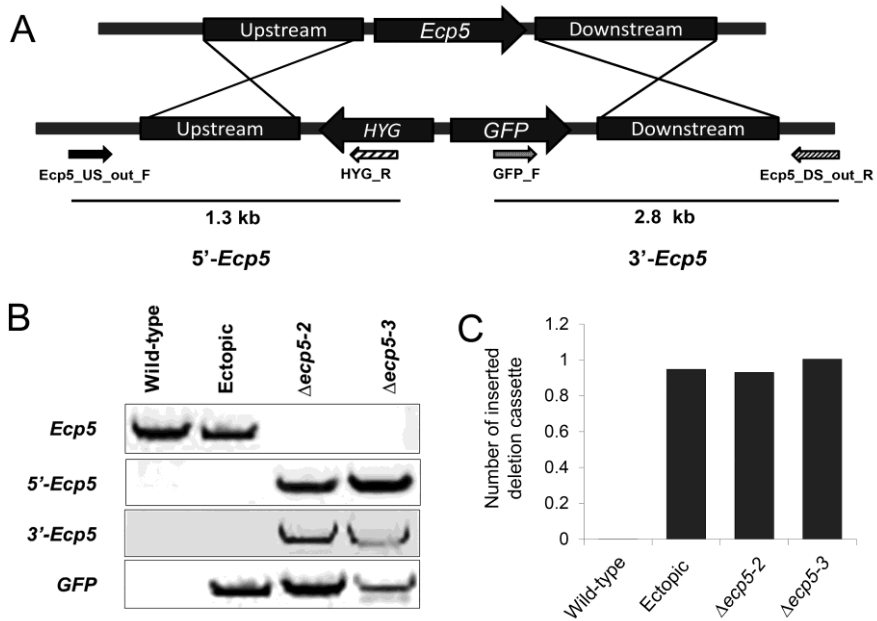
Supporting information Fig. S3. Molecular analysis of Δ*ecp5* mutants of *Cladosporium fulvum*. (A) Representation of the *Ecp5* locus in the wild-type and the Δ*ecp5* deletion mutant of *C. fulvum* after homologous recombination. The *Ecp5* gene is replaced by *hygromycin resistance* (*HYG*) and *green fluorescent protein* (*GFP*) genes. Oligonucleotides used to screen for the mutants are indicated below. **(B)** Targeted gene deletion of *Ecp5* was confirmed by PCR using oligonucleotides shown in (A). **(C)** Single insertion event of the gene deletion cassette was confirmed by quantitative PCR using genomic DNA of each transgenic strain. The *HYG* gene was used as a measure for number of insertion events, together with the *actin* gene for normalization and the *Avr4* gene as a single copy reference gene, using the $2^{-\Delta Ct}$ method.

Supporting information Table S1. 60 *Cladosporium fulvum* candidate effector proteins analyzed in this study.

JGI ID	Length	Cys #	Description	e-values	Distance to Repeats <5 kb
C. fulvum-specific proteins (20)					
184265	135	4	None		3'-3 kb
186373	165	10	None		5'-0.2 kb; 3'-0.2 kb
186477	167	9	None		5'-1.2 kb
186598	210	9	None		5'-0.2 kb; 3'-0.2
186625	297	7	None		3'-1 kb
187184	184	8	None		
187601	268	10	None		
190169	104	7	None		
190347	211	9	None		5'-3.5 kb
190392	89	10	None		5'-0.8 kb
190906	201	6	None		5'-2.5 kb
191227	164	4	None		5'-0.4 kb
191661	197	7	None		3'-1.6 kb
193226	238	8	None		3'-0.9 kb
193798	195	5	None		
193896	162	8	None		5'-2 kb
194586	130	8	None		5'-0.5 kb
195059	236	10	None		3'-1.8 kb
195437	82	9	None		5'-3.5 kb
196507	131	8	None		3'-0.1kb; 5'-0.3 kb
C. fulvum candidate effector proteins with homology with those present in other fungi (18)					
183790	195	2	Hypothetical protein MYCGRDRAFT_105677 [Zymoseptoria tritici IPO323]	3,6E-20	
183882	215	5	Hypothetical protein MYCGRDRAFT_34196 [Zymoseptoria tritici IPO323]	4,9E-72	5'-1.3 kb
184933	105	8	Hypothetical protein MPH_03145 [Macrophomina phaseolina MS6]	6,6E-16	5'-0.4 kb
185651	187	7	Hypothetical protein MYCGRDRAFT_99676 [Zymoseptoria tritici IPO323]	2,1E-18	3'-0.4kb

JGI ID	Length	Cys #	Description	e-values	Distance to Repeats <5 kb
188178	196	4	Hypothetical protein MYCGRDRAFT_41315 [*Zymoseptoria tritici* IPO323]	2,9E-54	
189051	160	11	Similar to small secreted protein [*Nectria haematococca* mpVI 77-13-4]	8,3E-22	
189382	155	8	Hypothetical protein MYCGRDRAFT_93838 [*Zymoseptoria tritici* IPO323]	2,1E-8	5'-0.2 kb
189833	242	5	Hypothetical protein MYCGRDRAFT_95537 [*Zymoseptoria tritici* IPO323]	3,8E-7	3'-0.9 kb
190522	241	9	Hypothetical protein [*Paracoccidioides brasiliensis* Pb01]	8,3E-12	
190687	106	6	Similar to Pc22g06530 [*Penicillium chrysogenum* Wisconsin 54-1255]	1,2E-32	5'-2.3 kb
190787	183	5	Similar to predicted protein [*Magnaporthe grisea* 70-15]	2,9E-75	
193612	141	6	Similar to small secreted protein [*Nectria haematococca* mpVI 77-13-4]	8,7E-24	
194693	114	6	Hypothetical protein MYCGRDRAFT_46900 [*Zymoseptoria tritici* IPO323]	4,2E-43	
194955	198	5	Hypothetical protein MYCGRDRAFT_96436 [*Zymoseptoria tritici* IPO323]	3,8E-31	
195050	248	11	Similar to predicted protein [*Pyrenophora tritici-repentis* Pt-1C-BFP]	1,5E-44	
195873	227	4	Similar to Pc16g03280 [*Penicillium chrysogenum* Wisconsin 54-1255]	2,2E-65	5'-0.4kb; 3'-0.4 kb
196849	149	4	Hypothetical protein MYCGRDRAFT_105182 [*Zymoseptoria tritici* IPO323]	8,2E-22	5'-0.2 kb
197226	128	2	Hypothetical protein NECHADRAFT_106217 [*Nectria haematococca* mpVI	6,5E-27	
C. fulvum candidate effector proteins with a conserved domain (22)					
183787	297	8	Similar to CFEM domain protein	1,0E-4	
183917	223	7	Similar to cutinase precursor [*Aspergillus terreus* NIH2624]	2,4E-70	5'- 0.2 kb; 3'-0.1 kb
184408	289	5	Glycosyl hydrolases family 17	3,0E-14	
184928	246	5	Exo-beta-glucanase	1,1E-38	
186144	146	4	Similar to Epl1 protein [*Trichoderma atroviride*]	9,5E-49	
186485	263	8	Similar to CFEM domain protein [*Aspergillus clavatus* NRRL 1]	1,3E-12	
186520	228	5	Similar to NAD-dependent deacetylase sirtuin-5 [*Pyrenophora tritici-repentis*]	3,7E-87	
186906	226	4	Similar to acetylxylan esterase 2 precursor, putative [*Penicillium marneffei*]	8,8E-61	
187834	146	4	Similar to GPI anchored serine-threonine rich protein [*Penicillium marneffei*]	1,1E-29	
188403	291	5	Similar to expansin family protein [*Flammulina velutipes*]	3,7E-27	
189372	195	4	Similar to PhiA protein [*Emericella nidulans*]	1,0E-25	
190869	197	4	Similar to IgE-binding protein [*Aspergillus fumigatus* Af293]	8,3E-35	5'-0.5 kb
191039	230	4	SCP-like extracellular protein	1,1E-47	3'-1.5 kb
191043	276	16	LysM containing protein	5,6E-36	

JGI ID	Length	Cys #	Description	e-values	Distance to Repeats <5 kb
192236	285	5	SCP-like extracellular protein	1,8E-62	3'-0.7 kb
192503	180	4	Similar to ML domain protein [*Aspergillus fumigatus* Af293]	4,8E-69	
192888	208	6	Chitin binding protein, putative [*Aspergillus clavatus* NRRL 1]	8,7E-56	
194142	152	4	Extracellular guanyl-specific ribonuclease	7,6E-28	
194152	249	6	Similar to malate dehydrogenase [*Aspergillus fumigatus* Af293]	8,4E-76	3'-0.4 kb
194257	211	5	SCP-like extracellular protein	4,8E-52	
194863	158	8	WSC domain-containing protein	1,2E-20	
195452	229	6	Similar to ThiJ/PfpI family protein [*Neosartorya fischeri* NRRL 181]	7,8E-41	

Supporting information Table S2. Oligonucleotides used in this study.

Primer name	Sequence (5'-3')
qCf_Actine_F	GGCACCAATCAACCCAAAG
qCf_Actine_R	TACGACCAGAAGCGTACAG
qAvr2_F	GCAGCAGCCAAAAAACTACC
qAvr2_R	TTCCTCCCCTCGTCAACTTC
qAvr4_F	CCCCAAAACTCAACCATACAAC
qAvr4_R	GCTTCGCATTGCCAACTTC
qAvr4E_F	GCAATCAAGCCGAATGGAG
qAvr4E_R	ATGTGACCGAACATCCCAG
qCfAvr9_F	TTGCTACTACTCTCCCACTTTGC
qCfAvr9_R	AGTGGACACATTGTAGCTTATGAAA
qEcp1_F2	AACCAGAACTGCCAGCAAAT
qEcp1_R2	TTAAAGGCACTTGGGGTTTG
qEcp2_F	CACCTACAACCAAATTGTCTCC
qEcp2_R	TGAACTCTGACCTGACCACC
qEcp4_F	GTTGCATTGTCAAGCTGTTC
qEcp4_R	CTGCCATCCACCAACAATC
qEcp5_F	TACGACACGACTGGAGAAC
qEcp5_R	CGAACATCAAACGTCAAATGC
qEcp6_F	TAACCCCGACAACAAGTCC
qEcp6_R	GTCGAGCGTGATGTTGAAG
qEcp7_F	AACATCGGTTGTCGAAAAGG
qEcp7_R	GGCATGCATTGTCGTCATAG
qCf_Tubulin_F	CCTTCAGAGCTGTAACTGTCC
qCf_Tubulin_R	CCTCCTTCATAGATACCTTGCC
qHYG_F	GATGTAGGAGGGCGTGGATA
qHYG_R	ATAGGTCAGGCTCTCGCTGA
qCfNLP_F	TCCTGGTTCTGGCCTAAAGA
qCfNLP_R	GCCTCGTACCACTCAGGTTC
q183787_F	GCAATGCACTGTGACCAACT
q183787_R	TGTCGCGTTGTTGATGGTAT
q183882_F	GCATCAGTCAAGCACAGTTC
q183882_R	TGTAGTCCCCACACATTCC
q184408_F	GACTGCCATCAACCAACAC
q184408_R	CAACATGCCCAACCTCAAC
q184933_F	GAACAATGAAGCCACACAAAAG
q184933_R	AGCAGTGTCAAAGTCGCAG
q186144_F	CAATGGCAACAGCATCAAC
q186144_R	TGAGACCGCATTGAGACAC
q186373_F	TTACTGCTCTTACGACTCCC
q186373_R	AGCCTGAACGCTACAAATAC
q186598_F	ATCTCTCCATAGACCTCCCC
q186598_R	GCCATGTTCTTGCACTTCTC
q186625_F	ACCTCACGTCCATCATGACA
q186625_R	CTCGAAGTCAGACCCTACGC
q187184_F	TTCCCCTCACCGACAAGTTC

Primer name	Sequence (5'-3')
q187184_R	AACACAACCATGCAGCTCC
q188178_F	GCAAGAACAAGAGCCCAAG
q188178_R	CGATGACGGAATCGGAGTAG
q191227_F	AATCACTGAAGATCCGAGCC
q191227_R	CGTCCTCATCATTTGCCTCC
q191661_F	ATGTTCTTGCTCTCCGTTGG
q191661_R	ATCCACTTTGGCATGCTTCT
q192236_F	TCTGTCGGTTTGCTTGAGTG
q192236_R	GTATGGCTCCTCACCGTTGT
q192503_F	AGTACGGCCTCATCACCATC
q192503_R	CATCCTTCGTCACCACCTTT
q193226_F	TCCTCTACCCCTACATCACCTC
q193226_R	TTGTAGCCGCATTGGTCTC
q193612_F	GCGGAAAGAGCAAGAATGAC
q193612_R	CCATCAGGGAAAACACCAC
q193896_F	ATTCATCAACACCGCAGCC
q193896_R	GCCTTCTTGACATCTCCTTCAC
q194142_F	ACCCTCACTACTACGGCAAC
q194142_R	ACCATTCTTCTCGGCGATTAC
q194257_F	AGGTTGGGTGTTGGACTGAG
q194257_R	TAACTCCAGCTCTCCGTCGT
q195059_F	AAGTGGTTTACTGCCCGATG
q195059_R	GTTCCCTTCCCCTCACTCTC
q196507_F	AGTACGCCGCTACATTCTC
q196507_R	ATGGTCCCAAAGACAGCAG
AscI_PR1A_F	GGCGCGCCATGGGATTTGTTCTCTTTTCAC
ClaI_PR1A_F	ATCGATATGGGATTTGTTCTCTTTTCAC
PR1A_R	ATTTTGGGCACGGCAAGAGTG
183787_F	CTTGCCGTGCCCAAAATGCGAAACTGCCAGACTGC
183787_R	GCGGCCGCTCATCGCCGACCCCGGCCAT
183790_F	CTTGCCGTGCCCAAAATGAACCACTCCGCTTCACC
183790_R	GCGGCCGCCTAGACGTACTGGTATGCC
183882_F	CTTGCCGTGCCCAAAATAGCCCAATACCACGATCCG
183882_R	GCGGCCGCCTACCACCTGCCCAGCGC
183917_F	CTTGCCGTGCCCAAAATACCCCCCTCACAAAACGC
183917_R	GCGGCCGCTTATGCCTGCACAGTGCC
184265_F	CTTGCCGTGCCCAAAATATGCCAGAGCCTGCTATCAC
184265_R	GCGGCCGCCTACGAGCTCGGTGTTGGC
184408_F	CTTGCCGTGCCCAAAATTACTACCAAGGGTTCAACGTTG
184408_R	GCGGCCGCTTAGCACCCGTACAAATCG
184928_F	CTTGCCGTGCCCAAAATTCGCCGGTCGATCTTTCC
184928_R	GCGGCCGCCTAGCAAGCCTCATTCTTGAAC
184933_F	CTTGCCGTGCCCAAAATTGTGCTAACTACGAAGATTGC
184933_R	GCGGCCGCCTAATCGAAAGGTCCGAC
185651_F	CTTGCCGTGCCCAAAATGCAGCATTATCAACCCGC
185651_R	GCGGCCGCCTACCCCTGCTTCGGCCTC
186144_F	CTTGCCGTGCCCAAAATACCACAGTTAGCTACGATGAGG

Primer name	Sequence (5'-3')
186144_R	GCGGCCGCTTATGCGTTGAATTCGATCG
186373_F	CTTGCCGTGCCCAAAATCTCGACTGCAAGGCCGTAGC
186373_R	GCGGCCGCCTATTGAGAACCACAGTGAACG
186477_F	CTTGCCGTGCCCAAAATTCACCGGTCCAGACCACC
186477_R	GCGGCCGCCTAGTGATCTCCTAAGATCG
186485_F	CTTGCCGTGCCCAAAATCAGATGACGCTCAACGACAT
186485_R	GCGGCCGCTTACATGGCGGCAAGAGCT
186520_F	CTTGCCGTGCCCAAAATCTCAGCCAAATCGCGGAAC
186520_R	GCGGCCGCCTAGGTCTGCTTCTTCGCC
186598_F	CTTGCCGTGCCCAAAATGCTCCAATCCGCGAAGCA
186598_R	GCGGCCGCTCACCCGTCAGCTCCTCG
186625_F	CTTGCCGTGCCCAAAATGACCTTCCAATCAAACTAAG
186625_R	GCGGCCGCTCACCGTATCGAACGTCCC
186906_F	CTTGCCGTGCCCAAAATGCTCCTCTGGTCCCAAGACAG
186906_R	GCGGCCGCCTATGATGCTACGCCAAC
187184_F	CTTGCCGTGCCCAAAATGTAGCCGTTGCTCAAGGCG
187184_R	GCGGCCGCTCACAAAGCTCCGAAGGTGTC
187601_F	CTTGCCGTGCCCAAAATGCTGAGGCCGGTGAAGAAG
187601_R	GCGGCCGCTTAGAGGGTGACTGGTGCG
187834_F	CTTGCCGTGCCCAAAATTACACCCAGCCCAAACAACC
187834_R	GCGGCCGCTCAAGCCTTGAACATAGCC
188178_F	CTTGCCGTGCCCAAAATGTGCCAAATACTGCGTGG
188178_R	GCGGCCGCCTACGCCGTGCAACTCGAC
188403_F	CTTGCCGTGCCCAAAATGCTGTGCCAATGGCCGGC
188403_R	GCGGCCGCTTAGTTGAACTTCCACTTCATGCCG
189051_F	CTTGCCGTGCCCAAAATACCCTAAAACCCTGCGTC
189051_R	GCGGCCGCTCAGAAACAATCAACAAACGGC
189372_F	CTTGCCGTGCCCAAAATTCCCCAATGGATCCAATAG
189372_R	GCGGCCGCCTATGACGAGTCCGAGTACC
189382_F	CTTGCCGTGCCCAAAATGCCCCTCTCGCCGCGAGC
189382_R	GCGGCCGCTCAAGCCGATCTACAAAACCG
189833_F	CTTGCCGTGCCCAAAATGCAGCAGTTGCAGCACCC
189833_R	GCGGCCGCCTAGTCCTGGTGGTTTGCC
190169_F	CTTGCCGTGCCCAAAATGCACCAAACCCAATCGCC
190169_R	GCGGCCGCCTATGACAAGATACCGACG
190347_F	CTTGCCGTGCCCAAAATGCACCACAAGTATCGATCG
190347_R	GCGGCCGCCTAGCACTTGTTCAACGCC
190392_F	CTTGCCGTGCCCAAAATGTGGACACTGAATGCAAGTT
190392_R	GCGGCCGCCTAGAACATCCTCGGTGGA
190522_F	CTTGCCGTGCCCAAAATACGCCGCAGGTAAGTGAAC
190522_R	GCGGCCGCTCAACACCCAAAAAACCAATCC
190687_F	CTTGCCGTGCCCAAAATAATCTCCACTCCTACGGCGTC
190687_R	GCGGCCGCTCAATTCCCCACACTCTCC
190787_F	CTTGCCGTGCCCAAAATATCAACTTCAGCGATTGGGC
190787_R	GCGGCCGCCTACTTCTTGCTATCGCAAGC
190869_F	CTTGCCGTGCCCAAAATGCAGCAACCCCAGGAGCCG
190869_R	GCGGCCGCCTAGTTGTACTGCCATGCGC

Primer name	Sequence (5'-3')
190906_F	CTTGCCGTGCCCAAAATCTTCCCGTGCCCACTGAC
190906_R	GCGGCCGCCTACTTCCAACACGCCACC
191039_F	CTTGCCGTGCCCAAAATCTACCAGTGGAGGAGGCTAC
191039_R	GCGGCCGCCTAAGGTGTCCAATCCCAGC
191043_F	CTTGCCGTGCCCAAAATCAACGTTTCAGAGGTGGCAG
191043_R	GCGGCCGCCTACGCCTTGTACACATCG
191227_F	CTTGCCGTGCCCAAAATGCCCCTATGCCAGAAATCGTA
191227_R	GCGGCCGCTCAAACCCCTCCGCCGCA
191661_F	CTTGCCGTGCCCAAAATGCACCTGCCAATGACGAGTAC
191661_R	GCGGCCGCTTACTCGTGGACATGCTTG
192236_F	CTTGCCGTGCCCAAAATGTGCCCTACAACCAGGAGC
192236_R	GCGGCCGCCTACGACGAGCCCGTGTTC
192503_F	CTTGCCGTGCCCAAAATCGATCAACAGGCCTCTTCAC
192503_R	GCGGCCGCCTAAAGCCCCTGCTTGAAG
192888_F	CTTGCCGTGCCCAAAATCACGGCTACTTCCAATCCCC
192888_R	GCGGCCGCCTAAGCAAAGTCCCTAGCATGG
193226_F	CTTGCCGTGCCCAAAATCAAGCTCTATCTTCATCAGAAAC
193226_R	GCGGCCGCTCAAGCTGGCAAGCCACCC
193612_F	CTTGCCGTGCCCAAAATCGAGCAGATCGCCGTCAAAC
193612_R	GCGGCCGCTTAGCTAGGGGTGAAGCAGC
193798_F	CTTGCCGTGCCCAAAATCAGCAATACCAATACCCA
193798_R	GCGGCCGCTCAATTGCACTTCACGCTGC
193896_F	CTTGCCGTGCCCAAAATGCCAGGCTTGCCTCACGC
193896_R	GCGGCCGTCAGTCACGACCGTTGGAAG
194142_F	CTTGCCGTGCCCAAAATAACCCCATCCTCCTTGAGA
194142_R	GCGGCCGCCTACTTACACAGCTGGAACCC
194152_F	CTTGCCGTGCCCAAAATATGCCATGGGGTGACAAC
194152_R	GCGGCCGCTCAGTTGTAGAACCAGTACTCTG
194257_F	CTTGCCGTGCCCAAAATGCAGTCCACTCCTCCCACC
194257_R	GCGGCCGCCTACTGCGTATAACTCCAGCTC
194586_F	CTTGCCGTGCCCAAAATAATGGGTTGGTGAAACGCCAA
194586_R	GCGGCCGCCTAGGAGCTTTCAAAAGAGC
194693_F	CTTGCCGTGCCCAAAATCAATTCTTCAACTTCGGTAAC
194693_R	GCGGCCGCTCAAAGCAACCCTTTCCTC
194863_F	CTTGCCGTGCCCAAAATCAAGACACCAGCATCGTCTAC
194863_R	GCGGCCGCCTAAGCCCCCGCCCCCTT
194955_F	CTTGCCGTGCCCAAAATCAGGAGTGCACCACCCAGC
194955_R	GCGGCCGCTCATGCCTCGAAAGAGATCTC
195050_F	CTTGCCGTGCCCAAAATTCAGTAATCAAGGCACGCC
195050_R	GCGGCCGCTCACACCACTGTCTCGTTAC
195059_F	CTTGCCGTGCCCAAAATCTTTCCTTCAACACAACATC
195059_R	GCGGCCGCTCACTTTCTCTTCTTGCAC
195437_F	CTTGCCGTGCCCAAAATTTCTGCTACCTGCCAGGTT
195437_R	GCGGCCGCTCAGCCGTATTTGCAGTTC
195452_F	CTTGCCGTGCCCAAAATTGCGAAGTCTTTGGCAGCA
195452_R	GCGGCCGCTTATACAGTCCAACCCTCGG
195873_F	CTTGCCGTGCCCAAAATGGCGTTGTTCGCCGAGACTA

Primer name	Sequence (5'-3')
195873_R	GCGGCCGCTCAAGTATACGTGTGTGACTC
196507_F	CTTGCCGTGCCCAAAATGCAGCCGAGTACGCCGCTAC
196507_R	GCGGCCGCTCAGCATGTTCCCCTGGC
196849_F	CTTGCCGTGCCCAAAATTCGGAGCTAGCCGGGCCGAAA
196849_R	GCGGCCGCCTAGGCGACACCTGT
197226_F	CTTGCCGTGCCCAAAATACTCCGCTCTCAAGACGTGAC
197226_R	GCGGCCGCTCAGTACTCTTCCTCAGTTCTG
*Asc*I_CfNLP1_R	GCGGCCGCCTACAGAGCTGCCTTGCCCA
AttB1_PR1A_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGGATTTGTTCTCTTTTCAC
AttB2_CfNLP1_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTACAGAGCTGCCTTGCCCA
Cf_NLP1_F2	GCCAGATTCGACTGGGAGAGC
Cf_NLP1_R2	CCAGTCGAATCTGGCCCCTAG
Ecp4_DS_F	GGGGACAGCTTTCTTGTACAAAGTGGCAGGTCACTTGCCCGTAATC
Ecp4_DS_R	GGGGACAACTTTGTATAATAAAGTTGGTGAGGAAGTGACAACTCTTG
Ecp4_US_F	GGGGACAACTTTGTATAGAAAGTTGGCACCAAGATGTATCGTAGC
Ecp4_US_R	GGGGACTGCTTTTTTGTACAAACTTGGGAGGGAGGTTGGAATTTG
Ecp5_DS_F	GGGGACAGCTTTCTTGTACAAAGTGGCATCTACTGTCGCGGCAAC
Ecp5_DS_R	GGGGACAACTTTGTATAATAAAGTTGGAAAAGAAGCTTGCGGTG
Ecp5_US_F	GGGGACAACTTTGTATAGAAAGTTGCCTGTATGGACGATATAGCC
Ecp5_US_R	GGGGACTGCTTTTTTGTACAAACTTGGTTTGAGCAGAGGGAAAGAG
Ecp4_US_Out_F	ACCCGCAGGATGTAGACGC
Ecp4_DS_Out_R	GATGTTTCGCGGTGGATG
Ecp5_US_Out_F	TCGCAATGCTCGCAACAC
Ecp5_DS_Out_R	GGAAGAGAGGACACGACG
HYG_R	GTCCGAGGGCAAAGGAATAG
GFP_F	GATCACTCACGGCATGGAC

Chapter 3

Detoxification of α-tomatine by *Cladosporium fulvum* is required for full virulence on tomato

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ABSTRACT

α-Tomatine is an antifungal glycoalkaloid that provides basal defense to tomato (*Solanum lycopersicum* L.). However, tomato pathogens overcome this basal defense barrier by secretion of tomatinases that degrade α-tomatine into less fungitoxic compounds β-tomatine or tomatidine. Although pathogenic on tomato, it was reported that the biotrophic fungus *Cladosporium fulvum* is unable to detoxify α-tomatine. Here we present a functional analysis of the glycosyl hydrolase 10 (GH10), CfTom1, which is orthologous to fungal tomatinases. We show that *C. fulvum* hydrolyzes α-tomatine into tomatidine *in vitro* and during infection of tomato, which is fully attributed to the activity of CfTom1 as shown by heterologous expression of this enzyme in tomato. Accordingly, Δ*cftom1* mutants of *C. fulvum* are more sensitive to α-tomatine and are less virulent on tomato than the wild-type fungus. Although α-tomatine is thought to be localized in the vacuole, we show that it is also present in the apoplast where it is hydrolyzed by CfTom1 upon infection. Accumulation of tomatidine during infection appears to be toxic to tomato cells and does not suppress defense responses as suggested previously. Altogether, our results show that CfTom1 is responsible for detoxification of α-tomatine by *C. fulvum*, and is required for full virulence of this fungus on tomato.

INTRODUCTION

Plants have developed basal defense barriers that protect them from infection by potential pathogens. These include physical barriers such as lignin or callose and chemical barriers such as antimicrobial compounds (Hammerschmidt *et al.*, 1984; de Wit *et al.*, 2009). A well-known class of antimicrobial compounds present in plants comprises the saponins, which are glycosylated steroids or steroidal alkaloids representing a constitutive chemical barrier against a wide range of fungal pathogens (Bowyer *et al.*, 1995; Osbourn, 1996). Saponins cause loss of membrane integrity in target organisms by forming complexes with sterols, resulting in pore formation and cell lysis (Keukens *et al.*, 1995; Osbourn, 1996). Sensitivity to saponins is correlated

with the type of sterols present in the membranes of the potential pathogens. Fungal membranes that contain sterols with free 3β-hydroxy groups are sensitive to saponins, while plant cell membranes are insensitive due to the presence of sterol glycosides (Steel & Drysdale, 1988). Similarly, oomycetes are insensitive to saponins because their membranes lack 3β-hydroxy sterols (Steel & Drysdale, 1988).

In response to the inherent resistance mechanism present in plants, bacteria and fungi, which are pathogenic on saponin-producing plants, have developed a detoxification mechanism by secreting saponin-detoxifying enzymes (Ford *et al.*, 1977; Roldan-Arjona *et al.*, 1999; Kaup *et al.*, 2005). Saponin detoxification by pathogens has mainly been studied for avenacin and α-tomatine, which are present in oat and tomato, respectively. During infection of oat roots, the fungus *Gaeumannomyces graminis* var. *avenae* secretes the avenacinase enzyme that detoxifies avenacin, a triterpenoid saponin. Mutants deleted for the avenacinase gene are no longer able to infect oat, while they are still virulent on wheat, a host that does not produce saponins (Bowyer *et al.*, 1995). In tomato (*Solanum lycopersicum* L.), the major saponin is α-tomatine, a steroidal glycoalkaloid that is present in leaves and green fruits in concentrations as high as 1 mM (Roddick, 1977; Osbourn, 1996). α-Tomatine consists of the aglycon tomatidine and the tetrasaccharide (lycotetraose) (Fig. 1A). Toxicity of α-tomatine depends on presence of lycotetraose, because removal of one or all four sugar residues renders α-tomatine less toxic (Osbourn, 1996). During tomato infection, bacterial and fungal pathogens secrete various types of tomatinase enzymes that can detoxify α-tomatine by removing one or more sugar residues (Martin-Hernandez *et al.*, 2000; Kaup *et al.*, 2005; Pareja-Jaime *et al.*, 2008). Tomatinase enzymes secreted by *Septoria lycopersici*, *Botrytis cinerea*, and *Verticillium albo-atrum* belong to the glycosyl hydrolase family 3 (GH3) of carbohydrate-degrading enzymes (CAZY) (Martin-Hernandez *et al.*, 2000). They remove the terminal β-1,2-D-glucose or the β-1,3-D-xylose residues from α-tomatine (Osbourn *et al.*, 1995; Quidde *et al.*, 1998). Other tomato pathogens, such as the fungus *Fusarium oxysporum* f. sp. *lycopersici* and the bacterium *Clavibacter michiganensis* subsp. *michiganensis* secrete a tomatinase that

belongs to the glycosyl hydrolase family 10 (GH10) (Roldan-Arjona *et al.*, 1999; Kaup *et al.*, 2005). GH10 tomatinase enzymes remove lycotetraose from α-tomatine to form the aglycon tomatidine (Fig. 1A) (Roldan-Arjona *et al.*, 1999; Pareja-Jaime *et al.*, 2008). Although several knock-out studies have been performed to assess the role of tomatinase enzymes in the virulence of bacterial and fungal tomato pathogens (Martin-Hernandez *et al.*, 2000; Kaup *et al.*, 2005), only the GH10 tomatinase tom1 from *F. oxysporum* f. sp. *lycopersici* was shown to play a role in virulence of this vascular pathogen (Pareja-Jaime *et al.*, 2008). It has also been suggested that products resulting from tomatinase activity play an indirect role in virulence of tomato pathogens by suppressing plant defense responses. The different breakdown products of α-tomatine (β-tomatine, tomatidine and lycotetraose) were reported to suppress various types of defense responses, including the oxidative burst and the hypersensitive response (Bouarab *et al.*, 2002; Ito *et al.*, 2004).

The non-obligate biotrophic fungus *Cladosporium fulvum* is a well-studied tomato pathogen that causes leaf mould. *C. fulvum* enters tomato leaves through stomata and colonizes the apoplastic space surrounding mesophyll cells (Stergiopoulos & de Wit, 2009). Although pathogenic on tomato, it was previously reported that *C. fulvum* is not able to detoxify α-tomatine and the vacuolar location of α-tomatine was hypothesized to allow the fungus to infect tomato (Melton *et al.*, 1998). However, heterologous expression of the GH3 tomatinase gene from *S. lycopersici* in *C. fulvum* resulted in a transformant showing increased sporulation during infection of tomato as compared to wild-type (Melton *et al.*, 1998). These results suggested that *C. fulvum* might be exposed to α-tomatine during colonization of the apoplastic space of tomato, and that it does not produce functional tomatinase enzymes. However, recent sequencing of the *C. fulvum* genome revealed the presence of 19 genes encoding GH3 and two genes encoding GH10 enzymes (de Wit *et al.*, 2012). Here, we demonstrate that one of the two GH10 genes encodes a functional tomatinase, CfTom1, which degrades α-tomatine into less-toxic tomatidine both *in vitro* and during infection of tomato. Functional analysis of Δ*cftom1* mutants of *C. fulvum* showed that degradation of α-tomatine is required for full virulence of the fungus on tomato, which is likely due

to increased sensitivity of these mutants to α-tomatine rather than to suppression of basal defense responses by its breakdown products.

RESULTS

Cladosporium fulvum* secretes a functional tomatinase enzyme that degrades α-tomatine *in vitro* and *in planta

Unlike other tomato pathogens, it was previously reported that *C. fulvum* is unable to degrade α-tomatine (Melton *et al.*, 1998). During infection, *C. fulvum* colonizes the apoplastic space surrounding tomato mesophyll cells without entering them. It was hypothesized that *C. fulvum* does not need any tomatinase enzyme as α-tomatine was suggested to solely reside in the vacuole. However, apoplastic fluids (AFs) of tomato leaves have never been critically inspected for the presence of α-tomatine. In this study, we performed LC-MS analysis on both total leaf extracts and AFs to monitor the relative levels of α-tomatine and its degradation products. As a control, we also analyzed rutin, a glycoside of the flavonoid quercetin, which has been reported to be present in the cytoplasm and/or vacuole of plants (Marrs *et al.*, 1995; Markham *et al.*, 2001). As expected, rutin was abundantly present in the total leaf extracts, but it was undetectable in the AFs, while α-tomatine was detected in the AFs at a concentration of 0.02 ±0.005 µmol g^{-1} fresh leaf (Fig. 1B and supporting information Fig. S1). Although this concentration is lower than that of total leaf extracts (1.0 ±0.1 µmol g^{-1} fresh leaf), it indicates that α-tomatine is detected at a significant level in AFs. Similar distributions of rutin and α-tomatine were seen in both infected and mock-inoculated leaves (Supporting information Fig. S1). In contrast to what has been reported in the literature, this result shows that *C. fulvum* does encounter α-tomatine upon colonization of tomato apoplastic space. The ability of *C. fulvum* to degrade α-tomatine during infection of tomato was subsequently addressed. AFs from both *C. fulvum*-infected and healthy tomato leaves were isolated at different time points and analyzed by LC-MS. We observed again, in contrast to previous reports, a clear
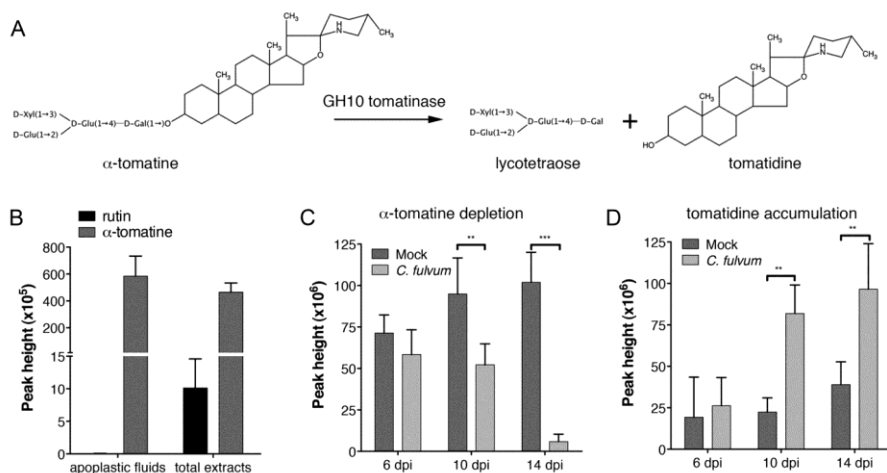
Fig. 1. Detoxification of α-tomatine by *Cladosporium fulvum*. (A) Tomatinase enzymes of the GH10 family hydrolyze α-tomatine into tomatidine and lycotetraose. **(B)** LC-MS detection of rutin, a compound that has similar polarity with α-tomatine and thought to be localized in the cell and/or vacuole, and α-tomatine, in apoplastic fluid (AF) and total extract (TE) from tomato leaves inoculated with *C. fulvum* at 6 days post inoculation (dpi). α-tomatine is present in total leaf extracts and in AFs at the concentration of 1.0 ±0.1 µmol g^{-1} and 0.02 ±0.005 µmol g^{-1} of fresh leaf, respectively. LC-MS detection of relative peak height of **(C)** α-tomatine and **(D)** tomatidine in AFs isolated at 6, 10 and 14 dpi from healthy (mock) and *C. fulvum*-inoculated tomato plants. A two-way ANOVA analysis was performed and followed by a Bonferroni test. Only significant differences are indicated (** $P<0.01$; *** $P<0.001$; n=4). Error bars represent standard deviation of at least three biological repeats.

depletion of α-tomatine with concomitant accumulation of tomatidine in AFs isolated from *C. fulvum*-infected plants from 10 days post inoculation (dpi) onwards (Fig. 1C and d). At 14 dpi, the concentration of α-tomatine in AFs of *C. fulvum*-infected tomato leaves was 17 times lower than in AFs obtained from mock-inoculated tomato leaves. In addition, LC-MS analysis also revealed depletion of dehydrotomatine, another glycoalkaloid in *C. fulvum*-infected tomato leaves (Supporting information Fig. S2A-B). Accordingly, accumulation of tomatidenol, the degradation product of dehydrotomatine, was also detected in AFs of *C. fulvum*-infected tomato leaves (Supporting information Fig. S2C). However, the observed depletion of α-tomatine could be due to the induction of plant-derived α-tomatine-degrading enzymes in

infected plants. In order to prove that the fungus is responsible for this degradation, we firstly incubated culture filtrate from *C. fulvum* grown *in vitro* with α-tomatine (100 µg ml^{-1}) for 24 h. Subsequently, the relative level of α-tomatine and its degradation product tomatidine were monitored by LC-MS**.** The concentration of α-tomatine after incubation with culture filtrate was reduced compared to the non-inoculated control medium and α-tomatine degradation coincided with the accumulation of tomatidine (Supporting information Fig. S3), which was never detected in the non-inoculated control medium. These results suggest that the α-tomatine degradation observed during tomato infection is likely due to the secretion of a tomatinase enzyme by *C. fulvum*. Noteworthy in all experiments, no β-tomatine could be detected, which suggests that *C. fulvum* does not secrete any functional GH3 tomatinase enzymes. Thus, a secreted GH10 enzyme is expected to be responsible for the degradation of α-tomatine by *C. fulvum*.

Identification of *Cladosporium fulvum* CfTom1, a GH10 tomatinase that degrades α-tomatine into tomatidine *in planta*

The availability of the genome sequence of *C. fulvum* facilitated the identification of GH10 enzyme-encoding genes. Only two genes belonging to this family were identified, but no substrate specificity could be assigned (de Wit *et al.*, 2012). However, one of the genes encodes a protein that shares 61% amino acid identity with the *F. oxysporum* f. sp. *lycopersici* GH10 tomatinase enzyme; this gene was named *CfTom1* (JGI protein ID: 188986). A phylogenetic analysis of selected GH3 and GH10 family enzymes confirmed that CfTom1 belongs to the clade of characterized fungal and bacterial GH10 tomatinases (Fig. 2). Although no GH3 tomatinase activity could be detected, it was also found that the *C. fulvum* genome contains genes related to the GH3 tomatinase of *S. lycopersici* and avenacinase of *G. graminis*. As enzymes in the GH3 clade have been shown to provide different substrate specificities, even when they share high similarity, the GH3 enzymes of *C. fulvum* are believed to target other tomato metabolites. Given that the toxicity of α-tomatine is

pH-dependent, the expression of *CfTom1* was assessed by quantitative PCR *in vitro* at different pH values and also in the presence or absence of α-tomatine. Consistent with the higher toxicity of α-tomatine at neutral pH, *CfTom1* is weakly expressed at low pH, whereas its expression is induced at pH 7 (Fig. 3) (Dow & Callow, 1978; Roddick & Drysdale, 1984). Similar to tomatinase induction by α-tomatine in *F. oxysporum* f. sp. *lycopersici* (Pareja-Jaime *et al.*, 2008), the presence of α-tomatine also induces the expression of *CfTom1*, even at pH 4 (Fig. 3). *CfTom1* shows a very low expression level during the early stages of infection of tomato, but its expression is significantly induced at 12 and 15 dpi (Fig. 3).
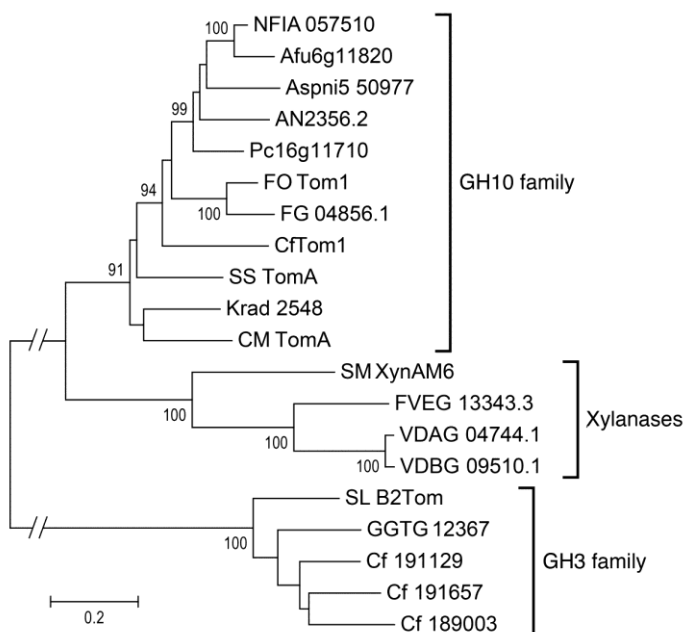
Fig. 2. Phylogenetic tree of GH3 and GH10 tomatinase enzymes from fungal and bacterial pathogens. Amino acid sequences were aligned using ClustalW2 and the phylogenetic tree was constructed using the minimum evolution algorithm and performing 1,000 bootstraps. Scale bar shows the genetic distance (substitutions per site). NFIA: *Neosartorya fischeri*; Afu6g: *Aspergillus fumigatus*; Aspni5: *Aspergillus niger*; AN: *Aspergillus nidulans*; Pc16g: *Penicillium chrysogenum*; FO: *Fusarium oxysporum* f. sp. *lycopersici*; FG: *Fusarium graminearum*; Cf: *Cladosporium fulvum*; SS: *Streptomyces scabiei*; Krad: *Kineococcus radiotolerans*; CM: *Clavibacter michiganensis*; SM: *Streptomyces megasporus*; FVEG: *Fusarium verticillioides*; VDAG: *Verticillium dahliae*; VDBG: *Verticillium albo-atrum*; SL: *Septoria lycopersici*; GGTG: *Gaeumannomyces graminis* var. *avenae*.
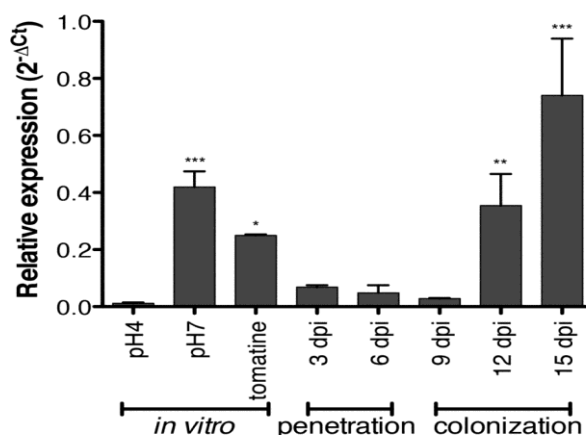
Fig. 3. Expression profile of the *Cladosporium fulvum CfTom1* gene. The *CfTom1* expression level under three *in vitro* conditions (B5 medium adjusted to pH 4 and pH 7, and B5 medium adjusted to pH 4, containing 50 µM α-tomatine) and during tomato infection, was measured by quantitative PCR. Expression was normalized using the *actin* gene of *C. fulvum* according to the $2^{-\Delta Ct}$ method. *CfTom1* expression is induced at pH 7, in the presence of α-tomatine at pH 4 and during colonization at 12 and 15 days post inoculation (dpi). A one-way ANOVA analysis was performed and followed by a Tukey-Kramer test. Only significant differences are indicated (* $P<0.01$, ** $P<0.001$, ***$P<0.0001$, n=3). Error bars represent standard deviation of at least three biological repeats.

In order to verify that CfTom1 is responsible for the degradation of α-tomatine into tomatidine, the *CfTom1* gene was constitutively expressed in tomato seedlings using the *Potato Virus X* (PVX) expression system (Hammond-Kosack *et al.*, 1995). Five weeks after agroinoculation, AFs were isolated from PVX::*CfTom1*- and empty PVX-expressing tomato plants. LC-MS analysis of AF from control plants showed detectable levels of α-tomatine, but also the presence of dehydrotomatine (Fig. 4). The control AF also contained limited but detectable levels of tomatidine, which points to a low level of α-tomatine degradation by plant enzymes. In contrast, clear accumulation of tomatidine was detected in AF obtained from *CfTom1*-expressing plants, demonstrating that CfTom1 has GH10 tomatinase activity. The LC-MS analysis also revealed accumulation of tomatidenol in AF from *CfTom1*-expressing tomato plants, indicating that CfTom1 can also degrade another glycoalkaloid present in tomato (Fig. 4 and Supporting information Fig. S2C).
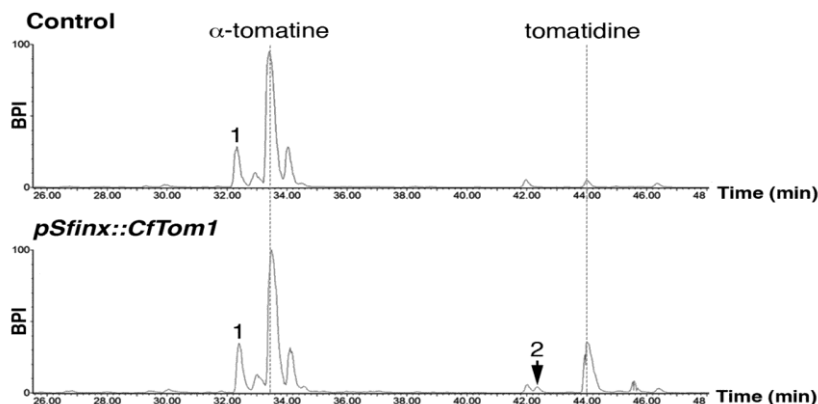
Fig. 4. CfTom1 is a GH10 tomatinase enzyme. Apoplastic fluids were isolated from tomato plants expressing either *CfTom1* or the empty vector (control) using the *pSfinx* system. The relative base peak intensity (BPI) of α-tomatine and tomatidine were measured by LC-MS. In addition, dehydrotomatine [1] and the corresponding degradation product, tomatidenol [2] were detected.

Δ*cftom1* mutants of *Cladosporium fulvum* can no longer degrade α-tomatine *in vitro*

Functional analysis of *CfTom1* was continued by targeted gene deletion in the sequenced *C. fulvum* strain. For this, the *CfTom1* gene was replaced by the *hygromycin resistance* (*HYG*) and *green fluorescent protein* (*GFP*) genes. Two independent Δ*cftom1* mutants (Δ*cftom1*-2.6 and Δ*cftom1*-5.1) for which gene replacement was confirmed by Southern blot analysis were selected (Supporting information Fig. S4). An ectopic transformant and the wild-type strain were used as controls in all experiments. Both the Δ*cftom1-2.6* and Δ*cftom1-5.1* mutants did not show any visible phenotype regarding morphology, *in vitro* growth and sporulation rates when compared to the wild-type strain and ectopic transformant. To check for altered sensitivity of the mutants to α-tomatine, the compound was tested in a conidium germination assay. Conidia of the wild-type strain, ectopic transformant and two Δ*cftom1* mutants were allowed to germinate in the presence of control solution (1% methanol) or 100 µM α-tomatine. In the absence of α-tomatine, no difference in germination of conidia was observed between all strains (about 90% of conidia germinated; Fig. 5A-B). However,

when incubated in 100 µM α-tomatine, the germination of conidia of both Δ*cftom1* mutants was reduced to 70% (Fig. 5A-B). The higher sensitivity of the mutants is likely due to their inability to degrade α-tomatine.
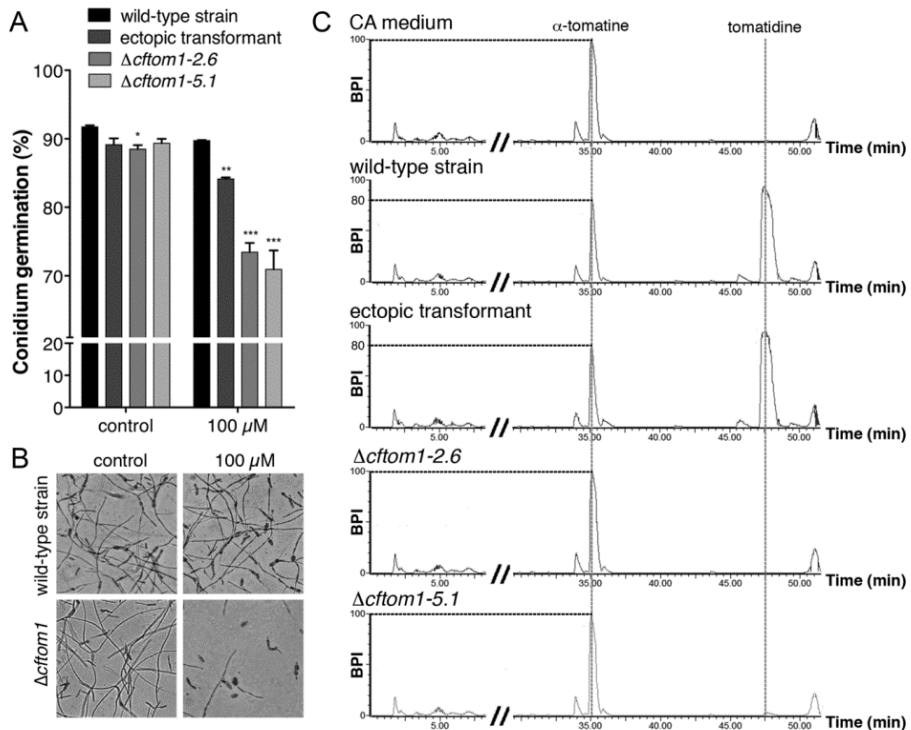

Fig. 5. Δ*cftom1* mutants are sensitive to α-tomatine. (A) Sensitivity of *Cladosporium fulvum* to α-tomatine was tested by measuring its effect on conidium germination. Conidia from the wild-type strain, an ectopic transformant and two independent Δ*cftom1* mutants were incubated in the absence or presence of 100 µM α-tomatine. The number of germinated conidia out of 100 conidia was determined. A two-way ANOVA analysis was performed and followed by a Bonferroni test. Only significant differences are indicated (* $P<0.05$; ** $P<0.01$; *** $P<0.001$). Error bars represent standard deviation of at least three biological repeats. **(B)** Representative pictures of the conidium germination assay to assess sensitivity to α-tomatine. **(C)** Sterile CA medium (control) and culture filtrates from the same *C. fulvum* strains as shown in (A) were supplemented with 100 µg ml^{-1} α-tomatine. After 24 h incubation, the relative base peak intensity (BPI) of α-tomatine and tomatidine were measured by LC-MS.

To verify this hypothesis, culture filtrates of the deletion mutants were incubated with α-tomatine (100 μg ml^{-1}), along with culture filtrates of the wild-type strain and ectopic transformant as positive controls, and non-inoculated culture medium as a negative control. After 24 h of incubation, both α-tomatine depletion and tomatidine accumulation were observed for the wild-type strain and ectopic transformant (Fig. 5C). In contrast, culture filtrates from both mutants were not able to degrade α-tomatine and no tomatidine accumulation was detected. These results show that the higher sensitivity of the Δ*cftom1* mutants to α-tomatine is due to their inability to degrade the compound *in vitro*.

In planta degradation of α-tomatine is required for full virulence of *Cladosporium fulvum* on tomato

The ability to degrade α-tomatine appears to be important for successful infection of tomato by fungal pathogens (Pareja-Jaime *et al.*, 2008). The role of *CfTom1* in *C. fulvum* virulence was analyzed by inoculating susceptible tomato plants with the wild-type strain, ectopic transformant and two Δ*cftom1* mutants. Virulence was measured by quantifying the fungal biomass by quantitative PCR. No significant difference in fungal biomass was observed for the wild-type, the ectopic transformant and two Δ*cftom1* mutants during the early stages of infection (until 8 dpi) (Fig. 6A). However, from 10 dpi onwards, the biomass of the two Δ*cftom1* mutants was reduced by approximately 63% when compared to the wild-type strain and ectopic transformant (Fig. 6A). Reduction in fungal biomass is correlated with a delay in disease progression at 10 dpi, indicating that *CfTom1* is required for full virulence of *C. fulvum* on tomato.

Depletion of α-tomatine and accumulation of tomatidine were measured at 10 dpi of the wild-type, the ectopic transformant and two Δ*cftom1* mutants during tomato infection. Similar to the *in vitro* results, no tomatidine accumulation was determined in AFs obtained from plants inoculated with the Δ*cftom1* mutants, whereas this compound was clearly accumulated in the AFs of plants inoculated with

the wild-type and the ectopic transformant (Fig. 6B). The reduction in fungal biomass production by the deletion mutants might be explained by their inability to degrade α-tomatine.

It has been previously reported that the breakdown products of α-tomatine, such as tomatidine and β-tomatine, can suppress plant defense responses (Bouarab *et al.*, 2002; Ito *et al.*, 2004), suggesting that the accumulation of tomatidine can also indirectly contribute to virulence. Thus, the reduced virulence of the Δ*cftom1* mutants could not only be due to their increased sensitivity to α-tomatine, but also due to their reduced ability to suppress plant defense responses as tomatidine does no longer accumulate. We assayed the presumed defense suppressing activity of tomatidine on H_2O_2 production in MSK8 tomato cell suspensions, but such activity was not confirmed in our experiments. On the contrary, MSK8 tomato cells that were incubated with α-tomatine or tomatidine showed higher H_2O_2 production when compared to the negative control (Supporting information Fig. S5).

Furthermore, α-tomatine or tomatidine even enhanced the H_2O_2 production after treatment of the cells with chitin showing that they were unable to suppress chitin-triggered basal defense responses. This unexpected effect of tomatidine on H_2O_2 production suggested that it could even be toxic to tomato cells. Indeed, Itkin *et al.* (2011) recently showed that down-regulation of the glycoalkaloid metabolism-1 (*GAME1*) gene, which is responsible for the glycosylation of the aglycone tomatidine to α-tomatine, results in growth retardation of this tomato mutant as a consequence of the high accumulation of tomatidine. To further investigate this observation, different concentrations of α-tomatine and tomatidine (100, 250 and 500 μM) were injected in the intercellular space of tomato leaves. Results clearly show that tomatidine induces cell death in tomato leaves at concentrations of 250 and 500 μM, while α-tomatine does not cause cell death even at the highest concentration (Fig. 7). These results do not point to a role in plant defense suppression for tomatidine, but rather indicate that the compound is toxic to tomato. Thus, the decrease in virulence of the Δ*cftom1* mutants is caused by their inability to degrade α-tomatine.

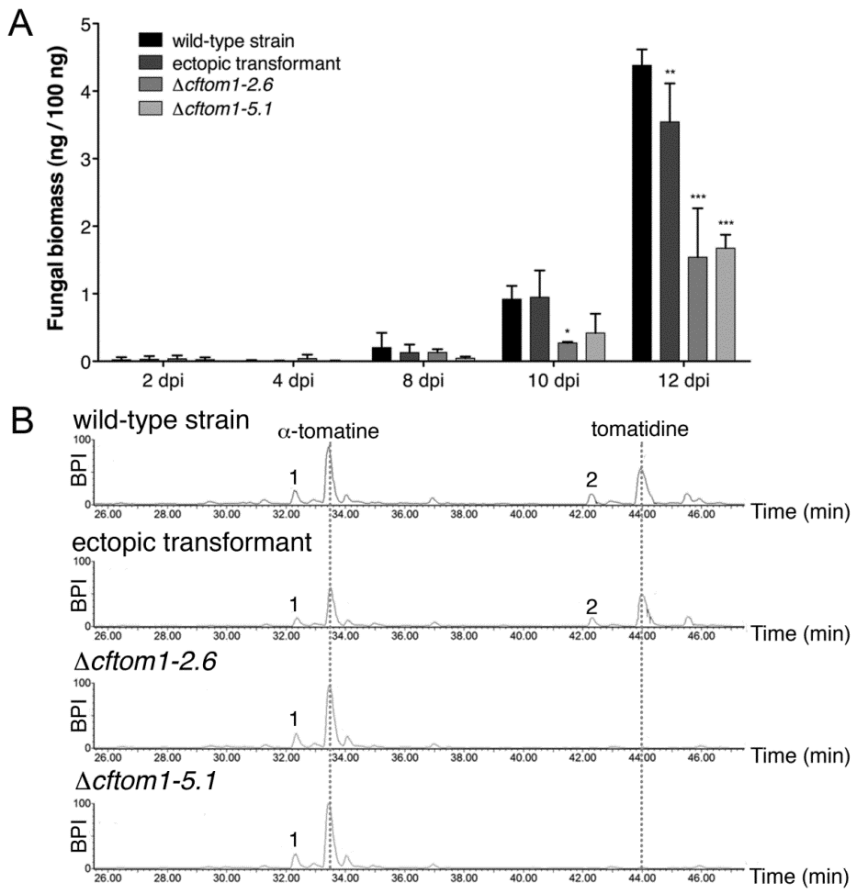**Fig. 6. Δ*cftom1* mutants show reduced virulence. (A)** Virulence of the wild-type *Cladosporium fulvum* strain, an ectopic transformant and two independent Δ*cftom1* mutants was assessed by quantification of fungal biomass at different time points after inoculation of susceptible tomato plants. Quantitative PCR was performed on genomic DNA using the *actin* gene from *C. fulvum* as a standard. The fungal biomass was deduced from a standard curve. Tissues of infected tomato leaves from 2 to 12 days post inoculation (dpi) were harvested and analyzed. A two-way ANOVA analysis was performed and followed by a Bonferroni test. Only significant differences are indicated (* P<0.05; ** P<0.01; *** P<0.001). Error bars represent standard deviation of at least three biological repeats. **(B)** Apoplastic fluids were isolated from tomato plants inoculated with the same *C. fulvum* strains as indicated in (A) at 10 dpi. The depletion of α-tomatine and accumulation of tomatidine were monitored by LC-MS. In addition, dehydrotomatine [1] and the corresponding degradation product, tomatidenol [2], were detected as well. Both mutants have lost their ability to detoxify α-tomatine during infection of tomato.
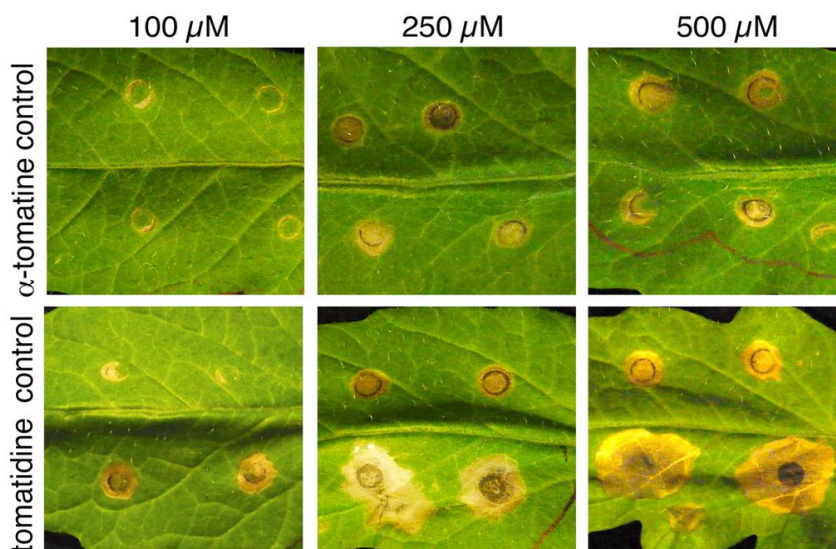
Fig. 7. Phytotoxic effects of α-tomatine and tomatidine on tomato leaves. Tomato leaves were infiltrated with different concentrations of α-tomatine and tomatidine (100, 250 and 500 μM) and with the control solution containing the same dilution rate of methanol (2%, 5% and 10% v/v). Pictures were taken at 3 days post inoculation.

DISCUSSION

Saponins are antimicrobial compounds present in plants that provide constitutive protection against a broad range of pathogens (Osbourn, 1996). In tomato, the major characterized saponin is α-tomatine, a compound that disrupts fungal membranes. Tomato pathogens, however, can overcome this chemical barrier by production of tomatinase enzymes that detoxify α-tomatine into β-tomatine (by GH3 enzymes) or tomatidine and lycotetraose (by GH10 enzymes) (Roldan-Arjona *et al.*, 1999; Martin-Hernandez *et al.*, 2000).

The concentration of α-tomatine in tomato leaves can reach levels as high as 1 mM, assuming a uniform distribution in cells (Arneson & Durbin, 1967). In previous reports it was assumed that most of the α-tomatine is localized in the vacuoles (Roddick, 1977). For this reason, and the fact that *C. fulvum* grows biotrophically in the intercellular space (Stergiopoulos & de Wit, 2009), it was expected that this fungus

would not need to detoxify α-tomatine during infection. However, the assumed vacuolar localization is questionable as the original conclusion of Roddick (1977) was mainly based on the fact that α-tomatine was present in the supernatant after sequential centrifugation, including ultracentrifugation, without taking into account the possibility for possible localization in the apoplast. Our analysis reports for the first time the concentration of α-tomatine in AFs as 0.02 ±0.005 µmol g^{-1} fresh leaf. This quantification seems to be reliable because the concentration of α-tomatine in total leaf extract (1.0 ±0.1 µmol g^{-1} fresh leaf) is comparable to what has been reported by others (Melton *et al.*, 1998). This higher concentration suggests that α-tomatine is indeed more abundant inside the plant cells, but it is also significantly present in the AFs. It is likely that the concentration of α-tomatine *C. fulvum* encounters around the mesophyll cells inside tomato leaves is higher than that we measured. Monitoring α-tomatine degradation and tomatidine accumulation during tomato infection by *C. fulvum* revealed that this fungus is able to detoxify this saponin. This result contrasts with previous reports, which could possibly be explained by a lower sensitivity of the methods used at the time. In their analysis, Melton *et al*., (1998) performed thin-layer chromatography (TLC), which is less sensitive than LC-MS, to detect breakdown products of α-tomatine after incubation with proteinaceous extracts isolated from cultures filtrates of *C. fulvum* (Melton *et al.*, 1998). More important, the culture filtrates they used in this assay originate from *C. fulvum* grown on B5 medium, for which the pH is approximately 4.5. Our expression data showed that *CfTom1* is barely expressed in the same medium at pH4, suggesting that the absence of tomatinase activity in this previous study is due to a limited expression of *CfTom1*.

Analysis of the *C. fulvum* CAZymes revealed two GH10 and 19 GH3 genes in its genome (de Wit *et al.*, 2012). A phylogenetic analysis showed that only one of the GH10 enzymes belongs to the GH10 tomatinase clade (CfTom1), while three GH3 enzymes belong to the GH3 tomatinase/avenacinase clade. Our results showed that *C. fulvum* degrades α-tomatine into tomatidine both *in vitro* and during infection of tomato, suggesting that CfTom1 is responsible for the observed activity. A specific

search for the presence of β-tomatine was unsuccessful both *in vitro* and during infection of tomato, suggesting that the enzymes encoded by the three putative GH3 genes cannot degrade α-tomatine into β-tomatine. Alternatively, CfTom1 could also degrade β-tomatine to tomatidine. However, this is unlikely because the presence of β-tomatine was neither observed *in vitro* nor *in planta* when α-tomatine degradation was determined for the Δ*cftom1* mutants. These results indicate that CfTom1 is probably the only enzyme responsible for degradation of α-tomatine by *C. fulvum* and those three putative GH3 tomatinases cannot complement Δc*ftom1* mutants for α-tomatine degradation. The predicted protein sequences suggest that the GH3 enzymes are functional, but they might be involved in the degradation of other (secondary metabolite) compounds present in tomato. Indeed, although the homology between the GH3 enzymes is high, they could have different substrate specificities. For example the amino acid identity between *S. lycopersici* GH3 tomatinase and *G. graminis* var. *avenae* GH3 avenacinase is 53% (Osbourn *et al.*, 1995). In addition, recent RNA-seq analysis performed on *C. fulvum*-infected tomato leaves revealed that these three GH3 genes are poorly expressed both *in vitro* and *in planta* conditions (unpublished results, P.J.G.M. de Wit).

Our results showed a good correlation between α-tomatine depletion (Fig. 1C, 6A), *CfTom1* gene expression (Fig. 3) and *C. fulvum* growth (Fig. 6A, wild type). α-Tomatine depletion was measured at 10 dpi, which is the time point when the *CfTom1* gene starts to be significantly induced and the fungal biomass to significantly increase. At later time points, the fungal biomass is much higher (Fig. 6A, wild-type) and the α-tomatine depletion is also higher (Fig. 1C). Therefore, we assume that the depletion of α-tomatine is related to fungal biomass and expression level of *CfTom1*.

The ability of tomato pathogens to specifically degrade α-tomatine suggests that tomatinase enzymes play an important role in the infection process. Several tomatinase enzymes have been characterized from bacterial and fungal tomato pathogens (Martin-Hernandez *et al.*, 2000; Sandrock & Vanetten, 2001; Kaup *et al.*, 2005; Seipke & Loria, 2008). However, neither GH3 tomatinase knock-out mutants in *S.*

lycopersici (Martin-Hernandez *et al.*, 2000) nor GH10 tomatinase knock-out mutants in *C. michiganensis* subsp. *michiganensis* (Kaup *et al.*, 2005) showed significant decrease in virulence as compared to wild-type strains. These results could be due to only subtle effects on virulence that were difficult to detect, or due to the presence of additional tomatinase-encoding genes in the genomes of these pathogens (Sandrock & Vanetten, 2001; Pareja-Jaime *et al.*, 2008; this study). So far, the only strong evidence for the involvement of a tomatinase enzyme in pathogenicity was found for the GH10 *tom1* gene of *F. oxysporum* f. sp. *lycopersici*, although the genome of this fungus also contains putative GH3 tomatinase genes (Pareja-Jaime *et al.*, 2008). Our study shows that the GH10 *CfTom1* gene of *C. fulvum* is involved in virulence. Colonization of tomato leaves by Δ*cftom1* mutants was reduced as shown by the significant reduction in fungal biomass from 10 dpi onwards. No significant difference in biomass was observed between the mutants, wild-type strain and ectopic transformant at the early stages of infection (2-8 dpi), at which time points the *CfTom1* gene was also only weakly expressed. The *CfTom1* gene expression is induced after 9 dpi, which explains the difference in growth between the mutants and controls at later stages of infection. Accordingly, degradation of α-tomatine to tomatidine during infection was found to occur from 10 dpi onwards. This study confirms the previous finding that heterologous expression of the *S. lycopersici* GH3 tomatinase gene in *C. fulvum* causes increased sporulation in susceptible tomato plants, and supports a role for α-tomatine degradation in *C. fulvum* virulence (Melton *et al.*, 1998).

Since it was reported that breakdown products of α-tomatine, such as β-tomatine and tomatidine, suppress plant defense responses (Bouarab *et al.*, 2002; Ito *et al.*, 2004), the absence of this indirect activity may also contribute to the reduction in virulence of the Δ*cftom1* mutants, in addition to increased sensitivity of the fungus to α-tomatine. However, suppression of plant defense responses by tomatidine could not be confirmed in our experiments. On the contrary, we found that tomatidine was toxic to tomato cells. A similar effect was also found by Itkin *et al*. (2011) in mutant tomato plants accumulating tomatidine (Itkin *et al.*, 2011). These results suggest that

the reduction in virulence of the Δ*cftom1* mutants is only due to increased sensitivity to α-tomatine.

Altogether, our results clearly show that the intercellular tomato pathogen *C. fulvum* encounters α-tomatine during colonization of the apoplastic space of tomato leaves and that CfTom1 is the major and possibly only α-tomatine detoxifying enzyme that contributes to full virulence of this fungus on tomato. This activity certainly contributed to the adaptation of *C. fulvum* to tomato after divergence from its close relative *Dothistroma septosporum* that is pathogenic on pine and lacks the *CfTom1* gene (de Wit *et al.*, 2012). The present work shows how powerful genome mining and sensitive assays are to solve discrepancies reported in previous studies.

MATERIALS AND METHODS

Fungal and plant materials

The *Cladosporium fulvum* race 0WU (CBS131901) (de Wit *et al.*, 2012) was grown on half-strength potato dextrose agar (19.5 g l^{-1} PDA and 15 g l^{-1} technical agar, Oxoid, Cambridge, UK) at 20°C for 2-3 weeks for conidia production and DNA isolation. Stocks of conidia were maintained in 25% glycerol at -80°C. For tomatinase activity assays, the fungus was grown in liquid CA medium (10 g l^{-1} casamino acids, 10 mM ammonium sulfate and 0.5 g l^{-1} yeast nitrogen base, pH 6.5). To induce the expression of the *CfTom1* gene, *C. fulvum* was pre-incubated in Gamborg B5 medium supplemented with 20 g l^{-1} sucrose at 22°C and incubated in an orbital shaker at 200 rpm for 6 days. Mycelium was then transferred to several B5 induction media at pH 4, pH 7, in the absence or presence of 50 µM α-tomatine, and cultured for 24 h.

Susceptible Money Maker (MM) Cf-0 tomato (*Solanum lycopersicum* L.) was used for inoculation experiments. Plants were grown in the greenhouse at 70% relative humidity, at 23-25°C during daytime and at 19-21°C at night, with a light/dark regime of 16/8 h and 100Watt m^{-2} supplemental light when light intensity was less than 150 Watt m^{-2}.

Nucleic acid methods

DNA was isolated from freeze-dried mycelia of *C. fulvum* strains, which were scraped from PDA plates, or from ground *C. fulvum*-inoculated tomato leaves frozen in liquid nitrogen, using the DNeasy plant mini kit (Qiagen Benelux bv, Venlo, The Netherlands) according to the manufacturer's instructions. Total RNA was isolated from ground mycelia or *C. fulvum*-inoculated tomato leaves using the hybrid method as described by van Esse *et al*. (2008).

cDNA was synthesized from 5 µg of total RNA using the SuperScript II reverse transcriptase kit (Invitrogen) as previously described by van Esse *et al*., 2008. Quantitative PCR was performed with the 7300 System (Applied Biosystems, Foster City, U.S.A.): each reaction was performed in 25 µl containing 12.5 µl Sensimix (Bioline, London, UK), 1 µl of each forward and reverse oligonucleotides (5 µM), 100 ng of template cDNA and 9.5 µl ddH$_2$O. The thermal profile included an initial 95°C denaturation step for 10 min followed by denaturation for 15 s at 95°C and annealing/extension for 45 s at 60°C for 40 cycles. qCfActin_F and qCfActin_R ; and qCfTom1_F and qCfTom1_R oligonucleotide pairs were designed with Primer3 Plus (Supporting information Table S1) (Untergasser *et al.*, 2007). The efficiency and specificity of the oligonucleotide pairs were determined with a dilution series of genomic DNA prior to use. The *C. fulvum actin* gene was used as a reference gene for normalization and results were analyzed using the $2^{-\Delta Ct}$ method (Livak & Schmittgen, 2001). Results are the average of three biological repeats.

All PCRs were performed in 25 µl using Pfu or GoTaq DNA polymerase (Promega) following the manufacturer's recommendations, and using 100 ng of genomic DNA or cDNA as template. The PCR program was initiated by a denaturation step at 94°C for 5 min, followed by 32 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 45 s, and extension at 72°C for 1 min, with a final extension step at 72°C for 7 min.

Southern blotting was performed with digoxigenin-labeled probes using a DIG DNA Labeling and Detection Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. The probe was generated using the pCfTom1_US_F and pCfTom1_US_R primer pair (Supporting information Table S1).

Identification and cloning of *CfTom1* gene and its expression by the *Potato Virus X* expression system in tomato

Amino acid sequences of GH3 and GH10 tomatinases were aligned with ClustalW2 (Larkin *et al.*, 2007) and edited in GeneDoc software (Nicholas *et al.*, 1997). A consensus phylogenetic tree was constructed using the minimum-evolution algorithm with default parameters and 1000 bootstrap replications in the MEGA5 software (Tamura *et al.*, 2011).

The GH10 tomatinase gene (*CfTom1*; jgiID 188986) from *C. fulvum* was amplified by PCR using the following oligonucleotide set: the CfTom1_F oligonucleotide excludes the native signal peptide sequence of the tomatinase gene and includes a 15-nucleotide overhang sequence corresponding to the *PR1A* signal peptide sequence; the CfTom1_R oligonucleotide contains the *Not*I restriction site (Supporting information Table S1). A second PCR was performed to amplify the *PR1A* signal peptide sequence by using the PR1A_F oligonucleotide containing the *Cla*I restriction site and PR1A_R oligonucleotide. Overlapping PCR was performed to fuse the *PR1A* signal peptide sequence to the *CfTom1* gene using the PR1A_F and CfTom1_R oligonucleotides (Supporting information Table S1). The amplified *PR1A-CfTom1* fragment was purified from the agarose gel using the Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK). The purified *PR1A-CfTom1* fragment was cloned into the pGEM-T Easy vector (Promega) according to the manufacturer's instructions. Recombinant pGEM-T vector was introduced into chemically competent *Escherichia coli* cells (DH5α) by the standard heat shock transformation protocol. The plasmid was retrieved from a positive clone using the Miniprep plasmid isolation kit (Qiagen) and the insert was sequenced by Macrogen Inc

(Amsterdam, NL). The correct *PR1A-CfTom1* insert was isolated using *Cla*I and *Not*I restriction enzymes (Promega) and was ligated (Promega) into the binary *Potato Virus X* (PVX)-based vector pSfinx (Hammond-Kosack *et al.*, 1995; Takken & Lu, 2001) that was digested with the same restriction enzymes. The ligation reaction was introduced into *E. coli* and the obtained *pSfinx::PR1A-CfTom1* plasmid was finally introduced into *Agrobacterium tumefaciens* (GV3101) by electroporation. A positive *A. tumefaciens* clone containing the *pSfinx::PR1A-CfTom1* construct was cultured on plates containing LB medium (10 g l^{-1} bacto-peptone; 5 g l^{-1} yeast extract; 5 g l^{-1} NaCl) supplemented with 100 μg ml^{-1} kanamycin and 25 μg ml^{-1} rifampicin for 48 h at 28°C. Heterologous expression of *pSfinx::PR1A-CfTom1* in tomato seedlings was performed as described previously (Stergiopoulos *et al.*, 2010).

Construction of the *CfTom1* targeted deletion plasmid

The gene replacement vector *pR4R3Δcftom1* was constructed using the MultiSite Gateway$^®$ Three-Fragment Vector Construction Kit (Invitrogen^{TM}, Carlsbad, CA, USA) according to the manufacturer's instructions. The upstream (US, 1.8kb) and downstream (DS, 1.55kb) flanking regions of *CfTom1* were amplified using oligonucleotides with overhang sequences homologous to the *AttB4, AttB1r, AttB2r* or *AttB3* recombination sites (Supporting information Table S1). Purified US and DS fragments were cloned into *pDONR*^{TM} *P4-P1R* and *pDONR*^{TM} *P2R-P3*, respectively, using 1 μl of BP clonase^{TM} II enzyme mix (Invitrogen^{TM}), 70 ng of insert DNA and 70 ng of pDONR^{TM} in a 5 μl reaction volume, and introduced into *E. coli*. The *p221_GFP_HYG* pENTRY vector was kindly provided by Rafael Arango Isaza (Plant Research Internationational, Wageningen). To construct the final replacement vector, an LR reaction was performed with 50 ng of *pDONR*^{TM} *P4-P1R_cftom1US*, 50 ng of *pDONR*^{TM} *P2R-P_cftom1DS*, 50 ng of *p221_GFP_HYG* and 70 ng of pDEST^{TM}R4-R3 destination vector in the presence of 1 μl of LR clonase^{TM} II enzyme mix (Invitrogen) in a 5 μl total volume. LR reaction was introduced into *E. coli* to obtain the final *pR4R3Δcftom1*

plasmid. Insertion of all fragments was confirmed by PCR using insert-specific primers (Supporting information Table S1).

Agrobacterium tumefaciens-mediated fungal transformation and mutant screening

The *pR4R3Δcftom1* construct was transformed into *Agrobacterium tumefaciens* AGL1 by electroporation. *A. tumefaciens*-mediated transformation of *C. fulvum* was performed as described previously (Zwiers & De Waard, 2001) with the following modifications. *A. tumefaciens* AGL1 cells containing the *pR4R3Δcftom1* construct were grown in minimal medium (MM) (10 mM K_2HPO_4, 10 mM KH_2PO_4, 2.5 mM NaCl, 2 mM $MgSO_4$, 0.7 mM $CaCl_2$, 9 µM $FeSO_4$, 4 mM NH_4SO_4, and 10 mM glucose; adjusted to pH 5.5) supplemented with 100 µg ml^{-1} spectinomycin and 10 µg ml^{-1} rifampicin for 2-3 days at 28°C. *A. tumefaciens* AGL1 cells were collected by centrifugation at 3363×g for 8 min and resuspended in induction medium (IM) [MM; 40 mM 2-(N-morpholino)ethanesulfonic acid, pH 5.3; 0.5% glycerol (w/v); 200 µM acetosyringone] supplemented with appropriate antibiotics to the final OD_{600} of 0.15. Prior to co-cultivation, the *A. tumefaciens* AGL1 culture was incubated at 28°C for 4-6 h to an OD_{600} of 0.25. *C. fulvum* conidia were collected from half-strength PDA plates with sterile water and passed through two layers of miracloth to remove mycelium. The *A. tumefaciens* AGL1 culture (200 µl; OD_{600} 0.25) was co-cultivated with 200 µl conidia suspension (1×10^7 conidia ml^{-1}). The co-cultivation mixture was plated on H Bond-nitrocellulose membrane that was previously placed on IM plates and incubated for 2 days at 20°C. After 2 days co-incubation, membranes were transferred to MM plates supplemented with 100 µg ml^{-1} hygromycin and 200 µM cefotaxime. Transformed fungal colonies (2-3-week-old) were transferred onto PDA plates supplemented with 100 µg ml^{-1} hygromycin and 200 µM cefotaxime, and incubated at 20°C for 2-3 weeks. Fungal transformants were sub-cultured twice on selective PDA plates and once on non-selective PDA plates. Subsequently, transformants were tested on PDA plates containing 100 µg ml^{-1} hygromycin.

α-Tomatine sensitivity assay

Conidia from wild-type *C. fulvum*, one ectopic transformant and two independent Δ*cftom1* mutants were collected and diluted to a final concentration of 2×10^4 conidia ml^{-1}. Agar plugs were prepared on sterile microscope slides by addition of 250 μl CA medium (CA medium supplemented with 1% technical agar). Conidia (20 μl) were mixed with 10 mM α-tomatine (in methanol) (Sigma Aldrich) to obtain a final concentration of 100 μM, and incubated on the CA agar plugs at 20°C for 2 days. As a negative control, conidia were incubated in methanol (1% v/v) but without α-tomatine. For each sample, the germination of 100 conidia was analyzed. The results are the average of two biological repeats.

Tomatinase activity monitoring by LC-MS

Extraction and Liquid Chromatography-Quadrupole Time of Flight-Mass Spectrometry (LC-QTOF-MS) analysis of semi-polar secondary metabolites were performed according to the protocols described before (De Vos *et al.*, 2007; Tikunov *et al.*, 2010). Briefly, 0.1 g of ground frozen tomato leaves and 2 ml freeze-dried apoplastic fluid (AF) (0.5 ml of AF is obtained from 1 g of leaves) were extracted with 300 μl [99.9% methanol with 0.1% formic acid (v/v)] and 200 μl [75%, methanol with 0.1% formic acid (v/v)], respectively and sonicated for 15 min. The mixture was centrifuged at 20,000×*g* for 10 min and the supernatant was filtered using a 0.45 μm inorganic membrane filter (Anotop 10, Whatman, NH, USA), fitted onto a disposable syringe, and transferred to a glass vial. Ionization was performed using electrospray ionization in positive mode. Collision energy of 10 eV was used for full-scan mass detection in the range of m/z 100 to 1500. Leucine enkaphalin, [M+H]$^+$= 556.6305, was used for online mass calibration (lock mass) using a separate spray inlet. For monitoring of rutin and α-tomatine in the AF and total extract (TE) of tomato leaflets, LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific) was used, with Xcalibur software to control the instrument, for data acquisition and analysis (van der Hooft *et al.*, 2011). The mass chromatograms that were generated in LTQ-Orbitrap

were processed (peak picking and baseline correction) using MetAlign software package (Lommen, 2009). From the processed data, information on the relative peak height of the representative masses [M+H]$^+$ for α-tomatine, dehydrotomatine and their degradation products, tomatidine and tomatidenol, was extracted at their recorded retention time for tomato fruit (Supporting information Table S2), in the MOTO database (Moco *et al.*, 2006) and the Komics database (http://webs2.kazusa.or.jp/komics/) (Iijima *et al.*, 2008). Absolute quantification of α-tomatine was done using a standard curve of commercial α-tomatine (Sigma Aldrich).

For *in vitro* α-tomatine degradation, 1×10^6 conidia of *C. fulvum* wild-type strain, ectopic transformant and Δ*cftom1* mutants were cultured in 30 ml liquid CA medium at 22°C with shaking at 200 rpm for 5-6 days, using three biological replicates. The fungal cultures were filtered to remove mycelia and the supernatants were used to determine tomatinase activity. Culture filtrate (4 ml) of each strain was incubated with 100 µg ml^{-1} α-tomatine (Sigma Aldrich) for 24 h with shaking at 28°C. After 24 h incubation, samples were prepared for LC-MS analysis to determine α-tomatine, and its degradation products tomatidine and β-tomatine, as described above. This experiment was performed with three biological replicates.

A. tumefaciens strains containing the *pSfinx::CfTom1* and the original *pSfinx* (empty virus) vectors were inoculated on 10-day-old MM-Cf-0 tomato seedlings for transient heterologous *CfTom1* gene expression. This experiment was performed in three biological replicates. After 5 weeks, *pSfinx::CfTom1* or *pSfinx::Empty* construct-transformed tomato leaves were collected for AF isolation. AF isolation was performed by vacuum infiltration according to the method described by de Wit and Spikman (1982). 15 ml of AF for each sample was freeze-dried and re-suspended in 6 ml methanol containing 0.1% formic acid. Prior to LC-MS analysis, 1 ml of each sample was filtered through 0.45 µm pore size filters and samples were analyzed by LC-MS in positive ionization mode to determine depletion of α-tomatine and accumulation of tomatidine. This experiment was also performed for wild-type, ectopic and Δ*cftom1* mutants-inoculated tomato plants. Here, AF was isolated from inoculated tomato

plants at 10 dpi with the different strains (this experiment was performed with two biological replicates).

Virulence assays

Wild-type *C. fulvum* (race 0WU; CBS131901), one ectopic and two independent Δ*cftom1* mutants (Δ*cftom1-2.6* and Δ*cftom1-5.1*) were grown on half-strength PDA plates for 2-3 weeks at 20°C. Conidia were collected from the plates with water and subsequently passed through one layer of miracloth to remove fungal mycelium. Conidia were diluted to a final concentration of 5×10^5 conidia ml^{-1}. Five-week-old MM-Cf-0 tomato plants were inoculated with conidia from the wild-type, ectopic and the two Δ*cftom1* mutants via spray inoculation on the abaxial side of the tomato leaves. *C. fulvum*-inoculated tomato plants were kept in plastic-covered cages for two days to ensure 100% relative humidity for conidial germination. After two days, plastic covers were removed and disease development of the wild-type, ectopic transformant and Δ*cftom1* mutants were assayed every two days. Inoculated leaves were collected at different days after inoculation (2, 4, 8, 10 and 12 dpi) (the experiment was performed with three biological replicates). Fungal biomass quantification was performed using quantitative PCR analysis as described previously but using genomic DNA. Genomic DNAs were isolated from wild-type, ectopic transformant and Δ*cftom1* mutants infected leaves and were diluted to final concentration of 100 ng µl^{-1}. A standard curve was constructed by using the serial dilutions of *C. fulvum* genomic DNA (10 ng µl^{-1}; 1 ng µl^{-1}; 0.1 ng µl^{-1}; 0.01 ng µl^{-1}; 0.001 ng µl^{-1}) using *actin* as a reference gene. Logarithms (base 10) of DNA concentrations were plotted against crossing point of Ct values.

The effect of tomatidine on H$_2$O$_2$ accumulation

Cell suspensions of MSK8 tomato (*Solanum lycopersicum* L.) were grown in Murashige and Skoog plant salt base (4.3 g l^{-1}) liquid medium supplemented with B5 vitamins (myo-inositol (100 g l^{-1}), nicotinic acid (1 g l^{-1}), pyridoxine.HCl (1 g l^{-1}) and

thiamine.HCl (10 g l^{-1}), sucrose (3%) and kinetin (0.1 mg ml^{-1}). The MSK8 cells were continuously rotated at 125 rpm in the dark at 25°C. Five-day-old MSK8 cell suspensions were used for the H_2O_2 accumulation assay. 2 ml of MSK8 cell suspensions were transferred to 12 well-plates and stabilized for two hours in a rotary shaker before further use. These cells were co-incubated either with α-tomatine (final concentration of 40 μM) or tomatidine (final concentration of 40 μM) in the presence of the histochemical stain DAB (100 μg ml^{-1}) and; with or without 1 μM chitin-hexamer. The MSK8 cell suspensions treated with same dilution rate of methanol (0.8% v/v) were used as a control. Pictures were taken 15 h post co-incubation. The reddish-brown precipitate indicates accumulation of H_2O_2.

Assessing phytotoxic effects of α-tomatine and tomatidine towards tomato

100, 250 and 500 μM of α-tomatine and tomatidine (diluted from 5 mM stock of α-tomatine and tomatidine in methanol) (Sigma Aldrich) were infiltrated into 5- or 6-week-old tomato leaves. The same dilution rates of methanol (2%, 5% and 10% v/v) were used as negative control. Pictures were taken 3 days after treatment with α-tomatine or tomatidine. The experiment was performed with three biological replicates.

## ACKNOWLEDGMENTS

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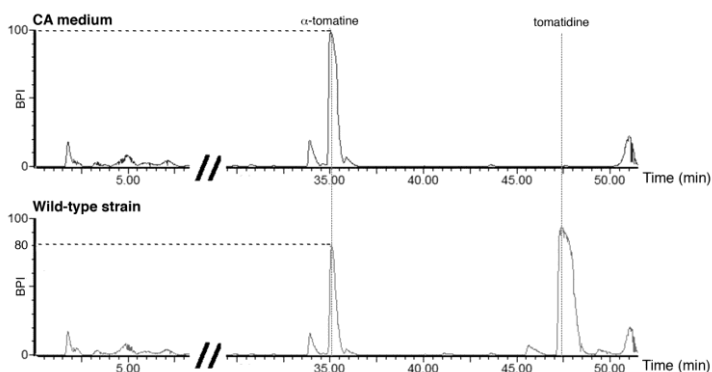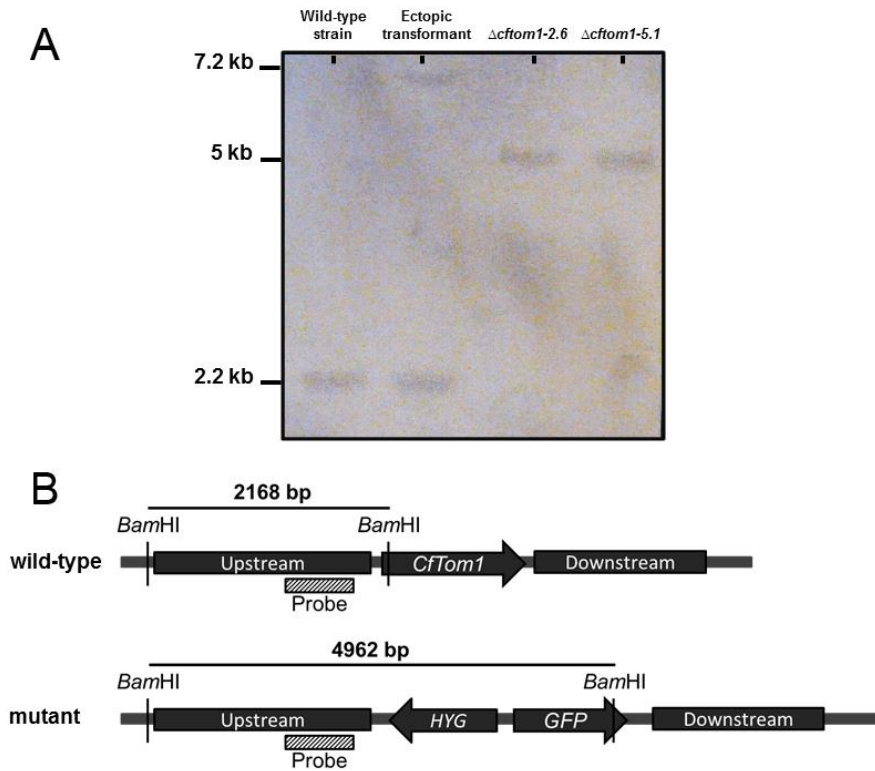
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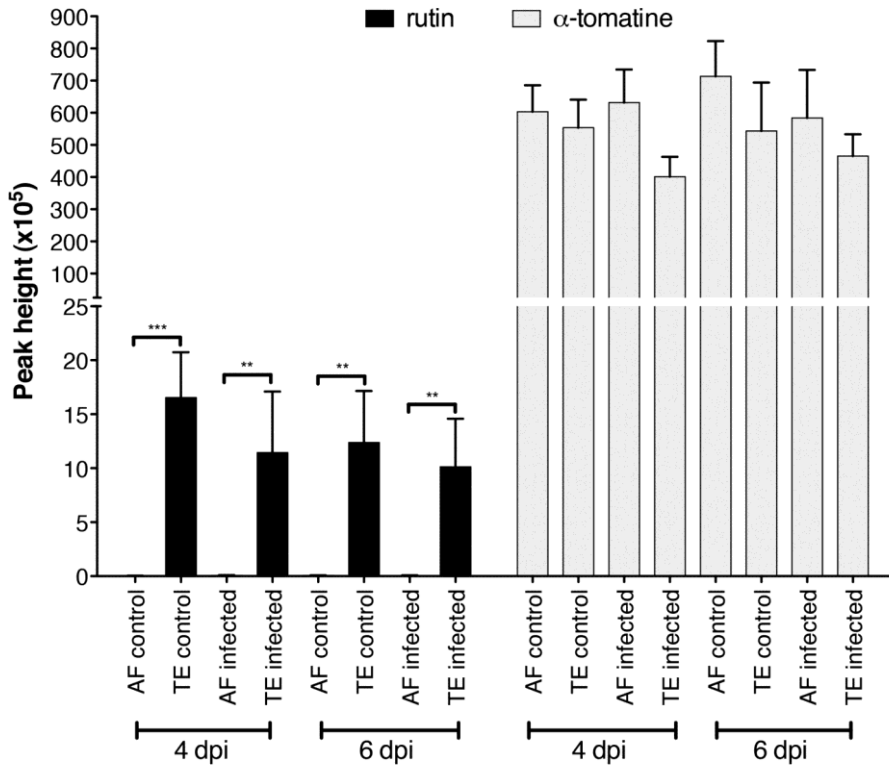
Supporting information Fig. S1. Localization of α-tomatine and rutin in tomato. LC-MS detection of rutin, a compound with similar polarity to α-tomatine that is thought to be localized in the cell and/or vacuole, and α-tomatine in apoplastic fluid (AF) and total extract (TE) from tomato leaves. Samples were collected at 4 and 6 days post inoculation (dpi) with mock (control) or a virulent *Cladosporium fulvum* strain (inoculated). Rutin is mainly detected in TEs, presumably originating from vacuoles, whereas α-tomatine is detected both in the AF and TE. Their localization is independent from the inoculation with *C. fulvum*. A one-way ANOVA analysis was performed and followed by a Tukey-Kramer test. Only significant differences are indicated (** P<0.001, ***P<0.0001, n=4). Error bars represent standard deviation of at least three biological repeats.
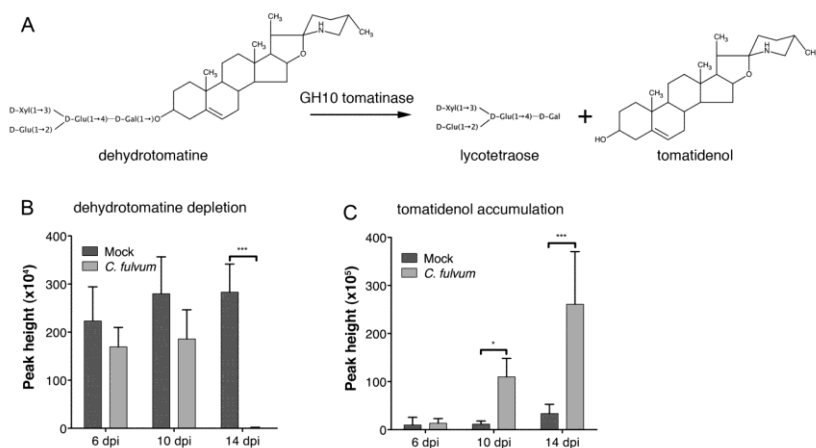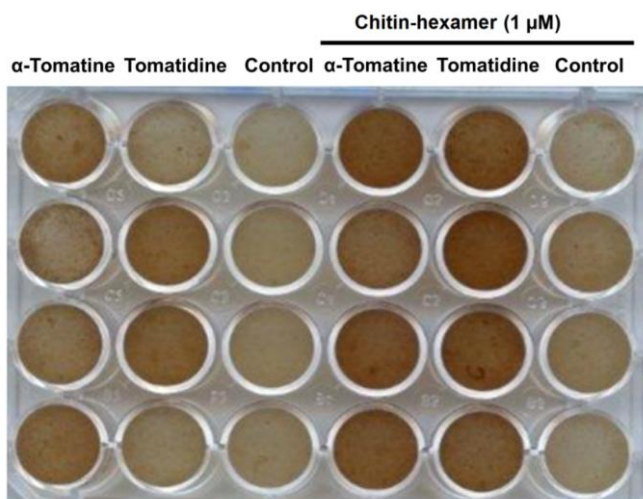
Supporting information Fig. S2. Detoxification of dehydrotomatine by *Cladosporium fulvum*. (A) Tomatinase enzymes of the GH10 family hydrolyze dehydrotomatine into tomatidenol and lycotetraose. LC-MS was performed to detect the relative peak height of **(B)** dehydrotomatine and **(C)** tomatidenol in apoplastic fluids isolated from healthy (mock) tomato plants or at 6, 10 and 14 days post inoculation (dpi) with *C. fulvum*. A two-way ANOVA analysis was performed and followed by a Bonferroni test. Only significant differences are indicated (* $P<0.05$; *** $P<0.001$). Error bars represent standard deviation of at least three biological repeats.

Supporting information Fig. S3. *In vitro* detoxification of α-tomatine by *Cladosporium fulvum*. Sterile CA medium and culture filtrate from the wild-type strain were supplemented with 100 µg ml^{-1} of α-tomatine. After 24 h of incubation, the relative base peak intensity (BPI) of α-tomatine and tomatidine were measured by LC-MS. The *C. fulvum* culture filtrate shows GH10 tomatinase activity. This figure was modified from Fig. 5C.

Supporting information Fig. S4. Southern blot analysis of the Δ*cftom1* mutants. (A) Targeted gene deletion of *C. fulvum CfTom1*. Genomic DNA preparations of wild-type *C. fulvum*, an ectopic transformant and two Δ*cftom1* mutants were digested with *Bam*HI, the obtained fragments were separated on gel, blotted and hybridized with the indicated probe. **(B)** *Bam*HI restriction map of the *CfTom1* genomic region in wild-type and Δ*cftom1* mutants. The *CfTom1*, *HYG* and *GFP* coding regions are shown as gray arrows indicating the orientation of the genes, while 5' and 3' flanking regions of *CfTom1* that are used for homologous recombination are shown as gray boxes.

Supporting information Fig. S5. Effects of α-tomatine and tomatidine on H_2O_2 accumulation in chitin-treated tomato MSK8 cell suspensions. Tomato MSK8 cell suspensions were incubated with 40 µM α-tomatine and 40 µM tomatidine in the absence or presence of 1 µM chitin-hexamer to induce H_2O_2 production for 15 hours. 3,3-Diaminobenzidine (DAB) (100 µg ml^{-1}) staining was performed to visualize the H_2O_2 production. The MSK8 cell suspension treated with same dilution rate of methanol (0.8%) was considered as a control. Images were taken 15 h post co-incubation. The reddish-brown color indicates the accumulation H_2O_2.

Supporting information Table S1. Oligonucleotides used in this study. The names and sequences of forward and reverse oligonucleotides used for all PCRs and quantitative PCRs.

Oligonucleotide name	Oligonucleotide sequences (5' - 3')
PR1A_F	ATCGATATGGGATTTGTTCTCTTTTCAC
PR1A_R	ATTTTGGGCACGGCAAGAGTG
CfTom1_F	CTTGCCGTGCCCAAAATACAGATGTTCCTAGCACAGGC
CfTom1_R	GCGGCCGCCTACTTGCCACCAATCTCCTC
qCfTom1_F	GTTGGGTTTACGAAGGTCCA
qCfTom1_R	CCGACAACTTGCACAGAAGA
qCfActin_F	TGACCCTCAGGTACCCAATC
qCfActin_R	TCGAGACGTAGAAGGCTGGT
CfTom1_DS_F	GGGGACAGCTTTCTTGTACAAAGTGGGAGATTTCAGGCATTGTCGGTG
CfTom1_DS_R	GGGGACAACTTTGTATAATAAAGTTGATTCCCTTGGTGCTCGGTCG
CfTom1_US_F	GGGGACAACTTTGTATAGAAAAGTTGACCAAGCCACGGACCCTTTAG
CfTom1_US_R	GGGGACTGCTTTTTTGTACAAACTTGGGTGTGACAGTGCTGGATCTTG
pCfTom1_US_F	ACATGCAAACGATCAGGGCTAC
pCfTom1_US_R	AACCACTTTTCGCTACCCCGAC

Supporting information Table S2. Accurate mass LC-MS and MS/MS data of the major tomato alkaloids affected by CfTom1 enzyme.

Compound	Molecular formula	Calculated $[M+H]^+$	Observed $[M+H]^+$	Mass deviation ppm	Observed MS/MS fragments $[M+H]^+$					
					-18 (-H$_2$O)	-132 (-Xyl)	-162 (-Glu)	-294 (-Xly-Glu)	-456 (-Xly-Glu-Glu)	AglC+H
α-Tomatine	C$_{50}$H$_{83}$NO$_{21}$	1034.5530	1034.5527	-0.64	1016.5436	902.5116	872.5013	740.4576	578.4046	416.3521
Dehydrotomatine	C$_{50}$H$_{81}$NO$_{21}$	1032.5374	1032.5356	-1.68	1014.5259	900.4940	870.4836	738.4401	576.3880	414.3359
					-18 (-H$_2$O)	-217 (-C$_8$H$_{19}$NO)	-255 (-C$_{15}$H$_{29}$NO$_2$)			
Tomatidine	C$_{27}$H$_{45}$NO$_2$	416.3523	416.3509	-3.27	398.3416	199.1487	161.1323			
Tomatidenol	C$_{27}$H$_{43}$NO$_2$	414.3366	414.3354	-3.02	396.3255	197.1322	159.1164			

Chapter 4

Functional analysis of the conserved transcriptional regulator CfWor1 in *Cladosporium fulvum* reveals diverse roles in the virulence of plant pathogenic fungi.

Bilal Ökmen, Scott Griffiths, Russell Cox, Jérôme Collemare and Pierre JGM de Wit. (Submitted)

ABSTRACT

Fungal Wor1-like proteins are conserved transcription regulators that are reported to regulate the virulence of several plant pathogenic fungi by affecting the expression of virulence genes. However, it is not known whether these genes are directly or indirectly affected. Here, we report the functional analysis of *CfWor1,* the homologue of Wor1 carried by *Cladosporium fulvum*. *CfWor1* deletion mutants are characterized by rough hyphae and sclerotium-like structures, which do not sporulate and are covered with a dark extracellular matrix. These mutants are developmentally defective and no longer virulent on tomato. A *CE.CfWor1* mutant that constitutively expresses *CfWor1* is not impaired in development, yet it also shows reduced virulence. Although a subset of known effector and secondary metabolism genes are differentially regulated in both Δ*cfwor1* and *CE.CfWor1* mutants, CfWor1 seems to primarily influence virulence through the fungal development. Complementation of a non-virulent Δ*fosge1* (Wor1-homologue) mutant of *Fusarium oxysporum* f. sp. *lycopersici* with *CfWor1* restored expression of the *SIX* effector genes, but not virulence. Chimeric proteins of CfWor1/FoSge1 also only partially restored defects of the Δ*fosge1* mutant, suggesting that these transcription regulators have functionally diverged. Altogether, our results suggest that CfWor1 regulates development of *C. fulvum* and in some extent it also affects the expression of a subset of genes involved in virulence.

INTRODUCTION

Establishment of compatible interactions between fungal pathogens and their host plants relies on diverse virulence factors, including secreted small proteins and secondary metabolites, which often act in concert. Virulence factors are diverse in action, and can be found acting at many layers of the infection process, such as penetration of the host, suppression or evasion of host immune defense, and nutrient acquisition, which may involve damaging or killing host cells (Schneider & Collmer, 2010). In addition, virulence of some plant pathogenic fungi, such as *Ustilago maydis* and *Zymoseptoria tritici*, requires switching from a yeast-like growth to a filamentous

growth, which is referred to as dimorphism (Nadal *et al.*, 2008). Indeed in a more general sense, plant pathogenic fungi invariably require a degree of morphological differentiation as infection cycles begin with the complete cell reprogramming of a germinating spore for the construction of runner hyphae. Some pathogens, such as *Magnaporthe oryzae* and *Colletotrichum higginsianum*, further differentiate their runner hyphae into appressoria, specialized dome-shaped cells to facilitate host penetration (Howard & Valent, 1996; Voegele *et al.*, 2001; Kankanala *et al.*, 2007; O'Connell *et al.*, 2012). In the case of *M. oryzae*, once the penetration hypha has entered a host cell, the fungus differentiates yet again into invasive bulbous hyphae that are in close contact with the host cell (Kankanala *et al.*, 2007). Similarly, *C. higginsianum* first differentiates a bulbous biotrophic primary hypha, which then differentiates a thin secondary hypha during the necrotrophic phase of the infection (O'Connell *et al.*, 2012). Other plant pathogens like rust fungi differentiate more specialized feeding structures named haustoria (Voegele *et al.*, 2001). It appears as though parasitic growth of most plant pathogenic fungi has evolved to generate structurally distinct invasive hyphae that are adapted to colonize host tissues. Although most plant pathogenic fungi are not dimorphic *per se*, the generation of specific structures or hyphae differentiation for parasitism dictates that species-specific regulatory networks exist, which control those morphological processes.

Morphological transitions involved in pathogenicity have been studied in detail in human pathogenic fungi, such as *Histoplasma capsulatum*. This fungus switches from filamentous growth in the soil to parasitic budding-yeast growth in the human host in a temperature dependent manner (Liu *et al.*, 1994; Klein & Tebbets, 2007; Nguyen & Sil, 2008), a trait required for successful infection. This morphological switch involves the transcriptional regulator Ryp1 (Nguyen & Sil, 2008), which is the ortholog of Wor1 of *Candida albicans* (Huang *et al.*, 2006; Srikantha *et al.*, 2006; Zordan *et al.*, 2006; Nguyen & Sil, 2008). In *C. albicans* and *Candida tropicalis*, Wor1 is involved in the white to opaque cell type switch, a transition required for both mating and pathogenicity (Huang *et al.*, 2006; Srikantha *et al.*, 2006; Zordan *et al.*, 2006;

Ramirez-Zavala *et al.*, 2008; Porman *et al.*, 2011). Wor1 is also conserved in *Saccharomyces cerevisiae,* where it is known as Mit1, a regulator of pseudohyphal growth (Cain *et al.*, 2012). Although Wor1 and Mit1 can bind the same DNA motif (Lohse *et al.*, 2010; Cain *et al.*, 2012), they appear to regulate different sets of genes that control different types of morphological changes in their respective species (Lohse *et al.*, 2010; Cain *et al.*, 2012).

Wor1 homologues have also been identified and studied in several plant pathogenic fungi, including *Fusarium oxysporum* f. sp. *lycopersici* (FoSge1), *Botrytis cinerea* (BcReg1)*, Fusarium graminearum* (Fgp1) and *Verticillium dahliae* (VdSge1) (Michielse *et al.*, 2009b; Michielse *et al.*, 2011; Jonkers *et al.*, 2012; Santhanam & Thomma, 2013). These fungi are not known to undergo morphological switches like human pathogenic fungi, and Wor1 homologue deletion mutants did not show obvious morphological phenotypes, except an impaired production of conidia. However, Wor1 homologues are important for host colonization, as all mutants showed reduced virulence on susceptible host plants (Michielse *et al.*, 2009b; Michielse *et al.*, 2011; Jonkers *et al.*, 2012; Santhanam & Thomma, 2013). In *F. oxysporum* f. sp. *lycopersici* reduced virulence of the Δ*fosge1* mutant was mainly correlated with loss of expression of *SIX* (Secreted In Xylem) effector genes (Michielse *et al.*, 2009b). This was also suggested to explain the loss of virulence of Δ*vdsge1* mutants of *V. dahliae* on tomato, where *VdSge1* was found to differentially regulate the expression of putative effector genes (Santhanam & Thomma, 2013). In *F. graminearum*, the inability of the Δ*fgp1* mutant to penetrate the rachis node of wheat ears was correlated with the loss of trichothecene toxin production (Jonkers *et al.*, 2012). Similarly, the reduced virulence of the Δ*bcreg1* mutant in *B. cinerea* was correlated with the loss of production of the botcinic acid and botrydial phytotoxins (Michielse *et al.*, 2011). These studies suggest that effector and secondary metabolism genes involved in virulence might be specific targets of Wor1-like proteins in plant pathogenic fungi. This contrasts with the developmental function of Wor1 homologues in *Candida* species, *H. capsulatum* and *S.*

cerevisiae (Huang *et al.*, 2006; Srikantha *et al.*, 2006; Zordan *et al.*, 2006; Nguyen & Sil, 2008; Cain *et al.*, 2012).

In this study, we identified and characterized the *Wor1* homologue of the non-obligate biotrophic fungus *Cladosporium fulvum*, the causal agent of tomato leaf mould. After penetration through open stomata, *C. fulvum* runner hyphae differentiate into larger hyphae that colonize the intercellular space of tomato leaf tissue without entering host-cells (Thomma *et al.*, 2005). *C. fulvum* is well known for the secretion of many effectors that are required for full virulence. Our functional analysis of *CfWor1* shows that the CfWor1 has a major effect on the development of *C. fulvum*, and reduced virulence of knock-out mutants is not only due to regulation of effector genes. We also provide evidence for a similar function of Wor1 homologues in other plant pathogenic fungi.

RESULTS

The *Cladosporium fulvum* genome contains *Wor1* homologues

The search for genes that encode Wor1-like proteins in the *C. fulvum* genome (de Wit *et al.*, 2012), by using the amino acid sequence of FoSge1 from *F. oxysporum* f. sp. *lycopersici*, retrieved two *Wor1* homologues (Clafu_183744 and Clafu_191897). A phylogenetic analysis showed that Clafu_191897 belongs to the Pac2 family of transcriptional regulators (Fig. 1), which is not required for pathogenicity in *F. oxysporum* f. sp. *lycopersici* and *F. graminearum* (Michielse *et al.*, 2009b; Jonkers *et al.*, 2012) and was not considered for further investigation. The other homologue, Clafu_183744, belongs to a clade that comprises all characterized Wor1-like proteins, suggesting that they all share the same origin (Fig. 1). However, the orthology of Wor1-like proteins is only resolved at the order level (Capnodiales, Pleosporales, Helotiales and Saccharomycetales) (Wang *et al.*, 2009). Paralogues might also be present, since FgFgp1 seems more closely related to VdSge1 than FoSge1. Both *Fusarium* species

belong to the same order of the Hypocreales while *V. dahliae* belongs to another order (Wang *et al.*, 2009). Hereafter we will refer to Clafu_183744 as CfWor1.
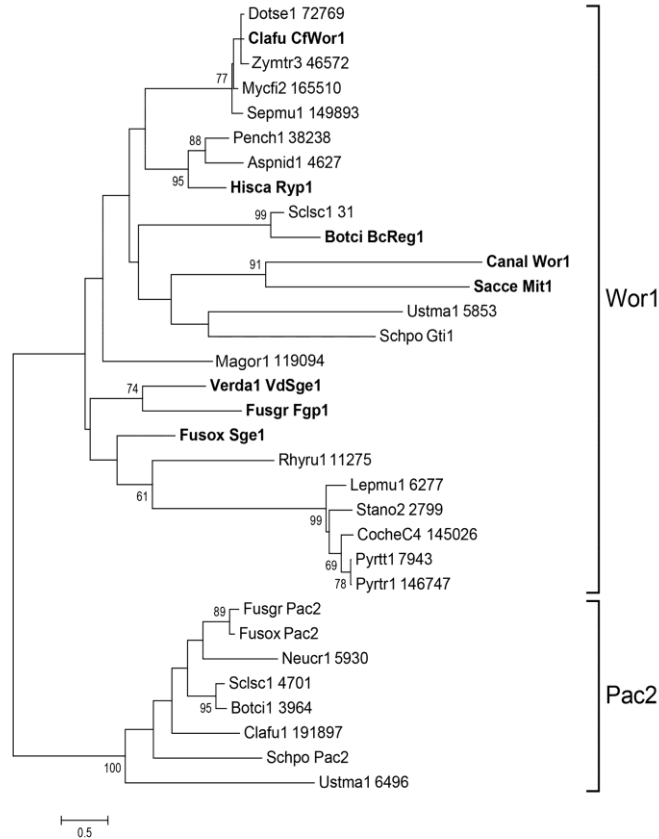

Fig. 1. Maximum likelihood phylogenetic tree of fungal Wor1-like proteins. The scale bar indicates the number of substitutions per site. Dotse: *Dothistroma septosporum*; Clafu: *Cladosporium fulvum*; Zymtr: *Zymoseptoria tritici*; Mycfi: *Mycosphaerella fijiensis*; Sepmu: *Septoria musiva*; Pench: *Penicillium chrysogenum*; Aspnid: *Aspergillus nidulans*; Hisca: *Histoplasma capsulatum*; Sclsc: *Sclerotinia sclerotiorum*; Botci: *Botrytis cinerea*; Canal: *Candida albicans*; Sacce: *Saccharomyces cerevisiae*; Ustma: *Ustilago maydis*; Schpo: *Schizosaccharomyces pombe*; Magor: *Magnaporthe oryzae*; Verda: *Verticillium dahliae*; Fusgr: *Fusarium graminearum*; Fusox: *Fusarium oxysporum* f. sp. *lycopersici*; Rhyru: *Rhytidhysteron rufulum*; Lepmu: *Leptosphaeria maculans*; Stano: *Stagonospora nodorum*; Coche: *Cochliobolus heterostrophus*; Pyrtt: *Pyrenophora teres* f. sp. *teres*; Pyrtr: *Pyrenophora tritici-repentis*; Neucr: *Neurospora crassa*.

The N-terminal part of CfWor1 is more conserved than its C-terminal part when compared with other Wor1-like proteins (Supporting information Fig. S1). The 220 amino acid residues from the N-terminus of CfWor1 share 65%, 59%, 52%, 46%, 42% and 39% of similarity with those from FoSge1, FgFgp1, VdSge1, BcReg1, Ryp1 and Wor1, respectively, which is consistent with evolutionary distance between these fungi and *C. fulvum*. The similarity is due to the presence of conserved DNA-binding domains (WOPRa and WOPRb boxes), phosphorylation site and nuclear localization signal (Supporting information Fig. S1) (Huang *et al.*, 2006; Srikantha *et al.*, 2006; Nguyen & Sil, 2008; Michielse *et al.*, 2009b; Lohse *et al.*, 2010). These observations suggest that CfWor1 functions as a transcriptional regulator and is most likely localized in the nucleus as reported for other Wor1-like proteins (Huang *et al.*, 2006; Srikantha *et al.*, 2006; Michielse *et al.*, 2009b). In contrast, the C-terminal domain is not conserved at the sequence level and greatly varies in length (Supporting information Fig. S1).

Quantitative RT-PCR was performed to determine the expression profile of *CfWor1* in wild-type *C. fulvum* grown under different *in vitro* conditions and during tomato infection. The results showed that *CfWor1* expression was five times higher *in vitro* than during tomato infection (Fig. 2).

Δ*cfwor1* mutants show developmental defects and are non-virulent on tomato

To assess the role of *CfWor1* during *in vitro* growth, four independent Δ*cfwor1* mutants were generated using *Agrobacterium tumefaciens*-mediated transformation. The wild-type *CfWor1* gene was replaced by the *hygromycine resistance* (*HYG*) and the *green fluorescence protein* (*GFP*) genes by homologous recombination. Replacement of *CfWor1* in the four deletion mutants was confirmed by PCR and single insertion event was confirmed by quantitative PCR (Supporting information Fig. S2).
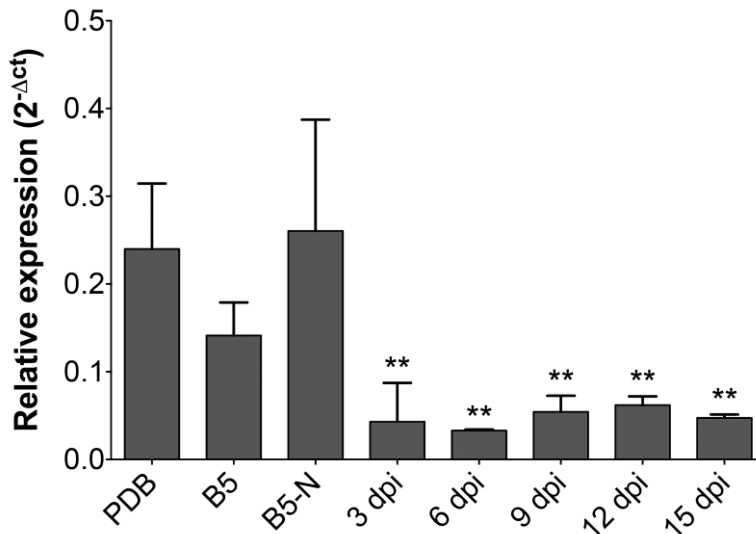
Fig. 2. Expression profile of *CfWor1* in *Cladosporium fulvum*. Quantitative PCR was performed to assess the expression of *CfWor1* in three *in vitro* conditions (PDB, B5 medium with and without nitrogen source) and during tomato infection from three to 15 days post inoculation (dpi). Expression was normalized using the *actin* gene of *C. fulvum*. A one-way ANOVA analysis was performed and followed by a Bonferroni test. Only significant differences compared to the expression in PDB medium are indicated (** $P<0.001$). Error bars represent standard deviation of three biological repeats.

The morphology of the Δ*cfwor1* mutants was remarkably different from wild-type (Fig. 3). Wild-type and ectopic transformant strains form green-brown and fluffy colonies (Fig. 3A), whereas Δ*cfwor1* mutants form dark-coloured rigid colonies (Fig. 3E). The colonies of the Δ*cfwor1* mutants are slightly larger in diameter than wild-type and have a different shape. Unlike the wild-type strain (Fig. 3B, 3C and 3D), Δ*cfwor1* mutants do not produce conidia and form many structures that resemble sclerotia (Fig. 3F and 3G). Finally, Δ*cfwor1* mutants secrete an extracellular matrix (ECM) of unknown composition that completely covers the hyphae and sclerotium-like structures (Fig. 3G and 3H). The presence of this ECM gives the Δ*cfwor1* mutant hyphae a rough appearance as compared to the smooth appearance of wild-type hyphae.
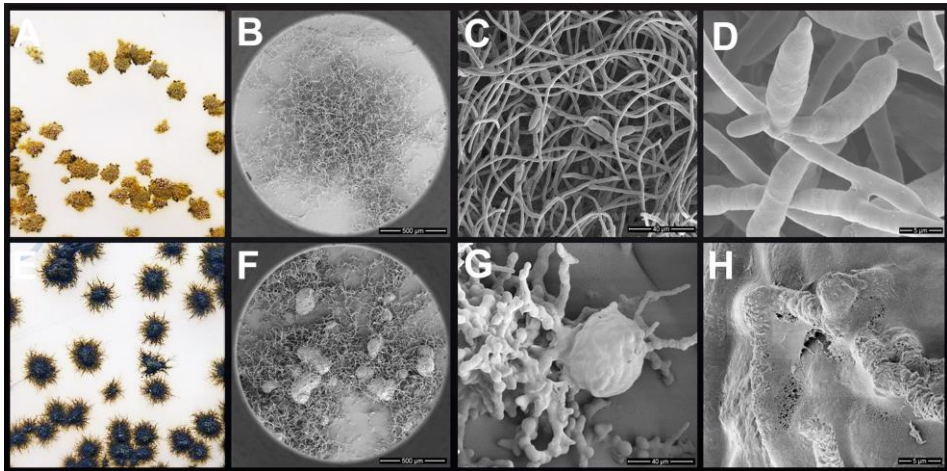
Fig. 3. Δ*cfwor1* mutants of *Cladosporium fulvum* show strong developmental defects. (A-D) Morphology of colonies from wild-type grown on PDA. **(B)** The same colonies were observed using scanning electron microscopy. Higher magnifications at **(C)** 2,000× and **(D)** 10,000× show smooth hyphae and conidia. **(E-H)** Morphology of colonies of Δ*cfwor1* mutant grown on PDA. **(F)** The same colonies were observed using scanning electron microscopy, showing sclerotium-like structures. Higher magnifications at **(G)** 2,000× and **(H)** 10,000× show rough hyphae and an extra-cellular matrix.

Because sporulation of the Δ*cfwor1* mutants was impaired, virulence of the mutants could not be analyzed by inoculating plants with conidia. Therefore wild-type, ectopic transformant and Δ*cfwor1* mutants were cultured in flasks and ground mycelia of each strain, which did not contain any spores, were used to inoculate tomato plants. The disease development was assessed at different time points after inoculation. Ground mycelia of wild-type and ectopic transformant did grow on the leaf surface and successfully penetrated the leaf through stomata at 6 days post inoculation (dpi) (Fig. 4). Tomato leaves were being colonized by 10 dpi and both strains completely invaded the host by 16 dpi (Fig. 4). In contrast, tomato plants inoculated with Δ*cfwor1* mutants were nearly symptomless until 16 dpi and only a few lesions could be observed at 18 and 20 dpi (Fig. 4). Although the initial inoculum of both wild-type and the mutants was similar (Supporting information Fig. S3), the hyphae of deletion mutants showed unusual shape compared to the hyphae of wild-type and ectopic transformant, and

were not able to properly grow on the leaf surface (Fig. 4 and Supporting information Fig. S3). This observation likely explains the fewer penetration events and reduced virulence of the *Δcfwor1* mutants.
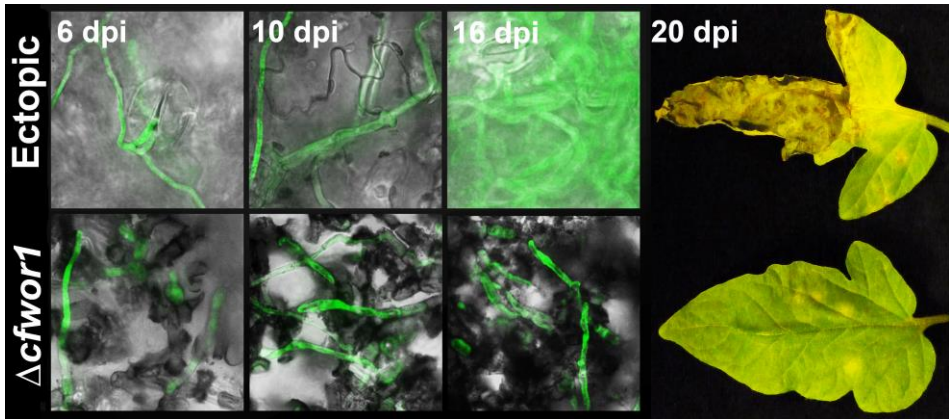

Fig. 4. Δ*cfwor1* mutants of *Cladosporium fulvum* show reduced virulence on susceptible tomato. Ground mycelia of an ectopic transformant and four independent **Δ***cfwor1* mutants were spray-inoculated on susceptible tomato plants. Disease establishment was monitored until 20 days post inoculation (dpi). Penetration and colonization of tomato leaves by both strains transgenic for GFP was followed by confocal fluorescence microscopy. Pictures representative of each stage are shown as an overlay of GFP fluorescence and visible light images.

Constitutive expression of *CfWor1* decreases virulence of *Cladosporium fulvum* on tomato

Because *CfWor1* had lower expression during infection compared to *in vitro*, we generated a mutant that expresses *CfWor1* under control of the constitutive *ToxA* promoter from *Pyrenophora tritici-repentis* (Ciuffetti *et al.*, 1997) in order to test the effects of a higher expression on morphology and virulence. Expression of *CfWor1* was three-fold higher in the obtained transformant (*CE.CfWor1*) compared to wild-type grown *in vitro* (Supporting information Fig. S4). The *CE.CfWor1* transformant did not show any obvious developmental defects *in vitro*, apart from a reduction in sporulation

(Fig. 5A). Inoculation of susceptible tomato plants with the *CE.CfWor1* transformant resulted in significantly reduced fungal biomass production compared to the wild-type strain as early as 3 dpi (Fig. 5B). The *CE.CfWor1* transformant expressed *CfWor1* at a significantly higher level than wild-type at three and six dpi, but the expression level was comparable to levels observed for wild-type at late stages of infection (Supporting information Fig. S4). These results suggest that the reduced virulence of *CE.CfWor1* transformant is due to the higher expression of *CfWor1* at the very early stages of infection.
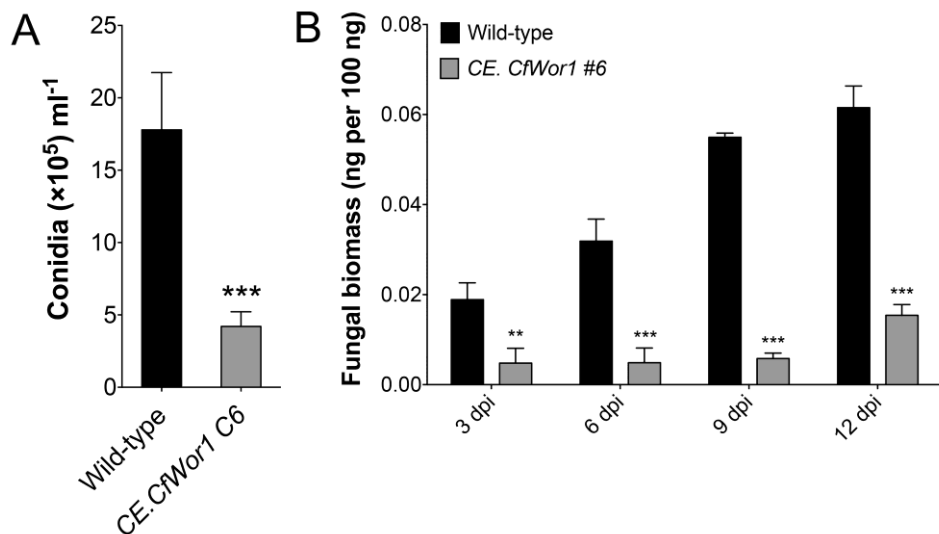

Fig. 5. Constitutive expression of *CfWor1* in *Cladosporium fulvum* results in reduced sporulation and virulence. (A) Conidia produced on PDA by wild-type and *CE.CfWor1* transformant. A t test analysis was performed to compare means (*** P<0.0001). Error bars represent standard deviation of at least four biological repeats. **(B)** Fungal biomass produced by wild-type and *CE.CfWor1* transformant during infection of tomato measured by quantitative PCR until 12 days post inoculation (dpi) using a standard curve calculated with the *actin* gene from *C. fulvum*. A multiple t test analysis was performed and followed by a Holm-Sidak test. Only significant differences between *CE.CfWor1* and wild-type are indicated (** P<0.001; *** P<0.0001). Error bars represent standard deviation of two biological repeats.

CfWor1 modulates the expression of a wide range of genes in *Cladosporium fulvum*

In *F. oxysporum* f. sp. *lycopersici,* reduced virulence of the *Δfosge1* mutant was suggested to be due to down-regulation of effector genes known to be required for full virulence (Michielse *et al.*, 2009b). *C. fulvum* is known as a secretor of many effector proteins that have been shown to play crucial roles in virulence (Stergiopoulos & de Wit, 2009), and we anticipated that CfWor1 might be involved in regulation of those effector genes. Therefore the relative expression of nine characterized effector genes was measured by quantitative RT-PCR in wild-type, Δ*cfwor1*, and *CE.CfWor1* strains grown *in vitro* under nitrogen starvation, a condition shown to induce the expression of *Avr9* (Van Den Ackerveken *et al.*, 1994). Expression of the tubulin gene (as a control) was not affected in any strain (Figure 6A). Of the nine effector genes that were tested, only *Ecp1* and *Ecp4* were not differentially expressed in both Δ*cfwor1* mutants and *CE.CfWor1* transformant compared to the wild-type strain, indicating that CfWor1 is not involved in their regulation (Fig. 6A). The expression of *Avr2*, *Avr9* and *Ecp6* was significantly up-regulated in the Δ*cfwor1* mutants, while they were down-regulated in the *CE.CfWor1* transformant compared to wild-type (Fig. 6A), suggesting that CfWor1 negatively affects the expression of these three effector genes. Expression of *Ecp5* was down-regulated in the *CE.CfWor1* transformant, but expression of *Avr4, Avr4E* and *Ecp2* genes was induced (Fig. 6A), suggesting that *CfWor1* acts as a negative or positive regulator of these genes. The regulation of *Avr9 in vitro*, and likely *in planta*, is under control of the transcription factor *Nrf1* (Perez-Garcia *et al.*, 2001). Consistent with the up-regulation of *Avr9*, *Nrf1* was also up-regulated in the Δ*cfwor1* mutants, while it was down-regulated in the *CE.CfWor1* transformant (Fig. 6A).

Expression profiling of a subset of the *C. fulvum* effectors was also performed during the infection of tomato by wild-type, Δ*cfwor1* and *CE.CfWor1* strains. Quantitative RT-PCR results showed that while expression of tubulin is stable over time and shows similar level in all strains (Fig. 6B), expression of all the tested effectors (*Avr2*, *Avr4E*, *Avr4*, *Avr9* and *Ecp6*) was significantly lower in the Δ*cfwor1* mutants

compared to the wild-type strain until 12 dpi (Fig. 6B). At 14 dpi, however, expression of these effector genes in the Δ*cfwor1* mutants reached the wild-type expression levels or even higher (Fig. 6B). These results suggest that positive regulators of effector genes were not induced in the Δ*cfwor1* mutants that only grew on the surface of the tomato leaf. Expression levels of *Avr4* and *Avr4E* in the *CE.CfWor1* transformant were not significantly different from the wild-type strain (Fig. 6B). However, similar to *in vitro* culture, *Avr2*, *Avr9* and *Ecp6* were lower expressed at 6 dpi in the *CE.CfWor1* transformant compare to the wild-type strain. These results were also verified at 9 dpi for *Avr9* and at 9 and 12 dpi for *Ecp6*. This suggests that down-regulation of these effectors might in part explain the reduced virulence of the *CE.CfWor1* transformant.

The dark-coloured mycelium of the Δ*cfwor1* mutants suggests the production of pigments, consistent with a role of Wor1-like proteins in regulation of fungal secondary metabolism (Michielse *et al.*, 2011; Jonkers *et al.*, 2012). The genome of *C. fulvum* contains 23 key genes that might be involved in the production of secondary metabolites (SMs) (de Wit *et al.*, 2012) (Collemare *et al.*, submitted). Expression of SM genes was assessed in the Δ*cfwor1* mutants and *CE.CfWor1* transformant. Compared to wild-type, *PKS1* was up-regulated and *PKS6* was down-regulated in the Δ*cfwor1* mutants (Fig. 6A), while the other SM genes remained unaffected (data not shown). In the *CE.CfWor1* transformant, *PKS6* was slightly induced, while the expression of *PKS1* was not affected, consistent with *CfWor1* being a repressor of *PKS1* and an activator of *PKS6*. *PKS1* was predicted to be involved in the production of a compound related to the toxin elsinochrome and *PKS6* was associated with the production of the pigment cladofulvin (Collemare *et al.*, submitted). SM profiling using LC-MS confirmed the absence of cladofulvin and the production of several new metabolites in the mycelium of the Δ*cfwor1* mutants, which are likely products of the *PKS1* biosynthetic pathway (Supporting information Fig. S5). Unfortunately, no molecular masses could be determined for any of these peaks. Thus, in addition to effector genes, CfWor1 also has an influence on the regulation of some SM genes in *C. fulvum*.
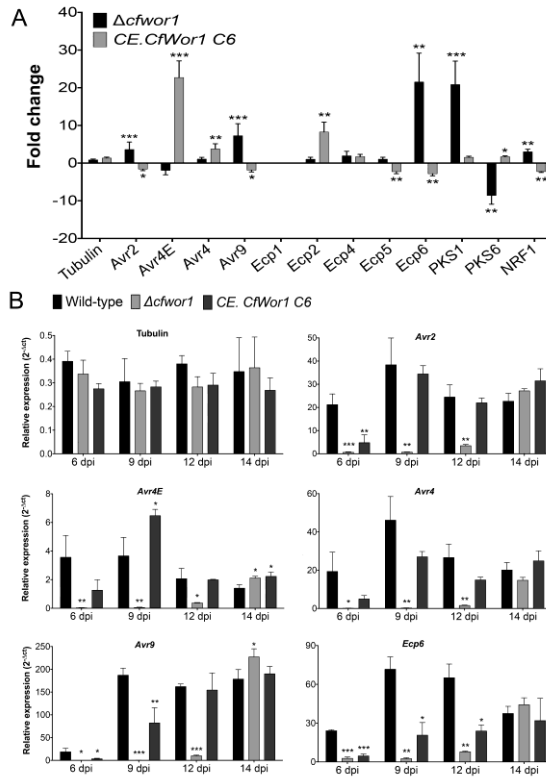
Fig. 6. Expression profile of effector and secondary metabolite genes in wild-type, Δ*cfwor1* and CE.CfWor1 strains of *Cladosporium fulvum*. (A) Quantitative PCR was performed to assess the expression level of nine known effector genes, and of the secondary metabolism key genes *PKS1* and *PKS6*, and *Nrf1* genes in wild-type, *CfWor1* deletion mutant and *CE.CfWor1* strains grown *in vitro*. Expression was normalized using the *actin* gene of *C. fulvum* and fold change compared to wild-type was calculated according to the $2^{-\Delta\Delta Ct}$ method. When the ratio was lower than one, the inverse number was taken to show the fold change in the graph. The *tubulin* gene was used as an internal control. A multiple t test analysis was performed and followed by a Holm-Sidak test. Only significant differences between wild-type and each transformant are indicated (* $P<0.05$; ** $P<0.001$; *** $P<0.0001$). Error bars represent standard deviation of at least three biological repeats. **(B)** Relative expression level of *tubulin*, *Avr2*, *Avr4E*, *Avr4*, *Avr9* and *Ecp6* genes are assessed with quantitative PCR for wild-type, *CfWor1* deletion and *CE.CfWor1* strains of *C. fulvum* during infection. Expression was normalized using the *actin* gene of *C. fulvum* according to the $2^{-\Delta Ct}$ method. A multiple t test analysis was performed and followed by a Holm-Sidak test. Only significant differences between wild-type and each transformant are indicated (* $P<0.05$; ** $P<0.001$; *** $P<0.0001$). Error bars represent standard deviation of three biological repeats.

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CfWor1 partially complements the function of FoSge1 in Δ*fosge1* mutants from *Fusarium oxysporum* f. sp. *lycopersici*

In order to determine whether *CfWor1* is a functional homologue of *FoSge1*, *CfWor1* was introduced into the Δ*fosge1 #32* deletion mutant of *F. oxysporum* f. sp. *lycopersici* (Michielse *et al.*, 2009b). In addition, chimeric *CfWor1/FoSge1* constructs encoding chimeric proteins were obtained, in which the N-terminal part of CfWor1 (amino acids 1-233) was fused to the C-terminal part of FoSge1 (amino acids 219-330) (denoted *WS*), or the N-terminal part of FoSge1 (amino acids 1-218) was fused to the C-terminal part of CfWor1 (amino acids 234-530) (denoted *SW*) (Fig. 7A). All constructs were expressed under the control of native *FoSge1* promoter and introduced into the Δ*fosge1 #32* mutant. Two independent transformants were selected for each constructs and analysed. As controls, the wild-type *F. oxysporum* f. sp. *lycopersici* 4287, Δ*fosge1 #32* mutant and *FoSge1 #5*-complemented strain were used (Michielse *et al.*, 2009b).

The Δ*fosge1* transformants complemented with *CfWor1* showed an intermediate colony phenotype between the original deletion mutant and the strain complemented with FoSge1 (Supporting information Fig. S6). Indeed, the colour of the *CfWor1*-complemented strains was similar to the Δ*fosge1* transformants complemented with *FoSge1*, but the colonies were as smooth as the original Δ*fosge1* mutant. In contrast, Δ*fosge1* transformants complemented with the chimeric genes (*SW* and *WS*) showed more aerial hyphae compared to the wild-type strain (Supporting information Fig. S6). Complementation of Δ*fosge1 #32* mutant with *CfWor1* did not restore sporulation of the deletion mutants, whereas the *SW* and *WS* chimeric genes partially and fully restored sporulation, respectively (Fig. 7B).

Previously, it was shown that FoSge1 is an activator of the expression of *SIX* effector genes in *F. oxysporum* f. sp. *lycopersici*, which was hypothesized to explain the decreased virulence observed for Δ*fosge1 #32* on tomato (Michielse *et al.*, 2009b).
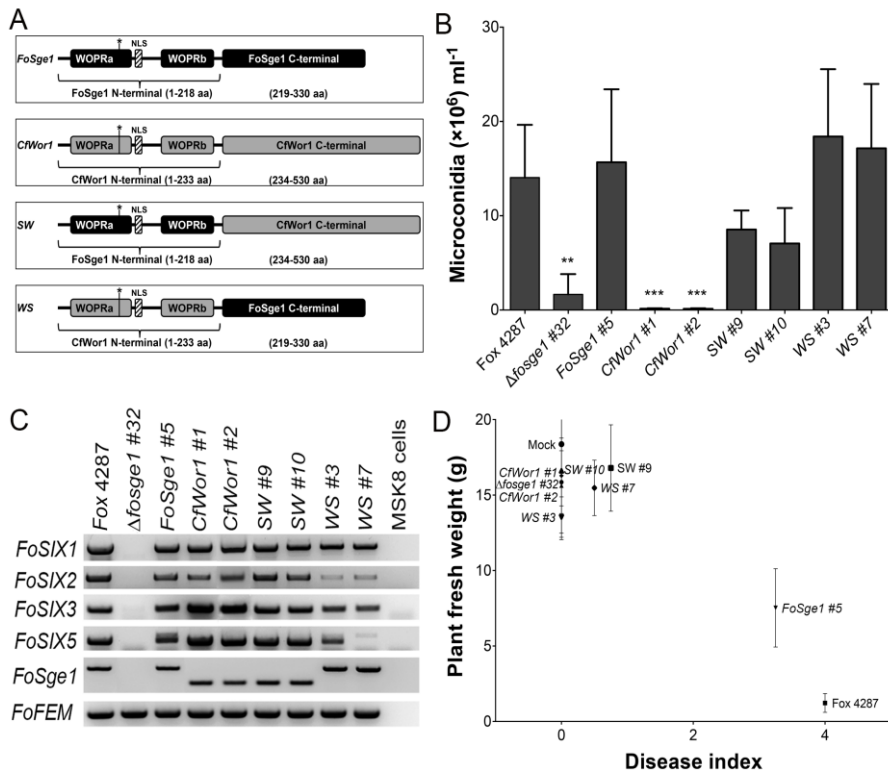
Fig. 7. CfWor1 partially complements the Δ*fosge1* mutant of *Fusarium oxysporum* f. sp. *lycopersici*.
(A) Chart representing the native and chimeric proteins that were used to complement the Δ*fosge1* mutant. The N- and C-terminal parts are indicated below each protein. The N-terminus contains the WOPRa and WOPRb DNA binding motifs, the NLS signature is shown as a dashed box and a star indicates the putative conserved phosphorylation site. **(B)** The number of microconidia produced was measured for the Fox 4287 wild-type strain, Δ*fosge1* #32 deletion mutant and transformant complemented with native FoSge1 (#5), two native CfWor1 and two of each chimeric protein (SW and WS). A one-way ANOVA analysis was performed and followed by a Bonferroni test. Only significant differences between wild-type and transformants are indicated (** $P<0.001$; *** $P<0.0001$). Error bars represent standard deviation of at least five biological replicates. **(C)** Expressions of *SIX* effector genes, *FoSge1*, *CfWor1*, *SW* and *WS* chimeric genes and *FoFEM*, as a positive control, were measured by RT-PCR in the same strains as in (B), which were grown *in vitro* in contact with MSK8 cells for effector gene induction. MSK8 cells alone were used as a negative control. **(D)** Virulence of wild-type, deletion mutant and all complemented transformants was assessed at 3 weeks after inoculation by measuring plant fresh weight and using a disease index. Error bars represent standard deviation of three biological replicates.

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Expression of the *SIX* genes was determined by RT-PCR in the wild-type and transgenic strains after co-incubation with MSK8 tomato cells. Consistent with the previous report (Michielse *et al.*, 2009b), the expression of all tested *SIX* genes was induced in the wild-type and FoSge1-complemented transformant, while their expression was not induced in the Δ*fosge1* mutant (Fig. 7C). Ectopic expression of full length *CfWor1*, *SW* and *WS* chimeric constructs in the Δ*fosge1 #32* mutant restored the expression of all tested *SIX* genes, although expression levels of some genes was slightly different from the *FoSge1 #5* complemented strain (Figure 7C). Although the expression of all tested *SIX* genes was restored in all *CfWor1*-, *SW*- and *WS*-complemented strains, none of them were virulent on susceptible tomato plants, except the FoSge1-complemented strain (Fig. 7D).

DISCUSSION

The role of Wor1 in the biology of *Cladosporium fulvum*

Wor1-like proteins are conserved transcriptional regulators involved in morphological switches of yeast-like fungal species (Huang *et al.*, 2006; Srikantha *et al.*, 2006; Zordan *et al.*, 2006; Nguyen & Sil, 2008; Cain *et al.*, 2012). Recently, it has been hypothesized that Wor1 homologues of several plant pathogenic fungi are involved in virulence by differentially regulating effector and secondary metabolism genes during infection (Michielse *et al.*, 2009b; Michielse *et al.*, 2011; Jonkers *et al.*, 2012; Santhanam & Thomma, 2013). In these plant pathogenic fungi, apart from a reduced sporulation there were no severe developmental defects reported.

In contrast to the five- to fourteen-fold up-regulation of *FoSge1* and *VdSge1* during infection of tomato by *F. oxysporum* f. sp. *lycopersici* and *V. dahliae*, respectively (Michielse *et al.*, 2009b; Santhanam & Thomma, 2013), *CfWor1* is not up-regulated during tomato infection. This expression profile is also opposite to the expression profile of *C. fulvum* effector genes, which are specifically up-regulated *in planta*. Although this observation does not suggest a role in virulence, both Δ*cfwor1*

and *CE.CfWor1* transformants were compromised in virulence. Observation of the shape and biomass quantification of the fungal hyphae during infection of tomato led us to hypothesize that *Δcfwor1* mutants did not properly recover from the grinding performed before the inoculation and thus could not grow normally on the leaf surface. This defect was only seen *in planta* because ground hyphae of both wild-type and mutant strains were able to recover and grow on plates. Surprisingly, biomass of the CE.CfWor1 transformant was significantly lower than wild type as early as three days post inoculation.

During tomato infection, all characterized *C. fulvum* effector genes share the same expression profile, in that they are positively regulated during fungal penetration and colonization of the plant leaf. Regulators of effector genes remain to be discovered in *C. fulvum* and it is likely that all effector genes are induced through the same regulatory network. CfWor1 does not seem to be a major regulator involved in the up-regulation of effector genes because it does not regulate their expression in the same manner. For example, CfWor1 seems to be a repressor of *Avr2, Avr9* and *Ecp6*, but an activator of *Avr4*, *Avr4E* and *Ecp2*. Down-regulation of three effector genes in the *CE.CfWor1* transformant at six days post inoculation might contribute to the reduced virulence since they are known to be virulence factors (Bolton *et al.*, 2008; van Esse *et al.*, 2008). However, it cannot explain the reduced fungal biomass as early as three days post inoculation. The lower expression of effector genes in the *Δcfwor1* mutants is likely due to an absence of plant specific induction of these genes, since the unusually shape mutant hyphae seemed unable to properly grow on the leaf surface and thus precluded penetration events. Few mutant hyphae managed to penetrate and colonize tomato tissues, and resulted in a few disease symptoms from 14 dpi onwards. This result suggests that the induction of effector genes at the late stages of tomato infection by *Δcfwor1* mutants is due to these penetration events, which allow the mutants to reach the same developmental stage as wild-type. Altogether, our data support the hypothesis that effector genes are under control of developmental signals, as it has also been reported for secondary metabolism genes (Calvo *et al.*, 2002).

Therefore, we conclude that CfWor1 is involved in the virulence of *C. fulvum* by regulating hyphal growth on the leaf surface.

The role of Wor1 in the virulence of plant pathogenic filamentous fungi

Modest developmental defects were observed when *Wor1* homologues were deleted in other filamentous fungi. Δ*vdsge1* mutants of *V. dahliae* and Δ*fgp1* mutants of *F. graminearum* showed reduced radial growth *in vitro* compared to wild-type strains (Jonkers *et al.*, 2012; Santhanam & Thomma, 2013) and all reported mutants of *Wor1* homologues showed reduced or loss of sporulation (Michielse *et al.*, 2009b; Michielse *et al.*, 2011; Jonkers *et al.*, 2012; Santhanam & Thomma, 2013). Although no *in vitro* developmental defect was reported for the Δ*fosge1* mutant of *F. oxysporum* f. sp. *lycopersici* (Michielse *et al.*, 2009b), in our study we observed that the wild-type strain produces more aerial hyphae than the mutant, which shows smooth colonies. These observations suggest that Wor1 also be involved in the development of these filamentous fungi.

We showed that CfWor1 or the chimeric proteins of CfWor1 and FoSge1 could not restore the virulence of the Δ*fosge1* mutant, while they could restore the expression of all *SIX* effector genes. Thus, the reduced virulence of the Δ*fosge1* mutant is not solely due to the loss of expression of *SIX* effector genes. Although it cannot be ruled out that expression of unknown virulence genes was not restored in the complemented strains, it might also be possible that a morphological switch between saprophytic and parasitic growth was not fully restored. This hypothesis is consistent with the observation that Δ*fosge1* mutants are neither impaired in root surface colonization nor in penetration, but only in growth within plant cells and the xylem (Michielse *et al.*, 2009b), which might require a morphological switch. Similarly, Δ*fgp1* and Δ*bcreg1* mutants are also unable to colonize plants, although the latter is still able to extensively grow saprophytically on the leaf surface (Michielse *et al.*, 2011; Jonkers *et al.*, 2012).

Functional diversification of a conserved regulator

Transcriptome analyses of Δ*fosge1, Δfgp1*, Δ*wor1* and Δ*mit1* mutants showed that Wor1 homologues regulate a wide range of genes (Cain *et al.*, 2012; Jonkers *et al.*, 2012). Remarkably, although both Wor1 of *C. albicans* and Mit1 of *S. cerevisiae* can recognize the same DNA binding motif (Lohse *et al.*, 2010; Cain *et al.*, 2012), they regulate only a limited number of genes in common, indicating that the gene sets regulated by Wor1 and Mit1 have changed since divergence of these fungal species (Cain *et al.*, 2012). Similarly, *FoSge1* and *Fgp1* regulate different sets of genes (Jonkers *et al.*, 2012), which is in agreement with the observation that neither regulator can functionally complement the other (Jonkers *et al.*, 2012). However, we found that both full length CfWor1 and chimeric versions of CfWor1 and FoSge1 proteins could fully restore the expression of all tested *SIX* effector genes in the Δ*fosge1* mutant. This suggests that these genes are likely regulated by direct binding of FoSge1 and CfWor1 to their promoter regions because the conserved N-terminus of Wor1 homologues is sufficient for DNA binding and transcriptional activation (Lohse *et al.*, 2010). These results also suggest that CfWor1 and FoSge1 can bind the same motif in the promoter of the *SIX* effector genes in *F. oxysporum* f. sp *lycopersici*. The DNA binding motifs recognized by Wor1 and Mit1 could not be identified in the promoter of genes regulated by Wor1 homologues in plant pathogenic fungal species, including *C. fulvum*, and no novel motif could be found in the promoters of all co-regulated genes (Michielse *et al.*, 2009b; Michielse *et al.*, 2011; Jonkers *et al.*, 2012; Santhanam & Thomma, 2013). Together with the results of Jonkers *et al.* (2012), domain swaps between CfWor1 and FoSge1 suggest that the non-conserved C-terminus plays another regulatory role, possibly through interactions with a diverse set of regulatory proteins. These might include homologues of Efg1 (StuA), a regulator inhibited by Wor1 in *C. albicans* (Zordan *et al.*, 2007), because they were shown to play a major role in conidiation in *Fusarium* species (Lysoe *et al.*, 2011). Indeed, we found that only proteins that contained the C-terminus of FoSge1 could fully restore sporulation of the Δ*fosge1* mutant. The lack of complementation of virulence might be due to the

inability of CfWor1 and the chimeric proteins to interact with different regulatory proteins that might be involved in differential expression of unknown virulence factors or differentiation of invasive hyphae. By diversifying both motif recognition and the ability to interact with other regulatory proteins, the conserved Wor1-like proteins appear to have evolved and functionally diverged in their regulation of different developmental processes in a species-species manner. A developmental defect in all Δ*wor1* mutants might be the main cause of their reduced virulence and should be further investigated in order to better understand the function of Wor1-like proteins in fungal biology.

MATERIALS AND METHODS

Fungal and plant materials

The wild-type *C. fulvum* race 0WU [CBS131901; (de Wit *et al.*, 2012)] was used for fungal transformation. *C. fulvum* was grown on half-strength potato dextrose agar (19.5 g l^{-1} PDA and 15 g l^{-1} technical agar, Oxoid) at 20°C for 2-3 weeks for conidia production. Stocks of conidia were maintained in 25% glycerol at -80°C. Nitrogen limited B5 medium was prepared as described by van den Ackerveken *et al.* (1994)*. C. fulvum* was pre-incubated in Gamborg B5 medium supplemented with 20 g l^{-1} sucrose at 22°C and incubated in an orbital shaker at 200 rpm for 6 days. Mycelium was then transferred to nitrogen limited B5 medium and cultured for 24 h.

F. oxysporum f. sp. *lycopersici* strain 4287, Δ*fosge1 #32* mutant and FoSGE1 *#5* complementation strain were kindly obtained from Dr. Martijn Rep (University of Amsterdam). Strains were grown in KNO$_3$ minimal medium (3% sucrose; 10 mM KNO$_3$; and 0.17% yeast nitrogen base without amino acids) at 25°C as shaking cultures (175 rpm) for 7 days for conidia production and DNA isolation.

Susceptible Money Maker Cf-0 tomato cultivar was used to perform infection experiments with wild-type and transformants. Tomato plants were grown in greenhouse at 70% relative humidity, at 23-25°C during daytime and at 19-21°C at

night, with a light/dark regime of 16/8 hours and 100 Watt m^{-2} supplemental light when the sunlight influx intensity was less than 150 Watt m^{-2}.

Nucleic acid methods

DNA was isolated from mycelia of fungal strains that were grown in PDB for 7 days, or from ground *C. fulvum*-infected tomato leaves, frozen in liquid nitrogen, using the DNeasy plant mini kit (Qiagen Benelux bv, Venlo, The Netherlands) according to the manufacturer's instructions. Total RNA isolation and cDNA synthesis were performed as previously described (van Esse *et al.*, 2008). Oligonucleotides for quantitative RT-PCR were designed using Primer3 Plus (Supporting information Table S1) (Untergasser *et al.*, 2007). Their efficiency and specificity were determined prior to their use. Quantitative RT-PCR was performed with the 7300 System SDS software (Applied Biosystems, Foster City, U.S.A.) using 100 ng cDNA or genomic DNA as template. Reactions were performed in 25 µl containing 12.5 µl Sensimix (Bioline, London, UK), 1 µl of each forward and reverse primer (5 µM) and 9.5 µl ddH$_2$O. The program used was an initial 95°C denaturation step for 10 min followed by denaturation for 15 s at 95°C and annealing/extension for 45 s at 60°C for 40 cycles. Results of at least three biological repeats were analyzed using the $2^{-\Delta Ct}$ method (Livak & Schmittgen, 2001). The number of insertion events of the deletion cassette in the mutants, reflected by the number of inserted *hygromycin* gene copies, was calculated using actin gene for normalization and the single copy gene *Avr4* as reference, using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

All PCRs were performed in 25 µl using Pfu polymerase (Promega) following the manufacturer's recommendations with 100 ng of genomic DNA as template. The PCR program was initiated by a denaturation step at 94°C for 5 min, followed by 32 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 45 s, and extension at 72°C for 1 min, with a final extension step at 72°C for 7 min.

Samples for expression of *SIX* genes in all *F. oxysporum* f. sp. *lycopersici* strains were obtained as described by Michielse *et al.* (2009) with some modifications. A 0.5 ml of 5×10^6 conidia ml^{-1} of each strain was co-incubated with 4 ml of a one week old MSK8 cell suspension culture for two days at 22°C. RT-PCR was performed for *SIX1*, *SIX2*, *SIX3* and *SIX5* effector genes, for *FoSge1*, *CfWor1*, and chimeric constructs (*SW* and *WS*) and for *FoFEM*, as a positive control. The RT-PCR program was initiated by a denaturation step at 94°C for 5 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 45 s, and extension at 72°C for 1 min, with a final extension step at 72°C for 7 min.

Construction of plasmids for CfWor1 functional analysis

Gene replacement construct *pR4R3Δcfwor1* was prepared by using MultiSite Gateway® Three-Fragment Vector Construction Kit (Invitrogen™, Carlsbad, CA, USA) according to the manufacturer's instructions. The upstream (US; 975 bp) and downstream (DS; 1034 bp) flanking regions of the *CfWor1* were amplified using primers (Supporting information Table S1) with overhang homologous to the *AttB4*, *AttB1r, AttB2r* and *AttB3* recombination sites. Purified US and DS fragments were cloned into *pDONR*™ *P4-P1R* and *pDONR*™ *P2R-P3* and the final plasmid was obtained as previously described (Ökmen *et al.* 2013).

CfWor1 gene for constitutive expression construct was amplified with the corresponding primers (Supporting information Table S1). *CfWor1* gene was fused to the native *FoSge1* promoter using overlapping PCR with the corresponding primers (Supporting information Table S1). The PCR fragment of *CfWor1* (1,600 bp) and *CfWor1* fused with the native *FoSge1* promoter (2,500 bp) was cloned into the pGEM-T Easy vector (Promega) according to the manufacturer's instructions. Recombinant pGEM-T vectors were introduced into chemically competent *Escherichia coli* cells (DH5α) by the standard heat shock transformation protocol. The plasmids were retrieved from a positive clone using the Miniprep plasmid isolation kit (Qiagen Benelux bv, Venlo, The Netherlands) and the inserts were sequenced by Macrogen Inc (Amsterdam, NL). The

correct *CfWor1* and *CfWor1* fused with the native *FoSge1* promoter constructs were digested using *Pac*I (New England Biolabs) and *Not*I restriction enzymes (Promega) and ligated (Promega) into pFBT0029, which contains a hygromycin resistance cassette and ToxA promoter (7.1 kb), and pFBT005 plasmid, of which ToxA promoter was removed and contains a nourseothricin resistance cassette (6.7 kb), that were also digested with same restriction enzymes by following the manufacturers' protocols, respectively (Plasmids were kindly obtained from Prof. Dr. Bart P.H.J. Thomma, Wageningen University). Similar overlapping PCRs were performed to construct the chimeric *CfWor1*/*FoSge1* genes in which the N- and C-terminal parts of both genes are swapped (*WS* and *SW* constructs) using the adequate oligonucleotides pairs (Supporting information Table S1) and *pFBT005_CfWOR1* plasmid or *F. oxysporum* genomic DNA as template. PCR fragments were digested by *Pac*I and *Not*I restriction enzymes and cloned into pFBT005 plasmid (without ToxA promoter) digested with the same endonucleases following the same procedure as above. All constructs were confirmed by sequencing (Macrogen, Inc, Amsterdam, NL).

Agrobacterium tumefaciens-mediated fungal transformation

Each construct was transformed into *Agrobacterium tumefaciens* AGL1 by electroporation. *A. tumefaciens*-mediated transformations of *C. fulvum* wild-type and *F. oxysporum* Δ*fosge1* strain were performed as described in Ökmen *et al.* (2013) and Takken *et al.* (2004), respectively. *C. fulvum* transformants were selected on PDA plates supplemented with 100 μg ml^{-1} hygromycin and *F. oxysporum* f. sp. *lycopersici* transformants on PDA plates supplemented with 50 μg ml^{-1} nourseothricin. Stable transformants were selected growing them on non-selective plates followed by a new round of culture on selective plates. Deletion mutants and complemented transformants were screened by PCR.

Scanning electron microscopy (SEM) was performed to monitor the phenotypic differences between wild-type and *CfWor1* deletion mutant that were grown on PDA. Both wild-type and mutant colonies were fixated by using 3% of

glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h, at room temperature. The samples were then three times washed with 0.1 M phosphate buffer (pH 7.2) for 20 min at 4°C. Subsequently, samples were incubated with 1% of osmium tetroxide in 0.1 M phosphate buffer (pH 7.20 for 1 h at room temperature. After three times washing step (20 min for each step) with water at room temperature, samples were dehydrated with graded acetone series (10% - 100% acetone). The samples were subsequently dried with carbon dioxide (CPD 030, BalTec, Liechtenstein) and attached on sample holders with CCC Carbon Adhesive (Electron Microscopy Sciences, Washington, USA). After evaporation of the solvent from the adhesive, samples were coated with a layer of 10 nm Tungsten (MED 020, Leica, Vienna, Austria) and analyzed at 2 KV at room temperature in a field emission scanning electron microscope (Magellan 400, FEI, Eindhoven, the Netherlands). The digitally recorded images were optimized with Photoshop CS.

To quantify the production of conidia for wild-type and *CE.CfWor1* transformant, 1,000 conidia were plated on PDA for each strain and incubated at 20°C for 3 weeks, with five biological replicates. After 3 weeks conidia were harvested with sterile water and counted in a Bürker-Türk haemocytometer. Quantification of conidia production was also performed for all *F. oxysporum* f. sp. *lycopersici* strains with five biological replicates. 1 ml of 10^6 conidia from each *F. oxysporum* f. sp. *lycopersici* strains were grown in 50 ml KNO_3 minimal medium (3% sucrose; 10 mM KNO_3; and 0.17% yeast nitrogen base without amino acids) at 25°C at 175 rpm for 6 days. After 6 days conidia were harvested and counted in a Bürker-Türk haemocytometer.

Phylogenetic tree

Amino acid sequences of Wor1 homologues were aligned with ClustalW2 (Larkin *et al.*, 2007) and edited in GeneDoc software (Nicholas *et al.*, 1997). A consensus phylogenetic tree was constructed using the maximum likelihood algorithm with JTT substitution model with default parameters and 250 bootstrap replications in the MEGA5 software (Tamura *et al.*, 2011).

Pathogenicity assays

Wild-type *C. fulvum* (race0WU; CBS131901), one ectopic (Δ*cfwor1-20*) and four independent Δ*cfwor1* mutants (Δ*cfwor1-1*, Δ*cfwor1-2, Δcfwor1-4* and Δ*cfwor1-5*) were grown on PDB liquid culture for 7 days at 20°C with 200 rpm shaking. Fungal mycelia from each strain were ground by using mortar and pestle and filtered through two-layers of miracloth. Each filtrate was checked for the presence of conidia. After adjusting the OD_{600} to 1.2 for each strain, mycelia were spray-inoculated on the abaxial side of Money Maker Cf-0 tomato leaves. Inoculated tomato plants were kept in enclosed plastic cages for two days to ensure 100% relative humidity. After two days, the cages were opened and plants were monitored every two days. Leaf samples were collected at 6, 9, 12 and 14 dpi and immediately frozen in liquid nitrogen and kept at -80°C for total RNA extraction. The experiment was performed with three biological replicates. Confocal fluorescence microscopy was performed to monitor the disease establishment of Δ*cfwor1* mutants at 6, 10, 16 and 20 dpi. Fungal biomass quantification in wild-type and Δ*cfwor1* mutants infected plants was performed using quantitative RT-PCR as described by Ökmen *et al.* (2013). cDNAs were isolated from infected leaves and were diluted to final concentration of 100 ng μl^{-1} and quantitative RT-PCR was performed by using *actin* as a reference gene.

Wild-type and *CE.CfWor1 C6* transformant of *C. fulvum* were grown on half-strength PDA plates for 2-3 weeks at 20°C. Conidia were collected from the plates with water and subsequently passed through one layer of miracloth to remove fungal mycelium. Five-week-old Money Maker Cf-0 tomato plants were inoculated with a final concentration of 3×10^5 conidia ml^{-1} from the wild-type and *CE.CfWor1 C6* transformant via spray inoculation on the abaxial side of the tomato leaves. The following procedures were performed as described for Δ*cfwor1* mutants pathogenicity assay. The experiment was performed with two biological replicates. Fungal biomass quantification in wild-type and *CE.CfWor1 C6* transformant infected plants was performed using quantitative PCR analysis as described by Ökmen *et al.* (2013). Genomic DNAs were isolated from infected leaves and were diluted to final

concentration of 100 ng µl^{-1} and quantitative PCR was performed by using *actin* as a reference gene.

Pathogenicity assays for *F. oxysporum* f. sp. *lycopersici* wild-type, Δ*fosge1* mutant and all complemented strains were performed using 10-day old Money Maker Cf-0 seedlings, following the root-dip inoculation methods (Michielse *et al.*, 2009a). Six tomato seedlings were used for each construct with at least three biological replicates. Disease index and fresh weight of infected plants were scored as described by Michielse *et al.*, (2009a).

Secondary metabolite extraction and LC-MS

The wild-type and Δ*cfwor1* mutant strains were grown in PDB medium for 12 days, which is a condition that allows the accumulation of *C. fulvum* secondary metabolism genes (Collemare et al., submitted). Secondary metabolites were extracted and analyzed by LC-MS following the procedure and conditions described in Collemare *et al.* (submitted). Briefly, mycelium and culture filtrate were separated and metabolites were extracted twice using ethyl acetate. Extracts were combined, concentrated under nitrogen flow and dissolved in methanol for LC-MS analysis.

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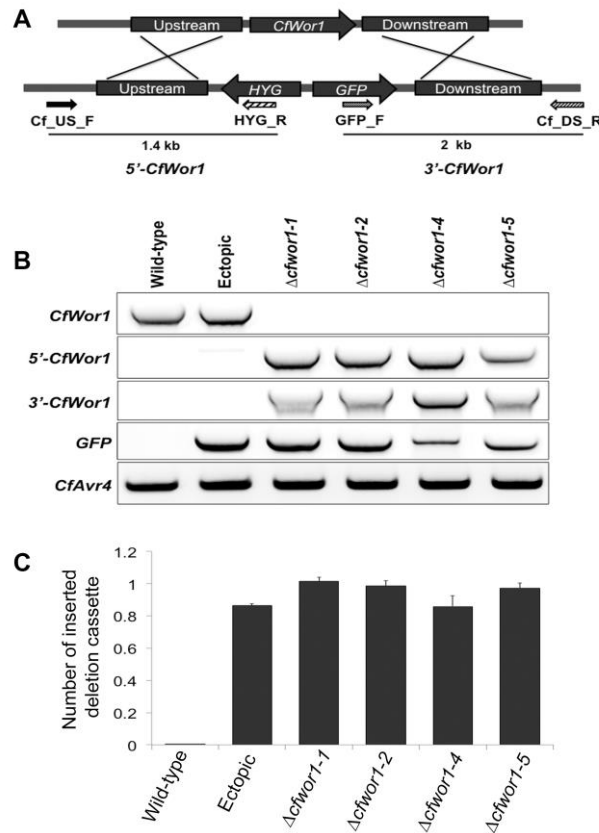
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SUPPORTING INFORMATION

Supporting information Fig. S1. Amino acid sequence alignment of Wor1 homologues in different fungi. Conserved residues are shaded in black and similar residues are shaded in grey. Conserved DNA binding boxes WOPRa and WOPRb are highlighted in red and green boxes, respectively, while putative nuclear localization signal is highlighted in a blue box. The asterisk indicates the conserved threonine residue for potential protein kinase A phosphorylation site. Blue arrow indicates the beginning of the C-terminal part of Wor1 proteins

Supporting information Fig. S2. Molecular analysis of Δcfwor1 mutants of *Cladosporium fulvum*. (A) Representation of the *CfWor1* locus in the wild-type and deletion mutant after homologous recombination. The *CfWor1* gene is replaced by hygromycin (*HYG*) and *GFP* genes. Oligonucleotides used to screen for the mutants are indicated below. **(B)** Targeted gene deletion of *CfWor1* was confirmed by PCR using oligonucleotides shown in (A). *CfAvr4* was used as a positive control. **(C)** Single insertion event of gene deletion cassette was confirmed by quantitative PCR using genomic DNA of each transgenic strain. The *HYG* gene was used as a measure for number of insertion events, together with *actin* gene for normalization and *Avr4* gene as a single copy reference gene, according to the $2^{-\Delta\Delta Ct}$ method.
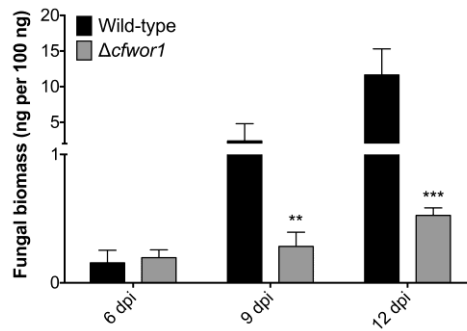
Supporting information Fig. S3. Δ*cfwor1* mutants of *Cladosporium fulvum* show reduced virulence on susceptible tomato. Ground mycelia of an ectopic transformant and four independent Δ*cfwor1* mutants were spray-inoculated on susceptible tomato plants. Fungal biomass produced by wild-type and Δ*cfwor1* mutants during infection of tomato measured by quantitative PCR until 12 days post inoculation (dpi) using a standard curve calculated with the *actin* gene from *C. fulvum*. A multiple t test analysis was performed and followed by a Holm-Sidak test. Only significant differences between wild-type and Δ*cfwor1* mutants are indicated (** P<0.001; *** P<0.0001). Error bars represent standard deviation of at least two biological repeats.
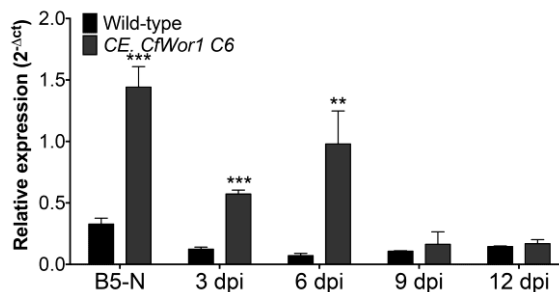
Supporting information Fig. S4. Expression profile of *CfWor1* in *CE.CfWor1* transformant of *Cladosporium fulvum*. Quantitative PCR was performed on *CE.CfWor1* transformant and wild-type grown *in vitro* and during infection of tomato from three to 12 days post inoculation (dpi). Expression was normalized using the *actin* gene of *C. fulvum* according to the $2^{-\Delta Ct}$ method. A multiple t test analysis was performed and followed by a Holm-Sidak test. Only significant differences between wild-type and *CE.CfWor1* transformant are indicated (** P<0.001; *** P<0.0001). Error bars represent standard deviation of three biological repeats from the *in vitro* experiment and two biological repeats from the *in planta* experiment.
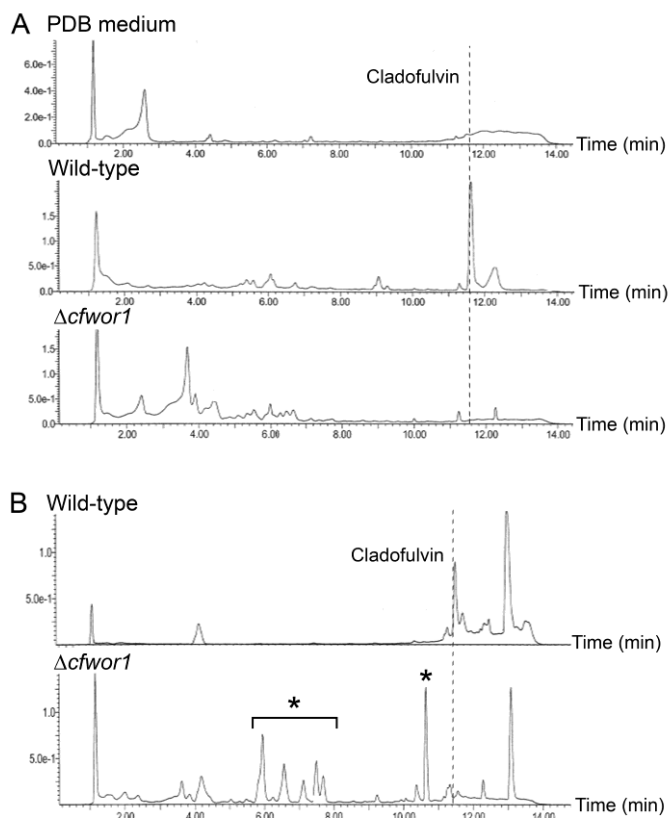
Supporting information Fig. S5. Secondary metabolite profiles of wild-type and Δ*cfwor1* mutant of *Cladosporium fulvum*. (A) LC-MS detection of secondary metabolites present in culture filtrates of wild-type and Δ*cfwor1* mutant of *C. fulvum*. PDB was used as a negative control. Cladofulvin was detected in the culture filtrate of wild-type strain only. **(B)** LC-MS detection of secondary metabolites present in mycelia of wild-type and Δ*cfwor1* mutant of *C. fulvum.* Cladofulvin was detected in the culture filtrate of wild-type strain only. Asterisks indicate additional unknown metabolites produced in Δ*cfwor1* mutant.
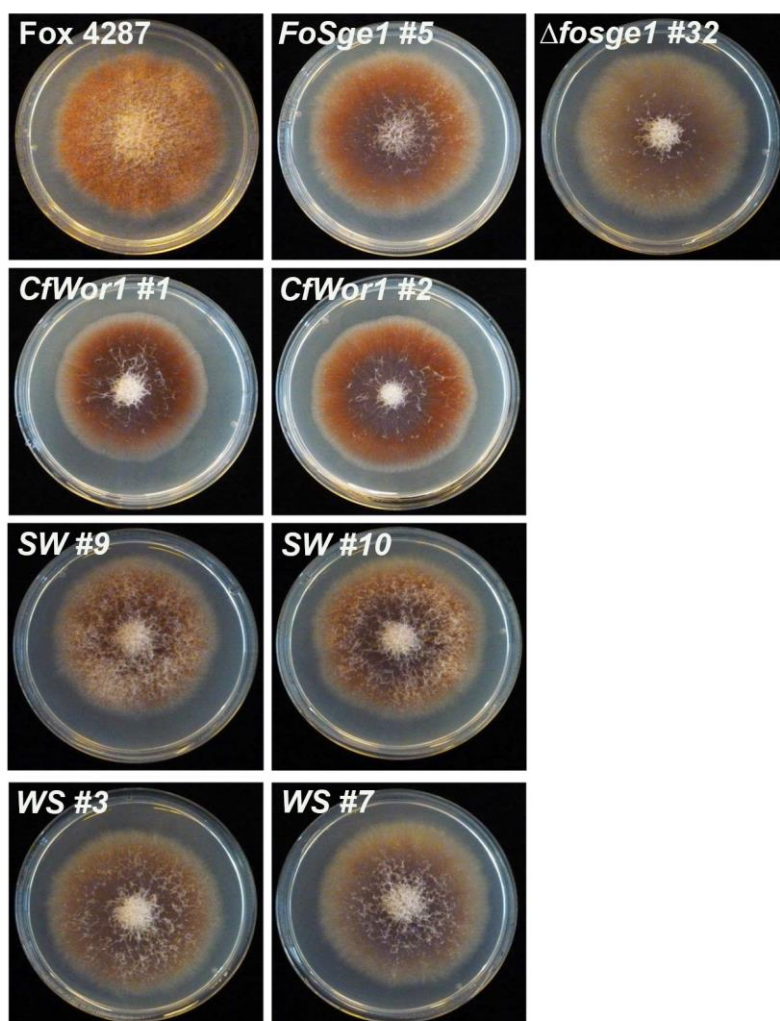
Supporting information Fig. S6. Complementation of Δ*fosge1* of *Fusarium oxysporum* f. sp. *lycopersici* with full length and chimeric versions of CfWor1 and FoSge1. Colony morphology of the Fox 4287 wild-type strain, Δ*fosge1* #32 deletion mutants and transformants complemented with native FoSge1 (#5), two native CfWor1 and two of each chimeric proteins (SW and WS) on PDA.

Supporting information Table S1. Primers used in this study.

Primer name	Primer sequences (5' - 3')
Primers for *CfWor1* knock-out	
CfWor1_US_F	GGGGACAACTTTGTATAGAAAAGTTGCCTGGCCGTTGCTCAACTC
CfWor1_US_R	GGGGACTGCTTTTTTGTACAAACTTGGGACGAGTCCATCCATGCTG
CfWor1_DS_F	GGGGACAGCTTTCTTGTACAAAGTGGCATCCACCAGAGGCTGTATC
CfWor1_DS_R	GGGGACAACTTTGTATAATAAAGTTGGGAATGGCGACTACCGGAG
HYG_R	GTCCGAGGGCAAAGGAATAG
GFP_F	GATCACTCACGGCATGGAC
Cf_US_F	CCGCTCAGCAGCAATACC
Cf_DS_R	GACGAGAGCGGCGACTTACG
Primers for constitutive expression and	
*Pac*I_CfWor1_Nterm _F	TTAATTAAATGAGCACTGGCGGCGGGGC
CfWor1_Nterm_R	TGGTGCCCGGAAATTTTG
CfWor1_Cterm_F	GAACTTCCGCGCCCCGGTCGAGGAGACAGAGCAC
*Not*I_CfWor1_Cterm_R	GCGGCCGCCTATGCTGGATATGGACCACTCG
FoSge1_Promoter_CfWor1 _F	CTTGAAGGCTATCCCTTCCAAGATGAGCACTGGCGGCGGGGC
*Pac*I_FoSge1_Promoter_F	TTAATTAACTTGAGTCCATCCATCTATGCC
FoSge1_Promoter_R	CTTGGAAGGGATAGCCTTCAAG
FoSge1_Nterm_R	CGGGGCGCGGAAGTTCTG
FoSge1_Cterm_F	CAAAATTTCCGGGCACCAATCGAGGAAGCTCAACAC
*Not*I_FoSge1_Cterm _R	GCGGCCGCCTACCACCACGTCTGACC
Primers for quantitative PCR	
qFoSix1_F	CTCAAGAGGCTGCGGTTC
qFoSix1_R	CAAGTTGCGCGATATGTGTT
qFoSix2_F	ATGCTCTTCAAAATCGCGTG
qFoSix2_R	CAACGCCGTTTGAATAAGCA
qFoSix3_F	TGAGCGGGCTGGCAATTC
qFoSix3_R	CAATCCTCTGAGATAGTAAG
qFoSix5_F	GCGCTTCGAGTACATCTCTG
qFoSix5_R	CTAGGCCGCATCACAATAGA
qFoSge1	TGAAGGCTATCCCTTCCAA
qFoSge1	CCAGAGAGACATGCCTCAAA
qFoFEM1_F	ATGAAGTACACTCTCGCTACC
qFoFEM1_R	GGTGAAAGTGAAAGAGTCACC
qCfWor1_F	CTGATTCCCAGACCGTTCAT
qCfWor1_R	TCAGCCACTCTCCTCATCCT
qAvr2_F	GCAGCAGCCAAAAAACTACC
qAvr2_R	TTCCTCCCCTCGTCAACTTC
qAvr4_F	CCCCAAAACTCAACCATACAAC
qAvr4_R	GCTTCGCATTGCCAACTTC
qAvr4E_F	GCAATCAAGCCGAATGGAG
qAvr4E_R	ATGTGACCGAACATCCCAG
qCfAvr9_F	TTGCTACTACTCTCCCACTTTGC
qCfAvr9_R	AGTGGACACATTGTAGCTTATGAAA
qEcp1_F2	AACCAGAACTGCCAGCAAAT
qEcp1_R2	TTAAAGGCACTTGGGGTTTG

Primer name	Primer sequences (5' - 3')
qEcp2_F	CACCTACAACCAAATTGTCTCC
qEcp2_R	TGAACTCTGACCTGACCACC
qEcp4_F	GTTGCATTGTCAAGCTGTTC
qEcp4_R	CTGCCATCCACCAACAATC
qEcp5_F	TACGACACGACTGGAGAAC
qEcp5_R	CGAACATCAAACGTCAAATGC
qEcp6_F	TAACCCCGACAACAAGTCC
qEcp6_R	GTCGAGCGTGATGTTGAAG
qCf_Actine_F	GGCACCAATCAACCCAAAG
qCf_Actine_R	TACGACCAGAAGCGTACAG
qCf_NRF1_F	GCAGCAGCAGTTTGTGTTC
qCf_NRF1_R	TCATCCTCCTCCTCATCCTC
qPKS1_F	TGCTGGTATCGTGGGTAACA
qPKS1_R	CAGAGTTCTCGGCCAGGTAG
qPKS6_F	CTGCATATCGGAGCAGTGAA
qPKS6_R	TTGCGTTTCTTGAAGTCGTG
qHYG_F	GATGTAGGAGGGCGTGGATA
qHYG_R	ATAGGTCAGGCTCTCGCTGA
qTubulin_F	CCTTCAGAGCTGTAACTGTCC
qTubulin_R	CCTCCTTCATAGATACCTTGCC

Chapter 5

General discussion

Bilal Ökmen

Effectors: versatile weaponry employed by plant pathogenic fungi

Plant pathogenic microbes secrete an arsenal of virulence factors, also called effectors, which quantitatively contribute to virulence in compatible interactions. Some effectors are delivered into the apoplastic space, whereas others are translocated in the cytoplasm of host cells to interfere with host physiology and facilitate successful infection. Effectors show diverse modes of action; some harbour an enzymatic activity that is required for detoxification of antifungal plant compounds, such as avenacin and α-tomatine (Bowyer *et al.*, 1995; Osbourn, 1996; Ökmen *et al.*, 2013); some have the ability to protect fungal cell walls from detrimental effects exerted by plant chitinases and glucanases by acting as a shield or an inhibitor, such as Avr4 of *Cladosporium fulvum* (van den Burg *et al.*, 2006; van Esse *et al.*, 2007) and the glucanase inhibitor protein of *Phytophthora sojae*, respectively (Rose *et al.*, 2002); others have the ability to suppress pathogen-associated molecular pattern-triggered immunity (PTI), such as AvrPiz-t of *Magnaporthe oryzae* and Avr3a of *Phytophthora infestans* (Bos *et al.*, 2010; Park *et al.*, 2012), or effector-triggered immunity (ETI), such as Avr1 of *Fusarium oxysporum* f. sp. *lycopersici* that suppresses I-2- and I-3-mediated resistance (Houterman *et al.*, 2008; Houterman *et al.*, 2009). However, effectors might interfere with the virulence of pathogens by being perceived and activating immunity of plants carrying the corresponding resistance (R) proteins. In these cases, effectors are not only virulence, but also avirulence factors. Discovery of avirulence and R proteins has resulted in the development of R-trait-based disease control strategies that have been successfully used to keep many crop diseases under control. As a consequence of selection pressure caused by the use of resistant cultivars in agriculture, new pathogen races that can overcome these *R*-traits have emerged by mutations in effector genes with avirulence activity (Stergiopoulos *et al.*, 2007). Because of effector's importance in virulence and avirulence of plant pathogenic fungi, identification and characterization of effector catalogues of pathogens can give new insights into our understanding of the molecular basis of host-pathogen interactions and can eventually lead to improvement of existing disease management strategies. In

this chapter, I will discuss several strategies that have been widely used for identification and functional analysis of effectors.

Hunting for effectors

Fishing in the apoplastic pool

Fungal pathogens secrete a cocktail of effectors into the apoplastic space of their host plants to promote disease. Such apoplastic fluid, rich in effectors, can be isolated and tested for their biological activities (Kim *et al.*, 2004; Thomma *et al.*, 2005; Kamoun, 2006; Vincent *et al.*, 2012). This strategy has led to the identification of most *C. fulvum* effectors because of their avirulence activity on plants containing the corresponding *R*-traits, which is easily scored by examining the induction of the hypersensitive response (HR). Indeed, the first fungal avirulence gene ever cloned, *Avr9* from *C. fulvum,* has been achieved using the amino acid sequence of the Avr9 protein obtained from apoplastic fluids of *C. fulvum*-infected plants (van Kan *et al.*, 1991). Similarly, the *Ecp1*, *Ecp2*, *Ecp4, Ecp5, Ecp6, Ecp7, Avr4*, and *Avr4E* effector genes from *C. fulvum* (van Den Ackerveken *et al.*, 1993; Joosten *et al.*, 1994; Bolton *et al.*, 2008) and *Avr1*, *Avr2*, and *Avr3* from *F. oxysporum* f. sp. *lycopersici* were also cloned by using this approach (using xylem fluids in the latter) (Rep *et al.*, 2004; Houterman *et al.*, 2008; Houterman *et al.*, 2009). Effectors can also be identified based on their biochemical activity, as exemplified for the glucanase inhibitor protein of *P. sojae* (Rose *et al.*, 2002) and several carbohydrate-degrading enzymes from the *Cochliobolus carbonum* that show hydrolase activity on plant cell walls (Walton, 1994). However, the quantities of proteins that can be isolated and identified from extracellular fluids is limited and it makes this approach only successful for the most abundant effector proteins. In addition, especially in the case of biological activity assays, loss of protein stability or activity during isolation and additional purification steps is another limitation of this approach. However, this strategy might still be of relevance in the

future thanks to the improvement in sensitivity of mass spectrometry instruments, coupled to the availability of increasing number of genome sequences.

Finding a needle in a haystack

Random insertional mutagenesis has been commonly used to identify novel virulence factors. T-DNA, plasmid or transposon insertion in genes or regulatory elements of gene expression might result in disabling genes that play a role in virulence (Seong *et al.*, 2005). Although an inactivated gene is tagged with an identifiable construct, it is sometime still difficult to retrieve the gene of interest from genomes that have not been sequenced yet. Since this type of approach also requires a robust quantitative pathogenicity assay, it is not suitable to identify effector genes that only slightly contribute to virulence, which is the case for most of the effectors.

Sieving genomes for gold

Availability of genome sequences from a wide range of fungal species has accelerated the identification of effector genes by different methods. Common effectors shared between fungal species can simply be identified using a BLAST tool. For example, a homology-based search for tomatinase-encoding genes in the genome of *C. fulvum*, as described in **chapter 3,** identified CfTom1, which belongs to glycosyl hydrolase family 10 (GH10). Functional characterization of CfTom1 **(Chapter 3)** revealed that, in contrast to a previous report (Melton *et al.*, 1998), *C. fulvum* requires this enzyme to detoxify the antifungal α-tomatine into the less-toxic metabolite tomatidine and lycotetraose during tomato infection and consequently to be fully virulent (Ökmen *et al.*, 2013). Although it is not an effector, this approach also allowed us to identify CfWor1, the homologue of FoSge1, which is a conserved transcriptional regulator that is required for expression of effector genes in *F. oxysporum* f. sp. *lycopersici* (Michielse *et al.*, 2009). However, functional characterization of CfWor1 suggested that this conserved regulator is not major regulator of the expression of effectors and virulence in *C. fulvum*, highlighting a possible role in fungal development

in plant pathogenic fungi **(Chapter 4)**. Previously, BLAST searches revealed the presence of homologues of chitin-binding proteins of *C. fulvum*, Avr4 and Ecp6, in different related fungal species (de Jonge & Thomma, 2009; Stergiopoulos *et al.*, 2010; de Wit *et al.*, 2012). Avr4 from *Mycosphaerella fijiensis* is a functional homologue since it binds to chitin and protects the fungus against deleterious effects of plant chitinases (Stergiopoulos *et al.*, 2010). In addition, both Avr4 from *M. fijiensis* and *Dothistroma septosporum* are recognized by the tomato Cf-4 resistance protein (Stergiopoulos *et al.*, 2010; de Wit *et al.*, 2012). These findings might be exploited for the development of R-mediated resistance against these two devastating pathogens of banana and pine, respectively. Ecp6 is a LysM domain-containing protein, which is conserved in many different fungal species. Functional characterization of LysM domain-containing proteins in *Zymoseptoria tiritici* (Mg3LysM) and *Magnaporthe oryzae* (Slp1) revealed that some of them also have the ability to scavenge chitin fragments and prevent chitin-triggered immunity (de Jonge *et al.*, 2010; Marshall *et al.*, 2011; Mentlak *et al.*, 2012). Homology-based screening is an easy and very efficient way to identify effectors that are conserved among fungal species or contain a conserved domain, but less so for identification of effectors that are specific to a given fungus.

For this purpose, characteristics that are common to most effectors can be used to identify genes encoding effector candidates at the whole genome level. Recently, several computational pipelines have been designed to filter out most promising effector candidates from whole genomes. The main criteria generally used in these filtering pipelines are (i) the presence of a signal peptide for secretion, (ii) a relatively small size (<300 amino acid), (iii) the presence of four or more cysteine residues, (iv) the presence of pathogenicity-related conserved motifs or domains, (v) the presence of amino acid sequence repeats, and (vi) a localization in gene-sparse or repeat-rich regions (Raffaele *et al.*, 2010; de Wit *et al.*, 2012; Hacquard *et al.*, 2012; Saunders *et al.*, 2012). In **Chapter 2,** by using only a similar computational pipeline, a catalogue of 271 putative effectors of *C. fulvum* was obtained. A subset of these effector candidates was screened on different tomato lines carrying different *R*-traits

to assess their avirulence activity, but none of them induced *R*-trait-specific plant cell death. Expression analysis revealed that many of these effector candidates are not or poorly expressed during tomato infection. These results indicate that using computational genome-wide prediction alone is not an efficient method to select biologically relevant effector candidates. Selection criteria for effector candidates should be less biased. For example, effectors are generally described as small and cysteine-rich proteins, but some effector proteins do not match these criteria (Catanzariti *et al.*, 2006; Gout *et al.*, 2006; Houterman *et al.*, 2007). Using this method, the *CfTom1* tomatinase gene would not have been identified (**Chapter 3**).

Comparative genomics is another approach that was successfully employed to identify new effector candidates, but it requires the genome sequence of closely related fungal species. For example, comparison of the genome of three *Fusarium* species, *F. oxysporum* f. sp. *lycopersici*, *F. graminearum* and *F. verticillioides*, resulted in the identification of four chromosomes specific to *F. oxysporum* f. sp. *lycopersici* (Ma *et al.*, 2010). Further investigations showed that these specific chromosomes primarily contain transposable elements and genes encoding known and putative effectors. Similarly, comparison of the genomes of *Ustilago maydis* and *Sporisorium reilianum*, two basidiomycete fungi that infect maize but cause different symptoms, revealed 43 polymorphic regions that contain primarily species-specific genes encoding putative effectors (Schirawski *et al.*, 2010). Functional characterization in *U. maydis* of some of these effector candidates showed that they are required for virulence (Schirawski *et al.*, 2010).

RNA-seq, where there is smoke there is fire

As discussed previously, mining fungal genomes for effector candidates by only bioinformatic tools might lead to selection of genes that are not involved in virulence because they may not be expressed during plant infection. Thus, information about the expression profile of putative effector genes during infection is important in order to evaluate their role in virulence **(Chapter 2)**. RNA-seq data obtained from both

in vitro and during plant infection, in combination with computational prediction of effector genes might be a better approach for identification of novel effector genes involved in virulence and avirulence. Such a comparative genomics analysis was performed using multiple race 1 and race 2 strains of *Verticillium dahliae* to identify the avirulence gene recognized by the *Ve1* tomato resistance gene (de Jonge *et al.*, 2012). This study revealed the presence of a 50 kb genomic region specific for race 1 strains and deep transcriptome analysis showed that only one gene was highly expressed in that region. This gene was convincingly demonstrated to be the race-specific *Ave1* effector gene that is recognized by Ve1 and is also involved in virulence (de Jonge *et al.*, 2012). A transcriptome analysis that has been employed in the *Colletotrichum higginsianum*-Arabidopsis pathosystem resulted in the identification of genes that are linked to infection stage-specific transitions, including effector candidates (O'Connell *et al.*, 2012). Furthermore, RNA-seq is also useful to identify effector gene candidates that might be missed during annotation of fungal genomes.

Mechanisms leading to biotrophy

C. fulvum is a non-obligate biotrophic pathogen that exclusively colonizes the extracellular space of tomato leaves and causes some chlorosis or necrosis only at late stages of infection (Thomma *et al.*, 2005). However, the analysis of the *C. fulvum* genome revealed that, in contrast to other biotrophic fungal pathogens, such as *Blumeria graminis* and *U. maydis*, *C. fulvum* has as many genes encoding carbohydrate-degrading enzymes as hemi-biotrophic and necrotrophic fungi (de Wit *et al.*, 2012). In particular, the number of genes encoding pectin-degrading enzymes is more similar to those present in fungi with a hemi-biotrophic or necrotrophic life style (Sprockett *et al.*, 2011; de Wit *et al.*, 2012). These results are contradictory with the infection strategy of *C. fulvum*. This observation also holds for the number of secondary metabolite biosynthetic pathways (de Wit *et al.*, 2012). However, detailed investigation of those genes revealed that most of them are not expressed during infection or are pseudogenized (de Wit *et al.*, 2012). During screening of the *C. fulvum*

effector catalogue **(Chapter 2),** we identified a glycoside hydrolase family 17 (GH17) member, predicted to be an exo-β-1,3-glucanase, that non-specifically induced necrosis in all tested tomato lines but *Cf-Ecp3*, indicating that the observed necrosis is unlikely the result of plant cell wall degradation (maceration). The necrosis-inducing activity of this protein could be due to its direct recognition or recognition of its enzymatic activity because it might release damage-associated molecular patterns (DAMPs) from the host cell wall. This GH17-encoding gene is differentially up-regulated at late stages of infection when the fungal biomass increases dramatically, suggesting it is likely used for the release of mono/oligosaccharides from the host cell wall that can be used as a carbon source. Since *C. fulvum* has a biotrophic lifestyle, the necrosis-inducing activity of this protein is likely suppressed by other effectors via scavenging of DAMPs during natural infection. Effector candidates predicted to have carbohydrate-binding or a LysM domain are potential candidates for suppression of defense responses by oligosaccharides **(Chapter 2)**.

In addition to the presence of hydrolytic enzymes discussed in **chapter 2,** *C. fulvum* carries a gene encoding a biologically active necrosis-inducing protein, NLP (NEP1-like protein), which is mostly found in hemi-biotrophic or necrotrophic phytopathogens where it plays a role in the transition from a biotrophic to a necrotrophic stage (Qutob *et al.*, 2002; Kanneganti *et al.*, 2006; Kleemann *et al.*, 2012). Genome mining of the obligate biotroph *Hyaloperonospora arabidopsidis* revealed the presence of 12 *NLP*-encoding genes, but none of them produces a protein with necrosis-inducing activity, suggesting that they might have evolved new functions relevant for a biotrophic lifestyle (Cabral *et al.*, 2012). In contrast, CfNLP1 can trigger necrosis, but this protein is expressed neither *in vitro* nor during infection of tomato **(Chapter 2)**. Likely, the *CfNLP1* gene is only expressed under very specific environmental conditions, possibly during its saprophytic phase. Similarly, we identified another effector candidate that triggers non-specific necrosis on tomato, but this candidate appeared only expressed *in vitro*. Overall, these results indicate that *C. fulvum* sustains its biotrophic lifestyle by down-regulating the expression of genes

encoding proteins that could otherwise induce chlorosis or necrosis during infection of tomato.

Concluding remarks

In the past, most of the effectors were identified by conventional proteomics and genetics approaches. So far, many bacterial, fungal and oomycete effectors have been identified and characterized and the number is gradually increasing. However, we have only touched the top of the iceberg with respect to effector identification and effector functions. The number of sequenced fungal genomes and transcriptomes is exponentially increasing and this will enable genome-wide cataloguing of fungal effectors involved in virulence and avirulence.

The *C. fulvum*-tomato interaction is still a very useful research model system for future studies on fungal effectors and corresponding plant resistance genes. Presence of additional *R*-traits (*Cf-1*, *Cf-3*, *Cf-5*, *Cf-6*, *Cf-9B*, *Cf-11* and *Cf-Ecp3*) in tomato, for which the corresponding *C. fulvum* effector genes have not yet been identified, suggests that many more novel effectors of *C. fulvum* are to be identified. Their identification and functional characterization will allow further dissection of *C. fulvum*'s infection strategies. The results of this PhD thesis show that, although computational prediction of effector candidates is a high throughput method, alone it does not give a true representation of the real effector catalogue. A combined bioinformatics and transcriptomics approach, which provides valuable information about expression profile of candidates, is best-suited for the future identification of novel effector genes. Using expression profiling as a primary criterium to study effectors should allow the identification of effector proteins that suppress PTI and/or ETI in the *C. fulvum*-tomato pathosystem.

Deep-transcriptome analysis at different stages of infection will provide stage-specific expression profiles of novel effectors that could be interesting candidates for further functional characterization. Some types of effectors, including cell wall and

secondary metabolite degrading enzymes, proteins involved in nutrient uptake and transcription factors involved in regulation of effectors have been studied less extensively and their functions in virulence still remain to be explored.

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THESIS SUMMARY

In order to establish disease, plant pathogenic fungi deliver effectors in the apoplastic space surrounding host cells as well as into host cells themselves to manipulate host physiology in favour of their own growth. *Cladosporium fulvum* is a non-obligate biotrophic fungus causing leaf mould disease of tomato. For decades, this fungus has been a model to study the molecular basis of plant-pathogen interactions involving effector proteins. Characterization of these effectors revealed their roles in both virulence and avirulence as they facilitate colonization of the host in the absence of cognate tomato *Cf* resistance genes, but also trigger Cf-mediated resistance in the presence of these genes. The availability of the genome sequence of *C. fulvum* is a great resource allowing us to dissect and better understand the molecular interaction between this fungus and tomato, particularly with regards to identification of new effectors. Such knowledge is of important to improve current strategies not only for disease resistance breeding of tomato against *C. fulvum*, but also for other host plants that are attacked by pathogenic fungi with similar infection strategies and lifestyles.

In **chapter 1** we give an introduction to the *C. fulvum*-tomato pathosystem. In a compatible interaction, *C. fulvum* secretes small cysteine-rich effectors that positively contribute to fungal virulence. Two of these effectors are chitin-binding proteins including Avr4, which protects fungal cell walls against hydrolysis by plant chitinases, and Ecp6, which sequesters released small chitin fragments, thereby preventing induction of basal defense responses associated with their recognition by plant receptors. Another effector, Avr2, is an inhibitor of four tomato cysteine proteases that are also important for basal plant defense. However, in an incompatible interaction, these effectors are directly or indirectly perceived by corresponding resistance proteins (encoded by *Cf* resistance genes that belong to the class of receptor-like proteins; RLPs) mediating race-specific plant defense responses also known as effector-triggered immunity.

In **chapter 2** we exploit the availability of the genome sequence of *C. fulvum* to identify novel effectors involved in virulence and avirulence of this fungus. An *in silico* search was performed using common features of characterized *C. fulvum* effectors: they (i) contain a signal peptide, (ii) are small (<300 amino acids) and (iii) contain at least four cysteine residues (SSCPs). This search identified 271 SSCPs in the *C. fulvum* genome. A subset of 60 of these predicted effectors was heterologously expressed in tomato lines carrying different *R*-traits, including *Cf-1*, *Cf-3*, *Cf-5*, *Cf-9B*, *Cf-11* and *Cf-Ecp3* in order to identify the corresponding effectors that are recognized by the RLPs. Although the screen of this subset of SSCPs did not result in identification of a new avirulence gene, two non-specific necrosis-inducing proteins were identified. In addition, a homology search identified *CfNLP1*, a gene encoding a functional NEP1-like protein that triggers non-specific necrosis in plants. However, quantitative PCR showed that these three genes are lowly or not expressed during tomato infection, which was also true for the *in planta* expression of some of the effector candidates that were tested for recognition by Cf proteins. In contrast, all genes from *C. fulvum* encoding the effectors that have been reported so far are highly up-regulated during infection where they play an important role in establishing disease. Like Avr2, Avr4, Ecp2 and Ecp6, we report that Ecp4 and Ecp5 also are involved in virulence of *C. fulvum* on tomato. Finally, we discuss the limitations of only using bioinformatics approaches to identify novel effectors involved in virulence.

In **chapter 3** we describe the identification and characterization of a novel effector secreted by *C. fulvum*. *CfTom1* encodes a functional tomatinase enzyme, which belongs to family 10 of glycoside hydrolases (GH10). Bacterial and fungal pathogens of tomato secrete this enzyme to detoxify the toxic saponin, α-tomatine, into the less toxic compounds tomatidine and lycotetraose. Similarly, CfTom1 is responsible for α-tomatine deoxification by *C. fulvum* both *in vitro* and during infection of tomato. Accordingly, Δ*cftom1* mutants are more sensitive to α-tomatine because they can no longer detoxify α-tomatine. They are less virulent on tomato plants than wild-type as reflected by a delay in disease symptom development and reduced fungal

biomass production. In addition, tomatidine appears to be more toxic to tomato cells than α-tomatine, but it does not suppress plant defense responses as previously suggested in literature. Altogether, our results clearly indicate that CfTom1, the major or possibly only tomatinase enzyme produced by *C. fulvum*, contributes to full virulence of this fungus on tomato by detoxifying α-tomatine.

Hardly anything is known about *in planta* regulation of effector genes. In **chapter 4** we describe the functional characterization of CfWor1, a homologue of FoSge1, a conserved transcriptional regulator of effectors in *Fusarium oxysporum* f. sp. *lycopersici*. CfWor1 is also homologous to Wor1/Ryp1/Mit1 proteins, which are involved in morphological switches in *Candida albicans*, *Histoplasma capsulatum* and *Saccharomyces cerevisiae*, respectively. In contrast to FoSge1, CfWor1 is unlikely a positive regulator of effector genes because it is weakly expressed during infection of tomato. Compared to wild-type, Δ*cfwor1* mutants show strong developmental and morphological defects. Δ*cfwor1* mutants do not produce any conidia, but differentiate sclerotium-like structures and secrete an extracellular matrix that covers fungal hyphae. *Δcfwor1* mutants are no longer virulent on tomato, likely because of developmental defects. Although constitutive expression of *CfWor1* in *C. fulvum* did not cause any obvious developmental defects, except reduced conidia production, the transformants showed reduced virulence. Quantitative PCR on known effector and secondary metabolism genes in both Δ*cfwor1* mutants and constitutive expression transformant revealed that the effect of CfWor1 on the expression of these genes is likely due to developmental defects rather than direct regulation. Complementation of a non-virulent Δ*fosge1* mutant of *F. oxysporum* f. sp. *lycopersici* with full length CfWor1 or chimera of CfWor1 and FoSge1 restored expression of *SIX* effector genes, but not virulence, indicating that reduced virulence observed for the Δ*fosge1* mutant is not solely due to loss of expression of these effector genes. Altogether, our study suggests that CfWor1 is a major regulator of development in *C. fulvum* which indirectly affects virulence.

Chapter 5 provides a general discussion of the present work on *C. fulvum* effectors, with particular emphasis on comparative genomics and transcriptomics approaches to identify novel effectors involved in fungal virulence and avirulence. Our findings are put in a broader perspective including a discussion on how identification of effectors will improve our understanding of molecular interactions between plants and pathogenic fungi and how we can use this knowledge to develop new strategies for sustainable disease resistance breeding.

SAMENVATTING

Om planten aan te kunnen tasten scheiden ziekteverwekkende schimmels effectoren uit in de apoplast en/of cytosol van waardplantcellen om de fysiologie van de waardplant in hun voordeel te veranderen. *Cladosporium fulvum* is een niet-obligate biotrofe schimmel die de bladvlekkenziekte van tomaat veroorzaakt. Reeds een aantal decennia staat deze schimmel model voor de bestudering van werkingsmechanismen van effectoren bij plant-schimmel interacties. Karakterisering van een aantal van deze effectoren toonde aan dat zij een rol spelen in zowel virulentie als avirulentie van schimmels daar zij enerzijds de kolonisering van de waardplant faciliteren in afwezigheid van bijpassende *Cf* resistentiegenen in tomaat, maar anderzijds ook Cf-gemedieerde resistentie induceren in aanwezigheid van deze *Cf* genen. De genoomsequentie van *C. fulvum* biedt een rijke bron aan informatie die ons in staat stelt om de moleculaire basis van de interactie tussen deze schimmel en tomaat te ontrafelen en beter te leren kennen, in het bijzonder met betrekking tot de identificatie van nieuwe effectoren en hun functies. Deze kennis is van belang om niet alleen strategieën van ziekteresistentieveredeling van tomaat tegen *C. fulvum* te verbeteren maar ook om ziekteresistentieveredeling van andere waardplanten die geïnfecteerd worden door pathogene schimmels met vergelijkbare infectiestrategieën en levenscycli te verbeteren.

In **hoofdstuk 1** wordt het pathosysteem *C. fulvum*-tomaat geïntroduceerd. *C. fulvum* scheidt tijdens infectie van tomaat een groot aantal kleine cysteïne-rijke effectoren uit die een positieve bijdrage leveren aan de virulentie van de schimmel. Twee van deze effectoren zijn chitine-bindende eiwitten met inbegrip van Avr4, dat chitine in de schimmelcelwand tegen hydrolyse door chitinases van de plant beschermt, en Ecp6, dat kleine chitinefragmenten, die tijdens infectie van tomaat door *C. fulvum* vrijkomen, wegvangt, waardoor deze geen basale afweerreacties, gemedieerd door chitine-receptoren van de plant, meer kunnen induceren. Een andere effector van *C. fulvum*, Avr2, is een remmer van tomatencysteïne-proteasen die belangrijk zijn voor de basale afweer van de plant. Echter, in een resistente plant

worden genoemde effectoren direct of indirect herkend door bijpassende resistentie-eiwitten (gecodeerd door *Cf* resistentiegenen die tot de klasse van receptor-achtige eiwitten behoren; RLPs) die ras-specifieke afweerreacties, ook bekend als effector-geïnduceerde immuniteit, mediëren.

In **hoofdstuk 2** benutten we de beschikbaarheid van het genoom van *C. fulvum* om nieuwe effectoren te identificeren die betrokken zijn bij virulentie en avirulentie van de schimmel. Een *in silico* zoekopdracht in het genoom van *C. fulvum* naar gemeenschappelijke kenmerken van effectoren zoals (i) aanwezigheid van een signaalpeptide, (ii) het molecuulgewicht (< 300 aminozuren), en (iii) de aanwezigheid van ten minste vier cysteïne-residuen (SSCPs). Deze zoekopdracht leidde tot de voorspelling van 271 SSCPs in het genoom van *C. fulvum.* Zestig van deze voorspelde effectoren werden tot expressie gebracht in tomatenlijnen met verschillende *Cf*-genen, met inbegrip van *Cf-1, Cf-3, Cf-5, Cf-9B, Cf-11* en *Cf-Ecp3* om zodoende de bijpassende RLP op te sporen. Hoewel het screenen van 60 van deze voorspelde SSCPs niet leidde tot de identificatie van een nieuwe effector met avirulentie functie, werden wel twee niet-specifieke necrose-inducerende effectoreiwitten geïdentificeerd. Bovendien leidde een vergelijkende genoomstudie tot opsporing van *CfNLP1*, een gen dat codeert voor een functioneel NEP1-achtig eiwit dat niet-specifieke necrose in planten induceert. M.b.v. kwantitatieve PCR bleek echter dat *CfNLP1* niet tot expressie komt tijdens infectie van tomaat hetgeen ook het geval voor de meeste van de andere 60 kandidaat effectorgenen die zijn getest voor erkenning door RLPs van tomaat. Daarentegen komen alle tot nu toe reeds bekende effectorgenen hoog tot expressie tijdens infectie van tomaat, waar ze een belangrijke rol spelen bij de aantasting van de plant. Evenals Avr2, Avr4, Ecp2 en Ecp6 hebben we ook gevonden dat Ecp4 en Ecp5 betrokken zijn bij virulentie van *C. fulvum*. Tot slot, bespreken we de beperkingen van het gebruik van louter bioinformatica om nieuwe effectoren op te sporen.

In **hoofdstuk 3** beschrijven we de identificatie en karakterisering van een basale effector uitgescheiden door *C. fulvum* die gecodeerd wordt door *CfTom1.* Het product betreft een enzym, tomatinase, dat tot familie 10 van glycoside hydrolases

(GH10) behoort. Ziekteverwekkers van tomaat veroorzaakt door bacteriën en schimmels scheiden dit enzym om uit om het giftige saponine, α-tomatine, af te breken tot de minder toxische verbindingen tomatidine en lycotetraose. CfTom1 is ook verantwoordelijk voor α-tomatine afbraak door *C. fulvum in vitro* en tijdens infectie van tomaat. Dienovereenkomstig zijn Δ*cftom1* mutanten van *C. fulvum* gevoeliger voor α-tomatine omdat ze niet langer α-tomatine kunnen afbreken. Ze zijn minder virulent op tomatenplanten dan de wild-type stam hetgeen zich uit in een vertraagde ziektesymptoom ontwikkeling en productie van minder schimmelbiomassa tijdens infectie. Bovendien lijkt tomatidine giftiger voor suspensiecellen van tomaat dan α-tomatine, terwijl het de basale afweerreacties van de plant niet onderdrukt zoals eerder gerapporteerd is in de literatuur. Over het geheel genomen geven onze resultaten duidelijk aan dat CfTom1, het belangrijkste en misschien wel het enige tomatinase enzym is dat geproduceerd wordt door *C. fulvum,* en nodig is voor virulentie van de schimmel op tomaat omdat het α-tomatine kan afbreken.

Over de expressie van effectorgenen *in planta* is nauwelijks iets bekend. In **hoofdstuk 4** beschrijven we de functionele karakterisering van CfWor1, een homoloog van FoSge1, een geconserveerde transcriptionele regulator van effectorgenen in *Fusarium oxysporum* f. sp. *lycopersici*. CfWor1 is ook homoloog aan Wor1/Ryp1/Mit1 eiwitten, die fungeren als morfologische schakelaars in *Candida albicans, Histoplasma capsulatum* en respectievelijk *Saccharomyces cerevisiae*. In tegenstelling tot FoSge1 is CfWor1 waarschijnlijk geen positieve regulator van effectorgenen omdat het coderende gen nauwelijks tot expressie komt tijdens infectie van tomaat. Vergeleken met het wild-type, vertonen Δ*cfwor1* mutanten sterke ontwikkelings- en morfologische gebreken. Δ*cfwor1* mutanten produceren geen conidiën, maar sclerotium-achtige structuren waarop zich een extracellulaire matrix heeft afgescheiden evenals op de schimmeldraden. Δ*cfwor1* mutanten zijn niet langer virulent op tomaat, waarschijnlijk vanwege morfologische defecten. Hoewel constitutieve expressie van CfWor1 in *C. fulvum* geen duidelijk morfologische defecten geeft, behalve enigszins verminderde conidiën productie, zijn deze transformanten minder virulent. Kwantitatieve PCR op

bekende effector- en secundair metabolisme genen in de Δ*cfwor1* mutanten en transformanten die CfWor1 constitutief tot expressie brengen, bleek dat het effect van CfWor1 op de expressie van deze genen eerder te wijten is aan ontwikkelingsstoornissen dan aan een direct effect op de expressie van deze genen.

Complementatie van een niet-virulente Δ*fosge1* mutant van *F. oxysporum* f. sp. *lycopersici* met een volledig of chimeer eiwit van CfWor1 en FoSge1 herstelde de expressie van *SIX* effectorgenen volledig, maar niet de virulentie, hetgeen suggereert dat de gereduceerde virulentie van de Δ*fosge1* mutant niet volledig te herleiden is tot de afwezigheid van expressie van de *SIX* effectorgenen. Over het geheel genomen suggereert onze studie dat CfWor1 een belangrijke regulator is van de ontwikkeling van *C. fulvum* die indirect ook betrokken is bij virulentie.

In **hoofdstuk 5** wordt het huidige onderzoek aan *C. fulvum* effectoren bediscussieerd met bijzondere aandacht voor vergelijkbare genoom en transciptom benaderingen voor de identificatie van nieuwe effectoren die een rol spelen bij zowel virulentie als avirulentie. Ook worden onze bevindingen in een breder perspectief geplaatst, waarbij we tevens ingaan op de betekenis van de identificatie van nieuwe effectoren voor een beter begrip van moleculaire interacties tussen planten en pathogene schimmels, en hoe we deze kennis kunnen gebruiken bij de ontwikkeling van nieuwe strategieën voor duurzame ziekteresistentieveredeling.

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I consider myself very lucky to have studied at Wagenigen University, not only for its research quality, but also for its international environment that allowed me to meet very diverse and colorful people coming from all around the world. One simply could not ask for a better or friendlier environment. Therefore, I would like to express my deepest gratitude to all members of Laboratory of Phytopathology for their collaboration and friendship during these five years. My special thanks goes to Chunxu Song, Pieter van't Hof, and Daniela Sueldo for arranging international dinners with a wide diversity of delicious ethnic foods and for my former ultimate squash partners Koste Yadeta, Partha Santhanam, Anja Kombrink and Emilie van Dijk-Fradin for having so much fun during our games.

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CURRICULUM VITAE

Bilal Ökmen was born on December 1, 1981, in Nusaybin, Turkey. He moved to Izmir in 1992, where he went to secondary school and later to high school. After he obtained his high school diploma in 1998, he enrolled at Ege University, Izmir, where he studied Biology and in 2004 obtained his BSc with specialization in fundamental and industrial microbiology. Subsequently, he joined the Izmir Institute of Technology where he studied for his MSc degree in Biotechnology and Bioengineering. He performed his MSc thesis under the supervision of Prof. Dr. Sami Doganlar at the Molecular Biology and Genetic Department on the topic: 'QTL Analysis of Antioxidant Characters in Tomato'. After his MSc degree, Bilal Ökmen started his PhD studies on September, 15, 2008 at the Laboratory of Phytopathology of Wageningen University on the *Cladosporium fulvum*-tomato interaction. He has worked on "identification and characterization of novel effectors of *C. fulvum"* under the supervision of Prof. Dr. Pierre JGM de Wit. On October 1, 2013, he will start working as a postdoctoral researcher at the Max-Planck Institute of Terrestrial Ecology, Marburg, in the research group of Dr. Günther Doehlemann.

LIST OF PUBLICATIONS

<u>Ökmen B</u>, Etalo DW, Joosten MHAJ, Bouwmeester HJ, de Vos RCH, Collemare J, de Wit PJGM. 2013. Detoxification of α-tomatine by *Cladosporium fulvum* is required for full virulence on tomato. *New Phytologist* **198**(4): 1203-1214.

<u>Ökmen B</u>, de Wit PJGM. 2013. The *Cladosporium fulvum*-tomato pathosystem: fungal infection strategy and plant responses. In. Guido Sessa, ed. *Molecular Plant Immunity*. Chichester, UK: John Wiley and Sons, Inc., 211-224.

de Wit PJGM, van der Burgt A, <u>Ökmen B</u>, Stergiopoulos I, Abd-Elsalam K, Aerts AL, Bahkali AHA, Beenen H, Chettri P, Cox MP, Datema E, de Vries RP, Dhillon B, Ganley AR, Griffiths S, Guo Y, Hamelin RC, Henrissat B, Kabir MS, Jashni MK, Kema G, Klaubauf S, Lapidus A, Levasseur A, Lindquist E, Mehrabi R, Ohm RA, Owen TJ, Salamov A, Schwelm A, Schijlen E, Sun H, van den Burg HA, van Ham RCHJ, Zhang S, Goodwin SB, Grigoriev IV, Collemare J, Bradshaw RE. 2012. The genomes of the fungal plant pathogens *Cladosporium fulvum* and *Dothistroma septosporum* reveal adaptation to different hosts and lifestyles but also signatures of common ancestry. *PLoS Genetics* **8**(11): art. no.e1003088.

<u>Ökmen B</u>, Sigva HO, Gürbüz N, Ülger M, Frary A, Doganlar S. 2011. Quantitative Trait Locus (QTL) analysis for antioxidant and agronomic traits in tomato (*Solanum lycopersicum*). *Turk. J. Agric. For.* **35**: 501-514.

Stergiopoulos I, van den Burg HA, <u>Ökmen B</u>, Beenen HG, van Liere S, Kema GHJ, de Wit PJGM. 2010. Tomato Cf resistance proteins mediate recognition of cognate homologous effectors from fungi pathogenic on dicots and monocots. *Proceedings of the National Academy of Sciences of the United States of America* **107**(16): 7610-7615.

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Ökmen B, **Şığva HÖ, Mutlu S, Doğanlar S, Yemenicioğlu A, Frary A. 2009.** Diversity of total antioxidant activity and total phenolics content in Turkish eggplant (*Solanum melongena L.*) cultivars. *International Journal of Food Properties* **12**(3): 616-624.

Frary A, Keçeli M, Ökmen B, **Şığva HÖ, Yemenicioğlu A, Doğanlar S. 2008.** Water Soluble Antioxidant Potential of Turkish Pepper Cultivars. *HortScience* **43**(3): 631-636.

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: **Bilal Ökmen**
Date: **9 September 2013**
Group: **Phytopathology, Wageningen University & Research Centre**
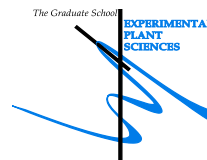
1) Start-up phase	*date*
► **First presentation of your project (higly recommended)**	
Functional Screening of Cladosporium fulvum cDNA Libraries for Secreted Effector Proteins	Mar 27, 2009
► **Writing or rewriting a project proposal**	
Characterization of the effector catalogue of the tomato pathogen Cladosporium fulvum	Jan 2009
► **Writing a review or book chapter**	
The Cladosporium fulvum-tomato pathosystem: fungal infection strategy and plant responses (In Molecular Plant Immunity, Guido Sessa, ed., John Wiley and Sons. Inc., Chichester, UK, pp 211-224)	2013
► **MSc courses**	
Molecular Aspects of Bio-interaction PHB 30806	Oct-Dec 2008
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*19.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day, Leiden University	Feb 26, 2009
EPS PhD Retreat, Cologne, Germany	Apr 15-17 2010
EPS PhD student day, Utrecht University	Jun 01, 2010
EPS PhD student day, Wageningen University	May 20, 2011
EPS PhD Retreat, Orsay, France	Jul 05-08 2011
EPS PhD Retreat, Norwich, UK	Aug 14-17, 2012
► **EPS theme symposia**	
EPS theme 2 'Interaction between Plants and Biotic Agents', Utrecht University	Jan 22, 2009
EPS theme 2 'Interaction between Plants and Biotic Agents', Utrecht University	Jan 15, 2010
EPS theme 2 'Interaction between Plants and Biotic Agents',Amsterdam University	Feb 03, 2011
EPS theme 2 'Interaction between Plants and Biotic Agents', Wageningen University	Feb 10, 2012
► **NWO Lunteren days and other National Platforms**	
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 06-07, 2009
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 19-20, 2010
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 04-05, 2011
ALW Platform Molecular Genetics Annual Meeting in Lunteren	Oct 2011
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 02-03, 2012
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 22-23, 2013
► **Seminars (series), workshops and symposia**	
Invited Seminars (Cryil Zipfel, T. Nurnberger)	Oct-Dec, 2008
Invited Seminars (Pieter van West, Rays Jiang)	Feb-Jun, 2009
Plant Sciences Seminars (Ton Bisselink, Harro Bouwmeester, Olaf van Kooten, Jack Leunissen, Pierre de Wit, Fred van Eeuwijk)	Sep-Nov 2009
1st Joint meeting of WUR-Marburg	Sep 2010
Invited Seminars (Paul Birch, Brigitte Mauch-Mani, Felix Mauch, Chris Hawes, Naoto Shibuya, Regine Kahmann, Richard Olivier)	May-Nov, 2010
KNPV spring meeting	Jun 16, 2010
Joint meeting CBS-Phytopathology on Bioinformatics and Medical Mycology	Nov 11, 2010
Invited Seminar Rosie Bradshaw	Aug 04, 2011
2nd Joint meeting of WUR-Marburg	Jan 30-31, 2012
Invited Seminar Brian Staskawicz	May 21, 2013
► **Seminar plus**	
► **International symposia and congresses**	
Europian Congress of Fungal Genetic 10 (ECFG10)	Mar 29-Apr 01, 2010
Gordon Research Congress	Jun 17-22, 2012
► **Presentations**	
Oral: ALW Lunteren	Apr 20, 2010
Oral: KNPV spring meeting: Functional Analysis of Cladosporium fulvum Effector Catalog	Jun 06, 2010
Oral:1st Joint meeting of WUR-Marburg: Caharacterization of novel effectors in C. fulvum	Sep 2010
Oral:2nd Joint meeting of WUR-Marburg: the role of tomatinase in C. fulvum pathogenicity	Jan 30, 2012
Oral: ALW Lunteren:Tomatinase from Cladosporium fulvum is required for full virulence by degrading α-tomatine during tomato infection	Apr 03, 2012
Oral: MPI Marburg:Identification and characterization of novel effectors of Cladosporium fulvum	May 16, 2013
Poster: ECFG10	Mar 29-Apr 01, 2010
Poster: Autumn school: Host-Microbe Interactomics	Nov 01-03, 2011
Poster Gordon Research Congress	Jun 17-22, 2012
► **IAB interview**	Feb 17, 2011
► **Excursions**	
Subtotal Scientific Exposure	*24.2 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Bioinformatics - a User's Approach	Mar 15-19, 2010
Autumnschool Host-Microbe Interactomics	Nov 01-03, 2011
Proteomics	Apr 23-26, 2013
► **Journal club**	
literature discussion group of Phytopathology	2009-2012
► **Individual research training**	
Subtotal In-Depth Studies	*6.8 credits**

4) Personal development	*date*
► **Skill training courses**	
Dutch course I	Sep 2010-Feb 2011
Techniques for Writing and Presenting Scientific Papers	Oct 18-21, 2011
EPS Career day	Nov 18, 2011
Project and Time Managment	Apr-Jun 2012
► **Organisation of PhD students day, course or conference**	
Phytopathology Lab outing organization	2011
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*5.1 credits**

TOTAL NUMBER OF CREDIT POINTS*	**55.6**

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

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