Identification and functional characterization of proteases and protease inhibitors involved in virulence of fungal tomato pathogens

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Chapter 1

General introduction and outline of the thesis

1. Plant-pathogen interactions

In nature, plants are exposed to various types of microbes, some of which are able to cause disease and retrieve nutrients to sustain their own growth and reproduction. Fungal plant pathogens have evolved different lifestyles. For example, saprotrophic fungi obtain their nutrients from dead organic materials, while other fungi establish interaction with their hosts. Obligate biotrophs, such as rust and powdery mildew fungi, entirely rely on host resources, and cannot survive outside living host tissue, except as resting structures (Catanzariti et al., 2006; Hacquard et al., 2013). Non-obligate biotrophic fungi, such as the tomato leaf mould pathogen *Cladosporium fulvum* (de Wit et al., 2012), thrive in nature for a significant part of their lifecycle on living host cells, but can also grow on artificial media. Both types of biotrophic fungal pathogens retrieve nutrients from living host cells using either intercellular hyphae or feeding structures such as haustoria (Catanzariti et al., 2006). Hemibiotrophs, such as the rice blast fungus *Magnaporthe oryzae* (Chen et al., 2012), and the leaf blotch fungus *Zymoseptoria tritici* (Goodwin et al., 2011), initially grow as a biotroph, but complete their life cycle as necrotroph. They initially colonize plant tissues without triggering any visible symptoms. After an extended incubation period, they gradually become necrotrophic by killing host cells, and subsequently feed on dead tissue (Mendgen and Hahn, 2002). The last group comprises necrotrophic fungi, such as the tomato grey mould fungus *Botrytis cinerea* (van Kan, 2006), that secretes enzymes and toxins to kill their host plants. Necrotrophic fungi degrade plant components and destroy host cells, feed on nutrients released from killed cells, and complete their life cycle on dead tissue. Biotrophic and hemibiotrophic fungi in their biotrophic phase secrete diverse virulence factors also known as effectors to suppress host defense, and orchestrate reprogramming of infected tissues to the pathogen's needs (Mendgen and Hahn, 2002; Chen et al., 2012). Necrotrophic and hemibiotrophic fungi (in their necrotrophic phase) also secrete diverse types of effectors including damage-eliciting or cell death-eliciting proteins and secondary metabolites to kill their hosts (van Kan, 2006). The plant defense machinery responds to infection by synthesizing Pathogenesis-Related (PR) proteins to prevent plant colonization (Houterman et al., 2008; Gan et al., 2010). The interaction between effectors secreted by pathogens and host proteins determines the success or failure of host colonization (Faulkner and Robatzek, 2012). In this interaction, plants and their pathogens are in an evolutionary "arms race," in which the pathogen develops new strategies to impede plant defense and the plant mounts new defensive mechanisms to resist the pathogen (Maor and Shirasu, 2005; Chisholm et

al., 2006). This arms race forces pathogens and their host plants to continuously adapt their strategies of infection and defense, respectively.

2. Co-evolutionary arms race between microbes and plants

2.1. Basal plant defense mechanisms

Plants have developed multiple layers of defense mechanisms against pathogens. The first layer is called basal defense including both pre-existing and inducible defense barriers against a wide range of potential pathogens. The pre-existing layer includes physical barriers such as cell walls, waxy epidermal cuticles, bark, lignin, and chemical barriers including antimicrobial compounds such as phenols, toxins and hydrolytic enzymes (Hückelhoven, 2007). The inducible layer of basal defense is triggered by Pathogen-Associated Molecular Patterns (PAMPs) such as chitin fragments that are released form fungal cell walls by constitutive plant chitinases during the initial phases of infection (Göhre and Robatzek, 2008; Spoel and Dong, 2012; Muthamilarasan and Prasad, 2013). Plants have developed transmembrane Pattern Recognition Receptors (PRRs), including Receptor-Like Kinases (RLKs) and Receptor-Like Proteins (RLPs) that contain an extracellular domain, that recognizes PAMPs, leading to PAMP-Triggered Immunity (PTI) (Jones and Dangl, 2006). For example, the rice CEBiP is an RLP and *Arabidopsis thaliana* CERK1 is a RLK that perceives chitin oligosaccharides to trigger defense responses (Kaku et al., 2006; Miya et al., 2007). PTI normally confers broad-spectrum resistance that restricts tissue colonization by pathogens. This layer of plant defense includes the activation of basal defense such as cell wall reinforcement, synthesis and accumulation of antimicrobial metabolites like phytoalexins, Reactive Oxygen Species (ROS), and PR proteins (Boller and Felix, 2009). PR proteins include antimicrobial enzymes such as chitinases, β-1,3-glucanases, peroxidases, proteases and protease inhibitors (PIs). β-1, 3-glucanase and chitinase act synergistically to inhibit the growth of many fungi (Mauch et al., 1988; Sela-Buurlage et al., 1993; Jongedijk et al., 1995) through hydrolysis of β-1,3-glucans and chitin (β-1,4-linked N-acetylglucosamine), respectively, which are major structural components of fungal cell walls (Balasubramanian et al., 2012; Grover, 2012). Perception of chitin oligomers released as a result of chitinase action activates additional basal defense responses.

2.2. Effector-triggered susceptibility and effector-triggered immunity

Pathogens have developed counter-defense strategies to overcome PTI. They secrete diverse sets of effectors to suppress PTI and cause Effector-Triggered Susceptibility (ETS). Depending on the type of pathogen, fungal plant pathogens either

secrete effectors to the intercellular space (apoplast) or deliver them inside plant cells. So far, many effectors, mainly from biotrophic and hemibiotrophic fungal pathogens, have been discovered that target apoplastic plant basal defense components induced by PAMPs. For example, Avr2, a cysteine protease inhibitor secreted into the tomato apoplast by *C. fulvum*, inhibits the cysteine protease Rcr3, a PR protein induced in tomato after infection (Rooney et al., 2005). *Ustilago maydis*, the corn smut fungus, secretes at least three effectors to inhibit apoplastic proteases involved in maize basal defense. Pit2 directly inhibits host cysteine proteases that are induced in infected plants (Mueller et al., 2013), while Pep1 induces the maize cystatin CC9 to inhibit endogenous apoplastic cysteine proteases (van der Linde et al., 2012b). In addition, Pep1 inhibits the maize peroxidase POX12 to disturb defense-associated oxidative bursts, which suppresses early immune responses in maize (Hemetsberger et al., 2012). Another group of effectors is translocated into plant cells to suppress induced basal defense. For example AvrPiz-t from *M. oryzae* targets the RING E3 ubiquitin ligase APIP6 and suppresses PTI in rice (Park et al., 2012). In addition, Tin2 form *U. maydis* targets anthocyanin biosynthesis, likely to suppress cell wall lignification (Tanaka et al., 2014).

Plants have evolved resistance (R) proteins that recognize effectors. R proteins include intracellular and extracellular immune proteins encoded by resistance genes (Piedras et al., 2000; Bent and Mackey, 2007; Han and Jung, 2013). Recognition of effectors by corresponding R proteins often results in a high level of resistance termed Effector-Triggered Immunity (ETI), which is often associated with the hypersensitive response (HR). Effectors can be recognized directly or indirectly. Direct interactions between effectors and R proteins are the exception rather than the rule. In independent studies have shown the direct interactions between effectors with their corresponding effectors such as direct interaction between effectors of the rice blast fungus *Magnaporthe grisea* and effectors of flax rust fungus *Melampsora lini* and their matching R proteins (Ravensdale et al., 2012; Dodds et al., 2006; Jia et al., 2000). Indirect interaction, also known as the guard model, involves recognition of effectors via perturbation of R-protein-guarded host targets (Jones & Dangl, 2006; Stergiopoulos and de Wit, 2009; Van'T Klooster et al., 2011). ETI is typically more pathogen-specific than PTI and is often associated with the HR (Heath, 2000; Lam et al., 2001; Jones and Dangl, 2006; Coll et al., 2011). HR is defined as a rapid cell death that occurs in response to pathogen attack to confine intruding (obligate) biotrophic pathogens to the site of penetration (Heath, 2000; Lam et al., 2001; Jones and Dangl, 2006; Coll et al., 2011).

Cytoplasmic R proteins recognize effectors secreted into host cells by haustoria or intercellular hyphae of biotrophic and hemibiotrophic pathogens such as *Blumeria graminis* (powdery mildew), *Puccinia striiformis* (yellow rust), and *M. oryzae* (rice blast). Effectors that localize to the cytoplasm are recognized via intracellular R proteins known as NLR (nucleotide-binding domain leucine-rich repeat) proteins, which contain a C-terminal leucine-rich repeat (LRR) domain, a central nucleotide-binding (NB) domain, and a variable N-terminal domain (Takken and Goverse, 2012; Han and Jung, 2013). The variable N-terminal domain is mostly represented by either a Toll-interleukin 1 receptor (TIR) motif or a coiled-coil (CC) motif, which subdivides plant NLRs into CC-NLRs and TIR-NLRs. In some cases, two different NLRs work in concert, such as the *Arabidopsis thaliana* RRS1 and RPS4 TIR-NLR pair (Sohn et al., 2014), and the rice RGA4 and RGA5 CC-NLR pair (Cesari et al., 2014).

Most apoplastic pathogens, including *C. fulvum*, that do not differentiate haustoria, secrete effectors into the apoplast, and their recognition is mediated by RLKs /or RLPs (Liebrand et al., 2013). Similar to RLKs, RLP receptors contain a short cytoplasmic tail, a membrane-spanning domain, and extra cytoplasmic leucine-rich repeats (LRRs). However, unlike RLKs, RLPs lack a C-terminal cytoplasmic kinase domain and require a signalling partner such as the RLK SOBIR1 to initiate downstream signalling (Liebrand et al., 2013). Recognition of extracellular effectors by RLPs and their responses are somewhat different from those observed during PTI and ETI (Thomma et al., 2011). This led to the birth of the term Effector-Triggered Defense (ETD). Unlike ETI, which operates against pathogens that secrete effectors into the host cytoplasm, ETD operates against extracellular pathogens that secrete effectors into the apoplast (Stotz et al., 2014), whereas PTI operates against both types of pathogens. ETD is somewhat slower than ETI and does not always lead to HR (Stotz et al., 2014). However, all three resistance mechanisms (PTI, ETI and ETD) partly overlap, which makes them less distinctive than initially suggested (Naito et al., 2007; Thomma et al., 2011; Stotz et al., 2014).

Unlike that for biotrophic and hemibiotrophic fungal plant pathogens, induction of cell death is beneficial for necrotrophic fungi as they derive their nutrients from killed host cells. Necrotrophic pathogens might exploit host resistance proteins for their own needs. For instance, necrotrophic pathogens such as *Cochliobolus victoriae* evolved effectors such as victorin, a host-selective toxin (HST) that activates the NB-LRR receptor-like LOV1 to trigger cell death (Lorang et al., 2012). Recognition of necrotrophic effectors such as HSTs is often called an inverse gene-for-gene relationship because recognition leads to disease susceptibility instead of resistance.

Thus, presence of a susceptibility gene encoding a toxin receptor determines host susceptibility. For example, the wheat pathogens *S. nodorum* and *Pyrenophora tritici-repentis* produce several effectors such as SnToxA and SnTox1-4 that are recognized by the corresponding sensitivity gene-encoded receptors Tsn1 and Snn1-4, respectively, conferring virulence on wheat cultivars carrying those susceptibility genes (Oliver et al., 2012). In contrast to ETI, the interaction of toxin effectors with host sensitivity proteins leads to ETS (Jones and Dangl, 2006; Oliver et al., 2012). It has been shown that recognition of *P. tritici-repentis* (Ptr) ToxA by the wheat receptor Tsn1 rapidly triggers accumulation of PR proteins, ROS, and enzymes involved in the phenylpropanoid, jasmonic acid, and ethylene biosynthesis pathways (Ciuffetti et al., 2010). The rapid induction of defense responses does not restrict the growth of necrotrophic pathogens as they have developed strategies to overcome, mitigate, or even exploit them.

3. Fungal pathogens of tomato and defense strategies of tomato

In this thesis we studied several different fungal tomato pathogens. Tomato (*Solanum lycopersicum*) is a host of different fungal pathogens that employ diverse virulence factors to establish a compatible interaction. The types of virulence factors and their action at different stages during infection of plants have led to different lifestyles.

C. fulvum is a non-obligate biotrophic fungal pathogen that causes tomato leaf mould. *C. fulvum* enters tomato leaves through stomata, and colonizes the intercellular space without entering host mesophyll cells (de Wit et al., 2012). Developed hyphae start draining plant resources from the apoplast, and finally complete their life cycle by developing conidiophores emerging from stomata, producing numerous conidia that can re-infect plants. *C. fulvum* is well known for the secretion of effectors that follow the gene-for-gene relationship as most of them are recognized by matching *C. fulvum* (Cf) resistance proteins in tomato, triggering Cf-mediated defense responses including the HR (Stergiopoulos et al., 2010; de Wit et al., 2012; Mesarich et al., 2014). In a compatible interaction, secreted effectors function as virulence factors and promote disease (van Esse et al., 2007; Bolton et al., 2008; Van Esse et al., 2008; de Jonge et al., 2010b; Ökmen et al., 2013). However, in an incompatible interaction, tomato Cf resistance proteins perceive corresponding effectors of *C. fulvum* to trigger an HR and resistance to the fungus (de Wit et al., 2002). So far, 12 *C. fulvum* effector genes have been cloned, and all encode peptides with four or more cysteine residues containing an N-terminal signal peptide for secretion into the apoplastic space of tomato leaves

(Mesarich et al., 2014). These effectors include six Avr proteins whose perception is mediated by the corresponding Cf resistance proteins whose encoding genes have all been cloned (de Wit et al., 2012), and many additional effectors of which the matching *Cf* genes remain unknown (Bolton et al., 2008; Stergiopoulos et al., 2012; Ökmen et al., 2013). Of these effectors, an intrinsic virulence function has been experimentally proven only for Avr2, Avr4 and Ecp6. Avr2 and Avr4 function against host enzymes involved in basal defense, while Ecp6 sequesters chitin fragments released from fungal cell walls to prevent chitin-triggered immunity (de Jonge et al., 2010a; Sánchez-Vallet et al., 2013). To date, six *Cf* resistance genes have been cloned, including *Cf-2, Cf-4, Cf-4E, Cf-5*, *Cf-9*, and *Cf-9B*. These genes encode extracellular LRR receptors that can perceive *C. fulvum* Avr proteins (Dixon et al., 1998; Seear and Dixon, 2003; Kruijt et al., 2005).

Fusarium oxysporum f. sp. *lycopersici* and *Verticillium dahliae* are vascular tomato pathogens that enter the root system, where they colonize and proliferate in xylem vessels, causing vascular wilts (Ma et al., 2010; Klosterman et al., 2011). Upon infection of tomato, PR proteins are induced (Houterman et al., 2008). *F. oxysporum* f. sp. *lycopersici* (*F. oxysporum*) secretes a plethora of enzymes including polygalacturonases, pectate lyases, xylanases and proteases, that likely contribute to virulence. Targeted disruption of the genes encoding these enzymes did not compromise virulence of the fungus (Michielse et al., 2009). However, targeted disruption of SNF1, a carbon catabolite repressor, affected the expression of cell wall degrading enzymes and reduced fungal virulence (Ospina-Giraldo et al., 2003). In addition, a few other transcription factors such as Fnr1 and pacC, which are responsive transcription factors to nitrogen and pH, respectively, were shown to play a role in virulence (Michielse et al., 2009). Furthermore, *F. oxysporum* secretes 11 small Six (for secreted in xylem) effector molecules, likely to promote host colonization (Takken and Rep, 2010). *Six* genes can also be avirulence genes because their products are recognized by cognate R proteins (*I* genes). Based on the ability of the fungus to overcome individual *I* genes, the *F. oxysporum* population is subdivided in different races. Effectors Avr1 (Six4), Avr2 (Six3), and Avr3 (Six1) are recognized by I-1 (Houterman et al., 2008), I-2 (Houterman et al., 2009) and I-3 (Rep et al., 2004), respectively. I-2, is an NB-LRR resistance protein that mediates recognition of the Avr2 effector (Takken and Rep, 2010). *V. dahliae* also enters the vascular system of tomato and causes similar symptoms including wilting, chlorosis, premature leaf drop, and stunting. *V. dahliae* secretes various pectinolytic enzymes (Bidochka et al., 1999), proteases (Dobinson et al., 1997), toxins, proteinaceous effectors, and secondary

metabolites (Fradin and Thomma, 2006; Luo et al., 2014). Tomato plants carry *Ve1* and *Ve2* genes that encode RLPs similar to the Cf proteins (Kawchuk et al., 2001). It was shown that only Ve1, but not Ve2 confers resistance to *V. dahliae* race 1 (Fradin et al., 2009). Ve1-mediated defense is triggered by the *V. dahliae* Ave1 effector (de Jonge et al., 2012).

Fusarium solani is another important soilborne pathogen of tomato and many other crops. It shows an ubiquitous distribution and causes mainly damping-off and root rot diseases. *F. solani* is actually a species complex that infects many solanaceous plants (Coleman et al., 2009). For example, *F. solani* f. sp. *eumartii* causes disease on both potato and tomato. Species in this complex secrete enzymes that degrade PR proteins. For example, *F. solani* f. sp. *pisi* secretes proteases that target host chitinases and β-1,3-glucanases (Lange et al., 1996; Sela-Buurlage, 1996). Neither effectors nor resistance proteins are available from *F. solani* and its hosts, respectively.

Other tomato pathogens like *Alternaria solani, Alternatia arborescens* (syn: *A. alternata*), and *B. cinerea* are necrotrophs that destroy host tissues*. A. solani* causes early blight of tomato (Kumar et al., 2008) and *A. arborescens* causes leaf spot (Prasad and Upadhyay, 2010) and stem canker (Oka et al., 2006). *Alternaria* species secrete an array of enzymes and toxins that have been shown to kill host tissues and in this way contribute to virulence of these fungi (Kendra et al., 1989; Lange et al., 1996; Lawrence et al., 2008; Chandrasekaran et al., 2014). The genome of *A. arborescens* contains a dispensable chromosome (CDC) that carries gene clusters involved in the biosynthesis host specific toxins (HSTs) such as AF-toxin, AK-toxin, and ACT-toxin, which are required to infect particular host plants (Hu et al., 2012). There are also tomato cultivars that are resistant against *Alternaria alternata*, f.sp. *lycopersici* producing AAL toxin (Brandwagt et al., 1998, Brandwagt et al., 2001), which is mediated by the *asc-1* gene (Brandwagt et al., 2002). In addition, accessions of wild tomato species are known to have high levels of early blight resistance (Chaerani and Voorrips, 2006).

B. cinerea is a necrotrophic tomato pathogen that causes grey mould. This fungus forms pseudo-appressoria that secrete many enzymes to weaken the cuticle and cause collapse plant cell walls, facilitating penetration of host tissues (van Kan, 2006). *B. cinerea* secretes a plethora of molecules such as hydrophobins that are important for amphipathic membrane formation at a hydrophobic–hydrophilic interface (Zampieri et al., 2010), plant cell-wall degrading enzymes, such as BcPG2 (Joubert et al., 2007) and pectin methyl esterases such as BcPME2 (Kars et al., 2005), pectin lyases and aspartyl proteinases (Ten Have et al., 2010), likely to counteract host defense. It was shown

that RBPG1, an *A. thaliana* leucine-rich repeat receptor-like protein (LRR-RLP) functions as a receptor for fungal endo-polygalacturonases that are recognized as MAMPs. This recognition triggers necrotic responses in *A. thaliana* Col-0 (Zhang et al., 2014). The molecular mechanisms underlying *B. cinerea* defense responses observed in tomato are less well understood (Beyers et al., 2014).

4. Understanding the role of proteases and protease inhibitors in plant-pathogen interactions

Pathogens deliver many effectors to different cellular compartments of their hosts to manipulate host defense (Stergiopoulos and de Wit, 2009; Gan et al., 2010; de Jonge et al., 2011; Win et al., 2012; Gohari et al., 2015). Some pathogens have acquired effectors that act as protease inhibitors (PIs), which counteract host proteases and interfere with their functions (Sabotič and Kos, 2012). PIs are classified either by the type of protease they inhibit, or by their mechanism of inhibiting aspartic proteases, cysteine proteases, metalloproteases or serine proteases (Rawlings et al., 2013). However, PIs exhibit low specificity, and one PI can often inhibit different proteases of the same family (Turk, 2006). PIs can also be classified according to their mechanism of action. Canonical PIs bind the protease in a lock-and-key manner, such as serine protease inhibitors (serpins) (Silverman et al., 2001), or block the active site by covering it, as shown for cystatins and cysteine cathepsins (Stubbs et al., 1990), or a combination of both mechanisms (Gomis-Ruth et al., 1997). Plant pathogenic fungi also contain large numbers of protease genes in their genomes. During the interaction with their host plant, fungal proteases might inactivate or destroy deleterious host proteins to facilitate acquisition of nutrients. The secretion of PIs was shown to contribute to the virulence of different plant pathogens and is reviewed in chapter 6 (Karimi Jashni et al. 2015).

Similarly, plants produce proteases and PIs that potentially inhibit the growth of a variety of pathogens (Kim et al., 2009). Plants produce proteases that normally work in concert with other antimicrobial components (van der Hoorn and Jones, 2004). These proteases may play a role inside the plant cell to maintain proper physiological functions, or to activate target proteins, which enables the fine-tuning of components that are involved in plant defense (Pesquet, 2012). Furthermore, plant proteases can directly display antimicrobial activity by targeting pathogens themselves (Shindo and van Der Hoorn, 2008; Song et al., 2009; Kaschani et al., 2010). Plants also produce numerous PIs that inhibit proteases secreted by pathogens (Leo et al., 2002). Plant PIs are small proteins present in almost all parts of plants including storage tissues

embryonic and vegetative parts where they provide protection against potential pathogens (Kim et al., 2009).

5. Thesis outline

Genomes of both plants and pathogens comprise large numbers of putatively secreted proteases, of which only a few have been studied in some detail. For example, tomato and potato produce basal levels of proteases such as P69 (Song et al., 2009), Rcr3, Pip1, C14, and CP2 (Tian et al., 2007; Shabab et al., 2008; Kaschani et al., 2010; Bozkurt et al., 2011; van der Linde et al., 2012b; van der Linde et al., 2012a; Mueller et al., 2013). These plant proteases, along with other components of basal defense, are induced upon challenge by pathogens, both locally (Tian et al., 2005), and systemically (Tian et al., 2007; Shabab et al., 2008; Song et al., 2009). However, fungal proteins targeted by these proteases remain to be elucidated. Pathogenic fungi secrete various types of effectors into host plants that mostly target plant defense components to promote host colonization. Proteases can hydrolyse PR proteins and PIs can inhibit plant proteases that are part of the host defense system. For example, *F. solani* (Lange et al., 1996; Olivieri et al., 2002) and *F. verticillioides* (Naumann et al., 2011) secrete serine and metalloproteases that target host chitin-binding domain (CBD)-containing chitinases. In addition, fungi also secrete PIs such as Pit2 of *U. maydis* (Mueller et al., 2013), and Avr2 of *C. fulvum* (Rooney et al., 2005), which inhibit host cysteine proteases.

Despite the importance of proteases and PIs secreted by fungal pathogens, little information about their role in virulence is available. Recent advances in genomics, bioinformatics, transcriptomics and proteomics have facilitated the identification and functional analysis of proteases and PIs that are relevant to plant-fungus interactions.

The aim of this thesis was to address the role of proteases and PIs in fungal virulence, using a few model fungi pathogenic on tomato. In **chapter 2**, we compared the number of putatively secreted proteases in the genomes of fungi with different lifestyles. We observed that fungi with a saprotrophic lifestyle contain more genes encoding putatively secreted protease than those with a biotrophic lifestyle. Surprisingly, the number of protease genes present in the genome of *C. fulvum* is comparable with those observed in hemibiotrophs and necrotrophs. We analyzed the *C. fulvum* protease gene complement both at the transcriptome and proteome level in order to understand this apparent discrepancy. In **chapter 3**, using an alignment-based gene prediction tool, we identified pseudogenes that contain disruptive mutations (DMs), which lead to the production of nonfunctional proteins. We found that

many putatively secreted proteases from *C. fulvum* are encoded by pseudogenes, suggesting that difference in pseudogenization of proteases between various pathogens might partially explain their different lifestyles. In **chapter 4**, we investigated the ability of tomato fungal pathogens to cleave CBD-containing chitinases and the role of this mechanism in fungal virulence. We showed that three fungal tomato pathogens secrete proteases that cleave extracellular CBD-chitinases, resulting in complete or partial removal of their CBD and reduction of their chitinase and antifungal activity. Analysis of proteolytic enzymes present in culture filtrate of *F. oxysporum* f. sp. *lycopersici* (*F. oxysporum*) identified a metallo protease, FoMep1, and a serine protease, FoSep1, whose synergistic activity removed the CBD of two extracellular CBD-chitinases. Targeted deletion of these two proteases genes showed that both encoded proteases are important for virulence of *F. oxysporum* on tomato. In **chapter 5**, we tried to identify the host target(s) of the Avr9 effector from *C. fulvum*. We assumed that the plant's virulence target of Avr9 might be an apoplastic protease because Avr9 shows a structure similar to carboxypeptidase inhibitors. Using biotinylated Avr9, we performed pull-down and far-western blotting assays with apoplastic fluids from tomato inoculated with a *C. fulvum* race lacking the *Avr9* gene. However, no Avr9-interacting proteins were identified. We then hypothesized that glycosylation of Avr9 might be important for its interaction with a host target. Indeed, using mass spectrometry, we showed that the primary product of Avr9 secreted by *C. fulvum* is glycosylated. Future studies to identify the intrinsic biological functions of (glycosylated) Avr9 and the encountered pitfalls are discussed. **In chapter 6**, we reviewed the role of protease and PIs involved in fungal virulence and plant defense in light of the results obtained in this thesis. We provide examples of proteases and PIs involved in the arms race between plants and their pathogens, and discussed their role in compromising PAMP-triggered immunity (PTI) and basal defense. **Chapter 7** is a general discussion about the challenges in future studies on fungal proteases and PIs. In particular, we discuss that genome studies are required, but not sufficient to draw conclusions on issues such as the lifestyle of fungi. Our results suggest that proteases may work synergistically against PR proteins, and that the functions of different types of proteases may partly overlap, making their studies more challenging. Finally, targeted proteomics approaches using known targets and multi-gene targeting of genes with unknown biological functions are proposed for functional analysis in future research on proteases and PIs.

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Chapter 2

Proteases in *Cladosporium fulvum*: genome mining, classification and expression

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ABSTRACT

Proteases are key components of the hydrolytic enzyme arsenal employed by fungi to invade their host plants. Despite the importance of proteases secreted by fungal pathogens, only a limited number has been characterized. The recent advances in -omics era have facilitated identification and functional analysis of proteases involved in plant-fungus interactions. Here we exploited the availability of fungal genomes with different lifestyles to get further insight into fungal proteases. Our analysis showed that fungi with a hemibiotrophic and saprotrophic lifestyle contain more secreted protease genes than those with a biotrophic lifestyle. Remarkably, the number of protease genes present in the genome of *Cladosporium fulvum*, a biotrophic tomato pathogen is comparable with that of hemibiotrophs and saprophytes. In order to identify host plant inducible protease genes, we performed transcriptome and proteome analyses of *C. fulvum* under *in vitro* and *in planta* conditions by means of RNA-Seq/RT-qrtPCR and mass spectrometry, respectively. Results show that most proteases of *C. fulvum* are not expressed during infection of tomato, likely to sustain the biotrophic growth of this fungus.

INTRODUCTION

Proteases are an important group of enzymes that catalyze the hydrolysis of peptide bonds. Based on their active sites and enzymatic mechanisms, proteases are classified into four major families: aspartic, cysteine, serine and metallo- proteases (Rawlings et al., 2013). They are involved in diverse biological functions including maturation and activation of other proteins (van den Ackerveken et al., 1993), regulation of signal transduction pathways and cellular localization (Ehrmann and Clausen, 2004), recycling of nitrogen (Šimkovič et al., 2008) and regulation of pathogenesis (Li et al., 2012). Because proteases play important physiological roles in modulating a wide range of cellular functions, all organisms contain genes to encode these enzymes in their genomes (Rao *et al.*, 1998). The exponentially growing number of sequenced genomes of fungal pathogens has promoted studies on virulence factors, including proteases (Cheng et al., 2015; Haiko et al., 2009; Yike, 2011). The role of secreted proteases in virulence of human pathogenic fungi is well studied. For example, aspartic proteases secreted by *Candida albicans* play a role in adherence of the fungus to its host, penetration into host tissues, and interaction with the immune system of the host (Monod et al., 2002). The role of proteases for bacterial pathogens is also well studied. For example, cysteine proteases are important for pathogenicity of *Leishmania tropica* on human and animals (Mahmoudzadeh-Niknam and McKerrow, 2004). Similarly, plant

pathogenic bacteria secrete proteases to cope with the host defense. For example, proteases secreted by plant pathogenic bacteria were shown to target defense pathways (Day et al., 2005) or host regulatory proteins (Shao et al., 2002). Plant fungal pathogens also secrete proteases during host colonization. Independent studies have previously shown the ability of fungal serine proteases and metalloproteases in targeting of host defense chitinase and glucanase proteins (Naumann et al., 2011; Olivieri et al., 2002). Improvements in genomics, bioinformatics, transcriptomics and proteomics have facilitated acquisition of knowledge on fungal proteases. Recently, the genomes of numerous fungi have been sequenced and revealed a considerable number of genes encoding protease in genomes of fungi with different lifestyles (Ohm et al., 2012). It is believed that necrotrophic, fungal pathogens secrete more hydrolytic enzymes to overcome host defense mechanisms and to get access to nutrients (Asis et al., 2009) than biotrophic fungal pathogens. Here, we performed comparative genome analysis of putatively secreted proteases across a set of fungi with different lifestyles. The genome of *Cladosporium fulvum*, which is a non-obligate biotrophic fungus causing leaf mould of tomato (*Solanum lycopesicum*) (de Wit et al., 2012), contains 59 putatively secreted protease genes, which is comparable to the number occurring in hemibiotrophs and necrotrophs. In order to get more insight into the role of secreted proteases of *C. fulvum*, we studied the annotation and expansion of its putatively secreted protease (PSP) genes; we subsequently analysed all PSP genes of *C. fulvum* at both transcriptome and proteome levels by means of RNA-Seq/RT-qrtPCR and mass spectrometry, respectively. Data showed that only a limited number of genes are expressed during infection of tomato. We discuss how the regulation of secreted protease genes may contribute to its biotrophic lifestyle and the beneficial effects of tight regulation of protease genes in its stealth pathogenesis.

MATERIALS AND METHODS

Plant and fungal materials

The susceptible tomato cultivar Money-Maker Cf-0 (MM-Cf-0) was used for all *in planta* studies including expression profiling and proteomics of foliar apoplastic fluid. Tomato plants were grown in a greenhouse at 70% relative humidity, 23-25 °C during daytime and 19-21 °C at night, with a light/dark regime of 16/8 hours and 100 W m^{-2} supplemental light when the sunlight influx intensity was less than 150 W m^{-2}. The fungal strain of *C. fulvum,* race 0 (WU-CBS131901), present in the stock of laboratory of phytopathology was used in all experiments.

Identification of proteases in fungal genomes

The Pfam domains corresponding to different subfamilies of fungal proteases (Table S1) were retrieved from the Merops database (http://merops.sanger.ac.uk/index.shtml) (Rawlings et al., 2013), and used as search criteria to retrieve putative proteases from thirty fungal genomes (Table 1) (Grigoriev et al., 2014). Signal peptide sequences of putative proteases were predicted using the SignalP 4.1 server (Petersen et al., 2011). Based on similarities in amino acid sequences, each putatively secreted protease was assigned to a family using the pfam database (Finn et al., 2014).

RNA-Seq data analysis

We used RNA-Seq data to assess the expression of protease genes during *in vitro* and *in planta* growth of *C. fulvum*. RNA isolation and analysis of RNA-Seq reads was as previously described (Mesarich et al., 2014). Transcript abundance representing the frequency of RNA-Seq reads for each gene was quantified with Cufflinks (http://cufflinks.cbcb.umd.edu) by calculating FPKM values (fragments per kilobase of exon per million fragments mapped), which are normalized values that account for differing gene lengths and the total number of RNA-Seq reads obtained from biological samples. FPKM values belonging to RNA samples isolated from fungal biomass grown in potato dextrose broth (PDB) medium for 7 days, and *in planta* at 4, 10 and 12 days post inoculation (dpi) were used.

Expression analysis of *Cladosporium fulvum* proteases

Expression of *C. fulvum* proteases was studied for both *in vitro* and *in planta* conditions. Biomass of fungus grown for 7 days in PDB liquid medium was harvested. In addition, leaf samples of MM-Cf-0 tomato plants inoculated with *C. fulvum* were collected at 6, 8, 10 and 12 dpi and were immediately frozen in liquid nitrogen. As previously described (de Wit et al., 2012), total RNA was isolated from fully ground samples and for each sample, 1 µg of total RNA was used for cDNA synthesis. Primer pairs for *C. fulvum* protease genes (Table S2) were designed with Primer3 Plus (Untergasser et al., 2007), and their efficiency and specificity were determined with a dilution series of genomic DNA of *C. fulvum.* Reverse transcription-quantitative real-time polymerase chain reaction (RT-qrtPCR) was performed as previously described (de Wit et al., 2012). Primer pairs were previously designed for *Cf-actin* and *Cf-β-tubulin* as reference genes (Mesarich et al., 2014), which were used as control and for normalizing the expression of other genes, respectively. Results were analysed using

the $E^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). Experiments included 2-3 biological replications.

Isolation of apoplastic fluids

Apoplastic fluid (AF) was isolated from MM-Cf-0 inoculated with *C. fulvum* at 12 dpi using a previously described method (de Wit and Spikman, 1982). AF was cleared by centrifugation (12,000xg; 20 min) at 4 °C and concentrated 3 times using 1 kDa cut-off Amicon filters.

Mass spectrometry analysis

To identify fungal proteases present in the apoplast of infected plants, 10 µL of AF was loaded on a 12% Tris SDS gel and the whole lane was excised from the SDS-PAGE gel and cut into cubes of one mm^3 in size. Protein samples were prepared for mass spectrometry as previously described (Karimi Jashni et al., 2015). Samples were then analysed by nLC MS/MS with a Proxeon EASY nLC connected to a LTQ-Orbitrap XL (Lu et al., 2011) at the Laboratory of Biochemistry of Wageningen University. The *C. fulvum* database was downloaded from the JGI website (http://genome.jgi-psf.org/Clafu1/Clafu1.home.html) and was used together with a contaminant database that contains sequences of common contaminants: BSA (P02769, bovin serum albumin precursor), trypsin (P00760, bovin), trypsin (P00761, porcin), keratin K22E (P35908, human), keratin K1C9 (P35527, human), keratin K2C1 (P04264, human) and keratin K1CI (P35527, human).

RESULTS

Genomes of fungi with different lifestyles contain different putatively secreted protease genes complements

We performed comparative genomics analysis of putatively secreted proteases (PSP) across a set of fungi with different lifestyles. Thirty different fungal species including 9 hemibiotrophs, 8 saprotrophs, 6 biotrophs, 4 human pathogens and 3 necrotrophs were selected and used to retrieve the genes encoding proteases across their genomes. All retrieved PSPs were screened for presence of a signal peptide using online prediction software (Petersen et al., 2011). Comparison of genomes showed that serine and metalloproteases are the most abundant proteases (up to 80% in *Magnaporthe oryzae* and *Schizosaccharomyces pombe*) in nearly all fungal species. Exceptions are *Phanerochaete chrysosporium, Laccaria bicolor* and *Candida albicans* that contain more aspartic proteases than metalloproteases (Fig. 1). Often, the number

of serine protease genes in individual genomes is similar or moderately higher than the number of metalloproteases, with the exception of saprotroph *Hysterium pulicare* that contains 40 serine protease and 10 metalloprotease genes. In contrast, another saprotroph *Coprinopsis cinerea* comprises only 18 serine protease genes compared to 47 metalloproteases. The hemibiotrophic fungus *Phanerochaete chrysosporium* and human pathogen fungus *Candida albicans* contain high numbers of aspartic protease genes in their genomes. In general, number of cysteine proteases is limited to 1-2 genes per genome, while the biotrophic fungus *Laccaria bicolor* contains 6 cysteine protease genes, and the hemibiotrophic fungus *Phanerochaete chrysosporium*, the obligate biotrophic fungus *Puccinia graminis* and the biotrophic fungus *Ustilago maydis* contain no cysteine protease gene. The overall median number of PSP genes in a fungal genome is 47.5. For hemibiotropics fungi, this number is higher (58), while it is lower (34.5) for biotrophic pathogens. Results showed that hemibiotrophic fungi such as *M. oryzae, Fusarium verticillioides*, *Fusarium graminearum*, *Fusarium oxysporum* contain more than 58 PSP genes. In contrast, biotrophic fungi *Tuber melanosporum, Ustilago maydis* and obligate biotrophic *P. graminis* contain less than 34.5 PSP genes in their genomes (Fig. 1). Saprotrophs and necrotrophs have median number of 48 and 40 PSP genes, respectively, but in each group there are large variation among their members. The median number of PSP genes for human pathogens is 29, which is similar to the number found for biotrophs. There was no correlation between genome size and total number of gene models with the lifestyle of these fungi (Fig. S1). For example, *Ustilago maydis* and *Tuber melanosporum* are both biotrophs with a lower number models compared to another biotroph *Laccaria bicolor.* However, there seems to be a tendency for hemibiotrophs and saprotrophs to contain a higher number of PSP genes than biotrophs (Table S3). In general, the percentage of PSP genes in fungal genomes is correlated with lifestyles (Fig. S2). For example, the percentage of PSP genes in hemibiotrophs is 0.44% of all gene models, which is significantly higher than the 0.27% of all gene models in biotrophs. Remarkably, *C. fulvum* comprised 59 PSP genes, which is 0.4% of the all gene models in this fungus, which is comparable to that of hemibiotrophic fungi.

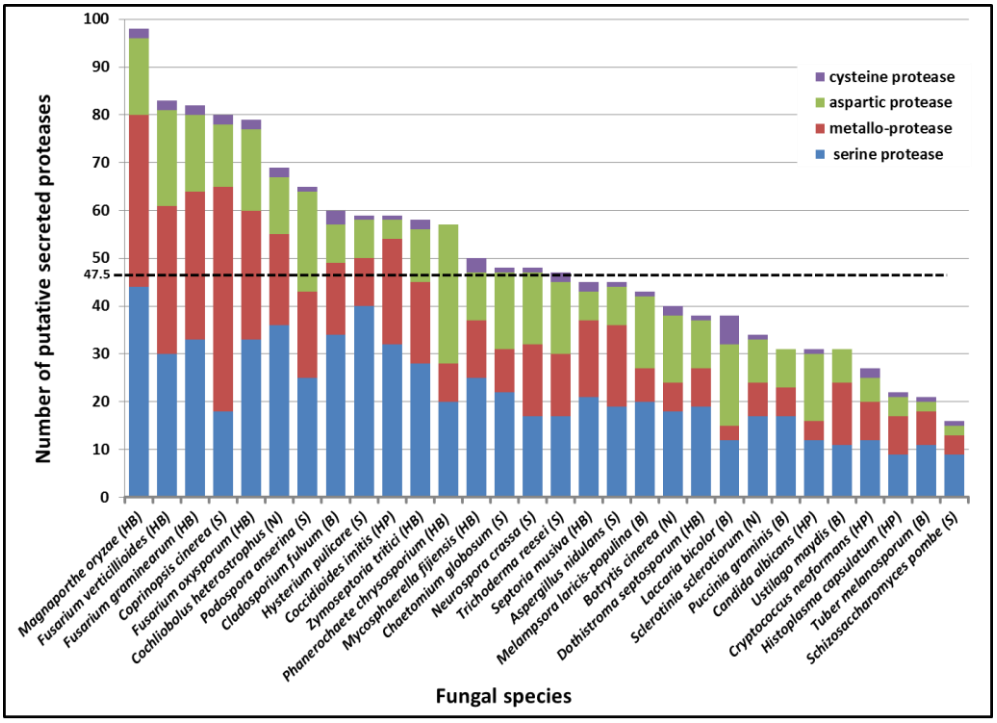

Figure 1: Numbers of predicted secreted proteases in fungi with different lifestyles. For each fungus, the number of putatively secreted proteases (predicted by Signalp 4.0) belonging to serine, metallo-, aspartic and cysteine proteases is indicated. The different lifestyles of fungal species are indicated: B for biotroph, HB for hemibiotroph, N for necrotroph, S for saprotroph, and HP for human pathogen. The dashed line indicates the median number of putatively secreted proteases in the selected fungi.

Genome of *Cladosporium fulvum* comprises 59 putatively secreted proteases

The number of PSP genes in the genome of *C. fulvum* is comparable with that of hemibiotrophs and saprotrophs. We studied the annotation and expansion of *C. fulvum* PSP genes. Out of 147 predicted protease genes in *C. fulvum,* 59 contained a signal peptide for secretion into vacuole or extracellular compartments (Table 1), which is comparable to the average number of PSPs in the genomes of other fungal pathogens (Fig. 1 and (Ohm et al., 2012)). These genes were named *CfPro1 to CfPro59,* and based on pfam database encode proteases belonging to different subfamilies of proteases (Table 1). Thirty five of the *C. fulvum* PSP genes encode serine proteases belonging to subfamilies S1 (n=1), S8 (n=16), S9 (n=2), S10 (n=14), S28 (n=1), S41 (n=1). Metalloproteases comprise fifteen genes encoding subfamilies M12 (n=1), M14 (n=4), M20 (n=1), M28 (n=4), M35 (n=1), M36 (n=1) and M43 (n=3). Seven PSP genes encode aspartic proteases corresponding to subfamily A1, and two PSP genes encode cysteine proteases corresponding to subfamilies C13 and C69. Overall, most

PSP genes in *C. fulvum* belong serine peptidase S8 and S10, metalloproteases M14 and M28, and aspartic protease A1. The *C. fulvum* protease genes are not clustered on particular scaffolds (Table 1).

Table 1. Characteristics of *Cladosporium fulvum* putatively secreted protease genes.

Gene name	Protein ID at JGI	Location on scaffold	Protein size(aa)	Cysteine (#)	Proteomic support	Family	Pfam domain
CfPro1	192213	scf7180000130553:199931..202218	410	4	-	Peptidase-A1	PF00026.18
CfPro2	189675	scf7180000130280:104362..106999	336	4	-	Peptidase-A1	PF00026.18
CfPro3	194655	scf7180000130782:160926..163334	447	4	-	Peptidase-A1	PF00026.18
CfPro4	196312	scf7180000130908:42179..45461	474	4	-	Peptidase-A1	PF00026.18
CfPro5	184463	scf7180000127143:61750..65133	571	4	-	Peptidase-A1	PF00026.18
CfPro6	186749	scf7180000128717:21846..26153	467	6	-	Peptidase-A1	PF00026.18
CfPro7	191012	scf7180000130368:186659..189261	398	4	+	Peptidase-A1	PF00026.18
CfPro8	196569	scf7180000130912:32925..35254	411	2	-	Peptidase-C13	PF01650.13
CfPro9	189132	scf7180000130132:237044..242347	527	3	-	Peptidase-C69	PF03577.10
CfPro10	191719	scf7180000130427:37593..41785	829	45	-	Peptidase-M12	PF13688.1
CfPro11	193898	scf7180000130743:23605..26632	240	0	-	Peptidase-M14	PF00246.19
CfPro12	185983	scf7180000127956:2973..7572	550	1	-	Peptidase-M14	PF00246.19
CfPro13	195657	scf7180000130859:67563..70250	405	2	-	Peptidase-M14	PF00246.19
CfPro14	188917	scf7180000130116:1345..4173	584	5	-	Peptidase-M14	PF00246.19
CfPro15	197234	scf7180000130985:74322..76759	413	1	-	Peptidase-M20	PF01546.23
CfPro16	194614	scf7180000130782:61447..64863	712	2	-	Peptidase-M28	PF04389.12
CfPro17	195127	scf7180000130799:80588..83784	524	4	-	Peptidase-M28	PF04389.12
CfPro18	196054	scf7180000130885:40914..45869	389	3	-	Peptidase-M28	PF04389.12
CfPro19	184060	scf7180000126623:42645..45166	408	2	-	Peptidase-M28	PF04389.12
CfPro20	185858	scf7180000127883:38529..41071	364	9	-	Peptidase-M35	PF02102.10
CfPro21	193187	scf7180000130681:47745..50832	624	5	-	Peptidase-M36	PF02128.10
CfPro22	183971	scf7180000126554:23457..25349	279	8	-	Peptidase-M43	PF05572.8
CfPro23	190978	scf7180000130368:95545..97696	278	7	-	Peptidase-M43	PF05572.8
CfPro24	192241	scf7180000130553:270988..272921	310	7	-	Peptidase-M43	PF05572.8
CfPro25	193444	scf7180000130692:4989..6979	321	5	-	Peptidase-S1	PF00089.21
CfPro26	197426	scf7180000130988:255819..258991	495	6	-	Peptidase-S8	PF00082.17
CfPro27	185597	scf7180000127726:15351..19057	840	10	-	Peptidase-S8	PF00082.17
CfPro28	197538	scf7180000131008:7629..9931	374	5	-	Peptidase-S8	PF00082.17
CfPro29	189824	scf7180000130291:43291..46449	624	11	-	Peptidase-S8	PF00082.17
CfPro30	184985	scf7180000127332:157873..160406	395	3	-	Peptidase-S8	PF00082.17
CfPro31	188106	scf7180000130004:4843..8520	600	14	-	Peptidase-S8	PF00082.17
CfPro32	196045	scf7180000130885:20363..23558	644	9	-	Peptidase-S8	PF00082.17
CfPro33	195779	scf7180000130861:96108..99367	633	7	-	Peptidase-S8	PF00082.17
CfPro34	190480	scf7180000130327:14564..19454	397	4	-	Peptidase-S8	PF00082.17
CfPro35	194207	scf7180000130764:49445..52233	595	7	-	Peptidase-S8	PF00082.17
CfPro36	188663	scf7180000130095:27433..32013	615	6	-	Peptidase-S8	PF00082.17
CfPro37	189334	scf7180000130183:129133..134228	390	2	-	Peptidase-S8	PF00082.17
CfPro38	192396	scf7180000130573:41722..45865	574	13	-	Peptidase-S8	PF00082.17
CfPro39	191659	scf7180000130420:101754..106081	898	20	-	Peptidase-S8	PF00082.17
CfPro40	188827	scf7180000130099:121219..123986	506	3	+	Peptidase-S8	PF00082.17
CfPro41	195145	scf7180000130799:116134..119226	617	7	-	Peptidase-S8	PF00082.17
CfPro42	190628	scf7180000130340:46366..50171	756	1	-	Peptidase-S9	PF00326.16
CfPro43	184779	scf7180000127307:51250..55032	722	5	-	Peptidase-S9	PF00326.16
CfPro44	186960	scf7180000128972:35296..38050	546	6	-	Peptidase-S10	PF00450.17
CfPro45	191132	scf7180000130384:73302..76913	724	9	-	Peptidase-S10	PF00450.17

CfPro46	184492	scf7180000127203:13575..17099	633	7	-	Peptidase-S10	PF00450.17
CfPro47	185697	scf7180000127843:5464..8369	544	8	-	Peptidase-S10	PF00450.17
CfPro48	192067	scf7180000130544:72747..75832	525	10	-	Peptidase-S10	PF00450.17
CfPro49	191799	scf7180000130456:22942..25746	549	10	-	Peptidase-S10	PF00450.17
CfPro50	186241	scf7180000128138:67941..72381	478	10	-	Peptidase-S10	PF00450.17
CfPro51	191625	scf7180000130420:22654..25641	305	9	-	Peptidase-S10	PF00450.17
CfPro52	191773	scf7180000130440:26462..29611	595	11	-	Peptidase-S10	PF00450.17
CfPro53	195806	scf7180000130862:27322..31023	607	7	-	Peptidase-S10	PF00450.17
CfPro54	193034	scf7180000130675:54210..57317	611	10	-	Peptidase-S10	PF00450.17
CfPro55	185189	scf7180000127485:70275..76323	1054	15	-	Peptidase-S10	PF00450.17
CfPro56	197471	scf7180000130996:4758..8143	642	9	-	Peptidase-S10	PF00450.17
CfPro57	194708	scf7180000130785:36860..39874	555	11	+	Peptidase-S10	PF00450.17
CfPro58	194320	scf7180000130767:7816..10752	556	9	-	Peptidase-S28	PF05577.7
CfPro59	195456	scf7180000130841:136270..139794	769	6	-	Peptidase-S41	PF03572

The protein IDs were obtained from JGI Mycocosm portal (http://genome.jgi.doe.gov/Clafu1/Clafu1.home.html). Proteome support refers to LC-MS data obtained from apoplastic fluids isolated from tomato plants inoculated with *Cladosporium fulvum* race 0. In the family column, A, C, M and S refer to subfamilies of aspartic, cysteine, metallo- and serine proteases.

RNA-Sequencing and RT-qrtPCR data reveal differential expression of putatively secreted proteases in *C. fulvum* during *in planta* infection

RNA-Seq analysis was performed to determine the expression of proteases under *in vitro* and *in planta* conditions (Mesarich et al., 2014). Results show that the majority of genes exhibit no/low expression across all the studied time points both under *in vitro* and *in planta* conditions. FPKM values of PSP genes varied between 0 and 535 during *in planta* growth (Fig. 1A). Out of the 59 PSP genes, 46 had FPKM values lower than 30, eight between 30 and 100 and five between 100 and 500. RNA-seq data showed that some of the PSP genes such as *CfPro7* and *CfPro57* showed similar expression *in vitro* and *in planta*. However, some PSP genes showed significant expression *in vitro* only such as *CfPro18* and *CfPro40*. Conversely, *CfPro19* was higher expressed *in planta* than *in vitro*. Interestingly, *CfPro41* was specifically induced under *in planta* condition with the highest level of expression at 8 dpi. To confirm the RNA-Seq data and quantify the expression of *C. fulvum* PSP genes by RT-qrtPCR, susceptible tomato cultivar MM-Cf-0 was inoculated with *C. fulvum,* and the expression of the 59 PSP genes (*CfPro1-59*) was analysed at 6, 8, 10 and 12 dpi. Expression of PSP genes was normalized to that of the *C. fulvum* β*-tubulin* gene. RT-qrtPCR data generally confirmed the expression patterns obtained by the RNA-seq approach. It also showed expression for some of the PSP genes such as CfPro4 to 6, CfPro8, CfPro27, CfPro39 and CfPro53-56, for which no expression was detected by RNA-Seq analysis. However RT-qrtPCR analysis did not confirm the expression of *CfPro3* at 4 dpi. In addition, RT-qrtPCR analysis showed that the majority of PSP genes was not expressed, whereas some were induced at a moderate level during plant colonization such as *CfPro14, CfPro15, CfPro18, CfPro19,*

CfPro27, CfPro46 and *CfPro56* (Fig. 2). Some of the PSP genes such as *Cfpro7, CfPro40, CfPro41* and *CfPro57* were expressed at the same level as *actin*. Of those, *CfPro40* was induced from 6 dpi onwards, while the expression of *Cfpro41* was high at 6 dpi, and gradually decreased by 50% at 20 dpi. The specific induction of *CfPro41* and *CfPro54* during plant infection was confirmed by RT-qrtPCR suggesting a role in virulence.

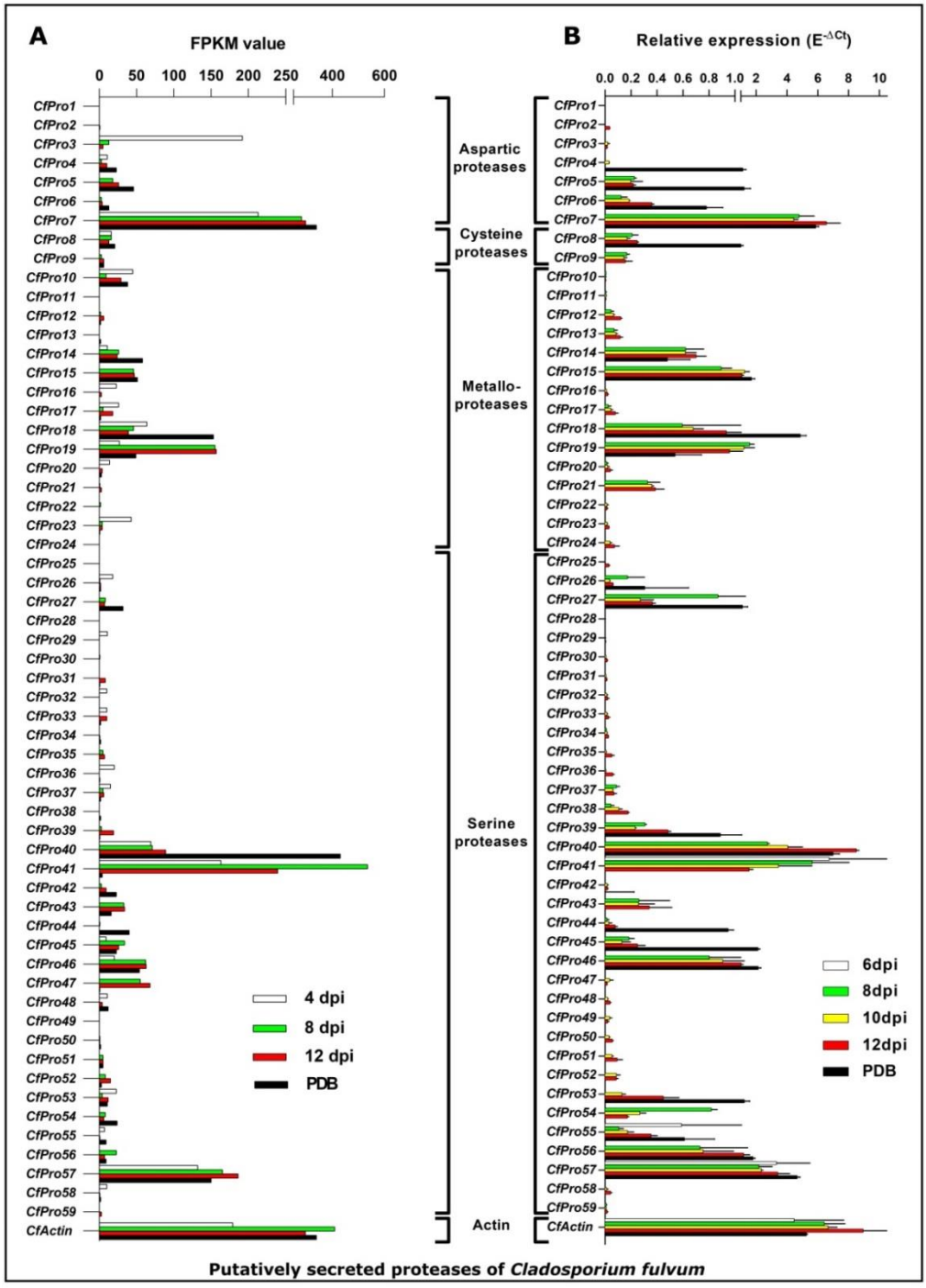

Figure 2: Expression profile of *Cladosporium fulvum* genes encoding putatively secreted proteases under *in vitro* and *in planta* conditions. Susceptible tomato cultivar

Money-Maker Cf-0 was inoculated with *Cladosporium fulvum* race 0. Leaf samples of *C. fulvum*-inoculated plants were collected at different days post inoculation (dpi) for RNA extraction and subsequent RNA-sequencing and RT-qrtPCR. Expression was also measured in *C. fulvum* grown *in vitro* in potato dextrose broth (PDB) liquid medium. (**A**) RNA-Seq analysis (FPKM (fragments per kilobase of exon per million fragments mapped) values) at 4, 8, and 12 dpi as well as after 7 days of growth *in vitro* on PDB. (**B**) RT-qrtPCR showing the expression of *C. fulvum* genes *CfPro1-59* during at 6, 8, 10 and 12 dpi as well as *in vitro* on PDB. Expression of genes is normalized to that of *C. fulvum β-tubulin* gene using the $E^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). Bars show the average of three biological replications with standard deviation. Protease family assigned to CfPro1-59 is indicated.

Proteome analysis shows secretion of some Cladosporium *fulvum* proteases into the apoplast during colonization of tomato

To detect PSPs at proteome level, apoplastic fluid was isolated from tomato cultivar Heinz at 12 dpi with race 0 of *C. fulvum* and analysed by mass spectrometry. Only Three proteases were detected using proteomics approach. In accordance with this finding, genes encoding these proteases were highly expressed when analysed by RNA-Seq and RT-qrtPCR. They include aspartic protease CfPro7 and the serine proteases CfPro40 and CfPro57 (Table 1). Altogether, these results show that *C. fulvum* secretes at least three fungal proteases, suggesting a role in fungal virulence.

DISCUSSION

Comparative genome analysis can provide a better insight in the the role of proteases in the complex lifestyles of fungal pathogens. Here, we analyzed and compared putatively secreted proteases (PSP) present in the genomes of thirty fungi with different lifestyles. To reduce the effects of genome annotation methods on the analysis, for each different lifestyle several fungi were selected. Overall, the genome of studied fungi contained many PSP genes encoding proteases belonging to aspartic, cysteine, serine and metallo- proteases. Although genes encoding serine and metalloproteases represent the majority of PSP genes in most genomes, some genomes showed different expansion patterns of PSP genes. For example, the saprotroph *Coprinopsis cinerea* contains only 18 serine protease genes compared to 47 metalloprotease genes. Hemibiotrophic fungus *Phanerochaete chrysosporium* contains 29 aspartic protease genes and only 20 serine and 8 metalloprotease genes. This difference might reflect adaptation to their ecological niche and might be of interest for more detailed future studies.

Among all proteases, subfamilies S8, S9, S10, M14, M20, M28, and A1 dominate in the genomes of fungal pathogens (Di Cera, 2009; O'Connell et al., 2012; Ohm et al., 2012). However, due to the limited number of functionally characterized proteases of

expanded subfamilies (Monod et al., 2002; Olivieri et al., 2002) and relatively unique subfamilies (such as M28, M35, M36 and M43) (Muszewska et al., 2011; Naumann et al., 2011), it is difficult to predict whether a protease family will be involved in virulence.

Our analysis showed that fungi with a hemibiotrophic or saprotrophic lifestyle contain significantly more PSP genes than biotrophic pathogens. This is in line with the observation that biotrophs employ less hydrolytic enzymes during plant colonization than hemibiotrophs (Spanu et al., 2010). For necrotrophs this distinction is less clear. The correlation of genome content with fungal lifestyle was also shown for *genes* encoding carbohydrate-active enzymes (CAZymes). They occur in lower numbers in symbionts and biotrophs as compared to hemibiotrophs and necrotrophs (Zhao et al., 2013).

We found that the biotrophic pathogen *C. fulvum* contains a number of PSP genes comparable with that of hemibiotrophs. Interestingly, *C. fulvum* also contains a large number of CAZyme genes (de Wit et al., 2012) as well as secondary metabolism genes (Collemare et al., 2014) which is not consistent with its biotrophic lifestyle. For CAZymes, it was found that many are not expressed during plant infection and many are pseudogenes in the genome of *C. fulvum* (de Wit et al., 2012). Expression studies of secondary metabolism genes of *C. fulvum* also showed most of them are down-regulated during plant infection (Collemare et al., 2014). Consistent with these observations, our results showed that more than 50% of the *C. fulvum* PSP genes were not expressed during plant infection, suggesting that this might be associated with a biotrophic lifestyle. Regulation of protease genes in fungi has been studied in human pathogens only. PrtT is a transcription factor of the fungal human pathogen *Aspergillus niger* and has a homolog in *Aspergillus fumigatus* that regulates the expression of fungal proteases involved in pathogenicity (Bergmann et al., 2009; Sharon et al., 2009). A homolog of PrtT with 43.2% identity is present in the genome of C. fulvum that might transcriptionally regulate the expression of protease genes in *C. fulvum* to sustain its biotrophy.

Eighteen PSP genes of *C. fulvum* were expressed at moderate level or comparable with the expression level of *actin* during both *in vitro* and *in planta* growth, meaning that these PSPs are unlikely involved in fungal virulence. However, a few protease genes such as *CfPro41* and *CfPro54* were specifically induced during plant infection and *CfPro19* that was higher expressed *in planta* than *in vitro*, and might therefore play a role in pathogenicity. To study the potential involvement of these proteases in pathogenicity their presence in apoplastic fluid of infected tomato leaves was analysed

by mass spectrometry. The PSPs CfPro7, CfPro40 and CfPro57 were identified, which all showed high levels of expression *in vitro* and *in planta.* Unexpectedly, the three proteases CfPro19, CfPro41 and CfPro54 that were mainly induced during plant infection were not identified at the proteome level. It is likely that they were secreted in early stages of infection as supported by their expression profile, and their concentration could have remained below the detection level at the studied time point. Alternatively, they could be absent in the apoplast if they would have been targeted into another compartment of fungal cells.

We found that S8 and S10 are two well expanded subfamilies of serine proteases in the genome of *C. fulvum*. S8 subfamily is also known as subtilisin-like serine protease and duplication and subsequent functional divergence of subtilisin-like serine protease has been shown in different groups of fungi (Bryant et al., 2009; Li et al., 2010). Gene duplication might have played an important role in the evolution of fungi and contributed to the development of pathogenicity and adaptation to different hosts or ecological niches (Hu and Leger, 2004). The S10 (serine carboxypeptidases) family also was expanded in *C. fulvum* as well as in other in fungi, but their role in pathogenicity is unknown (Ohm et al., 2012). Proteases *CfPro27, CfPro40* and *CfPro41* are members of subtilisin-like serine protease family, and *CfPro56* and *CfPro57* are members of S10 serine carboxypeptidase family, and their transcription and translation pattern suggests an active role in nutrition or virulence.

Our analysis showed that fungi with a hemibiotrophic and saprotrophic lifestyle contained more secreted protease genes than biotrophs. Overall, fungi show expansions of PSP genes encoding serine and metalloproteases. Remarkably, the number of protease genes in the genome of the biotroph *C. fulvum*, is comparable with that of hemibiotrophs and saprotrophs. However, transcriptome and proteome analyses showed most proteases are not expressed during plant infection, likely to contribute to its stealthy and biotrophic lifestyle. The few that were highly expressed during plant infection or detected by proteome analysis might play a role in virulence, but this needs to be further studied.

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Supplementary information:

Supplementary table S1. The Pfam domains corresponding to different subfamilies of proteases

PFAM domain	family	PFAM domain	family	PFAM domain	family
PF09668	Peptidase_A1	PF02127	Peptidase_M18	PF13688	Peptidase_M84
PF13650	Peptidase_A1	PF01244	Peptidase_M19	PF00089	Peptidase_S1
PF00026	Peptidase_A1	PF07687	Peptidase_M20	PF00082	Peptidase_S8
PF03051	Peptidase_C1	PF01546	Peptidase_M20	PF00326	Peptidase_S9
PF01650	Peptidase_C13	PF00814	Peptidase_M22	PF02897	Peptidase_S9
PF00656	Peptidase_C14	PF00557	Peptidase_M24	PF00450	Peptidase_S10
PF00648	Peptidase_C2	PF04389	Peptidase_M28	PF00574	Peptidase_S14
PF02902	Peptidase_C48	PF02102	Peptidase_M35	PF02129	Peptidase_S15
PF03568	Peptidase_C50	PF02128	Peptidase_M36	PF02190	Peptidase_S16
PF03577	Peptidase_C69	PF01434	Peptidase_M41	PF05362	Peptidase_S16
PF01433	Peptidase_M1	PF05572	Peptidase_M43	PF00717	Peptidase_S24
PF01400	Peptidase_M12	PF01435	Peptidase_M48	PF10502	Peptidase_S26
PF00246	Peptidase_M14	PF03571	Peptidase_M49	PF05577	Peptidase_S28
PF08367	Peptidase_M16	PF13398	Peptidase_M50	PF03572	Peptidase_S41
PF00675	Peptidase_M16	PF09768	Peptidase_M76	PF13365	Peptidase_S7-S46

The Pfam domains were retrieved from the Merops database (http://merops.sanger.ac.uk/index.shtml) (Rawling et al. 2013). In the family column, A, C, M and S represent the subfamilies of aspartic, cysteine, metallo and serine proteases.

Reference:

Rawlings, N.D., Waller, M., Barrett, A.J. and Bateman, A. 2013. MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Res.*, D503–D509.

Supplementary table S2. Primers used for RT-qrtPCR study.

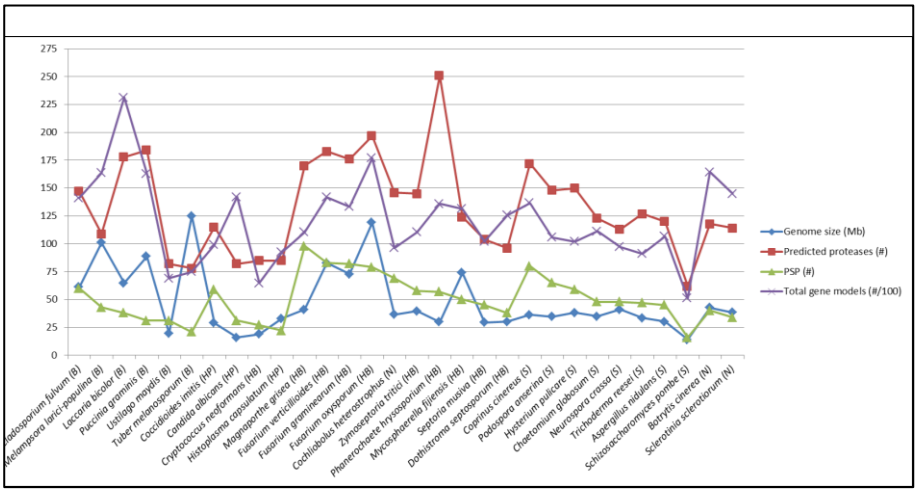
name	protein ID at JGI	protein ID at home website	primer efficiency	forward primer (5'-3')	reverse primer (5'-3')
CfPro1	192213	CFU-839018	2.0	GGTATGGTTCGCAAGAATGG	GCGGAGCCAGTATCAAAGTC
CfPro2	189675	CFU-834418	2.1	TCGGTACCCCTCATTGACTC	CTGAAGATCTGCGATGTGGA
CfPro3	194655	CFU-841396	2.0	CAATGAACGCAAAGAGGTCA	ATCGGCTCTCTTCCAGTTGA
CfPro4	196312	CFU-836875	2.1	GCTACGCAAACGACACGATA	AAGAGTTTGGGGCGTAGGTT
CfPro5	184463	CFU-837316	2.1	CTGGAGGCGCATACTACACA	GGGAGAAGCTTCGACTTCCT
CfPro6	186749	CFU-832191	2.0	TCACATAGAGCGGCAAGATG	TAGTCTCGCTGCCTTCCTGT
CfPro7	191012	CFU-827371	2.1	GCGAGATGTTCAAGGAGACC	CTAGGCACCCACAAGTTGGA
CfPro8	196569	CFU-837474	2.1	ATCGCGTTTCTGGTTCAACT	CGTGCCTTTGTCATCGTAGA
CfPro9	189132	CFU-838244	2.1	TCCAGATATTCCCAGCCAAG	TCATGTCCCACAGCTCAGTC

CfPro10	191719	CFU-830335	1.8	CCTTTGCATTGTCCCTGTTT	AGCTGTGCCAGTCCAGAGTT
CfPro11	193898	CFU-833585	1.9	TGACCCTCAACATCCTCTCC	GGCACTCCATTGGTCGTAGT
CfPro12	185983	CFU-836307	1.8	AGCCCCGGTTGAGTTTTACT	TTCGTGATCGTCAGTGCTTC
CfPro13	195657	CFU-828148	1.8	AGAACAAAGCCCTCGTAGCA	GCCTTGCTGCCTAGATCAAC
CfPro14	188917	CFU-829902	1.8	GCTCTACCGGCACTGCTATC	TGTCACTATGCGCTCTGTCC
CfPro15	197234	CFU-827618	2.0	TTCCACAAAACGCTTGTACG	CTCAGCATCCCTCGTCTTTC
CfPro16	194614	CFU-841355	2.0	CAGAAGCTGGCTTCGAGACT	CTCCCAGCCAGTAACATCGT
CfPro17	195127	CFU-839641	1.8	TCGACAGCGTGATTGAGAAC	CCAAGCAGACCGAACTCTTC
CfPro18	196054	CFU-840956	2.1	CATCGTCAGCGATAGCTTCA	CTCCCACTTCTCGTCCTCTG
CfPro19	184060	CFU-831357	1.8	TTCGTTCTTCTGGACCTGCT	CCGACCATCGGTCTGTATCT
CfPro20	185858	CFU-831635	1.9	CTTACACACTGCCCCGGTAT	AACTATCCGCGTTCAACACC
CfPro21	193187	CFU-837141	1.9	GCTCTACCGGCACTGCTATC	TGTCACTATGCGCTCTGTCC
CfPro22	183971	CFU-832535	2.1	GGTCTTTTCCACGTCTTCCA	GCAGTAGCCCTCTCAACCTG
CfPro23	190978	CFU-840590	2.1	ACGTGGCGTTCAACCTAGTC	CACCGAGGTCAGAGAGGAAG
CfPro24	192241	CFU-839046	2.1	CTAGCACGCACTTCAACGAG	GATTGGGGTGTATGCCTTGT
CfPro25	193444	CFU-833942	2.0	CGCCATAGCACAAGCATCTA	AGTGGTGGTCTCGGTATTGG
CfPro26	197426	CFU-838479	2.1	AACAAGGACGCTCCAATCAC	GCTAGGAGGTCAAGGCAGTG
CfPro27	185597	CFU-838618	2.2	GCTTTCGAACAAGCAAAACC	CAGATGCCATTGCTCTTGAA
CfPro28	197538	CFU-833170	2.1	TTGGCTCGGAAATCTCTCAT	TGAGTTTCTTCGGCTCGTTT
CfPro29	189824	CFU-832284	2.1	AATCTGCTGAAGGCGGAGTA	ACCGGTACCAACTCTGATCG
CfPro30	184985	CFU-834992	2.1	CCAAAGCGTCGGTATGAGAT	GCGCTGTCGTCGTAAACATA
CfPro31	188106	CFU-835521	2.1	TCTCCTCTCGCCACTGTCTT	CTCGATGCTCTTGATTGCAC
CfPro32	196045	CFU-840947	2.1	CACCTCTTGCAGGTGTCTGA	CCATCTTCGTGCTGGTAGGT
CfPro33	195779	CFU-830669	2.0	CCATCTTCGTGCTGGTAGGT	AGCCGACGAGATCAAGAAGA
CfPro34	190480	CFU-832156	2.1	CCCTCGAAGACATCGTTGAT	CAATCTCCTGGCTCTTCTGC
CfPro35	194207	CFU-829590	2.0	TGCTTCAGGCAACATCTGTC	CCTGCCTTTTCGTAGTCTCG
CfPro36	188663	CFU-836653	2.0	TACGAGAATGCCAATGGTGA	TGGAGGAAGCTGGTTCAACT
CfPro37	189334	CFU-834084	1.8	CGTCAGTCTGCACTGTTGGT	GTGCCGGAGATAGTGTTGGT
CfPro38	192396	CFU-827898	1.8	CGCAGCTACCAAAGTCATCA	CGCCCACTAGTACCGTTGTT
CfPro39	191659	CFU-838015	2.1	GGTTCACAGCACAAAGCTCA	ATCGCTAGCCCTGGAAAGAT
CfPro40	188827	CFU-835370	2.1	ACGACTGGGTCAAGGACATC	TCGAAGTGGCCAGAGTATCC
CfPro41	195145	CFU-839659	2.1	CAGCATTGTGGATGATGGAG	CCATAGTCTCGCTGTCGTGA
CfPro42	190628	CFU-836618	2.0	GGAGACGAAGATGGCCAATA	GAATGGCGATACTGCTTGGT
CfPro43	184779	CFU-835905	2.3	CTCTGCCTGCACCATACTCA	GCTCCTTGGTGATCCAGGTA
CfPro44	186960	CFU-840044	2.1	AAGGCACCAGCGAAAGTAAA	GCCGATGTCGAATGGTACTT
CfPro45	191132	CFU-834858	2.1	CATGTACAACCGCTTTCACG	CTCATTGAACAGCCCAAACA
CfPro46	184492	CFU-832559	1.8	CATGACCTCCATGAAACACG	GACCTCCGACTGAGACTTCG
CfPro47	185697	CFU-836710	2.0	CACGAATCTGGCATCTTCAA	AGCACGTCAGGAAAGAGGAA
CfPro48	192067	CFU-830406	2.0	TTCCTGAAGCCTCGACAAGT	TAGCATGGGCCAATCTCTTC
CfPro49	191799	CFU-840360	2.1	GAGCTTTCAAGGACCTGCAC	TAAGCATACGGCCAATGTCA
CfPro50	186241	CFU-832947	2.1	CTCGTTTCAACGACAGCGTA	GCCGGAGAACCACAAAGATA
CfPro51	191625	CFU-837981	1.9	CATCGGATACAAAGCAACCA	CTTGGTGGATCCGTAGGAGA
CfPro52	191773	CFU-837782	2.0	TCAGATCACAACGGCACAAT	CTTGAGAACAAGCCGACCAT
CfPro53	195806	CFU-834186	2.1	CCAGCGAACGTCACAGACTA	GTCGGCTCTCAAAGAACCAG
CfPro54	193034	CFU-834173	2.1	CTACCTTCGCCCAATTCGTA	GTGCCTCGAAGAACCAGAAG
CfPro55	185189	CFU-840016	1.8	TTTATGGCACAGACCACCAA	ACCGAGATGTTGCTGTACCC
CfPro56	197471	CFU-840496	2.1	GCTGGATTCCAAGTTTCACC	CAAATCACAAGGGGAGCATT
CfPro57	194708	CFU-828945	1.8	CAAGCTCCAGACATCGTTCA	GTTGGACTTCCTGGTGTGCT
CfPro58	194320	CFU-831196	1.9	TCCCCAGTAGTCCGAAACAG	CCTCACATTGGACAATGCAC
CfPro59	195456	CFU-832077	2.2	AGTTACCCAGTCGCCACAAC	AGAAGCCGTACGGAGAGTGA
Cf-actin	189818	CFU_832278	2.0	GGCACCAATCAACCCAAAG	TACGACCAGAAGCGTACAG
Cf-β-tubulin	186849	CFU_831764	2.0	CCTTCAGAGCTGTAACTGTCC	CCTCCTTCATAGATACCTTGCC

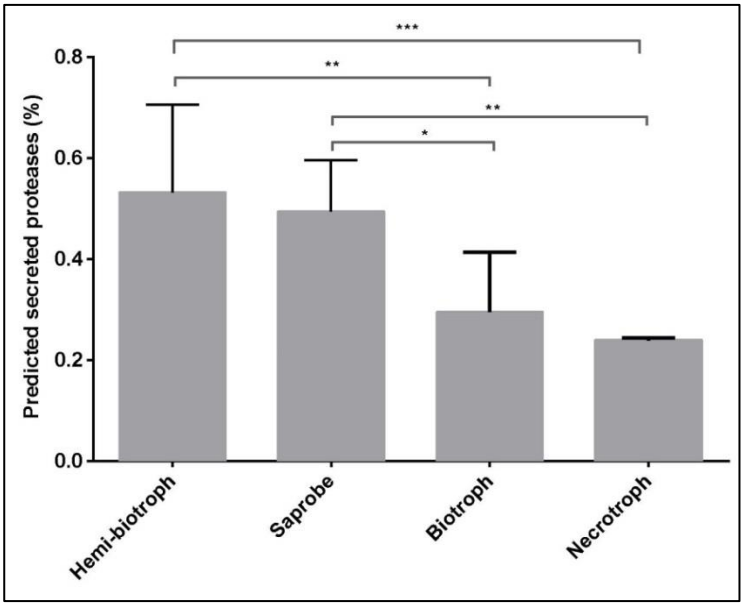
Supplementary table S3. Protease gene content present in fungal genomes.

No	fungus	lifestyle	Genome size	Gene models (#)	proteases per genome (#)	Secreted proteases per genome (#)	proteases per genome (%)	Secreted proteases per genome (%)	S (#)	M (#)	A (#)	C (#)
1	*Magnaporthe grisea*	HB	41.03	11054	170	98	1.537905	0.886557	44	36	16	2
2	*Fusarium verticillioides*	HB	82.88	14188	183	83	1.289822	0.585001	30	31	20	2
3	*Fusarium graminearum*	HB	72.91	13322	176	82	1.321123	0.615523	33	31	16	2
4	*Coprinus cinereus*	S	36.19	13657	172	80	1.259427	0.58578	18	47	13	2
5	*Fusarium oxysporum*	HB	119.16	17708	197	79	1.112492	0.446126	33	27	17	2
6	*Cochliobolus heterostrophus*	N	36.46	9633	146	69	1.515623	0.716288	36	19	12	2
7	*Podospora anserina*	S	34.72	10588	148	65	1.397809	0.613903	25	18	21	1
8	*Cladosporium fulvum*	B	61.1	14127	147	60	1.040561	0.424719	34	15	8	3
9	*Hysterium pulicare*	S	38.2	10200	150	59	1.470588	0.578431	40	10	8	1
10	*Coccidioides imitis*	HP	29.02	9910	115	59	1.160444	0.595358	32	22	4	1
11	*Zymoseptoria tritici*	HB	39.69	11044	145	58	1.31293	0.525172	28	17	11	2
12	*Phanerochaete chrysosporium*	HB	29.84	13602	251	57	1.845 317	0.419056	20	8	29	0
13	*Mycosphaerella fijiensis*	HB	74.14	13131	124	50	0.94433	0.380778	25	12	10	3
14	*Chaetomium globosum*	S	34.89	11124	123	48	1.105717	0.431499	22	9	16	1
15	*Neurospora crassa*	S	41.04	9730	113	48	1.161357	0.49332	17	15	15	1
16	*Trichoderma reesei*	S	33.4	9120	127	47	1.392544	0.515351	17	13	15	2
17	*Septoria musiva*	HB	29.35	10233	104	45	1.01632	0.439754	21	16	6	2
18	*Aspergillus nidulans*	S	30.24	10680	120	45	1.123596	0.421348	19	17	8	1
19	*Melampsora larici-populina*	B	101.13	16380	109	43	0.665446	0.262515	20	7	15	1
20	*Botrytis cinerea*	N	42.74	16447	118	40	0.717456	0.243205	18	6	14	2
21	*Dothistroma septosporum*	HB	30.21	12580	96	38	0.763116	0.302067	19	8	10	1
22	*Laccaria bicolor*	B	64.88	23132	178	38	0.769497	0.164275	12	3	17	6
23	*Sclerotinia sclerotiorum*	N	38.53	14503	114	34	0.786044	0.234434	17	7	9	1
24	*Puccinia graminis*	B	88.72	16309	184	31	1.128211	0.190079	17	6	8	0
25	*Candida albicans*	HP	16	14217	82	31	0.576774	0.218049	12	4	14	1
26	*Ustilago maydis*	B	19.66	6910	82	31	1.186686	0.448625	11	13	7	0
27	*Cryptococcus neoformans*	HP	19	6475	85	27	1.312741	0.416988	12	8	5	2
28	*Histoplasma capsulatum*	HP	33.03	9251	85	22	0.91882	0.237812	9	8	4	1
29	*Tuber melanosporum*	B	124.95	7496	78	21	1.040555	0.280149	11	7	2	1
30	*Schizosaccharomyces pombe*	S	14.1	5134	62	16	1.207635	0.311648	9	4	2	1

In the last four columns, S, M, A and C represent the serine, metallo-, aspartic and cysteine proteases.



Supplementary figure S1. Correlation of genome size, gene models, predicted proteases and predicted secreted proteases (PSP) across genomes of 30 fungi with different lifestyle.



Supplementary figure S2. Percentage of predicted secreted protease genes in the genomes of fungi with different lifestyles. A 1-way ANOVA with a *post-hoc* Tukey's multiple comparisons test was performed. Black asterisks indicate significant differences between the percentage of predicted secreted protease genes in the genomes of fungi with different lifestyles. (* p-value < 0.5; ** p-value < 0.01; *** p-value < 0.001).

Chapter 3

Pseudogenization in pathogenic fungi with different host plants and lifestyles might reflect their evolutionary past

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ABSTRACT

Pseudogenes are genes with significant homology to functional genes, but contain disruptive mutations (DMs) leading to the production of nonor partially functional proteins. Little is known about pseudogenization in pathogenic fungi with different lifestyles. Here, we report the identification of DMs causing pseudogenes in the genomes of the fungal plant pathogens *Botrytis cinerea*, *Cladosporium fulvum*, *Dothistroma septosporum*, *Mycosphaerella fijiensis*, *Verticillium dahliae* and *Zymoseptoria tritici*. In these fungi, we identified 1740 gene models containing 2795 DMs obtained by an alignment-based gene prediction method. The contribution of sequencing errors to DMs was minimized by analyses of re-sequenced genomes to obtain a refined dataset of 924 gene models containing 1666 true DMs. The frequency of pseudogenes varied from 1% to 5% in the gene catalogues of these fungi, being the highest in the asexually reproducing fungus *C. fulvum* (4.9%), followed by *D. septosporum* (2.4%) and *V. dahliae* (2.1%). The majority of pseudogenes do not represent recent gene duplications, but members of multi-gene families and unitary genes. In general, there was no bias for pseudogenization of specific genes in the six fungi. Single exceptions were those encoding secreted proteins, including proteases, which appeared more frequently pseudogenized in *C. fulvum* than in *D. septosporum*. Most pseudogenes present in these two phylogenetically closely related fungi are not shared, suggesting that they are related to adaptation to a different host (tomato versus pine) and lifestyle (biotroph versus hemibiotroph).

INTRODUCTION

Pseudogenes show homology to functional genes, but contain disruptive mutations (DMs) leading to non or partially functional proteins (Yang et al., 2011). A pseudogenization event caused by a single DM can result in a premature stop, frameshift, defective splice junction or distortion of regulatory sequences required for transcription (Yang et al., 2011; Zhang et al., 2010). Similarly, a transposon insertion dramatically alters gene continuity, but also represents a single DM event leading to pseudogenization. Most eukaryotic pseudogenes are disabled copies of duplicated parental genes (Gerstein et al., 2007) and the majority will eventually disappear, whereas some will evolve new functions and might become fixed in an organism (Lynch and Conery, 2000). Unitary pseudogenes are single-copy genes that may become nonfunctional through loss-of-function (LOF) variation caused by various types of mutation (Balasubramanian et al., 2011; Zhang et al., 2010). A residual biological function might develop for genes encoding multi-domain proteins that have lost only

one or a few of their functional domains. However, when a lost domain in a unitary pseudogene is essential and is not compensated for by another protein, the LOF variant will affect the performance of an organism (Zhang et al., 2010). LOF variants and unitary pseudogenes have been reported to cause several inheritable human diseases (Zhang et al., 2010). However, in some cases, an organism might also profit from pseudogenization, as for pathogens and commensals which need to adapt and co-evolve with their hosts.

When only a few DMs are present, pseudogenes still bear all the hallmarks of a protein-encoding gene, and *ab initio* gene prediction software will probably predict gene models at these loci, as also in the case of the absence of splice sites or the presence of a premature stop codon. Therefore, DMs will often cause erroneous gene model predictions. This is also true for sequencing errors (SEs) in genomic sequences that introduce in-frame stops by erroneous base calling or distortion of reading frames by insertions or deletions (indels). Thus, SEs can cause incorrect assignment of DMs and incorrect assignment of pseudogenes (Balasubramanian et al., 2011).

The extent of pseudogenization in (plant pathogenic) fungi has not been studied at a whole genome scale to date, although numerous reports have described individual genes that have been subjected to pseudogenization. Selection pressure imposed on plant pathogenic fungi by plant disease resistance genes has led to the rapid development of pseudogenes, whose parental genes encode effectors that are recognized by matching resistance gene-encoded receptor-like proteins (Stergiopoulos et al., 2007a; Westerink et al., 2004). Repeat-induced point mutations (RIPs) can cause pseudogenization by introducing premature stop codons by C to T and G to A transitions. RIP occurs in sexually active fungi mainly belonging to the Ascomycetes, where it was first discovered in Neurospora crassa (Galagan and Selker, 2004). Genes directly adjacent to repeats are at risk of being pseudogenized when RIP activity slightly protrudes the repeat locus boundaries. This has been shown in the oil seed rape pathogen *Leptosphaeria maculans*, where pseudogenization of the AvrLm1 effector gene is caused by RIP (Gout et al., 2007).

Here, we report the identification of DMs causing pseudogenes in the fungal plant pathogens *Botrytis cinerea*, *Cladosporium fulvum*, *Dothistroma septosporum*, *Mycosphaerella fijiensis*, *Verticillium dahliae* and *Zymoseptoria tritici*. From these six fungi, we have identified many DMs obtained by an alignment-based fungal gene prediction method (van der Burgt et al., 2014). The frequency of pseudogenes was highest in the gene catalogues of the phylogenetically related fungi *fulvum* (4.9%) and *D. septosporum* (2.4%). There was no clear bias for pseudogenization of specific genes

in these two fungi, except for those encoding secreted proteins, including proteases, and genes involved in the production of secondary metabolites, such as dothistromin. The biotrophic tomato pathogen *C. fulvum* shares many genes with the hemibiotrophic pine pathogen *septosporum*, but the gene set affected by pseudogenization in the two fungi is not shared. A possible role of pseudogenization and, eventually, gene loss in adaptation to a different host and lifestyle is discussed.

EXPERIMENTAL PROCEDURES

Fungal genomes used in this study

The genomes, proteomes, annotations and available unigenes of five fungal species were downloaded from the Fungal Genome Initiative of the BROAD Institute (Cuomo and Birren, 2010) and the Fungal Genomics Program of the US Department of Energy Joint Genome Institute (JGI) (Grigoriev et al., 2012) in collaboration with the user community. The data from the *C. fulvum* genome and transcriptome were generated at Wageningen University (de Wit *et al*., 2012) and are also available on the JGI MycoCosm website (Grigoriev *et al*., 2012).

Alignment-based fungal gene prediction

Genes with predicted DMs were obtained by genome-wide gene model assessment of six fungi with the ABFGP method (van der Burgt et al., 2014). Gene loci from 29 different fungi, mainly belonging to the Ascomycetes, served as informant DNA sequences for alignment-based assessment of the genes in the six target genomes (Table S1).

Distinguishing SEs from true DMs

SEs among predicted DMs were identified by comparing a 200-nucleotide window around each predicted DM with base calling in Illumina-based assemblies from *Z. tritici* strains STIR04_A26b and STIR04_A48b (62-fold versus 8.9-fold coverage of the reference genome of *Z. tritici* IPO323) (Stukenbrock *et al*., 2011), *V. dahliae* strain JR2 (30-fold versus 7.5-fold coverage of the *V. dahlia* VdLs.17 genome) (de Jonge *et al*., 2013), *M. fijiensis* strain CIRAD139a (25-fold versus 7.1-fold coverage of the *M. fijiensis* CIRAD86 genome) and *B. cinerea* strain B05.10 (50-fold versus 4.5-fold coverage assembly of the same isolate) (Staats and van Kan, 2012). All DMs that could not be confirmed as truly occurring in the population of these fungi were removed from the DM dataset (Method S1). In addition, DMs that were discovered to be falsely predicted by analyses of EST data (11 DMs, Table S2) and gene models containing

short, contiguous stretches of n-characters directly adjacent to DMs (seven DMs, data not shown) were removed.

Determination of the closest protein homologue and gene family size

For each protein, the closest homologue (in the proteome of the fungal species) was determined as the protein with the highest bit score of concatenated alignments (BLASTP), requiring the alignment to span at least 60% of the length of both proteins. A simple estimation of gene family size was performed by counting the number of proteins with a score of at least 200 bits, requiring the same alignment length.

Third-party software

Predicted (pseudo-) protein sequences of the gene loci with DMs were searched for putative secretion signals using SignalP 3.0 (Emanuelsson *et al*., 2007) and known PFAM protein domains with InterproScan (Hunter *et al*., 2009). Unigenes were aligned to their genomes using Genome Threader version 1.1.1.2 (Kurtz *et al*., 2005).

Confirmation of base calling and mRNA splicing in *Cladosporium fulvum* genes

Five *C. fulvum* genes encoding secreted proteases with predicted DMs were selected for confirmation of genome base calling and intron splicing. The original sequences were obtained from the published genome of *C. fulvum* race 0WU (CBS131901) (de Wit *et al*., 2012). The sequences at and around a DM site were amplified with the primers provided in Table S3. The presence of the DMs was analysed in six different *C. fulvum* isolates from different geographical origins with different virulence spectra and mating types (Table S4). From *C. fulvum* CBS131901, total RNA was isolated from mycelium grown under different *in vitro* and *in planta* conditions (Table S3) and the amplified cDNA fragments (using the same primer pairs) were evaluated for the occurrence of splicing around the DMs.

Repeat identification and RIP analyses

Repeats were determined using mummer (-maxmatch -nosimplify) (Kurtz *et al*., 2004), as segments of at least 250 nucleotides that are present in at least five copies in a given genome. RIP analysis was performed as described for *C. fulvum* and *D. septosporum* in de Wit *et al*. (2012).

RESULTS

The genomes of *C. fulvum* and *D. septosporum* have recently been released (de Wit *et al*., 2012). The Alignment-Based Fungal Gene Prediction (ABFGP) method (van der Burgt et al., 2014) was applied to six fungal genomes in order to identify DMs that would cause pseudogenization. Gene models predicted by ABFGP represent exons which are chained by both introns and DMs. The ABFGP method recognized DMs in genes which resulted in frame shifts (non-3*n* indels) or an in-frame stop codon when compared with homologous informant genes from several different fungi lacking the DMs. In multiple protein sequence alignments, the DMs are recognized as extension of conservation (i) throughout annotated introns, (ii) upstream of annotated start codons or (iii) downstream of annotated stop codons (Fig. 1). In all cases, high sequence similarity is shared with corresponding exonic parts of informant genes. Predicted DMs coincided predominantly with incorrectly predicted introns (Fig. 1a,c), truncated predicted proteins (Fig. 1a,b) and, rarely, in a single gene split into two gene models (Fig. 1c).

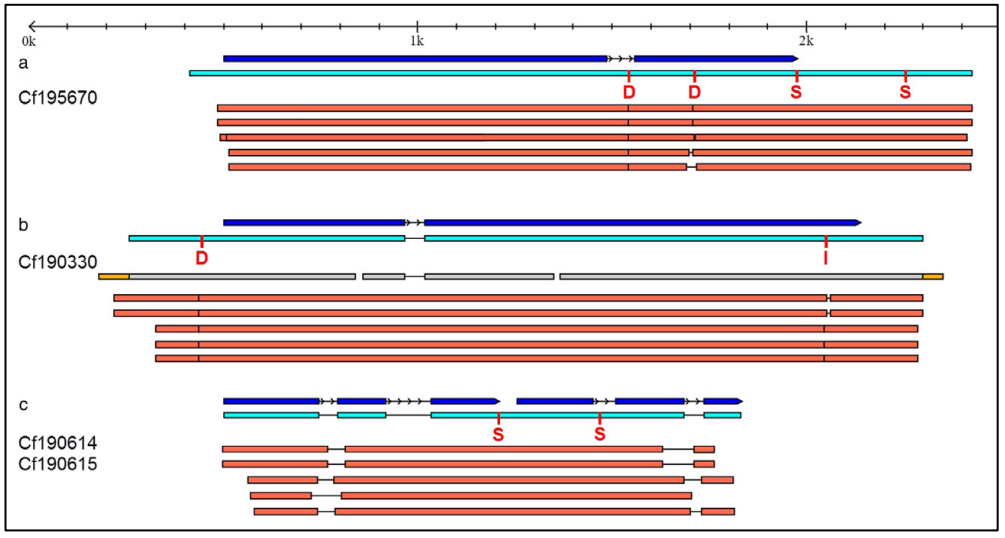

Figure 1. Examples of *ab initio*-predicted gene models that should have been designated as pseudogenes according to the Alignment-Based Fungal Gene Prediction (ABFGP) method. Three samples of GeneMark-ES-predicted gene models (blue) compared with ABFGP-predicted (van der Burgt et al., 2014) pseudogene models (cyan) containing disruptive mutations (DMs; marked in red). DMs are labelled as insertion (I), deletion (D) or in-frame stop (S). Available expressed sequence tag (EST) data were manually annotated as coding sequence (CDS) (grey) or untranslated region (UTR) (orange). (a) *Cf195670*, gene encoding an unknown protein; (b) *Cf190330*, gene encoding an oxidoreductase; (c) *Cf190614/Cf190615*, gene presumably encoding a glycosyl hydrolase. The genes are taken from the gene catalogue of *Cladosporium fulvum* (de Wit *et al*., 2012). Five randomly chosen BLASTX similarities (brown) against proteins from the nonredundant and Trembl database indicate approximate coding regions and, in all cases, support the pseudogene model extensions and the false gene split for *Cf190614* and *Cf190615*.

Table 1. Biological properties, genome sizes and gene content of six fungal species.

Species	Mode of reproduc	Life style	Size (Mb)	Genomic coverage (fold)	Ref	No. of genes	Studied genes
B. cinerea	Sexual	Necrotroph	43.4	4.5	Amselem et al. (2011)	16448	8504
C. fulvum	Asexual*	Biotroph	61.1	21.1	de Wit et al. (2012)	14127	7575
D. septosporum	Asexual†	Hemibiotroph	31.2	34.2	de Wit et al. (2012)	12580	8091
M. fijiensis	Sexual	Hemibiotroph	74.4	7.1	JGI	10313	7285
V. dahliae	Asexual‡	Hemibiotroph	34.4	7.5	Klosterman et al.(2011)	10535	8362
Z. tritici	Sexual	Hemibiotroph	40.3	8.9	Goodwin et al. (2011)	10952	7904

*Sexual stage unknown (Stergiopoulos *et al*., 2007b; Thomma *et al*., 2005).
†For *D. septosporum*, both mating types have been reported, but reproduction is predominantly asexual (Dale *et al*., 2011). The sequenced *D. septosporum* NZE10 was isolated from a population in New Zealand that contains only one mating type and has only reproduced asexually since its introduction in the 1960s.
‡For *V. dahliae*, both mating types have been reported, but reproduction is predominantly asexual (Usami *et al*., 2009).

Table 2. High-quality set of 1666 presumed true disruptive mutations (DMs) in 924 genes.

Species	All DMs	Genes with DMs	Genes (%)	Substitutions	Indels
Botrytis cinerea	130	82	1.0	66	64
Cladosporium fulvum	565	372	4.9	256	309
Dothistroma septosporum	497	194	2.4	247	250
Mycosphaerella fijiensis	97	60	0.8	47	50
Verticillium dahliae	308	173	2.1	121	187
Zymoseptoria tritici	69	43	0.5	34	35
Total	1666	924		771	895

Genes with predicted disrupted mutations

Around 8000 predicted gene models for each of the six selected fungi were assessed by ABFGP (van der Burgt et al., 2014) using informant genes from up to 28 different fungal species (Table S1, see Supporting Information). The biological properties and genome statistics of the six fungi belonging to the class of Ascomycetes are shown in Table 1. From this dataset, we retrieved the gene models with predicted DMs, resulting in a subset of 1713 genes (ranging from 68 to 567 affected genes per species) containing 2762 DMs in total for the six fungal species. The number of SEs occurring in sequenced genomes is expected to be inversely related to genome coverage. This renders the prediction of DMs in *Z. tritici*, *V. dahliae*, *M. fijiensis* and *B. cinerea* (in decreasing order

of genome coverage) less reliable than in the genomes of *C. fulvum* and *D. septosporum*, which have been sequenced using next-generation sequencing techniques at 21-fold and 34-fold coverage, respectively (de Wit *et al*., 2012). From those genomes with low coverage, DMs that could not be confirmed by resequencing (or sequencing related isolates) were scored as incorrect and removed from the DM dataset (Method S1, see Supporting Information). This accounted for 39 (34%), 453 (54%), 105 (46%) and 363 (72%) SEs in the four fungi, which indeed correlates with sequence coverage. A 100nucleotide window surrounding a predicted DM in *C. fulvum* was inspected in the genome assembly for coverage, correct base calling and the presence of poly pyrimidine tracts. No indication of SEs was observed (data not shown). This refinement yielded a final set of 1662 presumed true DMs in 924 genes which were used throughout this analysis (Table 2). The predicted ancestral protein products of the 924 genes are provided in Data file S1 (see Supporting Information).

As DMs recognized by ABFGP are located in exons of their functional homologues, we conclude that DMs are present in mature mRNAs and not in the introns. For five of six of the studied fungi, we aligned available unigene data to their genomes to verify whether predicted DMs overlapped with exons or introns (Table S2, see Supporting Information). Many of the identified pseudogenes appeared to be expressed (72% and 74% of the genes from *C. fulvum* and *D. septosporum*, respectively). In total, 572 DMs were covered by expressed sequence tags (ESTs), confirming that they occurred in exons. In all cases in which a DM was overlapping with a predicted intron (as in the first deletion in Cf195670 shown in Fig. 1), EST data indicated the absence of splicing. Only 11 DMs (1.9%) matched to introns and have therefore wrongly been predicted as DMs. The latter number reflects the false discovery of DMs by ABFGP. Interestingly, three of these 11 wrongly predicted DMs matched to alternatively spliced transcripts with intron retention around the DM site.

Although examination of unigene data indicated at least 98% accuracy in appointing DMs by ABFGP, we decided to more closely examine and experimentally confirm several of them. DMs were not chosen at random, but all predicted DMs in a particular class of genes in *C. fulvum*, namely secreted proteases, were selected. Five protease genes with predicted DMs (Fig. 2) were re-sequenced in the type strain and in six additional isolates of *C. fulvum* originating from different parts of the world (Tables S3 and S4, see Supporting Information). All DMs were confirmed and appeared identical in all seven isolates analysed: two collected in the Netherlands, two collected in Cuba and two collected in Japan. Seven of eight DMs coincided with introns predicted by GeneMark-ES (Ter-Hovhannisyan *et al*., 2008), which were all in conflict with observed expression data.

This suggests that the predicted introns are incorrect and represent DMs. To validate this, cDNA libraries from the sequenced *C. fulvum* reference strain (CBS131901) grown in different conditions were analysed (Table S3). The results confirmed that, except for Cf189824, all genes were clearly expressed and in none of the tested growth conditions was support for splicing of any of the wrongly predicted introns observed (data not shown). For the second DM leading to protein truncation of Cf186241, the cDNA covered the complete ancestral protein, suggesting that the parental gene locus once produced a functional transcript. All genes encode proteins with crucial functional domains interrupted by or downstream of the first encountered DM (Fig. 2). Based on these results, we conclude that none of them produce mRNAs that can be translated in a functional protease.

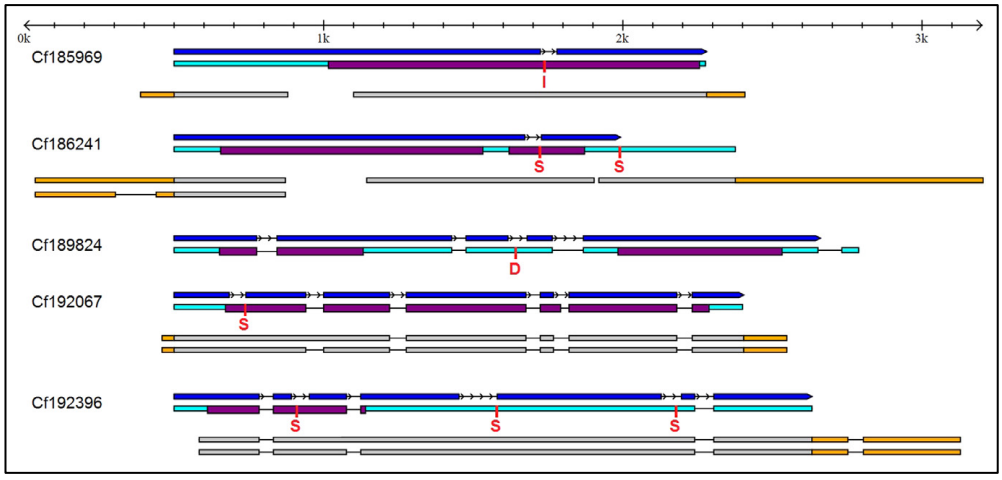

Figure 2. Pseudogenization of genes in *Cladosporium fulvum* encoding secreted proteases. Gene models and predicted PFAM domains (purple) of five pseudogenized secreted proteases of *Cladosporium fulvum*; for explanation of colors and symbols, see Fig. 1. For *Cf192067*, its fourth exon is incorrectly predicted by the Alignment-Based Fungal Gene Prediction (ABFGP) method, For *Cf189824*, an additional 3' exon is predicted by high-confidence sequence alignment to informant genes (data not shown).

Analysis of 1662 DMs in 924 genes

The 1662 DMs identified in 924 genes could be subcategorized as nucleotide substitutions (46%) and indels (54%) (Fig. 3a). Indels were based on the DNA sequences of informant genes estimated to represent nucleotide deletions (30%) and nucleotide insertions (24%). The frequencies of these subcategories appeared to be fairly similar for the different species; they varied from 39% to 50% for substitutions (Fig. 3b). The point mutations leading to the stop codons TAG, TGA and TAA accounted

for 49%, 27% and 23%of in-frame stops, respectively (Fig. 3c). These frequencies are as expected based on the notion that transitions occur more frequently than transversions and on the observed codon usage in *C. fulvum* and *D. septosporum* (Method S2, see Supporting Information).We conclude that the observed types of mutations result from random DNA mutations. Remarkably, only 14 pseudogene models contained long stretches of N-nucleotides which might represent repetitive sequence as a result of inserted transposons, as discussed later.

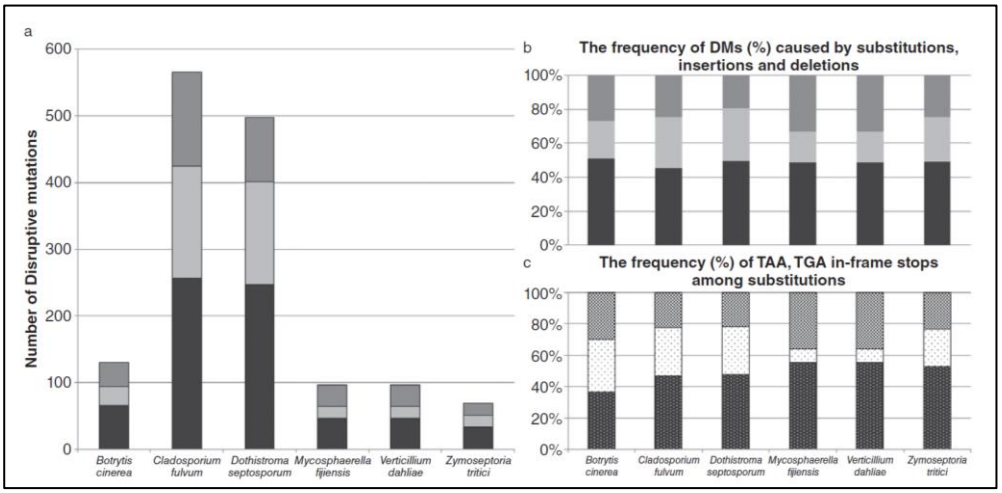

Figure 3. Disruptive mutations (DMs), types of DMs and their frequency. (a) Numbers of DMs caused by substitutions (black), insertions (dark grey) and deletions (light grey) after removal of sequencing errors in the six different fungi. (b) The frequency of DMs (%) caused by substitutions, insertions and deletions in the six different fungi. (c) The frequency (%) of TAG (black), TGA (white) and TAA (grey) in-frame stop codons observed in DMs representing substitutions from the six different fungi.

Pseudogenes with DMs are evenly distributed over the genome

If transposon insertion or RIP plays a significant role in the creation of pseudogenes, they will occur more frequently in the direct vicinity of repeats that might have undergone RIP. Other biased genomic distributions of pseudogenes could point to the preference of specific chromosomes, specific parts of chromosomes or gene clusters. Only 105 (11%) of the pseudogenes are located within a distance of 1 kb from a repeat or scaffold end (Fig. S1, see Supporting Information), and only 32 pseudogenes (3.4%) are located close to repeat areas that have undergone RIP (Fig. S2, see Supporting Information). Only 14 pseudogenes embedded a repeat within their

coding sequence (Data file S1), which most probably represents genes inactivated by transposon insertion. On average, pseudogenes were 26.3 kb apart from repeats and, for the extremely repeat-dense *C. fulvum* genome (de Wit et al., 2012), the average distance was 14.5 kb. Therefore, we conclude that the presence of repeats and RIP activity were of minor importance in the evolution of the pseudogenes studied here. The pseudogenes not only lacked a positional bias towards repeats, but no general trends for chromosome enrichment or positional enrichment towards other pseudogenes could be observed. In general, pseudogenes are evenly distributed over the chromosomes of *Z. tritici* and *D. septosporum*. (Tables S5 and S6, see Supporting Information). No enrichment of pseudogenes on the dispensable chromosomes of *Z. tritici* was observed. We observed a median distance of one pseudogene per 147 kb, with the exception of *C. fulvum*, where this number was, on average, one pseudogene per 34.8 kb (Fig. S3, see Supporting Information). The observed median and average inter-pseudogene distances indicate that pseudogenes do not tend to cluster together, although occasionally (nearly) adjacent gene pairs were pseudogenized. In *D. septosporum* and *C. fulvum*, the species with the most pseudogenes, in total 23 pairs of directly adjacent pseudogenes were observed (Tables S7 and S8, see Supporting Information). This is slightly more than that expected based on chance only (data not shown); therefore, all pairs were inspected for being a member of a gene cluster. In the pseudogene-rich *C. fulvum*, some clear examples of functionally related, adjacent pseudogenes were found: a quartet of four adjacent pseudogenes which are involved in carbohydrate metabolism (Cf186934–Cf186937) and a triplet that encodes a putative chitinase, amino acid transporter and phosphodiesterase/alkaline phosphatase (Cf191135–Cf191137), respectively (Table S8).

A bias for pseudogenization of members of multi-gene families and secreted proteins

For each gene and pseudogene, the (global) amino acid similarity to their most similar protein-encoding homologue in the complete protein catalogue was determined (Fig. 4a). In addition, the total number of potential homologues was counted to express membership and size of a multi-gene family. In total, 682 pseudogenes, representing 74% of all DM-containing pseudogenes, share 45%–75% similarity with at least a single homologous, non pseudogenized gene, which is more than the genomic average. The majority of this class of pseudogenes has more than one homologue (Fig. 4b), suggesting that multi-gene families seem to be more frequently affected by

pseudogenization. When comparing the multi-gene family size of this class with all multigene families, no significant difference, increase or decrease, in gene family size could be observed.

Genes encoding proteins that are less than 45% similar are less affected by pseudogenization (Fig. 4a). Based on these findings, we made an arbitrary distinction between recent gene duplicates (>75% similarity), single-copy genes (<45% similarity) and genes that share between 75% and 45% sequence similarity. Remarkably, the set of 924 pseudogenes is not enriched for recent gene duplications, as expected on the basis of the general observation made in other higher eukaryotes (Gerstein et al., 2007). Figure 4a shows that recent gene duplications not only occur rarely in the six studied genomes, but are also not enriched for pseudogenes. At the proposed threshold of, at most, 45% similarity, 22% of all pseudogenes (8–62 per species and 203 in total) can be classified as single-copy, unitary pseudogenes (Balasubramanian et al., 2011; Zhang et al., 2010). Pseudogenization of genes belonging to multi-gene families suggests that some members might be redundant. In contrast, pseudogenization of unitary genes most probably have a direct impact on the functional repertoire of an organism.

Because the studied fungi are all plant pathogens which manipulate their host by means of secreted proteins, pseudogenization of genes encoding this class of protein was studied in more detail. Between one and 51 genes encoding secreted proteins appeared to be pseudogenized (Fig. 5). On average, secreted proteins account for around 10% of all proteins in these pathogenic fungi. In *C. fulvum*, pseudogenes encoding secreted proteins are significantly overrepresented, but are significantly underrepresented in *M. fijiensis* (but it should be noted that only small numbers of pseudogenes are present in the latter fungus). Remarkably, the percentage of pseudogenes used to encode secreted proteins was twice as high in *C. fulvum* as observed in its close *relative D. septosporum.*

Estimation of LOF among the 924 genes with DMs

A pseudogene can cause LOF or a change in function of the encoded protein, but detailed functional analyses are required to draw reliable conclusions. To address this question by an *in silico* approach, we quantified protein length truncation and the number of lost PFAM domains which are located downstream of the most 5' DM in the gene (Table S9, see Supporting Information). The results are summarized in Fig. 6; 824 of the encoded proteins (89%) are truncated by more than 50% or have lost at least one functional domain. In contrast, only 75 proteins (8%) are truncated by less

than 30% without having lost a single known protein domain. Based on these numbers, we assume that the vast majority of genes with DMs no longer encode a functional protein, or encode a protein that no longer fulfils its ancestral function.

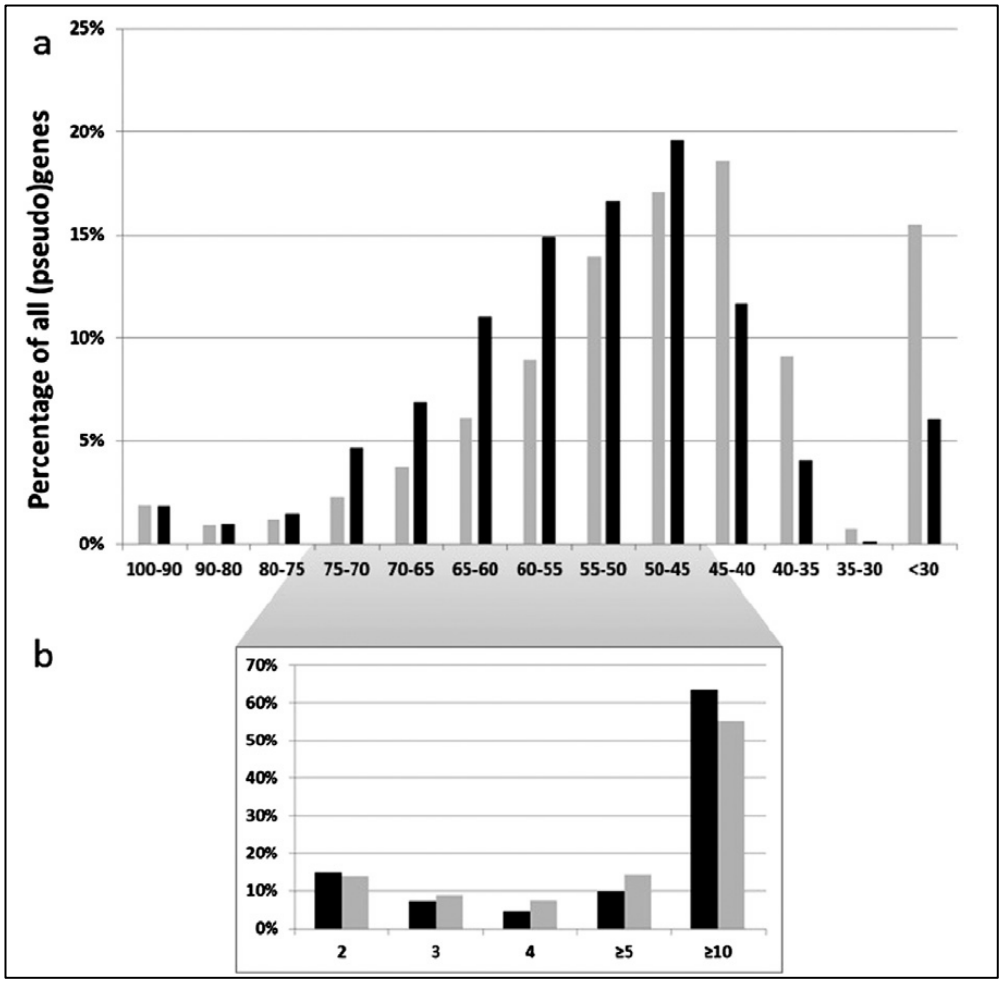

Figure 4. (a) Pairwise amino acid sequence similarity of proteins encoded by pseudogenes and their most similar functional homologue present in the predicted proteome. (b) A bias for pseudogenization of multi-gene families. Gene family size distribution of (pseudo)genes with at least a single functional homologue (between 45% and 75% homology). All proteins (n = 74 955) are compared with all pseudogenes (n = 924) in six species in the specified range of similarity. Gene family membership thresholds are set to a bit score of ≥200 (BLASTP) and similarity ≥60% of the protein's length.

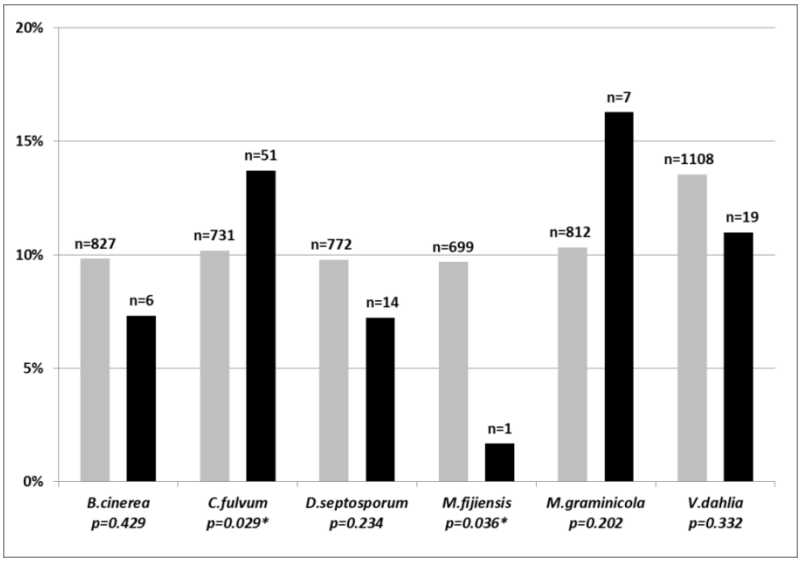

Figure 5. Pseudogenization of genes encoding secreted proteins in six different fungi. Frequency (%) of genes encoding secreted proteins (grey bars) and of pseudogenes encoding secreted proteins (black bars) for six different fungi. Total number of (pseudo)genes analysed per species is shown on top of the bars. Two-tailed Z-test statistics were calculated (http://socscistatistics.com); at $P < 0.05$.

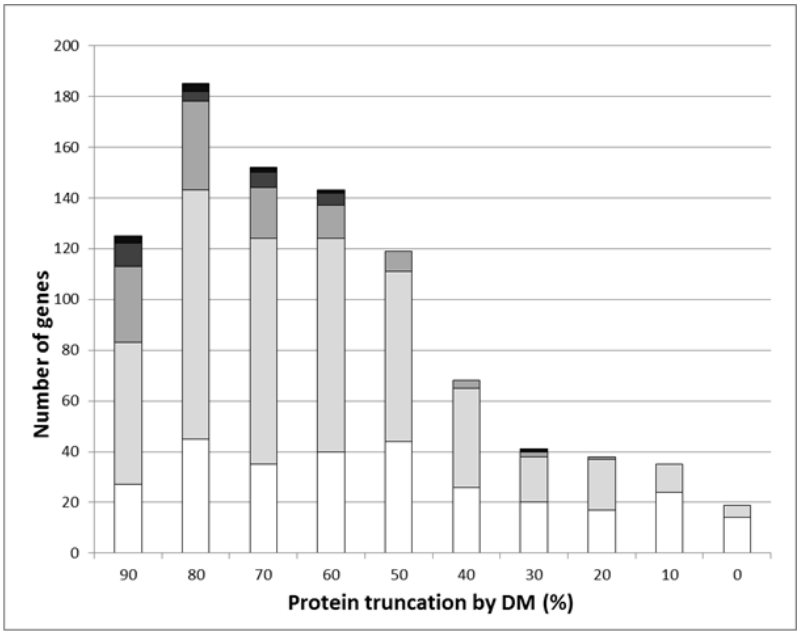

Figure 6. Protein truncation and number of PFAM domains lost by truncation caused by the first 5' disruptive mutation (DM). Truncation of proteins is expressed as a percentage of the total protein length; the number of PFAM domains lost by the first 5' DM is indicated ina greyscale from 0 (white), 1, 2, 3 to ≥4 (black).

DISCUSSION

The ABFGP method recognized many DMs in genes which resulted in frameshifts (non-3*n* insertions) or in-frame stop codons when compared with functional informant genes from fungi lacking these DMs. Closer inspections of the four re-sequenced genomes showed that a large fraction of DMs appeared to be SEs. Genes in genomes sequenced with low coverage contained considerable numbers of SEs in regions of protein-encoding genes (*B. cinerea*, *V. dahliae*, *M. fijiensis* and *Z. tritici*) which hampered the correct assignment of gene models. The occurrence of thousands of SEs in the original reference genomes was independently shown in *B. cinerea* (Staats and van Kan, 2012) (Method S1). This is probably also the case for many other fungal genomes that have been sequenced in the era of low-coverage Sanger sequencing.

Estimation of the extent of pseudogenes in fungi

In this study, we identified 924 pseudogenes in the gene catalogues of six different fungi, representing 0.5%–4.9% of their annotated genes. This number is probably a strong underestimation of the total extent of pseudogenization in these fungi because of the selection of informant genes. In this section, evidence for underestimation is provided and discussed. The examples provided are all chosen from *C. fulvum* and *D. septosporum*, where a high level of pseudogenization was observed during the analysis of their genomes (de Wit *et al*., 2012). As a start, only 7300–8500 of the annotated genes per species were analysed for the occurrence of pseudogenization of genes that were shared among 28 fungi. Genes that were not eligible for ABFGP were highly divergent, short, clade or species-specific-like effector genes. Only a few effectors are shared among fungi and most are species specific, but they can also be subject to pseudogenization when selection is imposed. A clear example of pseudogenization of a species-specific effector has been reported for the *Avr2* gene of the tomato pathogen *C. fulvum* (Luderer *et al*., 2002). Other examples are the homologues of the *C. fulvum* effector proteins *Ecp4* and *Ecp5*, which were identified as pseudogenes in *D. septosporum* because of the presence of in-frame stop codons (de Wit *et al*., 2012). These pseudogenes were not identified in this study because of the absence of close homologues in the gene catalogues of the 28 fungi used.

Furthermore, DMs were called by ABFGP (van der Burgt et al., 2014) in regions supported by strong sequence similarity to exons of informant genes. DMs in regions with poor similarity support, directly adjacent or even in splice sites, translational start sites or promoter regions were not addressed by ABFGP, but their contribution to

pseudogenization can be significant. An example is the key secondary metabolite (SM) pseudogene (*Pks9*) in *C. fulvum* (de Wit et al., 2012), which was not detected in our study, because of a single in-frame stop codon only 16 nucleotides upstream of the donor sequence of its third (EST-supported) intron. Finally, when several DMs or dramatic DMs (e.g. transposon insertions) are present in a gene, gene prediction software is likely to predict a fragmented (e.g. the third example in Fig. 1), highly truncated gene or predict no gene model at all. This might account for a rather large number of genes, which is emphasized by the failure of detection of six of seven manually annotated key SM pseudogenes in *C. fulvum* (de Wit et al., 2012). As a result of the occurrence of DMs, *Nps5* was predicted to be divided over two separate gene models and *Nps7*, *Nps10* and *Hps2* even over three separate gene models. *Nps1* is highly truncated, most probably by the transposon insertion (which concurrently marks the end of its contig), whereas *Pks4* has several adjacent DMs which resulted in a predicted, but not existing, 864-nucleotide intron. In all instances, recruitment of suitable informant gene loci failed for these (fragmented) gene models, explaining why the gene model was not present in the set of genes assessed by ABFGP.

In general, the (manual) annotation efforts invested in the gene catalogues of each of the six studied species vary significantly and might have affected the quality of the integral gene catalogue considerably, for instance by prior removal of obvious pseudogenes. This indicates that the practical delimitation to (somewhat properly) annotated gene models might have introduced a methodological bias in this study, resulting in the failure of detection of pseudogenes that contain more or dramatic DMs, which is the most obvious category to be resolved by manual curation of a gene catalogue. To further investigate this issue, we decided to perform an additional experiment which compared the gene catalogues of closely related species. In a pairwise comparison, genes unique to one species can be the result of gene gains in that species, gene losses in the other or unannotated genes caused by pseudogenization (Zhang et al., 2010). Such an analysis was performed on our dataset using the closely related Capnodiales species *C. fulvum*, *D. septosporum* and *Z. tritici*, and unambiguously identified 674 additional pseudogenes on loci lacking annotated gene models or containing misannotated fragments of longer genes (Method S3, Table S10, Data file S2, see Supporting Information). In this pseudogene dataset, *C. fulvum* again stands out in terms of the total number of pseudogenized genes. Among these genes were several (types of) pseudogenes that were expected. Approximately one-half of the pseudogenes are listed in the gene catalogue of these species, but as truncated, incorrectly predicted genes. Second, a small proportion was identified as

being disrupted by repetitive sequence(s), which most probably represent transposon insertions and can explain why they were not predicted as genes by the gene prediction software. A higher incidence of putative transposon insertions in *C. fulvum* relative to *D. septosporum* is probably correlated with the much higher repeat content of the former. The additionally identified pseudogenes do not alter the lower degree of pseudogenization in *Z. tritici* relative to *C. fulvum* and *D. septosporum*. Overall, we conclude that the actual number of pseudogenes in these six fungi is considerably higher than that described in this study as assigned by ABFGP (van der Burgt et al., 2014). Thus, the dataset described in this study represents only a subset of pseudogenes that are listed in current gene catalogues. DMs and SEs together account for a high error rate in current gene catalogues, which hampers *in silico* comparative genomics analyses.

Pseudogenes occur more frequently in asexually reproducing fungi

Significant differences in the frequency of DMs occur in the six different fungi. The highest frequency of DMs was observed in the two related fungi *C. fulvum* and *D. septosporum.* These large differences in the level of pseudogenization might be related to their mode of reproduction (Table 1). Species which, apart from asexual reproduction, also reproduce sexually, such as *B.* cinerea, *M. fijiensis* and *Z. tritici*, show lower numbers of pseudogenes when compared with those that reproduce predominantly asexually, such as C. fulvum (Stergiopoulos et al., 2007b; Thomma et al., 2005), *D. septosporum* (Dale *et al*., 2011) and *V. dahlia* (Usami et al., 2009). Deleterious DMs in sexually reproducing Ascomycetes can be either lost or restored after recombination and selection. It is assumed that haploid asexual fungi will initially adapt more rapidly to a new environment than sexually reproducing relatives. Pseudogenization of genes which are no longer required, not of advantage or even deleterious for a pathogen might enable it to quickly adapt to new environments, including new host plants.

The set of 924 pseudogenes that was identified in the six fungal genomes did not show a biased genomic distribution. The only exception to a random distribution over chromosomes is the occasional occurrence of (nearly) adjacent pseudogenes with related functions, suggesting that more than one gene of the same pathway is affected (Tables S7 and S8). Unexpectedly, no preference for pseudogenes in the vicinity of repeats, whether or not affected by RIP, was observed. A relationship between repeats and pseudogene/gene loss has been suggested in powdery mildew fungi as a result of retrotransposon insertions in the absence of RIP (Spanu et al., 2010), whereas RIP

activity in *Leptosphaeria maculans* clearly affected nearby located genes (Gout et al., 2007). In addition, in fungi that are assumed to reproduce asexually, RIP signatures have been observed (Oliver, 2012), as is the case in *C. fulvum* and *D. septosporum* (de Wit et al., 2012). This indicates that these fungi once were sexually active, but lost their ability to reproduce sexually, or sexual reproduction occurs only rarely (Dale et al., 2011). Extensive RIP activity will dramatically affect the continuity of a coding sequence, and ab initio gene prediction will probably not predict a gene on a RIP-affected locus. Because the dataset of 924 pseudogenes was retrieved from catalogues of predicted genes, it is expected to be underrepresented for pseudogenes caused by RIP. The same holds true for genes inactivated by transposon insertion; indeed, only 14 gene models with putative transposon insertions were identified. The apparent underrepresentation of pseudogenes with transposon insertions was further addressed by the additional *in silico* experiment on the three Capnodiales species discussed above (Table S10), where a small number of additional pseudogenes of this type were identified. However, even when these additional pseudogenes are taken into account, the contribution of transposon insertions to pseudogenization is of minor importance compared with indels and substitutions.

Pseudogenes in multigene families and unitary pseudogenes

Our analyses showed that fungal pseudogenes are not predominantly associated with recent gene duplications, but occur predominantly in multi-gene families; 74% of all pseudogenes have a closest homologue within the 45%–75% similarity range and, of these, 70% belong to multi-gene families of at least five members (Fig. 4b). One could argue that predominantly (partially) redundant genes become randomly pseudogenized. For example, high throughput gene knock-out studies in *Schizosaccharomyces pombe* (Decottignies et al., 2003; Spirek et al., 2010) showed that 17.5% of genes, when knocked out, caused a lethal phenotype. Most knock-out mutants gave no or weak phenotypes, suggesting some level of functional redundancy for many genes. However, these conclusions can only be drawn when supported by ecological studies performed on populations, enabling a comparison of the fitness of wild-type and knock-out mutants under different environmental conditions. Therefore, we expect that pseudogenization of members of multiple gene families is probably involved in subtle adaptations of fungi to different environmental conditions, whereas pseudogenization of unitary genes is expected to have more drastic effects on phenotypes, including even beneficial ones, when they would facilitate adaptation to a new environment. However, attributing pseudogenes in a multigene family to mere

redundancy is not supported by the reported functional diversification in gene families. Proteases cluster into several gene families based on sequence similarity in their functional domain(s), yet have very distinctive substrate specificities (Hedstrom, 2002; Monod et al., 2002; Yike, 2011). In Fig. 4a, they fall into the class of sharing intermediary similarity to a non-pseudogenized homologue and being putative members of a multi-gene family. Some, but not all, proteases have been reported to cause tissue necrosis, and their pseudogenization might suppress this phenotype (Gilroy et al., 2007).

Pseudogenization of genes encoding secreted proteins and key SM genes in *C. fulvum* might reflect host adaptation

In *C. fulvum*, genes encoding secreted proteins showed a higher frequency of pseudogenization and, among these, were five secreted proteases. The eight DMs in these five genes were confirmed by various approaches. It is tempting to speculate that pseudogenization of genes encoding secreted proteases could be related to the lifestyle of *C. fulvum*. Many proteases are known to induce senescence and sometimes cell death, which could facilitate some plant pathogenic fungi to kill plants and retrieve nutrients from necrotized or dead plant cells. *C. fulvum* is a biotrophic fungus thriving in the apoplast in close contact with mesophyll cells of tomato leaves, where it lives on nutrients released by the host, either passively or induced by fungal effectors. Only at very late stages of infection do host cells collapse. The ancestor of *fulvum* is not known, but it is closely related to *D. septosporum*, a pine pathogen that behaves as a hemibiotroph, killing host cells after a short biotrophic phase (de Wit et al., 2012).At the proteome level, they are remarkably similar, and their genomes share extended regions of mesosynteny, which accounts for about 70% of all genes and facilitates robust inference of orthology. When comparing the complete pseudogene catalogues of both species, only 22 pairs of closest homologues are pseudogenes in both species (Table S11, see Supporting Information). In these pairs, not a single individual DM is shared (compare with Data file S1, see Supporting Information). Over 85% of the pseudogenes in either of the two species have non pseudogenized closest homologues in the other species, many of which are indisputable orthologues based on their presence in mesosyntenic areas. In a few cases (fourand23, representing 2% and 6%), the pseudogenized gene is absent in one of the two species. These 23 pseudogenes in *C. fulvum* could represent quickly diversifying genes, C. fulvum-specific gene gains followed by pseudogenization, or genes which have been lost in *D. septosporum* at an early time point after species divergence, and in future might await the same fate in *C.*

fulvum. Given the fact that these genes have easily recognizable closest homologues in many more distantly related Ascomycetes, the latter hypothesis seems most likely.

These observations suggest that many, if not all, of the observed DM events leading to pseudogenization in *C. fulvum* and *D. septosporum* occurred post-speciation. After the occurrence of a first DM, the gene's locus is expected to exhibit neutral evolution, until it is lost from the genome. A discrepancy between the speed at which neutral evolution takes place and the speed at which small genomic segments are lost might explain why relatively large numbers of pseudogenes can accumulate in two genomes that have recently speciated. Pseudogenization of some of these genes could provide an advantage to these fungi in adapting to a new host, and the lack of a sexual cycle could accelerate the process of adaptation for these haploid fungi. The effector genes Ecp4 and Ecp5, known to be involved in the virulence of *C. fulvum* on tomato, are pseudogenes in *D. septosporum* (de Wit et al., 2012). Similarly, two crucial genes of the pathway that produces the toxin dothistromin during the infection of pine by *D. septosporum* are pseudogenes in *C. fulvum* (Chettri et al., 2013). Apparently, selection pressure on losing certain genes or, conversely, the necessity to maintain certain genes, may act on a different set of genes in these two fungi, which could reflect adaptation to different host plants. We speculate that the pseudogenization of genes involved in the production of SMs, secreted proteases and other damaging enzymes might be one of the reasons why the ancestor of C. fulvum might have been a hemibiotrophic tree pathogen, such as *D. septosporum*, but started to live as a biotroph when it became pathogenic on tomato because of the pseudogenization of some of the genes encoding these enzymes. It would be interesting to determine whether removal of the stop codons in a number of the pseudogenized protease genes would make *C. fulvum* more aggressive and possibly also hemibiotrophic on tomato. In addition, further research into the recent ancestry of *C. fulvum* and *D. septosporum* could date the pseudogenization events and therefore the speed at which pseudogenization takes place.

Our study not only supports the generally accepted fact that fungal genomes contain pseudogenes, it demonstrates that some actually have a larger number of pseudogenes than others, and that many pseudogenes are still listed as bona fide genes in gene catalogues. Therefore, we argue for the need to provide fungal (functional) gene annotations not only as a gene catalogue, but also with a pseudogene catalogue counterpart. Comparative genome studies rely heavily on the predicted gene catalogues, but these can be hampered by the occurrence of pseudogenes that fail to be identified. Moreover, the pseudogene arsenal of a species might still reflect an echo

of the legacy of an earlier, at that point in time, optimal gene repertoire. In particular, for plant pathogenic fungi, the comparison of several complete pseudogene repertoires might reveal interesting facts about their recent evolutionary past, which could provide an insight into their current host specificity and pathogenicity.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site (http://dx.doi.org/10.1111/mpp.12072)

Fig. S1 Distance of pseudogenes to repetitive sequence or scaffold boundary.

Fig. S2 Distance of pseudogenes from repeat-induced point mutation (RIP) loci.

Fig. S3 Inter-pseudogene distances.

Table S1 The 28 informant species used for Alignment-Based Fungal Gene Prediction (ABFGP).

Table S2 Expression evidence of pseudogenes and false discovery estimation of disruptive mutations (DMs) by Alignment-Based Fungal Gene Prediction (ABFGP) measured on unigene data.

Table S3 Sequences of primers used for resequencing disruptive mutations in secreted proteases of *Cladosporium fulvum*.

Table S4 Six strains of *Cladosporium fulvum* used to confirm the presence of predicted disruptive mutations (DMs) in secreted proteases.

Table S5 Pseudogene distribution over the chromosomes of *Zymoseptoria tritici*.

Table S6 Pseudogene distribution over the chromosomes of *Dothistroma septosporum*.

Table S7 Directly adjacent pseudogenized gene pairs and triplet in *Dothistroma septosporum.*

Table S8 Directly adjacent pseudogenized gene pairs and triplet in *Cladosporium fulvum*.

Table S9 Functional prediction of 924 pseudogenes by assignment of PFAM domains.

Table S10 Hundreds of additional pseudogenes in the Capnodiales species *Cladosporium fulvum*, *Dothistroma septosporum* and *Zymoseptoria tritici* detected on the basis of similarity to known proteins.

Table S11 Twenty-two pseudogenes shared between *Cladosporium fulvum* and *Dothistroma septosporum*.

Method S1 Removal of sequence errors from predicted disruptive mutations (DMs) by comparison with next-generation sequencing assemblies of four genomes with low coverage.

Method S2 Observed pattern of substitutions is explained by determinants of random DNA mutation alone.

Method S3 Additional pseudogenes in *Cladosporium fulvum*, *Dothistroma septosporum* and *Zymoseptoria tritici* identified by pairwise comparisons.

Datafile S1 Protein sequences of 924 pseudogenes including 1662 disruptive mutations.

Datafile S2 General Feature Format (GFF) annotation of 674 additional pseudogenes identified in the Capnodiales species *Cladosporium fulvum*, *Dothistroma septosporum* and *Zymoseptoria tritici.*

Chapter 4

Synergistic action of a metalloprotease and a serine protease from *Fusarium oxysporum* f. sp. *lycopersici* cleaves chitin-binding tomato chitinases, reduces their antifungal activity and enhances fungal virulence

Karimi Jashni, M., Dols, I.H.M., Iida, Y., Boeren, S., Beenen, H.G., Mehabi, R., Collemare, J., and de Wit, P.J.G.M. 2015. **Synergistic action of a metalloprotease and a serine protease from *Fusarium oxysporum* f. Sp. *lycopersici* cleaves chitin-binding tomato chitinases, reduces their antifungal activity and enhances fungal virulence.** Molecular Plant-Microbe Interaction (in press).

ABSTRACT

As part of their defence strategy against fungal pathogens, plants secrete chitinases that degrade chitin, the major structural component of fungal cell walls. Some fungi are not sensitive to plant chitinases because they secrete chitin-binding effector proteins that protect their cell wall against these enzymes. However, it is not known how fungal pathogens that lack chitin-binding effectors overcome this plant defence barrier. Here, we investigated the ability of fungal tomato pathogens to cleave chitin-binding domain (CBD)-containing chitinases and its effect on fungal virulence. Four tomato CBD-chitinases were produced in *Pichia pastoris* and incubated with secreted proteins isolated from seven fungal tomato pathogens. Of these, *Fusarium oxysporum* f. sp. *lycopersici, Verticillium dahliae* and *Botrytis cinerea* were able to cleave the extracellular tomato chitinases SlChi1 and SlChi13. Cleavage by *F. oxysporum* removed the CBD from the N-terminus, as shown by mass spectrometry, and significantly reduced the chitinase and antifungal activity of both chitinases. Both secreted metalloprotease FoMep1 and serine protease FoSep1 were responsible for this cleavage. Double deletion mutants of *FoMep1* and *FoSep1* of *F. oxysporum* lacked chitinase cleavage activity on SlChi1 and SlChi13 and showed reduced virulence on tomato. These results demonstrate the importance of plant chitinase cleavage in fungal virulence.

INTRODUCTION

Upon recognition of general elicitors, basal defences are induced in plants, which confer resistance to a wide range of pathogens. An important component of these defences is the accumulation of Pathogenesis-Related (PR) proteins (Sels *et al.*, 2008). Indeed, PR proteins have low or no expression in healthy plants, but their expression is strongly induced upon infection by various plant pathogens (Godoy *et al.*, 1996; Joosten and Wit, 1989). Many PR proteins show antifungal activities, with a major role for those that exhibit *β*-1, 3-glucanase and chitinase activities (Cota *et al.*, 2007; Sinha *et al.*, 2014). Both types of enzymes act synergistically to inhibit the growth of many fungi (Jongedijk *et al.*, 1995; Mauch *et al.*, 1988; Sela-Buurlage *et al.*, 1993) through the hydrolysis of *β*-1,3-glucans and chitin (*β*-1,4-linked *N*-acetylglucosamine), which are major structural components of fungal cell walls (Balasubramanian *et al.*, 2012; Grover, 2012). Hydrolysis of *β*-1,3-glucans and chitin leads to thinning of hyphal tips, followed by swelling and eventually bursting (Arlorio *et al.*, 1992). Antifungal activity of chitinases has been exploited to improve broad-spectrum resistance of plants. For example, plants such as tobacco, tomato, potato, peanut and cacao engineered to

over-express chitinases show enhanced resistance to fungal pathogens (Cletus *et al.*, 2013; Dana *et al.*, 2006; Iqbal *et al.*, 2012; Maximova *et al.*, 2006; Schickler and Chet, 1997).

Based on primary amino acid sequence of the catalytic domain, chitinases are grouped into two glycosyl hydrolase family 18 (GH18) or 19 (GH19). GH19 family is largely composed of the plant chitinases GH19, while GH18 are found throughout the tree of life (Grover, 2012; Hamid *et al.*, 2013; Li and Greene, 2010). Based on their amino acid sequence similarity, GH18 chitinases are divided into two structural classes, III and V (Ohnuma *et al.*, 2011), and GH19 chitinases into four classes I, II, IV and VII (Ohnuma *et al.*, 2012). Class I and class IV chitinases are distinct in sequence but they all contain a chitin-binding domain (CBD), which is absent in class II and class VII chitinases (Lu *et al.*, 2003). Binding of the CBD to chitin was reported to increase the enzymatic efficiency of CBD-chitinases and, expectedly, removal of the CBD diminishes their antifungal activity (Iseli *et al.*, 1993; Suarez *et al.*, 2001). All plant chitinases harbour a signal peptide to enter the secretory pathway. In addition, class I chitinases contain a C-terminal vacuolar localization signature that directs them to the vacuole (Neuhaus *et al.*, 1991; Wubben *et al.*, 1992). Other chitinases are extracellular, residing in the plant apoplast (Neuhaus *et al.*, 1991).

The role of chitinases in plant-fungal pathogen interactions has been well studied for the interaction between tomato (*Solanum lycopersicum*) and the leaf mould pathogen *Cladosporium fulvum*. This fungus is strictly extracellular and colonizes the apoplastic space between the mesophyll cells of tomato leaves (de Wit *et al.*, 2012). Although it has been reported that infection by *C. fulvum* induces the synthesis of tomato chitinases in the apoplast (Joosten and Wit, 1989), this fungus is not sensitive to plant chitinases (Joosten *et al.*, 1995). It was suggested that this is due to secretion of the chitin-binding effector protein CfAvr4 that binds to the chitin in the cell wall of *C. fulvum* (van den Burg *et al.*, 2006). Indeed, CfAvr4 was shown to bind chitin in the cell wall of *Trichoderma viride* and *Fusarium solani* f. sp. *phaseoli* and to protect them against the hydrolytic activity of plant chitinases (van den Burg *et al.*, 2006). Functional homologs of CfAvr4 were identified in other Dothideomycete plant pathogens, in which they also likely protect cell walls against plant chitinases (de Wit *et al.*, 2012; Stergiopoulos *et al.*, 2010). Noteworthy, vascular and necrotrophic tomato pathogens like *Fusarium oxysporum* f. sp. *lycopersici* (*F. oxysporum*), *Verticillium dahliae, F. solani* f. sp. *phaseoli*, *Botrytis cinerea*, and *Alternaria spp*. do not have a homolog of the *CfAvr4* gene in their genomes. Among these pathogens, *F. solani* f. sp. *phaseoli* was shown to be sensitive to chitinases (van den Burg *et al.*, 2006; van Esse

et al., 2007). Although these fungal pathogens lack a functional Avr4 homolog, they still overcome the deleterious effects of chitinases during plant infection. The fact that these fungal pathogens could overcome the resistance of plants over-expressing chitinases suggests that they employ mechanisms to compromise the deleterious effects of plant chitinases. Previously, proteolytic processing of a class IV chitinase during infection of bean roots by *F. solani* f. sp. *phaseoli* (Lange *et al.*, 1996) and *in vitro* proteolytic processing of a tobacco vacuolar class I chitinase by culture filtrate of the same fungus were reported (Sela-Buurlage, 1996). Also, an extracellular protease from *F. solani* f. sp. *eumartii* was shown to degrade PR proteins from the intercellular washing fluids of potato (Olivieri *et al.*, 2002). More recently, it was shown that *Fusarium verticillioides* and other maize pathogens, including *Bipolaris zeicola* and *Stenocarpella maydis*, secrete proteins that truncate maize class IV chitinases (Naumann, 2011; Naumann and Wicklow, 2010; Naumann *et al.*, 2009). Altogether these data strongly suggest that some fungal pathogens may inactivate induced host chitinases, and possibly other PR proteins, by proteolytic cleavage.

In the present study, we address the hypothesis that fungal pathogens lacking chitin-binding CfAvr4 homologs secrete proteases to cleave plant chitinases to overcome their deleterious effects. To this aim, we investigated the cleavage activity on antifungal CBD-chitinases of the model crop tomato by several of its fungal pathogens. We report the expression of tomato CBD-chitinases during infection by *C. fulvum* and *F. oxysporum* as representatives of an extracellular foliar (carrying *CfAvr4*) and a vascular tomato pathogen (lacking a *CfAvr4* homolog), respectively. We assessed the proteolytic activity of these two species, and five additional tomato pathogens, on four CBD-chitinases produced in *P. pastoris*. We show that three fungal tomato pathogens secrete proteases that cleave two of the four extracellular CBD-chitinases, resulting in complete or partial removal of the CBD and reduction of their antifungal activity. Finally, we identified the secreted proteases in *F. oxysporum* that are responsible for the cleavage of CBD-chitinases and showed that they play an important role in virulence of this fungus.

MATERIALS AND METHODS

Plant and fungal materials

The susceptible tomato cultivar Money Maker Cf-0 (MM-Cf-0) was used for all *in planta* studies including expression profiling, proteomics of foliar apoplastic fluid and xylem sap, and virulence assays. Tomato plants were grown in a greenhouse at 70% relative humidity, 23-25°C during daytime and 19-21°C at night, with a light/dark

regime of 16/8 hours and 100 W m^{-2} supplemental light when the sunlight influx intensity was less than 150 W m^{-2}. The fungal strains *Alternaria solani* (CBS 347.79), *Alternaria arborescens* (CBS 102605) and *Fusarium solani* (CBS 835.85) were obtained from CBS-KNAW Fungal Biodiversity Centre, Utrecht, Netherlands. Strains of *Cladosporium fulvum* (strain R97; race 0, WU-CBS131901), *Verticillium dahliae* (strain VdLs17; race 1), *Botrytis cinerea* (strain B05.10) and *Fusarium oxysporum* f. sp. *lycopersici* (strain 4287, VCG 0030) were present in the -80°C glycerol stock of our laboratory.

Virulence assays

Inoculation of tomato cultivar MM-Cf-0 with *C. fulvum* was performed as previously described (Ökmen *et al.*, 2013). Fungal spore suspensions calibrated at 10^6 spores/mL were sprayed on the abaxial side of tomato leaves. Leaf samples were collected at different days post inoculation for further studies. For virulence assays with *F. oxysporum,* roots of 10-day-old MM-Cf-0 tomato seedlings were dipped into a spore suspension of this fungus (calibrated at 10^7 spore/mL) for 1 minute, and then planted into 13 cm^2 pots. Plant height, disease index and fresh weight of upper part of plants were analysed four weeks after inoculation following a previously described method (Michielse *et al.*, 2009). Disease index was measured as follows: a slice of the hypocotyl was cut 1 cm above ground level and the number of brown vessels was counted, representing the disease index. Virulence assays for single and double deletion mutants were performed in a similar way as described for the wild type with three biological replications.

Genome mining of tomato chitinases

Amino acid sequences of plant chitinases belonging to each class were retrieved from the NCBI website (www.ncbi.nlm.nih.gov), and used as query sequences for tblastn searches against the tomato genome (http://solgenomics.net/). Furthermore, InterPro domains corresponding to different classes of plant chitinases (Table S2) were used for further genome mining. For each predicted gene potentially encoding a chitinase, signal peptide and pfam domains were predicted using the SignalP 4.1 server (Petersen *et al.*, 2011) and pfam database (Finn *et al.*, 2014), respectively. Domains, active sites, and catalytic residues were predicted for tomato chitinases based on similarity to previously described chitinases. For phylogenetic analysis, sequences of each class were aligned using Muscle (Edgar, 2004). Poorly aligned positions were removed using the Gblock server with less stringent parameters settings (Talavera and

Castresana, 2007). The curated alignments were used to build maximum likelihood phylogenic trees (JTT amino acid substitution model, four categories of gamma distributed substitution rates, using all sites and 500 bootstrap replications) with MEGA 5.1 (Tamura *et al.*, 2011).

Expression analysis of tomato CBD-chitinases

Expression of tomato CBD-chitinases was studied in leaves and roots of MM-Cf-0 tomato plants. Inoculations were performed with *C. fulvum* and *F. oxysporum* as described above. Leaf samples were collected at 0 (healthy plant), 4, 6, 8, 10 and 12 days post inoculation (dpi) with *C. fulvum* and were immediately frozen in liquid nitrogen. Root samples were collected at 0 (healthy plant), 4, 8, 12 and 20 dpi with *F. oxysporum,* properly washed in water and immediately frozen in liquid nitrogen. Total RNA was isolated from fully grinded samples using the Quick-RNA miniprep kit (Zymo Research) as provided by the company. RNA concentration and integrity was checked with a NanoDrop^{TM} 1000 spectrophotometer (Thermo Scientific) and on 1% agarose gels, respectively. For each sample, 1 µg of total RNA was used for cDNA synthesis using the First-Strand cDNA Synthesis Kit (Promega) according to manufacturer's instructions. Primer pairs for tomato chitinase genes (Table S3) were designed with Primer3 Plus (Untergasser *et al.*, 2007), and their efficiency and specificity were determined with a dilution series of genomic DNA of MM-Cf-0. Quantitative real-time (qrt) PCR was performed using the 7300 System (Applied Biosystems, Foster City, CA, USA): each reaction consisted in 25 µL, containing 12.5 µL Sensimix (Bioline, London, UK), 1 µL of each forward and reverse primer (7.5 µM), 1µL of 10x diluted template cDNA and 9.5 µL of double-distilled water. 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. A previously designed primer pair was used to amplify the reference gene *LeTub* (accession: DQ205342) (Aimé *et al.*, 2013), which allowed to normalize the expression of other genes. Results were analysed using the $E^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). Experiments included three biological replications.

Isolation of apoplastic fluids and xylem sap

Apoplastic fluids were isolated from MM-Cf-0 inoculated with *C. fulvum* at 12 dpi using a previously described method (de Wit and Spikman, 1982). Apoplastic fluids were cleared by centrifugation (12,000xg; 20 min) at 4 °C and were concentrated 3 times using 1 kDa cut-off Amicon filters. For xylem sap isolation, 10 plants of MM-Cf-0

were inoculated with *F. oxysporum* and stems were cut off at 2 cm above ground level five weeks later. Xylem sap was harvested using a previously described method (Rep *et al.*, 2002). Fifty mL xylem sap were cleared by centrifugation (12,000xg; 20 min) at 4 °C and dialyzed against 10 mM sodium acetate pH5.2 buffer using standard dialysis membrane (spectrum®labs) cut-off 3.5 kDa. The sample was then freeze-dried and dissolved in 0.5 mL of 10 mM sodium acetate pH5.2 buffer.

Cloning of CBD-chitinase genes, yeast transformation and protein production

Coding sequences of the six genes encoding CBD-chitinases (SlChi1, SlChi2, SlChi3, SlChi4, SlChi13 and SlChi14) were amplified from cDNA of healthy leaves using the primer pairs indicated in Table S4. Purified PCR fragments were ligated into the *pPIC9* expression vector following the method previously described (Kombrink, 2012). Sequence and orientation of insertion was confirmed for each construct. The different expression vectors were then linearized and transformed into *P. pastoris* GS115 strain (Kombrink, 2012).

Three positive transformants of *P. pastoris* for each construct were screened for protein production in small induction flasks and western blots were performed with the crude protein culture to select the transformant with the highest protein expression. Only colonies transformed with *SlChi1*, *SlChi2*, *SlChi4* and *Slchi13* produced sufficient amounts of the corresponding proteins. This transformant was used for large scale protein production in a fermenter (Bioflo 3000) as previously described (Kombrink, 2012). Yeast culture filtrates were centrifuged (4,000xg; 20 min). Supernatants were retrieved and centrifuged again (12,000xg; 20 min) at 4°C. Cell-free supernatants were concentrated using a 10 kDa cut-off filter (Pall Nanosep®) to 50 mL and run on a 10 mL Ni-NTA column (Qiagen) at 4°C. The column was washed with 20 mL washing buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, and 10 mM imidazole) and proteins were eluted with 20 mL elution buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, and 250 mM imidazole). Collected fractions containing the protein of interest were combined, dialyzed against 10 mM sodium acetate pH 5.2 buffer, concentrated using 3 kDa Amicon filters, and adjusted to a concentration of 1 mg/mL.

Preparation and fractionation of fungal secreted protein extracts

All fungal species were grown on 1% Oxoid™ Potato Dextrose Agar (PDA) plates containing 100 µg/mL of streptomycin sulfate salt (Sigma-Aldrich). Spores were harvested from PDA plates using sterile water for all fungi apart from *F. oxysporum* and *F. solani*. For these two fungi, 20 mL of Difco™ Potato Dextrose Broth (PDB) liquid

medium containing 100 µg/mL streptomycin sulfate salt (Sigma-Aldrich) was inoculated with a mycelium plug taken from PDA plates and incubated in an orbital shaker incubator at 22 °C and 200 rpm for 3 days. All fungal mycelia were filtered out using Miracloth (Calbiochem) and subsequently spores were collected by centrifugation at 3,700xg for 15 min, re-suspended in sterile water and adjusted at 10^6 spores/mL. Erlenmeyer flasks containing 25 g of fresh leaves from 4-week-old susceptible MM-Cf-0 tomato plants were autoclaved (15 p.s.i., 30 min), inoculated with 3 mL of calibrated spore suspensions and incubated as a still culture for 7 days at 22°C. Secreted proteins were isolated using a previously described method (Naumann *et al.*, 2011). Briefly, 5 g of inoculated autoclaved tomato leaves were incubated with 5 mL of extraction buffer (50 mM sodium acetate pH 5.2, 1 mM EDTA, 2 mM PMSF, 2 mM ascorbic acid) at 4°C for 30 min. The mixture was centrifuged at 4,000xg for 5 min at 4°C and supernatants were centrifuged again at 12,000xg for 20 min at 4°C. The protein extracts were then diluted to a concentration of 1 mg/mL. Prior to fractionation, the 100 mL protein extract isolated from the Δ*fomep1* deletion mutant was concentrated using a 3 kDa cut-off filter (Pall Nanosep®). The concentrated fraction of 3 mL was separated on a Superdex G-75 column that had been equilibrated with 50 mM Tris-HCl, pH 7.5 running buffer. The column flow rate was 1 mL/min and 30 fractions of 2 mL were collected, freeze-dried, and dissolved in 200 µL of 10 mM sodium acetate pH 5.2. Fractions were stored at -20°C for further analysis.

Chitinase modifying assay

A chitinase modifying assay was performed using a previously described method (Naumann *et al.*, 2011). Each reaction consisted of 10 µL total volume containing 5 µg of purified tomato chitinase and 2 µg of fungal secreted protein extract in 50 mM sodium acetate buffer pH 5.2. Reactions were incubated for 20 hours at 25°C and were stopped by adding SDS-PAGE loading buffer and a heat treatment of 5 min at 95°C. Proteins in the reaction mixture were analysed by 16% tricine SDS-PAGE. To identify fractions containing chitinase-modifying proteases, similar chitinase modifying assays were performed using fractionated protein extracts obtained from deletion mutants of the fungalysin metalloprotease gene (FOXG_16612) of *F. oxysporum*. In each reaction volume of 10 µL, 10 µg of purified tomato chitinase SlChi1 was incubated with 1 µL of each fraction in 50 mM sodium acetate buffer pH 5.2 and was analysed as mentioned above.

Mass spectrometry analysis

To determine the cleavage site in the targeted extracellular tomato CBD-chitinases, SlChi1 and SlChi13 were incubated with protein extracts of *F. oxysporum*, as described in the chitinase modifying assay section. Ten µL of extract with 3.5 µL of SDS gel loading buffer were boiled for 5 min and run on a 16% tricine SDS-PAGE gel. Intact and processed SlChi1 and SlChi13 were excised from the tricine SDS-PAGE gel and cut into cubes of one mm^3. Proteins were reduced with 50 mM dithiothreitol dissolved into 50 mM ammonium bicarbonate, carboxamidomethylated with 100 mM iodoacetamide in 50 mM ammonium bicarbonate and digested by adding 25 µL 10 ng/µL sequencing grade trypsin or chymotrypsin in 50 mM ammonium bicarbonate. Gel pieces were washed with water in between each step. Peptide samples were cleared by loading them onto a C18+ Stage tip (Lu et al., 2011) followed by elution.

To identify proteases in the active fractions obtained from fractionation of protein extracts, 10 µL of each active fraction were loaded on a 16% tricine SDS-PAGE gel and five bands were excised and prepared for mass spectrometry analysis by reduction and alkylation of cysteine residues, followed by trypsin or chymotrypsin digestion as described above. Samples were then analysed by nano scale LC MS/MS with a Proxeon EASY nLC connected to a LTQ-Orbitrap XL (Lu et al., 2011) at the Laboratory of Biochemistry of Wageningen University.

LC-MS runs with all MS/MS spectra obtained were analysed with MaxQuant 1.3.0.5 (Cox and Mann, 2008) using default settings for the Andromeda search engine (Cox *et al.*, 2011) except that extra variable modifications were set for de-amidation of N and Q. The *F. oxysporum* f. sp. *lycopersici* database (4287-FO2) was downloaded from the Broad institute (https://www.broadinstitute.org) and the tomato database ITAG2.3 was downloaded from the Solgenomics website (http://solgenomics.net). These were used together with a contaminant database that contains sequences of common contaminants e.g. BSA (P02769, bovin serum albumin precursor), trypsin (P00760, bovin), trypsin (P00761, porcin), chymotrypsin (P00766, bovin), keratin K22E (P35908, human), keratin K1C9 (P35527, human), keratin K2C1 (P04264, human) and keratin K1CI (P35527, human).

The "label-free quantification" as well as the "match between runs" (set to 2 min) options were enabled. Using de-amidated peptides was allowed for protein quantification and all other quantification settings were kept default. Filtering and further bioinformatic analysis of the MaxQuant/Andromeda workflow output and the analysis of abundances of the identified proteins were performed with the Perseus 1.3.0.4 module (available in the MaxQuant suite). Peptides and proteins with a false

discovery rate (FDR) of less than 1% and proteins with at least two identified peptides of which at least one unique and one unmodified were considered as reliable identification. Reversed hits were deleted from the MaxQuant result table.

Chitinase activity assay

Insoluble chitin azure was used as substrate to assay chitinase activity; in 2 mL low protein binding tubes (LoBind tubes, Eppendorf), 1 mg of chitin azure (Sigma) was washed once with 1 mL potassium-phosphate buffer (100 mM, pH 6.0) by brief centrifugation. The washed chitin azure pellet was re-suspended in 0.9 mL of the same buffer and then mixed with purified proteins SlChi1, SlChi2, or SlChi13 at a final concentration of 0.1 mg/mL, and incubated for 20 h at 37 °C. Reactions were centrifuged at 12,000xg for 10 min at room temperature and the optical density of 0.750 mL supernatants was measured at 560 nm. Similar chitinase activity assays were performed with SlChi1, SlChi2 and SlChi13 chitinases treated with protein extracts of wild type and protease mutant (ΔΔ*fomep1*::*fosep1*) of *F. oxysporum.*

Determination of the antifungal activity of full-length and cleaved chitinases

Fungal spores of *F. oxysporum* f. sp. *lycopersici* were harvested in sterile Milli-Q® water as described above and spore concentration was adjusted to 10^5 spores/mL. Ten µL of spore suspension were mounted on a microscopy slide and first incubated for four hours at room temperature inside a moist covered box. Then, 20 µL of non-cleaved SlChi1 or SlChi13 chitinase (in 10 mM sodium acetate, pH 5.2) were added to the spore suspension at a final concentration of 1 µg/µL. After 16 hours, samples were observed using a Nikon eclipse 90i microscope controlled by the imaging software NIS-Element AR 2.30. The length of about 100 germ tubes was measured. Similar antifungal assays were performed with cleaved SlChi1 and SlChi13 chitinases. For this purpose, both chitinases were incubated with protein extracts of *F. oxysporum* as described above prior to incubation with the spore suspension.

Construction of single and double deletion mutants of *FoMep1*, *FoSep1,* and *FoSep2* protease genes in *F. oxysporum* f. sp. *lycopersici*

The gene replacement vectors pR4R3Δfomep1, pR4R3Δfosep1 and pR4R3Δsfosep2 were constructed using the Multisite Gateway® Three-Fragment Vector Construction Kit (Invitrogen™, Carlsbad, CA, USA) according to the manufacturer's instructions. Genomic DNA was extracted from *F. oxysporum* mycelium, that was grown in PDB for three days, frozen in liquid nitrogen, and using the DNeasy plant mini kit (Qiagen

Benelux bv, Venlo, the Netherlands) according to the manufacturer's instructions. The upstream (US) regions (1.4 kb) and the downstream (DS) regions (1.4 kb) of *FoMep1* (FOXG_16612), *FoSep1* (FOXG_09801) and *FoSep2* (FOXG_01145) were amplified from genomic DNA of *F. oxysporum* f. sp. *lycopersici.* The PCR reactions were performed using GoTaq® DNA polymerase (Promega) and oligonucleotides (Table S5) containing the specific overhang sequences *AttB4, AttB1r*, *AttB2r* or *AttB3*, which are recombination sites. The US and DS purified PCR fragments of each genes were introduced into pDONR™ P4-P1R and pDONR™ P2R-P3 vectors, respectively. Seventy ng of purified US or DS fragment, 1 µL of BP clonase™ II enzyme mix (Invitrogen), and 70 ng of the corresponding pDONR™ vector were combined in a total volume of 5 µL. Reactions were incubated overnight at 25 °C and used to transform DH5α *Escherichia coli* cells by heat shock treatment. The three replacement vectors were obtained performing LR reactions that contained 70 ng of pDONR™ P4-P1R_US, 70 ng of pDONR™ P2R-P_DS, 70 ng of p221_GFP_HYG (containing the green fluorescent protein and hygromycin resistance genes) (Ökmen *et al.*, 2013), 70 ng of pDESTTMR4-R3 destination vector and 1 µL of LR clonase™ II enzyme mix (Invitrogen, Carlsbad, CA, USA) in a total volume of 5 µL. Reactions were incubated overnight at 25 °C and were subsequently transformed into DH5α *E. coli.* To generate double knockout mutants, the vector pRM254 (Mehrabi *et al.,* 2015) containing GFP and geneticin resistance gene was used. The replacement vectors were obtained performing LR reactions using pDONR™ P4-P1R_US (carrying upstream fragments of *FoSep1* or *FoSep2*), pDONR™ P2R-P_DS (carrying downstream fragments of *FoSep1* or *FoSep2*), pRM254, and pDEST™R4-R3 destination vector. The correct orientation of fragments in the final constructs was confirmed by PCR and sequencing. The gene replacement vectors were introduced into *Agrobacterium tumefaciens* AGL1 cells by electroporation*. A. tumefaciens*-mediated transformation of wild type strain 4287 and single deletion mutants of *F. oxysporum* was performed using a method described before (Ökmen *et al.*, 2013). Transformants were selected on PDA plates supplemented with 100 µg/mL hygromycin or 150 µg/mL geneticin. Stable transformants were selected by growing them on selective PDA plates followed by a new culture on non-selective plates. Deletion mutants were screened and characterized by PCR and qrtPCR (Table S6).

RESULTS

The tomato genome contains 30 genes that encode predicted chitinases grouped in seven different classes

To study the molecular interaction between tomato chitinases and fungal proteases that might cleave them, we first set out to determine the full complement of genes encoding putative chitinases in the tomato genome. Tblastn searches with characterized plant chitinases from the six reported classes retrieved a total of 30 genes, named *SlChi1* to *SlChi30*, of which 23 were predicted to encode complete and functional proteins (Fig. 1, Fig. S1 and Table S1).

Chromosomal location analysis showed that genes encoding chitinases of a given class were often located on the same chromosome, which did not carry genes encoding a chitinase from another class. Noteworthy, genes encoding chitinases from the same class were frequently organized in clusters comprising both functional and non-functional genes (Fig. S2). This observation suggests frequent gene duplication events, which might indicate a role in co-evolution with fungal pathogens. Twenty-six predicted tomato chitinases were grouped into the six described classes based on sequence similarity, protein structure and phylogenetic analysis (Figs S3-12). The four remaining predicted GH18 chitinases were related to TBC-2 from *Tulipa* species, and were previously assigned as a class IIIb chitinase (Yamagami and Ishiguro, 1998) (Fig. 1, Fig. S5, Fig. S7, and Fig. S9). However, their sequences did not seem to be related to chitinases from class III, and therefore we propose to assign these chitinases as well as TBC-2 to a new class VIII (Fig. S11). In addition, two chitinases from *Picea* species that were reported to belong to class VII (Kolosova *et al.*, 2014), lack two conserved regions compared to class VII chitinases and share high sequence similarity to class IV chitinases throughout the entire chitinase domain (Fig. S12). A phylogenetic analysis of GH19 chitinases also showed that *Picea* chitinases belong to a monophyletic clade that comprises class IV chitinases only (Fig. S13). Therefore, we propose they are members of class IV chitinases that lost the CBD independently from class II chitinases. Furthermore, the GH19 chitinase phylogenetic tree also shows that class I and class II share a common origin, indicating a single loss event of the CBD for these two families.

Out of 30 chitinase genes in tomato, a number that is comparable to the 25 chitinase genes in *Arabidopsis thaliana* and 49 chitinase genes in rice (*Oryza sativa*) (Grover, 2012), six encode CBD-chitinases (four class I and two class IV genes). Because of the presumed importance of the CBD for enzymatic and antifungal activities of chitinases (Iseli *et al.*, 1993; Suarez *et al.*, 2001) and because of the reported CBD cleavage by fungal proteases, these six genes represent good candidates that might

play a role in defence of tomato against fungal pathogens, and were selected for further analyses.

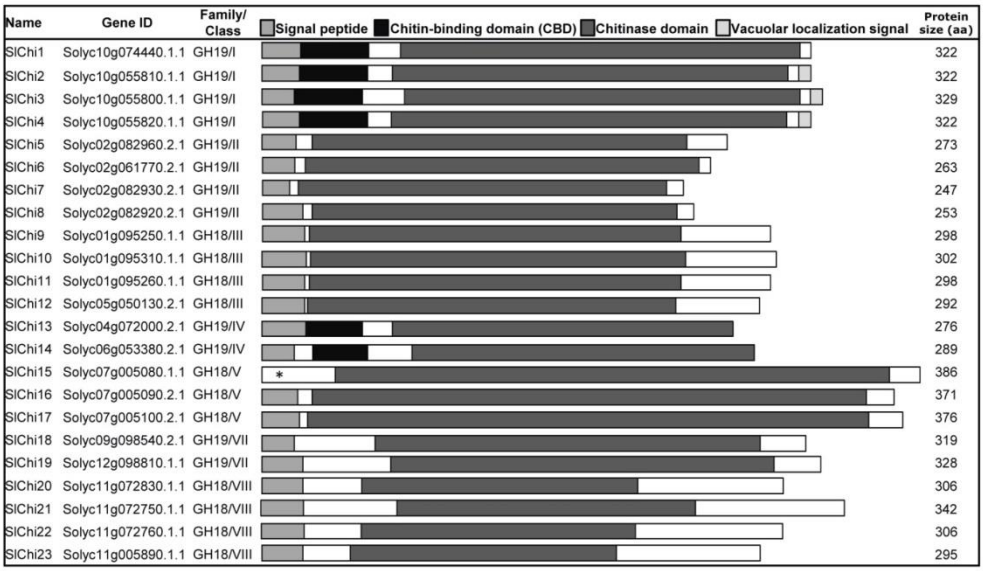

Figure 1. Characteristics of predicted functional chitinases from tomato. The tblastn search of the tomato genome identified 23 genes that encode likely functional chitinases, named SlChi1 to SlChi23. The family and class of each predicted chitinase is indicated based on sequence similarity and presence of glycosyl hydrolase conserved domains. Their protein structure is represented on scale. Asterisk indicates the absence of a signal peptide in SlChi15, indicating that it is unclear whether this predicted chitinase is functional or not. SlChi1 lacks a vacuolar localization signal and thus might be extracellular.

Induction of CBD-chitinase genes in tomato after inoculation with *Cladosporium fulvum* and *Fusarium oxysporum* f. sp. *lycopersici* suggests a role in plant defence

Previously, it was found that expression of *SlChi2* is induced when tomato plants are challenged with the protective strain Fo47 (Aimé *et al.*, 2013) or a pathogenic strain of *F. oxysporum* (Houterman *et al.*, 2007). In addition, *SlChi2, SlChi6, SlChi7* and *SlChi8* were up-regulated upon infection by *C. fulvum* (Danhash *et al.*, 1993; Joosten and Wit, 1989). We hypothesized that tomato extracellular and vacuolar CBD-chitinases might be differentially regulated in response to the attack by fungal pathogens with different infection styles. To test this hypothesis, susceptible tomato cultivar MM-Cf-0 was inoculated with the foliar pathogen *C. fulvum* and the vascular pathogen *F. oxysporum*, respectively. Expression profiles of the six genes encoding CBD-chitinases were analysed using RT-qrtPCR. All six genes exhibited a low basal expression level at 0 dpi in both leaves and roots (Fig. 2). None of them were induced

during early infection (0-6 dpi) by *C. fulvum*, but *SlChi2*, *SlChi3* and *SlChi4*, were all significantly up-regulated at a late stage of infection (12 dpi). *SlChi2* was significantly induced from 8 dpi onwards (Fig. 2A), which corresponds to massive colonization of the apoplast by *C. fulvum* (de Wit *et al*., 2012). All three induced genes encode predicted vacuole-localized class I chitinases that are expected to become active after release into the extracellular space at later time points of infection when vacuoles collapse. Mass spectrometry analysis of apoplastic fluids obtained from tomato leaves 12 dpi with *C. fulvum* revealed the presence of the CBD-chitinase proteins SlChi1, SlChi2, SlChi3 and SlChi13.

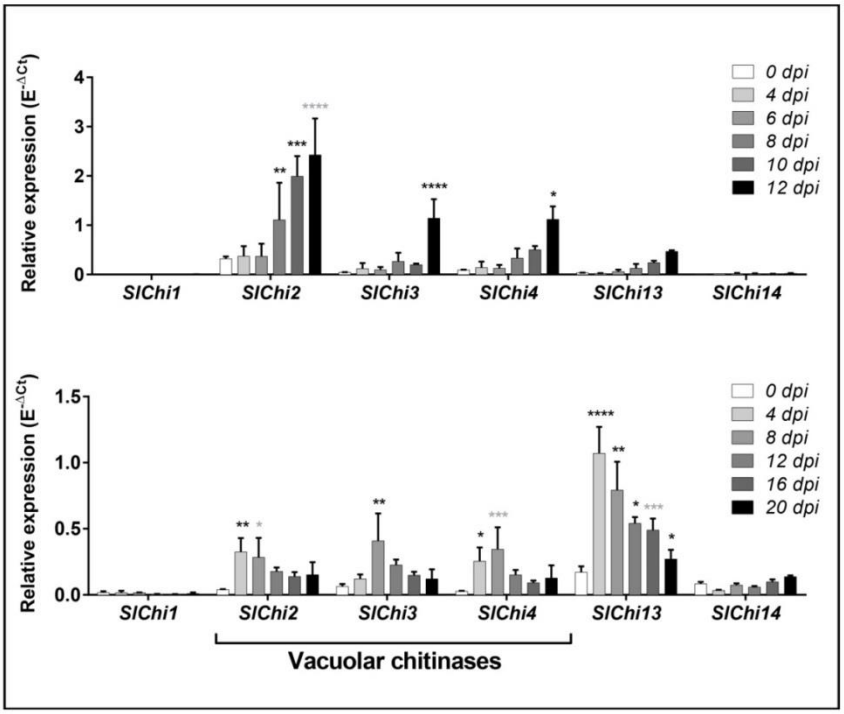

Figure 2. Expression of tomato genes encoding chitin-binding domain-containing chitinases is induced upon fungal infection. Susceptible tomato cultivar Money-Maker Cf-0 was inoculated with (**A**) *Cladosporium fulvum* or (**B**) *Fusarium oxysporum* f. sp. *lycopersici* (*F. oxysporum*). Leaf samples of *C. fulvum*-inoculated plants, and root samples of *F. oxysporum*-inoculated plants were collected at different days post inoculation (dpi) for RNA extraction and subsequent RT-qrtPCR. Expression of chitin-binding domain (CBD)-chitinase genes is normalized to that of the tomato β-tubulin gene (*LeTub*) using the $E^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). The bars show the average of three biological replications with standard deviation. A 2-way ANOVA with a *post-hoc* Tukey's multiple comparisons test was performed. Black asterisks indicate significant differential expression compared to the previous time point. When not applicable, grey asterisks indicate significant differential expression compared to 0 dpi (* *p*-value < 0.5; ** *p*-value < 0.01; *** *p*-value < 0.001; **** *p*-value < 0.0001).

In contrast, in roots inoculated with *F. oxysporum,* the predicted extracellular chitinase *SlChi13* was highly induced from 4 dpi onwards (Fig. 2B), while thereafter its expression decreased gradually to reach at 20 dpi the same expression level as at 0 dpi. In addition, the vacuolar chitinases *SlChi3* and *SlChi4* were transiently up-regulated at 8 dpi only, and to a lower extent than SlChi13. Altogether, these results showed that *SlChi3* and *SlChi4* were slightly and transiently up-regulated in both leaves and roots in response to fungal infection, suggesting a minor role in tomato defence. *SlChi2* and *SlChi13* were highly up-regulated in response to infection by *C. fulvum* and *F. oxysporum*, respectively. These two chitinases might provide a higher level of protection in response to fungal pathogens with different infection strategies.

Three fungal pathogens of tomato secrete proteins that cleave extracellular CBD-chitinases which reduces their chitinase activity

It has previously been shown that fungi secrete proteases that cleave CBD-chitinases (Olivieri *et al.*, 2002; Sela-Buurlage, 1996). Chitinase-modifying assays with pure maize CBD-chitinase and a metalloprotease from *F. verticillioides* (Naumann *et al.*, 2011) showed the truncation of this chitinase and determined the cleavage site. To assess whether tomato fungal pathogens also exhibit such activity, we performed a similar chitinase-modifying assay. For this purpose, seven tomato fungal pathogens with different infection strategies including *C. fulvum*, *A. solani*, *A. arborescens*, *B. cinerea* (foliar pathogens), *V. dahliae*, *F. oxysporum*, and *F. solani* (vascular pathogens) were grown on autoclaved tomato leaves and their secreted proteins were isolated. The six CBD-chitinases of tomato were expressed in *P. pastoris*, but only SlChi1, SlChi2, SlChi4, and SlChi13 could be produced and purified in sufficient amounts. These four CBD-chitinases were incubated for 20 hours with secreted protein extracts from the seven fungal tomato pathogens and their possible cleavage was analysed by SDS-PAGE. None of the secreted protein extracts could cleave the vacuolar CBD-chitinases SlChi2 and SlChi4, including extracts from *C. fulvum* (Fig. 3A). However, a slight increase in electrophoretic mobility was observed for SlChi4 after incubation with extract from *B. cinerea*, suggesting that this fungus might cleave this CBD-chitinase. In contrast, secreted protein extracts from *F. oxysporum, V. dahliae* and *B. cinerea* clearly modified the two extracellular CBD-chitinases SlChi1 and SlChi13 into forms that migrated faster on the SDS-PAGE gels, indicating that they were cleaved (Fig. 3A). Extract from *F. solani* also appeared to cleave SlChi13, albeit to a lesser extent. Secreted protein extracts from *C. fulvum, A. solani,* and *A. arborescens* did not cleave the extracellular CBD-chitinases.

To determine the N-termini of the SlChi1 and SlChi13 products cleaved by the *F. oxysporum* extract, they were excised from the SDS-PAGE gel and analysed by LC-MS/MS. Mass spectrometric analysis showed full coverage of the C-termini of both cleaved SlChi1 and SlChi13. No peptide covered the first 37 amino acids at the N-terminus of SlChi1, while native SlChi1 was fully covered. The potential cleavage site is located in the middle of the CBD and the removed upstream sequence includes two cysteine residues that are predicted to form disulfide bonds with two other cysteine residues behind the cleavage site (Fig. 3B and Fig. S14). Such cleavage likely results in a non-functional CBD. Mass spectrometry analysis of the cleaved product of SlChi13 showed that no peptide covered the first 49 amino acids at the N-terminus (Fig. 3B and Fig. S15). The potential cleavage site is located at the beginning of the hinge between the CBD and catalytic domain, resulting in complete removal of the CBD. These results suggest that *F. oxysporum* removes CBDs from extracellular CBD-chitinases that might affect their chitinase and antifungal activity.

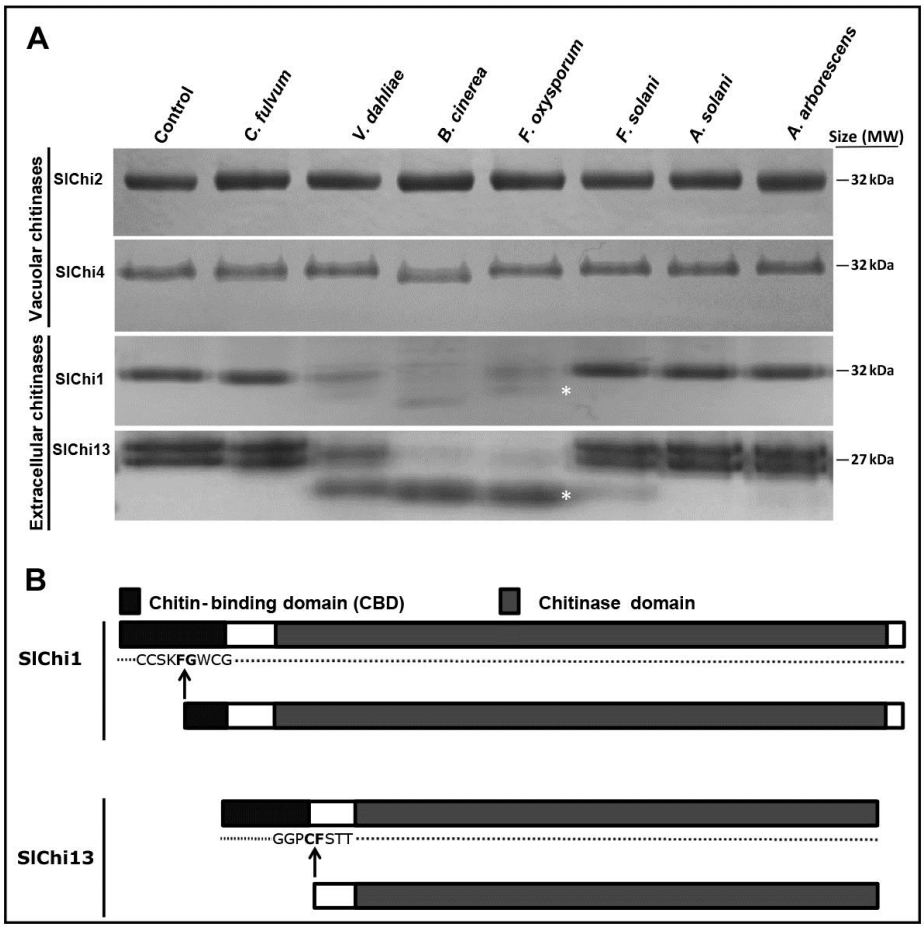

Figure 3. Secreted protein extracts from fungal pathogens remove the chitin-binding domain of extracellular tomato chitinases. (**A**) The four tomato chitin-binding domain (CBD)-chitinases SlChi1, SlChi2, SlChi4, and SlChi13 were produced by *Pichia pastoris*. Pure chitinases were incubated for 20 hours with secreted protein extracts isolated from the tomato pathogens *Cladosporium fulvum*, *Verticillium dahliae*, *Botrytis cinerea*, *Fusarium oxysporum* f. sp. *lycopersici* (*F. oxysporum*), *Fusarium solani* f. sp. *phaseoli* (*F. solani*), *Alternaria solani* and *Alternaria arborescens*. Results of the *in vitro* reactions were visualized on Coomassie blue-stained SDS-PAGE gels. Control is pure chitinase incubated in 10 mM sodium acetate buffer (pH 5.2) for 20 hours. The white asterisks indicate cleaved products of SlChi1 and SlChi13 that were isolated for mass spectrometry analysis. Chitinase modifying assays were performed at least three times. (**B**) Cleaved products of SlChi1 and SlChi13 after incubation with protein extract from *F. oxysporum* were sequenced by mass spectrometry to determine the cleavage sites. The cleavage site for SlChi1 is located within the CBD and the cleavage site for SlChi13 is located at the beginning of hinge between the CBD and chitinase catalytic domain. For each chitinase the upper bar represents the mature chitinase and the lower bar represents the cleaved chitinase. Arrows indicate the cleavage sites located between the two bold amino acid residues.

Removal of the CBD from SlChi1 and SlChi13 reduces their chitinase and antifungal activity

We assayed the chitinase activity of intact CBD-chitinases and chitinases that had lost their CBD after incubation with protein extracts from *F. oxysporum*. Loss of the CBD of SlChi1 and SlChi13 significantly reduced the chitinase activity using insoluble chitin azure as a substrate (Fig. S16).

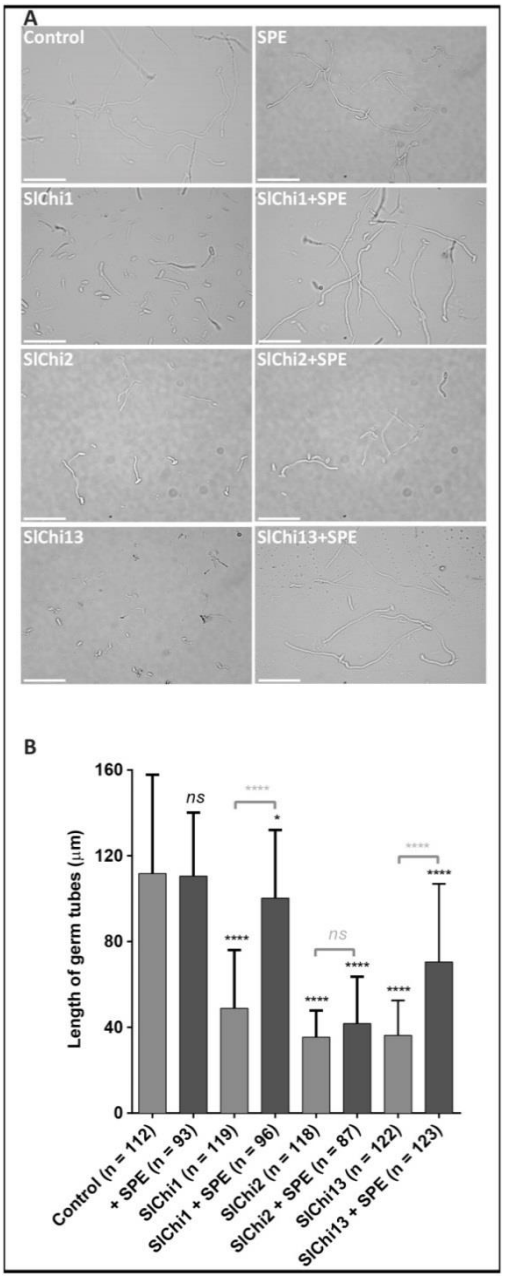

Figure 4. Cleavage of tomato chitin-binding domain containing-chitinases reduces their antifungal activity against *Fusarium oxysporum* f. sp. *lycopersici*. (**A**) Representative pictures of *F. oxysporum* f. sp. *lycopersici* (*F. oxysporum*) germinating spores that were incubated for 16 hours in 10 mM sodium acetate buffer (pH 5.2) alone or buffer containing 10 µg of secreted protein extract (SPE) isolated from *in vitro*-grown *F. oxysporum* as controls. Germinating spores were also incubated with intact or cleaved SlChi1, SlChi2 and SlChi13 chitinases. Scale bars indicate 50µm in size. (**B**) Average length and standard deviation of germ tubes after treatments shown in panel A. Number of measured germ tubes is indicated between brackets. A 1-way ANOVA with a *post-hoc* Tukey's multiple comparisons test was performed. Black asterisks indicate significant differences in length of germ tubes compared to control, and grey asterisks indicate significant differences between intact and cleaved chitinases. (* *p*-value < 0.5; **** *p*-value < 0.0001).

Chitinase activity of SlChi2, of which the CBD domain is not removed by protein extracts from *F. oxysporum* remained unchanged, stressing the importance of the CBD for chitinase activity on insoluble chitin. Antifungal activity of SlChi1 and SlChi13 was also significantly reduced after cleavage of the CBD by secreted protein extracts from *F. oxysporum.* Native SlChi1, SlChi2 and SlChi13 significantly inhibited the growth of germ tubes of *F. oxysporum* when compared to the controls (buffer and secreted protein extracts) (Fig. 4A). The average length of germ tubes was reduced by 32 to 44% after incubation with SlChi1, SlChi2 and SlChi13 (Fig. 4B). Mixing SlChi1 and SlChi13 CBD-chitinases with secreted protein extracts from *F. oxysporum* prior to incubation with fungal spores resulted in significant reduction of growth inhibition as showed by longer germ tubes (Fig. 4). These results demonstrate that removal of the CBD from SlChi1 and SlChi13 by *F. oxysporum* leads to a significant reduction of their chitinase activity and antifungal activity against this fungus. In contrast, no significant difference in growth inhibition was observed for germ tubes incubated with SlChi2 that was either treated or not treated with secreted protein extract from *F. oxysporum*.

Deletion of the fungalysin metalloprotease *FoMep1* partly reduces chitinase cleavage activity of *F. oxysporum*

The fungalysin metalloprotease encoded by the gene *FVEG_13630.0* of *F. verticillioides* was shown to cleave maize chitinases (Naumann *et al.*, 2011). A likely ortholog of this gene with 95.41% nucleic acid identity is present in the genome of *F. oxysporum* (*FOXG_16612*), (which we named *FoMep1*). Presence of FoMep1 in xylem sap extracted from MM-Cf-0 tomato infected by *F. oxysporum* was revealed by mass spectrometry analysis (Fig. S17). Therefore, we hypothesized that this metalloprotease might be responsible for cleavage of SlChi1 and/or SlChi13. Three targeted deletion mutants were obtained in which a cassette carrying the hygromycin resistance and green fluorescent protein (GFP) genes replaced *FoMep1*. All three *Δfomep1* deletion mutants did not show any *in vitro* phenotype different from wild type and ectopic transformants. As previously described, secreted protein extracts were isolated from the three Δ*fomep1* deletion mutants, wild type and ectopic transformant strain for incubation with SlChi1 and SlChi13. The chitinase-modifying assay showed that all protein extracts exhibited the same chitinase cleavage activity on SlChi1, but lost part of their activity on SlChi13 (Fig. 5A). This result indicates that the fungalysin metalloprotease encoded by *FoMep1* is required for partial modification of class IV CBD-chitinase SlChi13, but not for class I CBD-chitinase SlChi1.

In order to determine the type of protease that is responsible for additional chitinase-modifying activity in the Δ*fomep1* deletion mutants, secreted protein extracts in culture filtrates from these mutants were treated with inhibitors that are specific for cysteine proteases (E64 and cystatin), serine proteases (PMSF), metalloproteases (EDTA) and aspartic proteases (PepA). The chitinase-modifying assay showed that cleavage of SlChi1 and SlChi13 is inhibited by PMSF only at a concentration of 1 mM and higher (Fig. 5B and Fig. S18). This result indicates that serine proteases are likely responsible for additional cleavage of tomato CBD-chitinases.

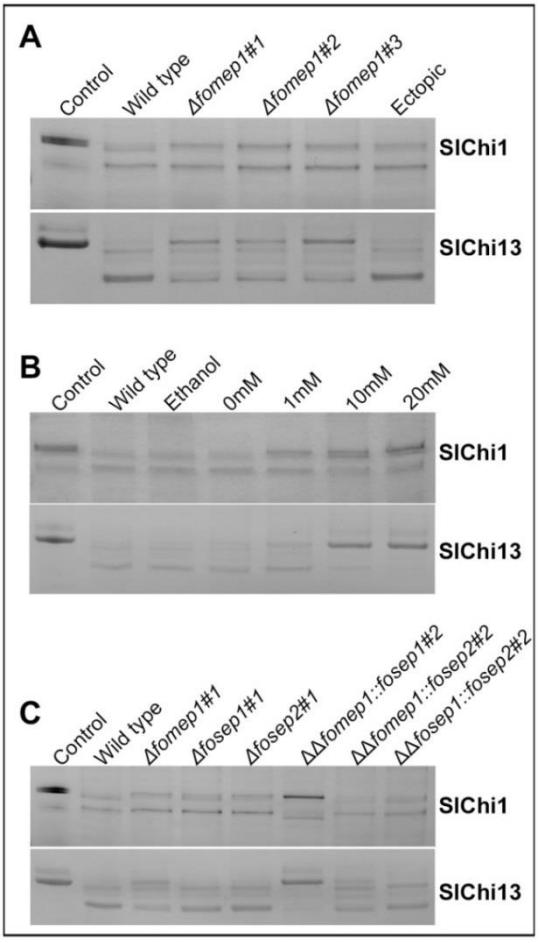

Figure 5. Synergistic action of a metalloprotease and a serine protease from *Fusarium oxysporum* f. sp. *lycopersici* cleaves tomato chitin-binding domain-containing chitinases. (**A**) SlChi1 and SlChi13 are cleaved after incubation with secreted protein extracts (SPEs) isolated from three independent *F. oxysporum* f. sp. *lycopsersici* (*F. oxysporum*) fungalysin metalloprotease *Δfomep1* deletion mutants. Control is 10 mM sodium acetate buffer (pH 5.2). (**B**) The serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) inhibits the cleavage of SlChi1 and SlChi13 by SPEs from the *F. oxysporum Δfomep1* deletion mutants in a concentration-dependent manner. (**C**) Only SPE from *F. oxysporum* double mutant ΔΔ*fomep1::fosep1* was not able to cleave SlChi1 and SlChi13.

Deletion of the serine protease *FoSep1* reduces chitinase cleavage activity of *F. oxysporum Δfomep1* deletion mutants

Identification of serine protease candidates was performed by fractionating secreted protein extracts from culture filtrates of the Δ*fomep1* deletion mutant and testing each fraction for chitinase-modifying activity. The culture filtrates were size-fractionated on a Superdex G-75 column and fractions were incubated with chitinase, and chitinase

modifying activity was monitored by PAGE. Protein bands present in active fractions were cut from the gel and analyzed by mass spectrometry. Two subtilisin serine proteases (FOXG_09801 and FOXG_01145) were identified in fractions 3 and 4 with chitinase-modifying activity (bands marked with asterisk; Fig. S19). Hereafter, the genes are named *FoSep1* and *FoSep2*. Deletion mutants, in which a cassette carrying geneticin or hygromycin resistance and GFP genes replaced the *FoSep1* and *FoSep2* genes, were generated in both wild type (single mutants) and *Δfomep1* deletion mutant (double mutants) strains (Fig. S20). In addition, both *FoSep1* and *FoSep2* were deleted in the wild type background. All single and double mutants showed normal vegetative growth and conidiation *in vitro* when compared to the wild type and ectopic transformants. As previously described, secreted proteins were isolated from each single and double mutant and tested in chitinase-modifying assays. The results showed that Δ*fosep1* and Δ*fosep2* single deletion mutants retained full activity against both chitinases SlChi1 and SlChi13 (Fig. 5C). However, protein extracts from the *ΔΔfomep1::fosep1* double mutant appeared to have lost chitinase cleavage activity towards both SlChi1 and SlChi13 nearly completely (Fig. 5C). The *ΔΔfomep1::fosep2* and *ΔΔfosep1::fosep2* double mutants showed the same activity as the *Δfomep1* single mutant and the wild type strain, respectively, indicating that FoSep2 does not display chitinase cleavage activity. Altogether, these results suggest that cleavage of both SlChi1 and SlChi13 chitinases is achieved by synergistic action of the fungalysin metalloprotease FoMep1 and serine protease FoSep1. Furthermore, we could show that chitinase activity of SlChi1 and SlChi13 treated with secreted protein extract of *ΔΔfosep1::fosep2* mutant of *F. oxysporum* was not reduced and its antifungal activity was not affected (Fig. S16).

Chitinase-modifying metalloprotease and serine protease are required for full virulence of *Fusarium oxysporum* on tomato

To determine the role of *FoMep1* and *FoSep1* in virulence, roots of MM-Cf-0 tomato seedling were inoculated with independent single and double deletion mutants of *FoMep1*and *FoSep1.* Virulence was quantified at four weeks post inoculation by measuring the height and fresh weight of the upper part of plants and by scoring the disease index. Results show that the plants inoculated with the single mutants Δ*fomep1* and Δ*fosep1,* performed slightly better growth than plants inoculated with the wild type and ectopic transformants (Fig. 6A-B) although, this reduction of virulence is not correlated with a lower disease index (Fig. 6C). Plants inoculated with the double mutants *ΔΔfomep1::fosep1* were as high as mock-inoculated plants (Fig. 6A) and were

significantly less diseased compared to those inoculated with the wild type and ectopic transformants (Fig. 6B-C). Altogether, these results indicate that synergistic action of *FoMep1* and *FoSep1* is required for full virulence of *F. oxysporum* on tomato.

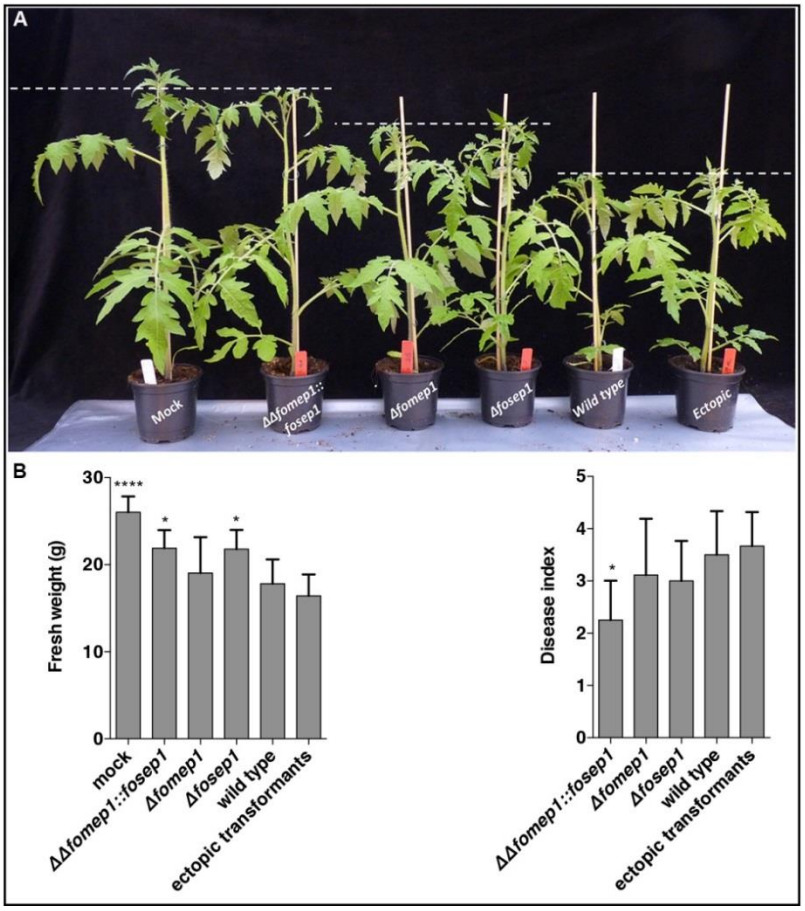

Figure 6. Synergistic action of a metalloprotease and a serine protease from *Fusarium oxysporum f. sp. lycopersici* is required for full virulence on tomato. (A) Representative picture showing MM-Cf-0 tomato plants four weeks after inoculation with *F. oxysporum* f. sp. *lycopersici* (*F. oxysporum*) wild type, ectopic transformants, single and double deletion mutants of *FoMep1* and *FoSep1* (Δ*fomep1*#1, Δ*fosep1*#1, ΔΔ*fomep1::fosep1*#2). Mock-inoculated plants serve as negative control. (**B)** Fresh weight of upper parts and **(C)** disease index of inoculated plants is presented as the average (and standard deviation) of three different plants from 2-3 independent strains. Results for Δ*fomep1*-ectopic and Δ*fosep1*-ectopic transformants are presented as ectopic transformants. A 2-way ANOVA with a *post-hoc* Dunnett's multiple comparisons test was performed. Black asterisks indicate significant differences compared to wild type (* *p*-value < 0.5; **** *p*-value < 0.0001).

DISCUSSION

Induction and accumulation of PR proteins constitute an important component of basal defence of plants against pathogens (Sels *et al.*, 2008; van Loon *et al.*, 2006; Wu *et al.*, 2014). Several PR proteins are chitinases and β-1,3-glucanases that have antimicrobial activity especially against fungi that contain chitin and β-1,3-glucans in their cell walls. Chitin-binding domain (CBD)-containing chitinases have reported to show high antifungal activity (Iseli *et al.*, 1993; Suarez *et al.*, 2001; Truong *et al.*, 2003). We analysed the tomato genome and identified six CBD-chitinase-encoding genes corresponding to class I (*SlChi1, SlChi2, SlChi3* and *SlChi4*) or class IV chitinases (*SlChi13* and *SlChi14*). They all showed a basic level of expression in healthy tomato plants but a clear differential expression after inoculation with the extracellular leaf pathogen, *C. fulvum,* and the vascular pathogen *F. oxysporum* f. sp. *lycopersici*. Strong induction of the CBD-chitinase SlChi2 at late stages of infection by *C. fulvum* was shown both at the transcriptome and proteome level. SlChi2 is predicted to localize in the vacuole, but we detected significant levels in the apoplast, which suggests it is either actively secreted or leaking from the vacuole into the apoplast during infection. SlChi2 is not modified by *C. fulvum* and the fungus is expected to protect itself against deleterious effects of this enzyme by secretion of the chitin-binding effector protein CfAvr4 (de Wit *et al.*, 2012; Stergiopoulos *et al.*, 2010; van den Burg *et al.*, 2006). In contrast, after inoculation of tomato roots with *F. oxysporum*, the extracellular CBD-chitinase SlChi13 is strongly induced at early stages of infection when the fungus colonises the cortical and vascular tissues. However, SlChi13 could not be detected in xylem sap by mass spectrometry. This might be due to its local accumulation, as reported for spruce seedlings infected by *Rhizoctonia spp.* (Benhamou *et al.*, 1990; Nagy and Fossdal, 2013). Our finding that *F. oxysporum* is able to cleave SlChi1 and SlChi13 is consistent with proteolytic cleavage of plant chitinases as an alternative to secretion of chitin-binding effector proteins. In addition to *F. oxysporum*, another vascular pathogen, *V. dahliae,* and a necrotrophic pathogen, *B. cinerea*, which all lack functional orthologs of the *CfAvr4* gene, seem to employ the same strategy because they were also able to modify the extracellular CBD-chitinases SlChi1 and SlChi13, but not the vacuolar CBD-chitinases SlChi2 and SlChi4. A deletion mutant of aspartic protease 8 (*Bcap8*), which encodes the major secreted protease of *B. cinerea* (ten Have *et al.*, 2010), showed similar chitinase cleavage activity to wild type *B. cinerea,* meaning that Bcap8 is not responsible for the cleavage of CBD-chitinases (results not presented). Homologs of *Mep1* and *Sep1* genes exist in the genome of *V. dahliae*, but the genome of *B. cinerea* contains only one homolog of *FoSep1.* We did not further

attempt to identify the enzymes responsible for CBD-chitinase modification in these two fungi. Other pathogens like *A. solani*, *A. arborescens* and *F. solani* did not cleave any of these CBD-chitinases and it remains unknown how they deal with deleterious effects of plant chitinases. However, it has been previously shown that macroconidia of *F. solani* secrete proteases that modify extracellular CBD-chitinases (Sela-Buurlage, 1996). It is likely that growth of the different fungi on autoclaved tomato leaves did not induce the expression of the corresponding protease in *F. solani,* and possibly also not in *Alternaria* species.

The metalloprotease FoMep1 of *F. oxysporum* is the ortholog of the fungalysin metalloprotease of *F. verticillioides*, which infects maize (Naumann *et al.*, 2011). Fungalysin from *F. verticilloides* could completely modify class IV chitinase ChitA from maize, while FoMep1 could only partly modify class IV chitinase SlChi13 from tomato. Complete cleavage of the CBD from SlChi13 was only achieved by synergistic action of FoMep1 and FoSep1 in *F. oxysporum.* Both proteases were also required for complete modification of the class I chitinase SlChi1. A similar enzyme from *F. solani* was suggested to cleave class I chitinase Chi-I from tobacco (Sela-Buurlage, 1996). In this fungus and in *F. verticillioides*, the cleavage site was identified in the hinge between the CBD and the catalytic domain of targeted chitinases. In our study we found that only synergistic action between metalloprotease FoMep1 and serine protease FoSep1 could cleave the CBD domain of SlChi1 and SlChi13. Synergistic actions of proteases has been previously suggested to cleave target proteins (McGowan, 2013). We do not know the specificity of FoMep1 and FoSep1, but their synergistic action results in cleavage of class I chitinase SlChi1 and class IV SlChi13 at different locations of the N-terminus. It is likely that they act sequentially.

Both *F. oxysporum* and *F. verticillioides* comprise orthologous genes *FoMep1* and *FoSep1* in their genomes. In addition, *F. oxysporum* is able to truncate maize ChitA and ChitB (Naumann and Wicklow, 2013) and similarly *F. verticillioides* cleavages SlChi1 and SlChi13 chitinases (results not presented), suggesting a similar mechanism involved in both fungi. However, the effects of fungalysin of *F. verticilloides* on antifungal activity of class IV chitinase ChitA from maize and the effects on virulence have never been reported. In our study we could show that antifungal activity of cleaved chitinases was significantly reduced, demonstrating the importance of the CBD in this activity as also reported previously (Iseli *et al.*, 1993; Suarez *et al.*, 2001). Removal of the CBD also decreased chitinase activity on insoluble chitin azure as a substrate, which is likely due to a decreased affinity for the substrate. The identified proteases did not modify the CBD-chitinase SlChi2 and consequently its chitinase and

antifungal activity was not changed after incubation with protein extract from *F. oxysporum.* However, SlChi2 is not induced during infection by *F. oxysporum*, suggesting that this fungus does not need to protect itself against this chitinase. This observation also argues in favour of co-evolution between plant chitinases and pathogen proteases. It is expected that CBD-chitinases from different hosts exhibit antifungal activity and their cleavage by proteases from host-adapted pathogenic fungi will result in loss of antifungal activity and increase in virulence.

Processing of chitinase was shown to be correlated with accumulation of proteolytic activity in plants infected by *Fusarium* species (Lange *et al.*, 1996; Naumann *et al.*, 2011; Olivieri *et al.*, 2002). Requirement of proteases for virulence were suggested for several fungal pathogens including *Magnaporthe poae* (Sreedhar et al., 1999), *Pyrenopeziza brassicae* (Ball *et al.*, 1991), but no experimental evidence for this role has been reported so far. Targeted mutants of *F. oxysporum* (Di Pietro *et al.*, 2001) lacking *prt1,* encoding a serine protease different from FoSep1 and FoSep2, and trypsin-deficient mutants of *Stagonospora nodorum* (Bindschedler et al., 2003) and *Cochliobolus carbonum* (Murphy and Walton, 1996) were not affected in their virulence. In our study, single deletion mutants of *F. oxysporum* showed only slightly reduced virulence based on plant growth, but plant colonization was not significantly impaired. In contrast, the double deletion mutant ΔΔ*fomep1::fosep1* showed a clear reduction in virulence. These observations suggest that both proteases are only components of an arsenal dedicated to infection. In this regard, it is possible that FoSep2 also plays a role in virulence but its effect could not be measured. The difference between single and double deletion mutants might be explained by complete modification of both chitinases SlChi1 and SlChi13. However, we cannot rule out that FeMep1, FoSep1 and FoSep2 in *F. oxysporum* target other plant proteins, including additional PR proteins. Indeed, single proteases might target specific plant proteins and synergistic actions of these enzymes might expand the number of plant protein targets.

In summary, data presented in this and previous studies show that plant pathogenic fungi employ different mechanisms to defend themselves against deleterious effects of CBD- chitinases. They either secrete a chitin-binding protein that interferes with chitin-binding of CBD-chitinases (van den Burg *et al.*, 2006; van Esse *et al.*, 2007) or secrete proteases that cleave CBD-chitinases as shown in this study and previous studies (Naumann and Wicklow, 2013). There are also examples of plant pathogens that overcome deleterious effects of PR proteins like β-1,3-glucanases and proteases by secreting inhibitors of those enzymes (Ham *et al.*, 1997; Rooney *et al.*, 2005; Rose *et al.*, 2002; Tian *et al.*, 2005; Tian *et al.*, 2004). Here we show for the first time that full

cleavage of a class I and class IV tomato CBD-chitinase requires synergistic action of two proteases that are required for full virulence of *F. oxysporum* on tomato. Cleavage of the CBD from extracellular CBD-chitinases and reduction of their antifungal activity towards *F. oxysporum* demonstrated the importance of CBD-chitinases in basal defence of tomato. A major challenge with functional analysis of fungal proteases is their redundancy, which explains why single protease deletion mutants did not show changes in phenotypes (Bindschedler *et al.*, 2003; Murphy and Walton, 1996). Our study suggests that it is important to investigate synergistic actions of additional secreted proteases of plant pathogens to eventually show their possible roles in virulence.

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AUTHOR CONTRIBUTIONS

MKJ, JC and PJGMdW conceived the project. MKJ and JC performed the bioinformatics analyses. MKJ and SB have performed the mass spectrometry analysis. MKJ, ID, YI, RM, and HB carried out the experimental work. MKJ, JC and PJGMdW wrote the manuscript.

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SUPPLEMENTARY FUGURES

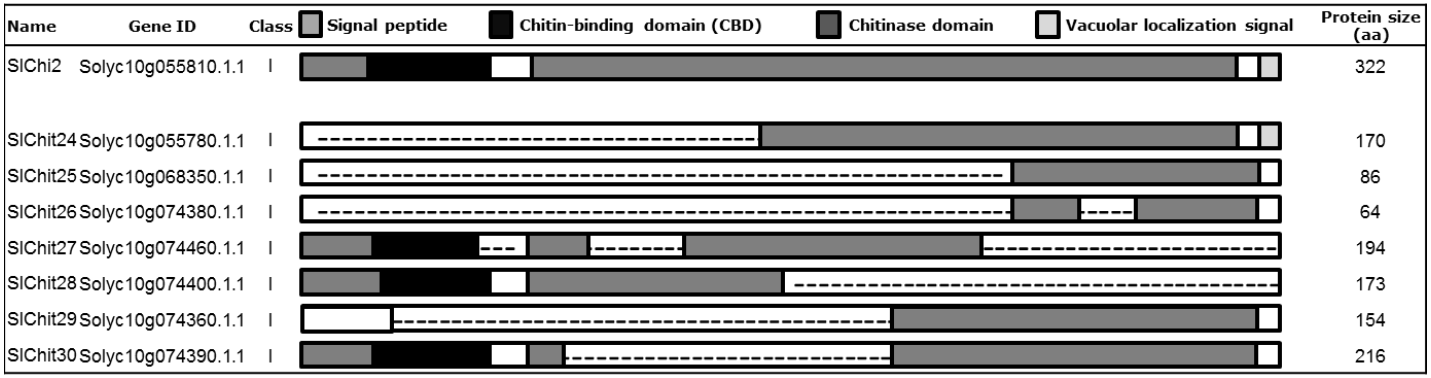

Figure S1. Schematic overview of the structure of predicted non-functional class I tomato chitinases. In the tomato genome, seven tomato genes are annotated as encoding GH19 chitinases with high similarity to class I chitinases. However, deletions and mutations seem to have accumulated in these seven genes, disrupting the coding sequence and consequently also the protein sequence. For comparison, the predicted functional SlChi2 chitinase is shown on top.

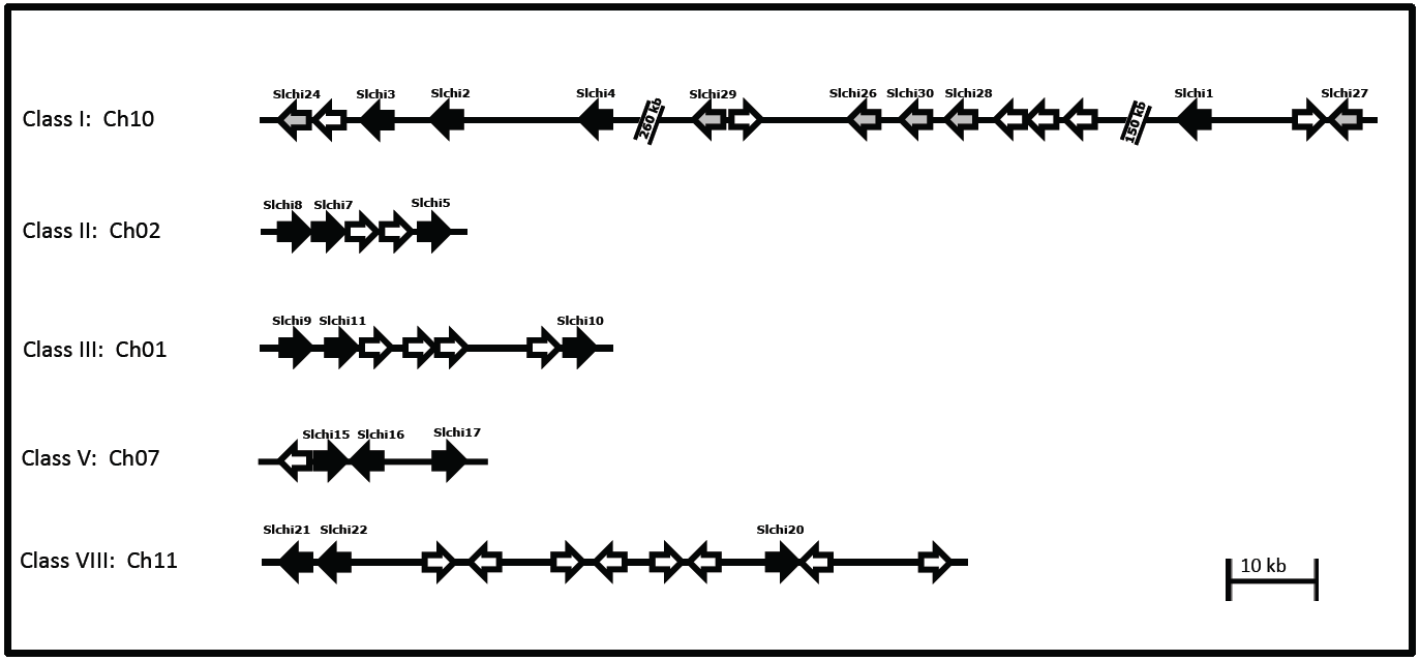

Figure S2. Chromosomes harbouring annotated tomato chitinase gene clusters. The majority of genes encoding a given class of tomato chitinases is often located on the same chromosome and frequently organized in clusters comprising both functional and non-functional genes. Different genes are indicated by the following colour codes: black and grey arrows depict complete and truncated chitinases, respectively; white arrows represent non-chitinase genes. Scaffold sizes range from 20 to 520 kb.

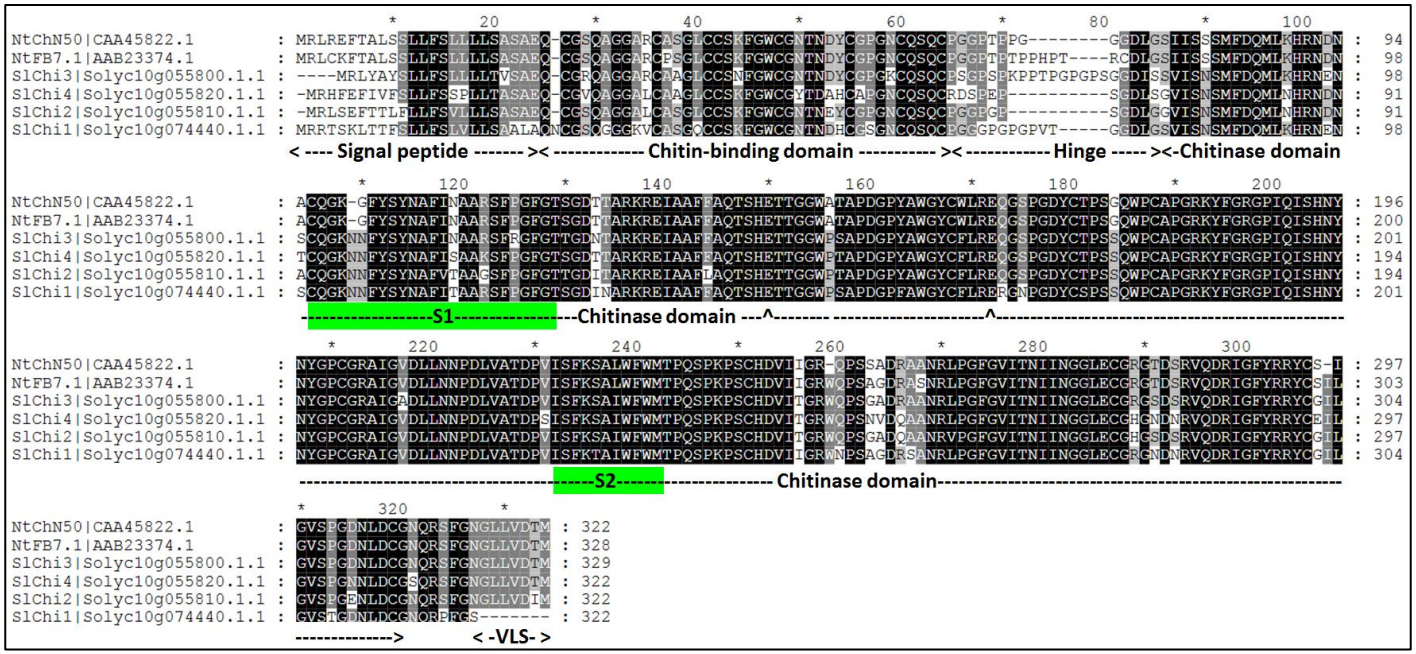

Figure S3. Multiple sequence alignment and structural features of tomato class I chitinases. The protein sequence of four tomato chitinases and two studied chitinases from *Nicotiana tabacum* (accessions CAA45822 and AAB23374) were aligned using Muscle on the EBI server (www.ebi.ac.uk/Tools/msa/muscle/), and were manually edited in GeneDoc. Gaps were introduced for optimal alignment and the degree of shading represents the level of similarity. Predicted annotations are shown under the alignment. "Signal peptide" is essential for secretion of the protein into the secretory pathway. Chitin-binding domain comprises signature type_1 (PS00026). Green-highlighted residues in the "chitinase domain" represent signature 1 (PS00773) shown as S1 and signature 2 (PS00774) shown as S2 in the GH19 family. The residues marked with a caret (^) are important for enzymatic activity (Iseli-Gamboni et al., 1998; Bishop et al., 2000). The NYNYG domain at position 205-209 is important for the activity of basic chitinases (Verburg et al., 1992). "VLS" indicates vacuolar localization signal.

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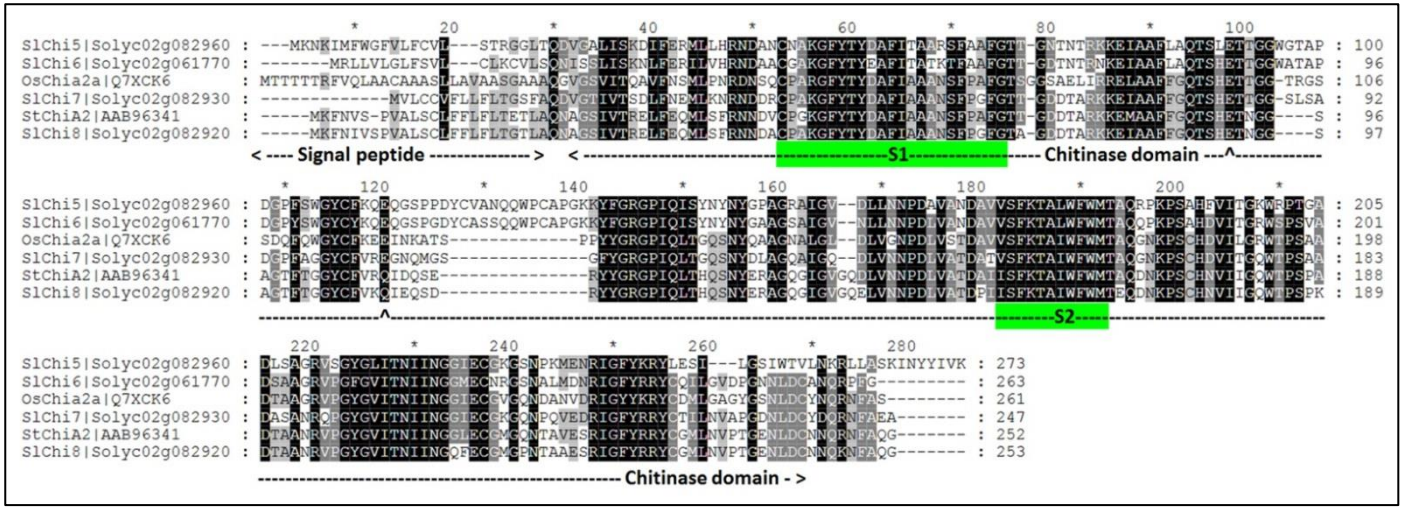

Figure S4. Multiple sequence alignment and structural features of tomato class II chitinases. Protein sequence of four tomato chitinases and two studied chitinases of *Solanum tuberosum* (accession AAB96341) and *Oryza sativa* (accession Q7XCK6) are aligned as described in Figure S3. Green-highlighted residues represent the S1 and S2 are as described in Figure S3. In class I, residues marked with a caret (^) are important for enzymatic activity (Büchter et al., 1997; Iseli-Gamboni et al., 1998; Bishop et al., 2000).

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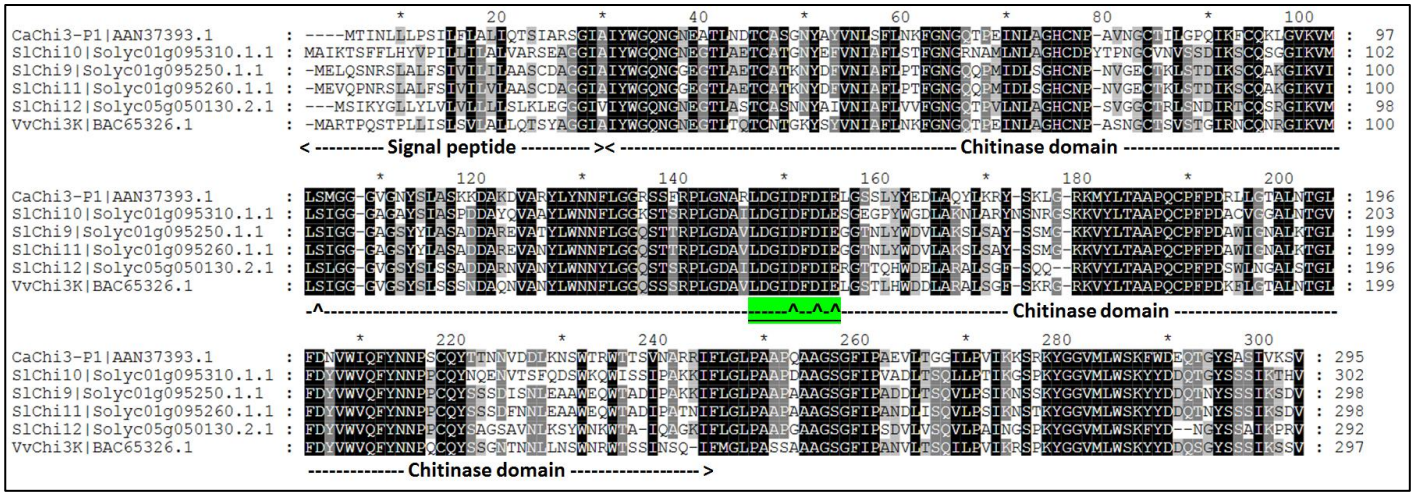

Figure S5. Multiple sequence alignment and structural features of tomato class III chitinases. Protein sequence of four tomato chitinases and two studied chitinases from *Capsicum annuum* (accession AAN37393) and *Vitis vinifera* (accession BAC65326) are aligned as described in Figure S3. Green-highlighted residues represent the PS01095 signature in the GH18 family. The residues marked with a caret (^) are important for enzymatic activity (Robert et al., 2002; Yeoh et al., 2013).

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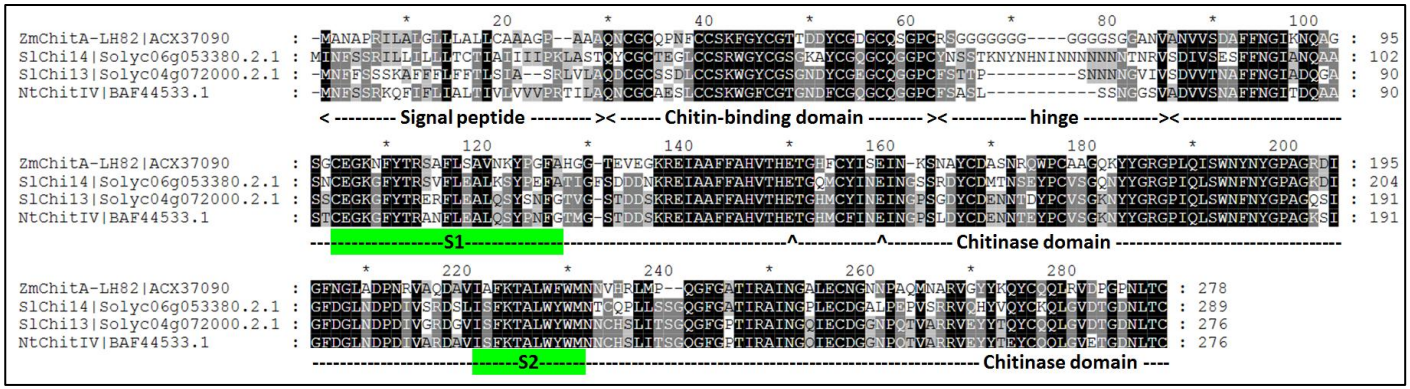

Figure S6. Multiple sequence alignment and structural features of tomato class IV chitinases. Protein sequence of two tomato chitinases and two studied chitinases of *Zea mays* (accession ACX37090) and *Nicotiana tabacum* (accession BAF44533) are aligned as described in Figure S3. Green-highlighted signatures S1 and S2 are as described in Figure S3. The residues marked with a caret (^) are important for enzymatic activity (Iseli-Gamboni et al., 1998; Bishop et al., 2000; Ubhayasekera et al., 2009; Chaudet et al., 2014).

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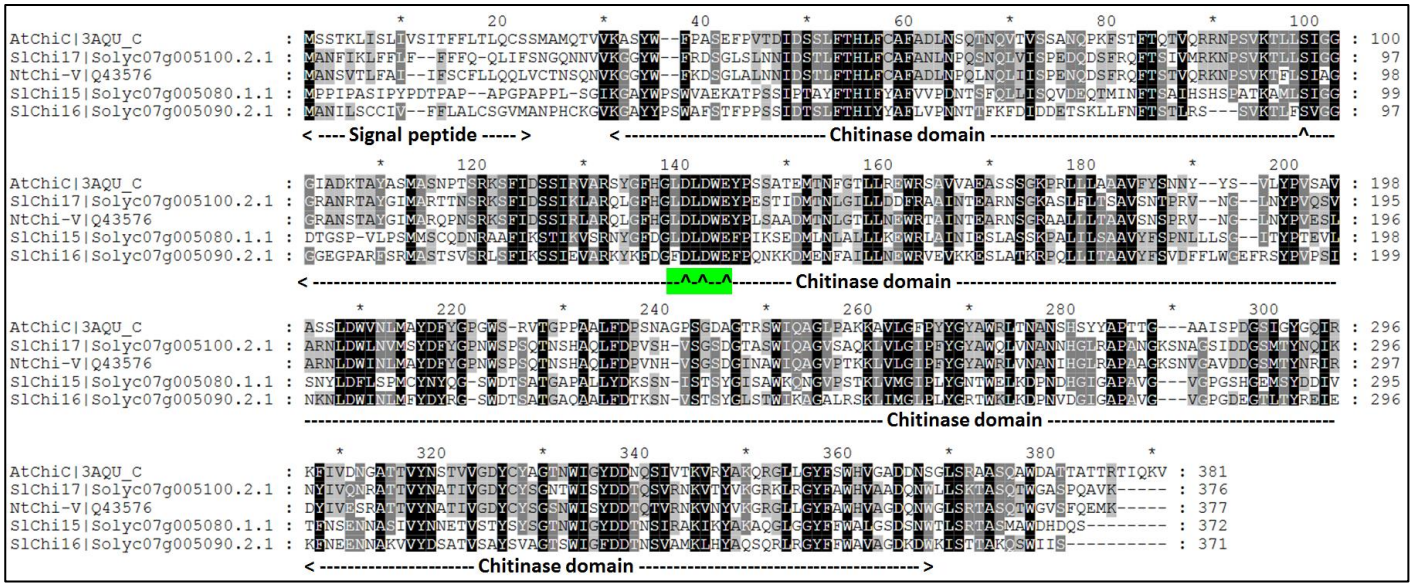

Figure S7. Multiple sequence alignment and structural features of tomato class V chitinases. Protein sequence of three tomato chitinases and two studied chitinases from *Arabidopsis thaliana* (accession C3AQU) and *Nicotiana tabacum* (accession Q43576) are aligned as described in Figure S3. Green-highlighted residues represent the PS01095 signature in the GH18 family. The residues marked with a caret (^) are important for enzymatic activity (Van Damme et al., 2007; Yeoh et al., 2013).

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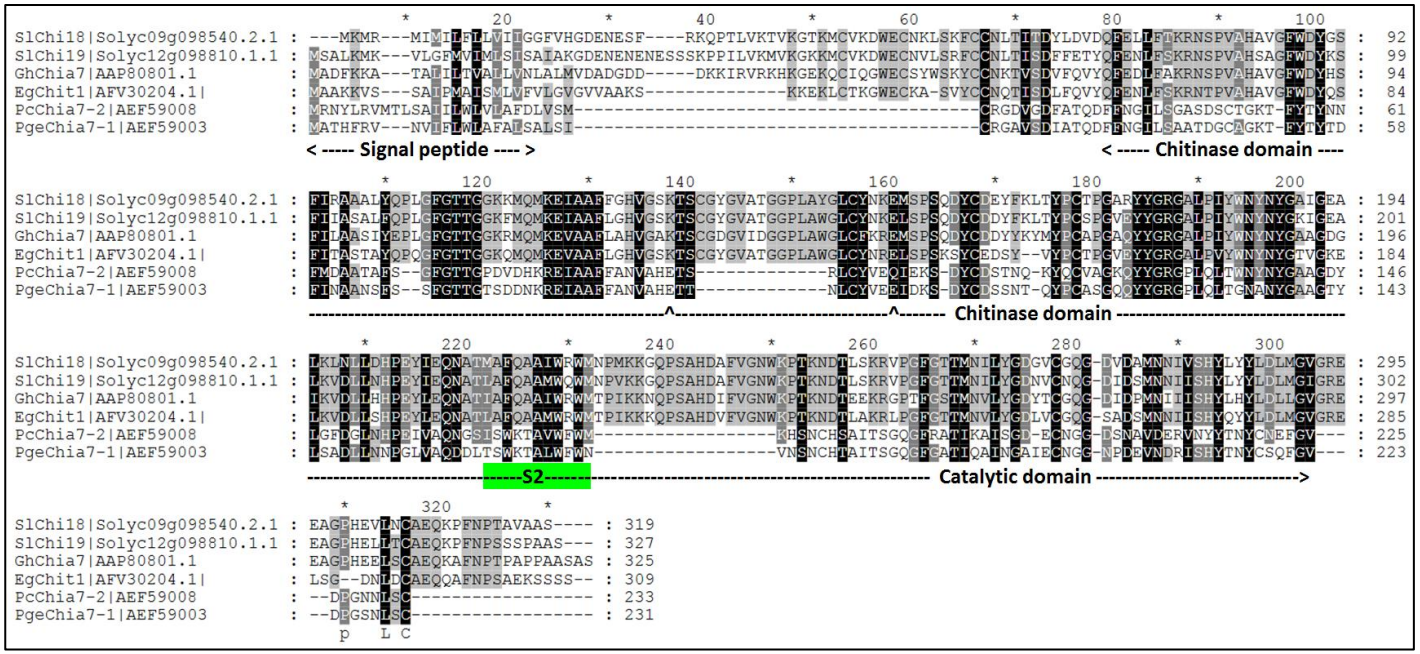

Figure S8. Multiple sequence alignment and structural features of tomato class VII chitinases. Protein sequence of two tomato chitinases and four studied chitinase of *Gossypium hirsutum* (accession AAP80801)*, Arabidopsis thaliana* (accession NP_188317) and *Picea* species (accessions AEF59003 and AEF59008) are aligned as described in Figure S3. Green-highlighted signature S2 is as described in Figure S3. The NYNYG domain at position 195-199 is important for activity of basic chitinases (Verburg et al., 1992). The residues marked with a caret (^) are important for enzymatic activity (Iseli-Gamboni et al., 1998; Bishop et al., 2000).

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Bishop, J.G., Dean, A.M., and Mitchell-Olds, T. 2000. Rapid evolution in plant chitinases: Molecular targets of selection in plant-pathogen coevolution. Proc. Natl. Acad. Sci. U.S.A. 97:5322-5327.

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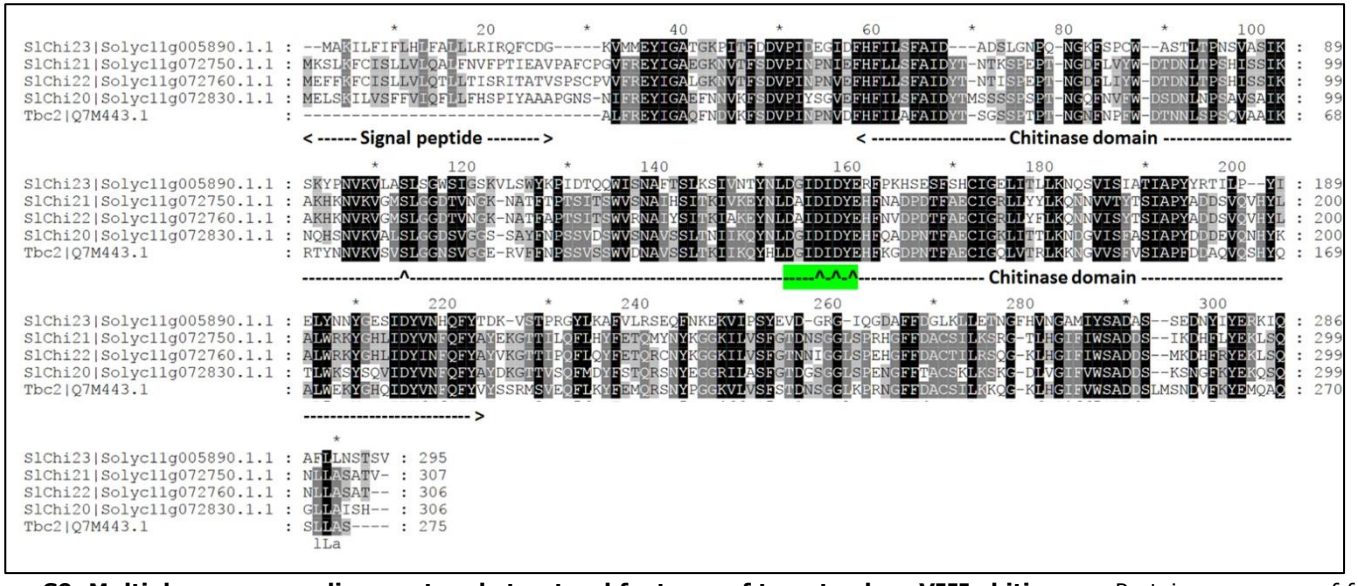

Figure S9. Multiple sequence alignment and structural features of tomato class VIII chitinases. Protein sequences of four tomato chitinases belonging to GH18 and TBC-2 from tulip bulb (accession Q7M443) are aligned as described in Figure S3. The green-highlighted residues represent the PS01095 signature in the GH18 family. The residues marked with a caret (^) are important for catalytic activity in class III and V of the GH18 family (Watanabe et al., 1993; Terwisscha van Scheltinga et al., 1994; Yamagami and Ishiguro, 1998; Robert et al., 2002).

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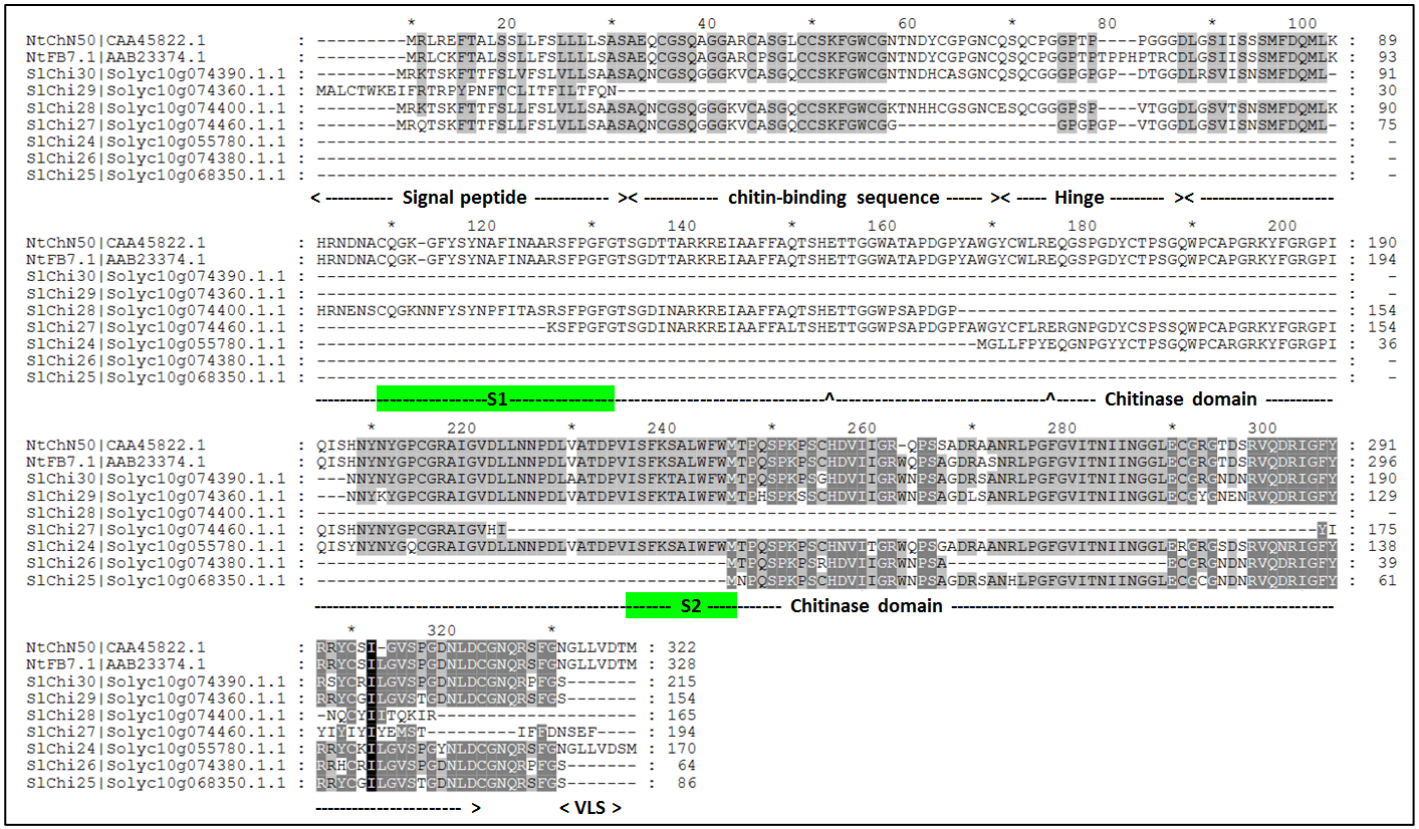

Figure S10. Multiple sequence alignment and structural features of truncated tomato class I chitinases. Protein sequence of seven tomato chitinases and two described class I chitinases of *Nicotiana tabacum* (accessions CAA45822 and AAB23374) are aligned as described in Figure S3. Green-highlighted signatures S1 and S2 are as described in Figure S3. The residues marked with a caret (^) are important for catalytic activity (Iseli-Gamboni et al., 1998; Bishop et al., 2000).

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Iseli-Gamboni, B., Boller, T., and Neuhaus, J.-M. 1998. Mutation of either of two essential glutamates converts the catalytic domain of tobacco class I chitinase into a chitin-binding lectin. Plant Sci. 134:45-51.

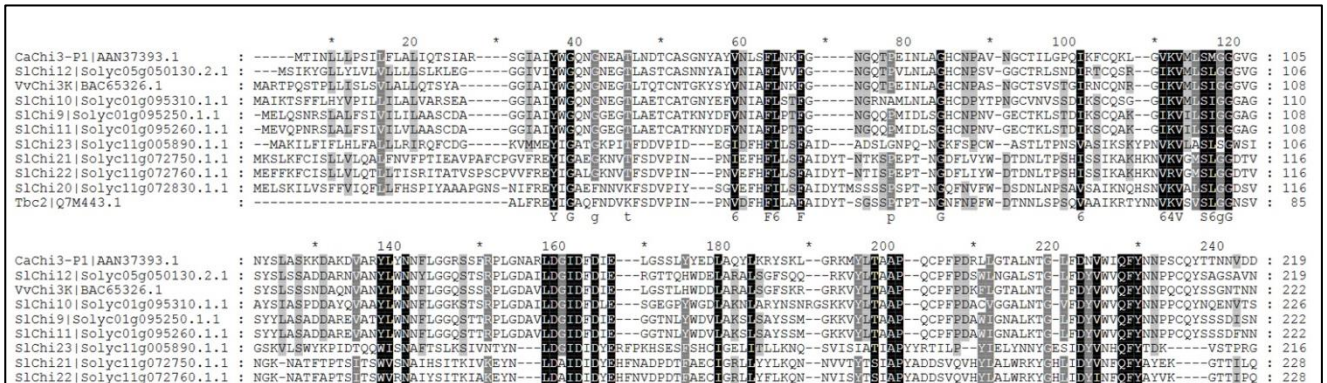

Figure S11. Multiple sequence alignment and structural features of class III and VIII of the GH18 family chitinases from selected organisms. Protein sequence of chitinase class III of *Vitis vinifera* (accession BAC65326) and chitinases class III (SlChi9-12) and class VIII (SlChi20-23) of tomato as well as TBC-2 from tulip bulb (accession Q7M443) are aligned as described in Figure S3. The conserved residues among TBC-2 and SlChi20-SlChi13 are different at several locations from class III chitinases. This suggests TBC-2 not to be a member of class IIIb but a member of a new class VIII.

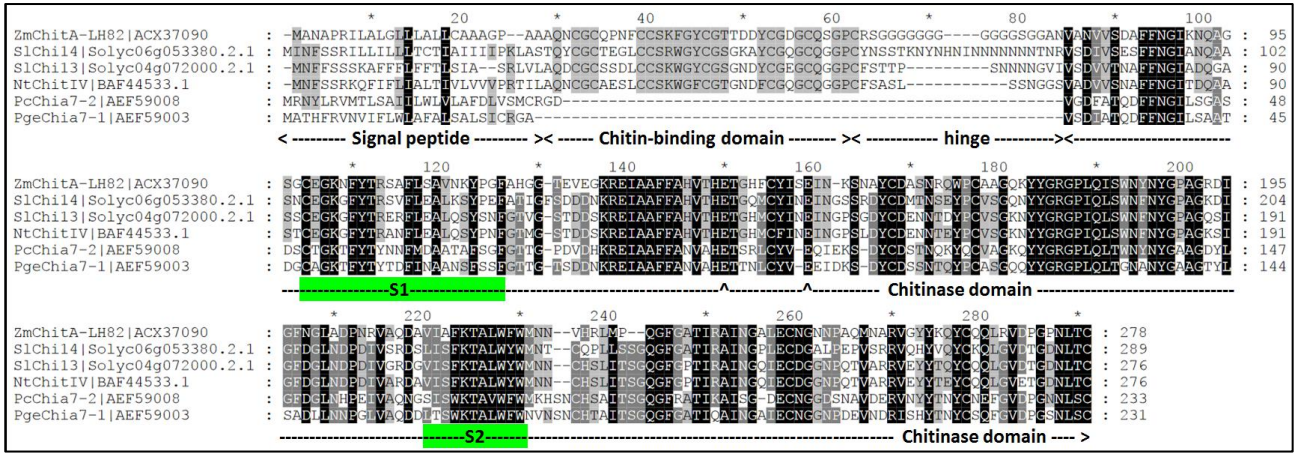

Figure S12. Multiple sequence alignment and structural features of class IV and two chitinases from *Picea*. Protein sequence of two class IV tomato chitinases and two studied chitinases of *Zea mays* (accession ACX37090) and *Nicotiana tabacum* (accession BAF44533) as well as studied chitinases of *Picea* species (accessions AEF59003 and AEF59008) are aligned as described in Figure S3. Chitinases of *Picea* species were reported to belong to class VII (Kolosova *et al.*, 2014), while they show higher sequence similarity in their chitinase domain to class IV than that to class VII chitinases. Therefore, we propose them to be members of class IV chitinases that lost the CBD independently from class II chitinases. Green-highlighted signatures S1 and S2 are as described in Figure S3. The residues marked with a caret (^) are important for enzymatic activity (Iseli-Gamboni et al., 1998; Bishop et al., 2000; Ubhayasekera et al., 2009; Chaudet et al., 2014).

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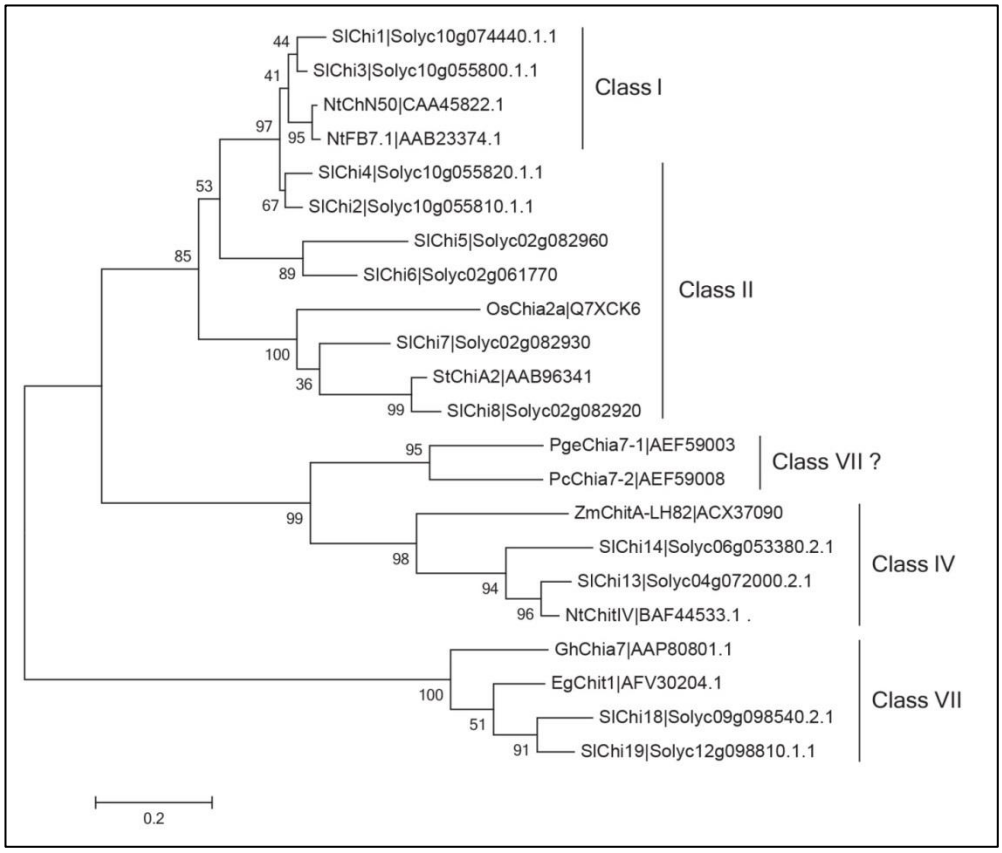

Figure S13. Phylogenetic tree of GH19 family chitinases from tomato and selected organisms. The maximum likelihood tree was generated with MEGA5.0 using a reliable alignment of all members of the GH19 family of tomato chitinases (sequences are in text file SlGH19-gb-less stringent phylogenies). The bootstrap numbers show the percentage of clustered trees within 500 replications. The scale represents the number of amino acid substitutions per site. The chitinase classes are shown on the right.

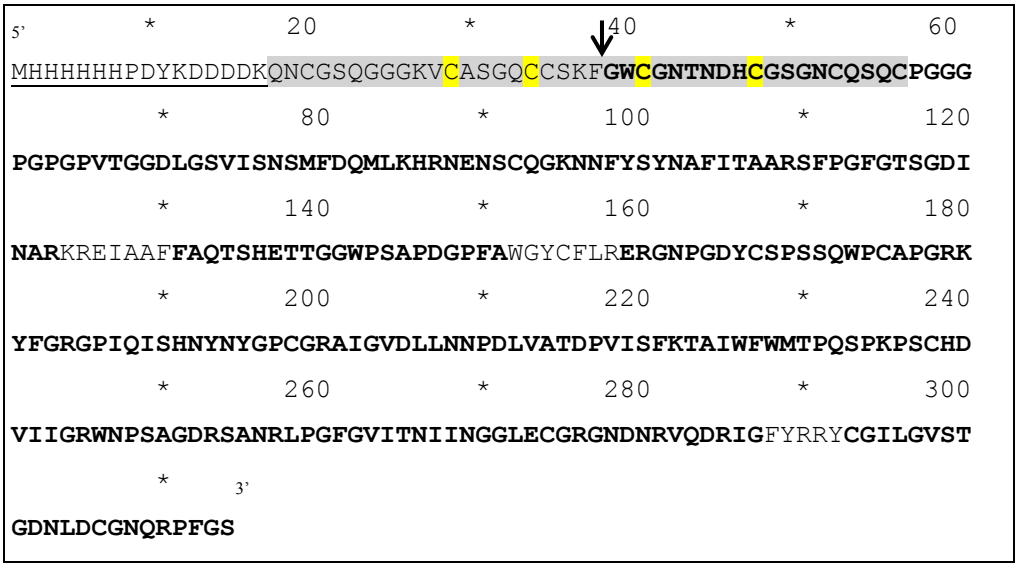

Figure S14. Amino acid sequence of the His_6-Flag-tagged mature protein of tomato chitinase SlChi1. The underlined residues 1-16 represent the His_6-Flag tags. The grey-shaded residues 17-56 are predicted to represent the chitin-binding domain. Residues in bold were covered by mass spectrometry of the cleaved SlChi1 product. The arrow shows the potential cleavage site of SlChi1 (behind Phe-37) after incubation with secreted protein extract from *Fusarium oxysporum* f. sp. *lycopersici*. Yellow-highlighted cysteine residues C12 and C24, and C17 and C31are predicted to form disulfide bonds in the 3D model obtained on the I-Tasser server (Yang et al., 2015).

Reference

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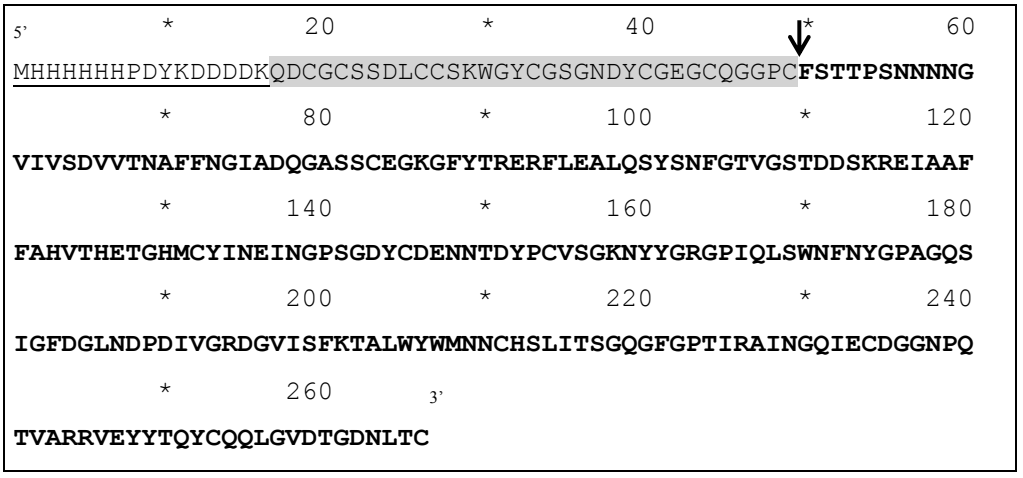

Figure S15. Amino acid sequence of the His_6-Flag-tagged mature protein of tomato chitinase SlChi13. The underlined residues 1-16 represent the His_6-Flag tags. The grey-shaded residues 17-49 are predicted to represent the chitin-binding domain. Residues in bold were covered by mass spectrometry of the cleaved SlChi13 product. The arrow shows the potential cleavage site of SlChi13 (behind Cys-51) after incubation with secreted protein extract from *Fusarium oxysporum* f. sp. *lycopersici*.

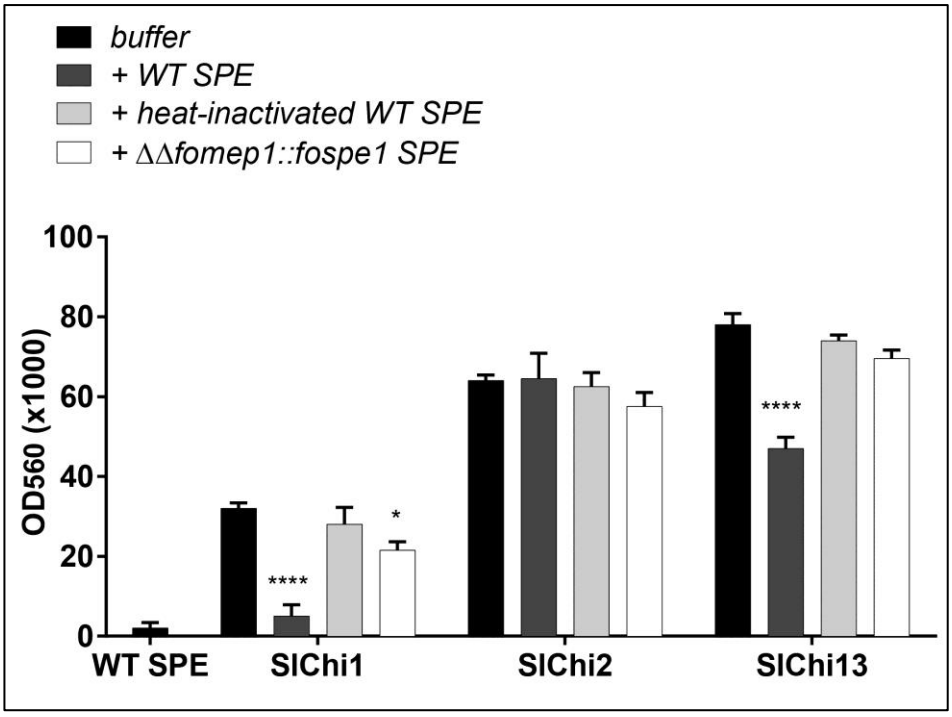

Figure S16. Chitinase activity of tomato SlChi1, SlChi2, and SlChi13 on chitin azure treated with secreted protein extracts (SPE) from wild type or *ΔΔfomep1::fosep1* mutant from *Fusarium oxysporum*. As previously (Shen et al., 2010) described insoluble chitin azure was used as substrate to assay chitinase activity of intact or cleaved (SPE-treated) tomato chitinases SlChi1, SlChi2, and SlChi13. Chitinase activity of intact chitinases is shown by black bars. Chitinase activity of chitinases, when were pre-treated with SPE of wild type *F. oxysporum* (+*WT SPE*), *heat-inactivated WT SPE* or SPE from double mutant *FoSep1* and *FoMep1* of *F. oxysporum* (*ΔΔfomep1::fosep1,*see Figure S20) are shown by dark grey, light grey and white bars, respectively. At the left, WT SPE represents the "chitinase activity" present in SPE of wild type *F. oxysporum.* Optical density of released azure from insoluble substrate is measured at 560 nm representing the chitinase activity. A 2-way ANOVA with a *post-hoc* Tukey's multiple comparisons test was performed. Black asterisks indicate significant differences in chitinase activity compared to the corresponding intact chitinases (incubated in buffer) (* p-value < 0.5; **** p-value < 0.0001).

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> FoMep1 (FOXG_16612)

MRFSDSLLLIGLSSLAGAHPSRRAPNPSPLSKRGLDLEAFKLPPMAEYVPQDEVPDDVS
AKVVTKRADYTETAKDLVKSTFPKATFRMVNDHYVGSNKIAHIHFKQTINGIDIDNADF
NVNIGADGEVFSYGNSFYEGKIPGPLTKRDEKDPVDALKDTVDVLSLPVEAEKAKAEKK
SKNHYTFTGTKGTVSKPEAKLTYLVDENKELKLTWRVETDIVDNWLLTYVNAAKTDEVV
GVVDYVNEATYKVYPWGVNDPSKGSRSTVENPWNLEASEFTWLSDGSNNYTTTRGNNGI
AQVNPSGGSTYLNNYRPDSPSLKFEYDYSTSTTTPTTYRDASIAQLFYTANKYHDLLYL
LGFTEQAGNFQTNNNGQGGVGNDMVILNAQDGSGTNNANFATPADGQPGRMRMYLWTYS
TPQRDCSFDAGVVIHEYTHGLSNRLTGGPANSGCLPGGESGGMGEGWGDFMATAIHIQS
KDTRASNKVMGDWVYNNAAGIRAYPYSTSLTTNPYTYKSVNSLSGVHAIGTYWATVLYE
VMWNLIDKHGKNDADEPKFNNGVPTDGKYLAMKLVVDGMSLQPCNPNMVQAR**DAIIDAD**
TALTKGANKCEIWKGFAKRGLGTGAKYSASSRTESFALPSGC

Figure S17. Mass spectrometry coverage of amino acid sequences from FoMep1. Presence of FoMep1 in xylem sap extracted from MM-Cf-0 tomato infected by *Fusarium oxysporum* f. sp. *lycopersici* was revealed by mass spectrometry. Residues in bold represent the amino acid sequence of the unique peptide identified by mass spectrometry present in the FoMep1.

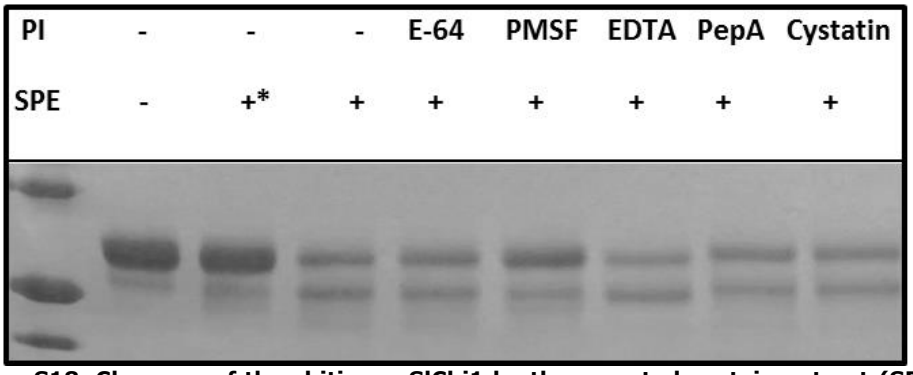
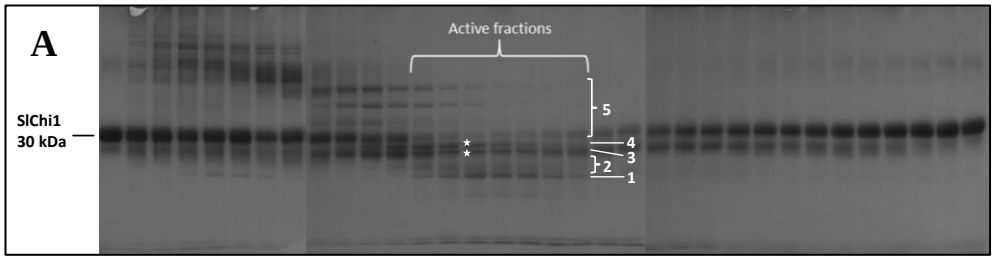


Figure S18. Cleavage of the chitinase SlChi1 by the secreted protein extract (SPE) from *Fusarium oxysporum* f. sp. *lycopersici* Δ*fomep1* mutant is inhibited by serine protease inhibitor PMSF. Protein extract of Δ*fomep1* was pre-incubated with different protease inhibitors before the chitinase cleavage assay on SlChi1: E-64 and cystatin inhibit cysteine proteases, PMSF inhibits serine proteases, EDTA inhibits metalloproteases, and PepA inhibits aspartic proteases. Protease inhibitors: E-64 (10 µM), PMSF (2 mM), EDTA (10 mM), PepA (10 µM), Cystatin (10 µg/ml). Incubation of SlChi1 with heat-inactivated protein extract of Δ*fomep1* is indicated by an asterisk.



B

> FoSep1 (FOXG_09801)

MTSIRRLALALGALLPAVLAAPADILSKRQAVPDKYIITLKPDASDSSVAAHLNWVGDV
HRRSLNKRDTSGVEKTFNISSWSAYSGEFDKSTIAEIKKSPEVAFVEPDYTMYLSYEES
EPELADR**ALTTQSGAPWGLGTISHRTSGSTSYIYDTTAGQGSYAYVVDSGVQVSHTNFG**
GRASLGYNAVGGAHEDTLGHGTHVAGTIAGTTYGVAKRANIISVKVFAGREGSTSTILA
GFNWAVNDITSKSRAGR**SVINLSLGGPASQTWTSAINAAYNSGVLSVVAAGNGDDAGRP**
LPVSGQSPANAPNALTVAAIDSSWRPASFTNYGAGVDVFGPGVNILSTWIGSNSATNTI
SGTSMACPHVAGLALYLQVLEGLSTPASVTNRIKSLATTGR**ITGTLSGSPNSVAYNGNG**
A

> FoSep2 (FOXG_01145)

MVNFKSLTFAATTLLGLVNAAPTTAKVDSSKIIPGKYIVTLKSDIAAADVESHLSWVED
VHKRGLNKRAEKGVERTYNGKYGFHGYAGSFDKSTIKEIKENPDVAIVEEDREWVINWV
EEEEEEAETLAKR**ALTTQSGATWGLGTVSHRSRGSTSYIYDTNAGTNTYAYVVDTGVRT**
THNEFEGRAQAVYTAFSGDNADSVGHGTHVSGTIAGKTYGVSK**KATIQAVKVFQGSSSS**
TSIILAGFNWAANDIISKSRTARSVVNMSLGGGYSASFNNAVDSASRSGIISAIAAGND
GANAANTSPASAASAITVGAIDSNWAIASYSNYGTVLDIFAPGSAVLSAWYTSNSATNS
ISGTSMATPHIAGLVLYGISVNGVSGVSGVTNWLTSTATSGQITGNLR**SSPNLIGNNGN**
TAQ

Figure S19. Identification of chitinase-modifying activity in protein extract from *Fusarium oxysporum* f. sp. *lycopersici* Δ*fomep1* mutant. (A) Proteins present in culture filtrates of Δ*fomep1* size-fractionated on a Superdex G-75 column and each fraction was incubated with SlChi1 as described in materials and methods. Active fractions displaying chitinase-modifying activity are indicated. Five samples, numbered from 1 to 5 were excised from the gel for mass spectrometry analysis. FoSep1 and FoSep2 were identified in samples 3 and 4 that contain two clear protein bands indicated by a white asterisk. (**B)** Residues in bold represent the amino acid sequence of the unique peptides identified by mass spectrometry in these two bands representing FoSep1 (sample 4) and FoSep2 (sample 3), the two serine proteases secreted by *Fusarium oxysporum* f. sp. *lycopersici*.

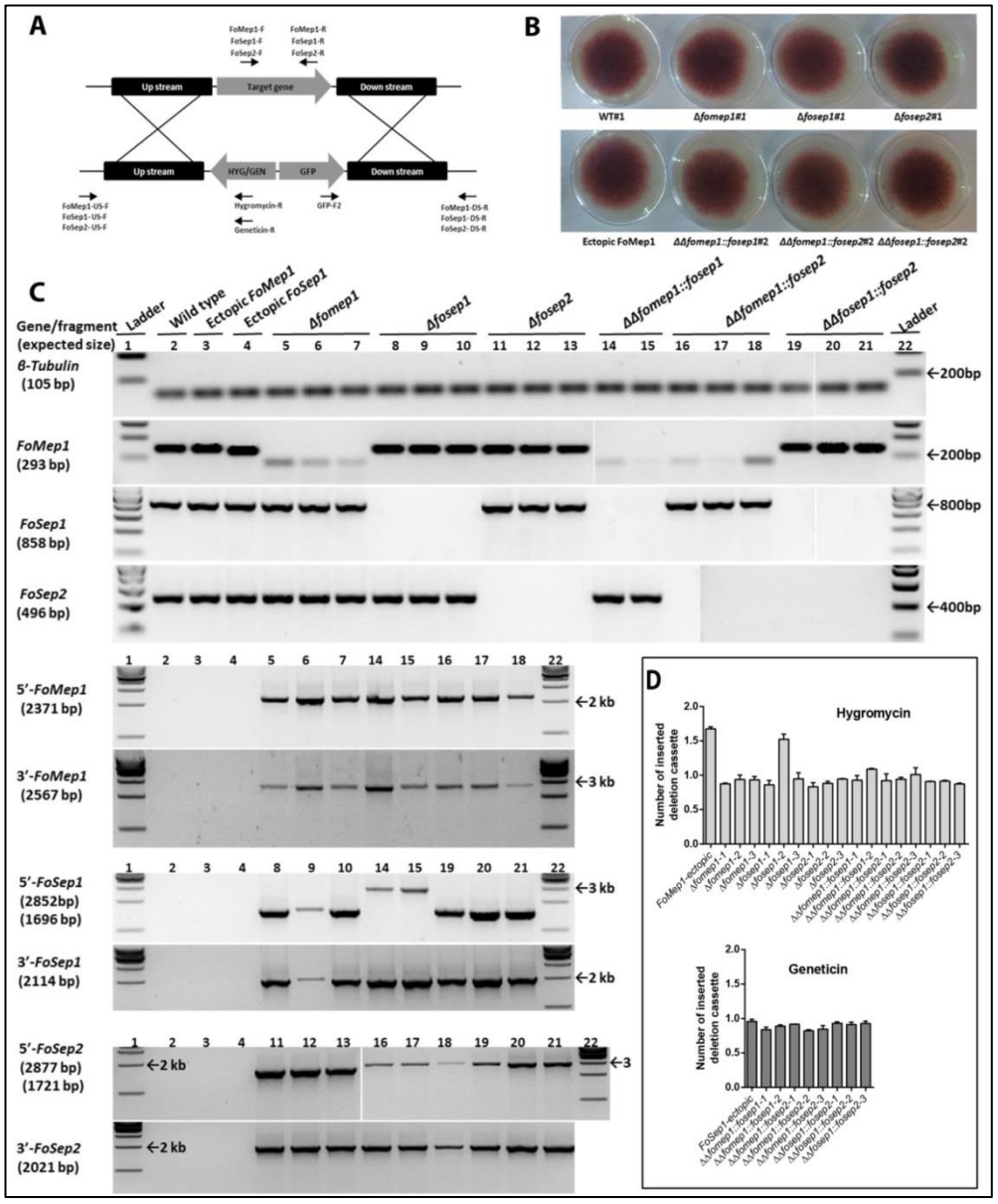


Figure S20. **Molecular analysis of single and double mutants of *FoMep1, FoSep1, FoSep2* protease encoding genes of *Fusarium oxysporum* f. sp. *lycopersici.* (A)** Representation of the *FoMep1, FoSep1, FoSep2* genes in the wild type and homologous recombinant deletion mutants. *FoMep1, FoSep1, FoSep2* genes were replaced by a cassette carrying hygromycin resistance (*Hph*) and *green fluorescent protein* (*Gfp*) genes. Double mutants were made by replacing *FoSep1, FoSep2* genes using a cassette carrying geneticin resistance (*Gen*) and *green fluorescent protein* (*Gfp*) genes in the background of Δ*fomep1.* **(B)** Representation of growth of the wild type, single and double deletion mutants of *FoMep1, FoSep1, FoSep2*. **(C)** Targeted gene deletion of *FoMep1, FoSep1*, and *FoSep2* was confirmed by PCR using oligonucleotides shown in panel A. On top of panel C, lanes are

numbered to simplify the presentation of lower gels. Lane 1 and 22 show markers of 200bp and 1kb ladders, respectively. Lanes 2 to 21 show the source of DNA that was used for PCR; lane 2: wild type *Fusarium oxysporum* f. sp. *lycopersici,* lanes 3 and 4 ectopic transformants for Δ*fomep1* and Δ*fomep1,* respectively, lanes 5, 6, and 7 independent deletion mutants of *FoMep1* (Δ*fomep1-1/2/3*), lanes 8, 9, and 10 independent deletion mutants of *FoSep1* (Δ*fosep1-1/2/3*), lanes 11, 12, and 13 independent deletion mutants of *FoSep2* (Δ*fosep1-1/2/3*), lanes 14 and 15 independent double deletion mutants of *FoMep1Sep1* (ΔΔ*fomep1::fosep1-1/2*), lanes 16, 17, and 18 independent double deletion mutants of *FoMep1Sep2* (ΔΔ*fomep1::fosep2-1/2/3*), and lanes 19, 20, and 21 independent double deletion mutants of *FoSep1Sep2* (ΔΔ*fosep1::fosep2-1/2/3*). On the left side of all gel pictures, the name of fragment/gene with the expected size in brackets is presented. First row shows the amplification of *β-Tubulin* for all strains. Second, third and fourth row shows the deletion of candidate genes in the corresponding single or double deletion mutants. Replacement was confirmed for all three genes by amplification of fragments at 5′ of candidate genes shown as 5′-*FoMep1,* 5′-*FoSep1,* 5′-*FoSep2* using forward primers outside the upstream of candidate genes and reverse primers inside the *Hyg* or *Gen* gene*.* Fragments 3′-*FoMep1,* 3′-*FoSep1,* 3′-*FoSep2* confirm the recombination at 3′ using forward primers inside the *Gfp* gene and reverse primers downstream of candidate genes. The reverse primer Hygromicin-R is used to confirm the 5′ of deleted candidate genes, except for 5′-*FoSep1* in lanes 14 and 15 and for 5′-*FoSep2* in lanes 16-21*,* as these two genes are replaced by cassettes carrying the geneticin gene, resulting in a larger PCR product using the geneticin-R primer*.* **(D)** Single insertion event of the gene deletion cassette was confirmed by quantitative PCR using genomic DNA of each transgenic strain. The *Hyg* and *Gen* gene were used as a measure for the number of insertion events, together with the *β-Tubulin* gene for normalization and the *Six1* gene as a single copy reference gene, using the E^-ΔCt method.

Table S1. Characteristics of tomato chitinase genes. In the genome of tomato, 30 genes are annotated as chitinase GH18 or GH19.

No	Name	Gene ID	Location on chromosome	Protein length (aa)	MW (kDa)	IPR domain	pI	Class	CBD	Predicted signal peptide (aa)	Protein family	Proteome (Tomato x *C. fulvum*)
1	SlChi1	Solyc10g074440.1.1	10	322	31.9	IPR000726	8.56	I	+	24	GH19	+
2	SlChi2	Solyc10g055810.1.1	10	322	31.9	IPR016283	6.21	I	+	22	GH19	+
3	SlChi3	Solyc10g055800.1.1	10	329	33.2	IPR016283	8.55	I	+	19	GH19	+
4	SlChi4	Solyc10g055820.1.1	10	322	32.4	IPR000726	6.32	I	+	22	GH19	-
5	SlChi5	Solyc02g082960.2.1	2	273	27.8	IPR000726	9.44	II	-	20	GH19	-
6	SlChi6	Solyc02g061770.2.1	2	263	26.4	IPR000726	8.45	II	-	19	GH19	-
7	SlChi7	Solyc02g082930.2.1	2	247	24.8	IPR016283	4.68	II	-	16	GH19	+
8	SlChi8	Solyc02g082920.2.1	2	253	25.0	IPR000726	5.63	II	-	24	GH19	+
9	SlChi9	Solyc01g095250.1.1	1	298	29.3	IPR001223	4.53	III	-	25	GH18	-
10	SlChi10	Solyc01g095310.1.1	1	302	29.4	IPR001223	4.96	III	-	26	GH18	-
11	SlChi11	Solyc01g095260.1.1	1	298	29.3	IPR001223	4.43	III	-	25	GH18	-
12	SlChi12	Solyc05g050130.2.1	5	292	28.7	IPR001223	8.21	III	-	23	GH18	+
13	SlChi13	Solyc04g072000.2.1	4	276	27.0	IPR016283	4.44	IV	+	26	GH19	+
14	SlChi14	Solyc06g053380.2.1	6	289	29.7	IPR016283	5.2	IV	+	19	GH19	-
15	SlChi15	Solyc07g005080.1.1	7	386	41.8	IPR011583	4.82	V	-	N	GH18	-
16	SlChi16	Solyc07g005090.2.1	7	371	39.3	IPR011583	8.84	V	-	21	GH18	+
17	SlChi17	Solyc07g005100.2.1	7	376	39.1	IPR011583	9.27	V	-	22	GH18	+
18	SlChi18	Solyc09g098540.2.1	9	319	33.2	IPR000726	7.1	VII	-	21	GH19	-
19	SlChi19	Solyc12g098810.1.1	12	328	33.9	IPR000726	5.72	VII	-	24	GH19	-
20	SlChi20	Solyc11g072830.1.1	11	306	30.9	IPR001223	4.69	VIII	-	24	GH18	+
21	SlChi21	Solyc11g072750.1.1	11	342	38.9	IPR001223	6.89	VIII	-	25	GH18	-
22	SlChi22	Solyc11g072760.1.1	11	306	31.8	IPR001223	6.13	VIII	-	25	GH18	-
23	SlChi23	Solyc11g005890.1.1	11	295	30.5	IPR001223	5.25	VIII	-	24	GH18	-
26	SlChi24	Solyc10g055780.1.1	10	170	18.8	IPR000726	9.5	I	-	N	GH19	-
24	SlChi25	Solyc10g068350.1.1	10	86	9.2	IPR000726	7.65	I	-	N	GH19	-
25	SlChi26	Solyc10g074380.1.1	10	64	7.2	IPR000726	9.6	I	-	N	GH19	-
27	SlChi27	Solyc10g074460.1.1	10	194	18.3	IPR000726	7.67	I	+	24	GH19	-
28	SlChi28	Solyc10g074400.1.1	10	173	15.0	IPR000726	8.57	I	+	31	GH19	-
29	SlChi29	Solyc10g074360.1.1	7	154	17.2	IPR000726	8.51	I	-	N	GH19	-
30	SlChit30	Solyc10g074390.1.1	10	216	20.2	IPR000726	7.6	I	+	24	GH19	-

Length, molecular weight (MW) and isoelectric point (pI) were determined using the Expasy online website (http://web.expasy.org/). Interpro domain (IPR) was determined using the Interpro-EBI (Mitchell *et al.*, 2015). The chitinase class and presence/absence of a chitin binding domain (CBD) were determined based on similarity to characterized chitinases and pfam prediction at Pfam database (Finn *et al.*, 2014). Signal peptides were determined by SignalP (V4) (Petersen *et al.*, 2011). Proteome support refers to LC-MS data obtained from apoplastic fluids isolated from tomato plants inoculated with *Cladosporium fulvum* race 0 (R0).

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Table S2. Reference protein sequences used for classification of tomato chitinases.

Name	Accession	Class	Source	Reference
NtChN50	CAA45822	I	*Nicotiana tabacum*	(Van Buuren *et al.*, 1992)
NtFB7.1	AAB23374	I	*Nicotiana tabacum*	(Neale *et al.*, 1990)
OsChia2a	Q7XCK6	II	*Oryza sativa*	(Truong *et al.*, 2003)
StChiA2	AAB96341	II	*Solanum tuberosum*	(Büchter *et al.*, 1997)
CaChi3-P1	AAN37393.1	III	*Capsicum annuum*	(Cheng *et al.*, 2002)
VvChi3K	BAC65326.1	III	*Vitis vinifera*	(Ano *et al.*, 2003)
ZmChitA-LH82	ACX37090	IV	*Zea mays*	(Naumann and Wicklow, 2010)
NtChitIV	BAF44533.1	IV	*Nicotiana tobacum*	(Shinya *et al.*, 2007)
AtChiC	3AQU_C	V	*Arabidopsis thaliana*	(Ohnuma *et al.*, 2011)
NtChi-V	Q43576	V	*Nicotiana tabacum*	(Melchers *et al.*, 1994)
GhChia7	AAP80801.1	VII	*Gossypium hirsutum*	(Kolosova *et al.*, 2014)
EgChit1	AFV30204.1	VII	*Elaeis guineensis*	(Yeoh *et al.*, 2013)

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Table S3. Primer pairs used for qrtPCR analyses.

Gene	Primer sequence (5'-3')		Efficiency
SIChi1	*F: CCATCGGAGTGGACCTTTTA*	*R: ACACCAAATCCAGGAAGACG*	**2.2**
SIChi2	*F:GGGACCTAGGCGGTGTTATT*	*R: CCCTTTTACGGGCAGTGATA*	**1.8**
SIChi3	*F: CCAGTGTCCATCTGGTCCTT*	*R: ACGAAAAGACCTTGCAGCAT*	**1.9**
SIChi4	*F: GAAATTGCTGCCTTCTTTGC*	*R: CTCCAATGGCTCTTCCACAT*	**1.9**
SlChi13	*F: CCCTTGTGTCTCAGGGAAAA*	*R: CTAATTGTTGGGCCAAATCC*	**2.0**
SIChi14	*F: GGCTTAACGACCCCGATATT*	*R: GCACCATCACACTCAAGAGG*	**1.8**
LeTub	*F:AACCTCCATTCAGGAGATGTTT*	*R: TCTGCTGTAGCATCCTGGTATT*	**1.9**
FoTub	*F: GTCTCACGAGCCAAGTCTACC*	*R: TTGTCGGGACGGAAGAGCTGA*	**1.8**
Hygromycin	*F: ATAGGTCAGGCTCTCGCTGA*	*R: GCGAAGAATCTCGTGCTTTC*	**2**
Geneticin	*F: ATGACTGGGCACAACAGACA*	*R: AGTGACAACGTCGAGCACAG*	**2**

Table S4. Primers used for expression of the six genes encoding CBD-chitinases in the *Pichia pastoris.*

Primer name	Primer sequence (5'-3')*
SlChi1-pichia-F	*AAAAAA**CCTAGG**CATCATCATCATCATCATCCCGACTACAAGGACGACGATGACAAG**CAGAATTGTGGTTCACAGG*
SlChi1-pichia-R	*AAAAAA**GCGGCCGC**ATGAAGTTATTAGATAGAA*
SlChi2- pipic9- F551	*AAAAAA**CTCGAG**AAAAGAGAGGCTGAAGCTCATCATCATCATCATCATCCCGACTACAAGGACGACGATGACAAGGAGCAATGTGGTTCACAGGC*
SlChi2- pipic9- R	*AAAAAAGCGGCCGCTATATGATGAAGTCGATCG*
Slchi13-pichia-F	***GAATTC**CATCATCATCATCATCATCCCGACTACAAGGACGACGATGACAAG**CAAGATTGTGGTTGTTCATCGG*
Slchi13-pichia-R	***GCGGCCGC**TCCAACTTGTTAATATATAA*
Slchi4-pichia-F	***CCTAGG**CATCATCATCATCATCATCCCGACTACAAGGACGACGATGACAAG**GAGCAATGTGGTGTGCAGG*
Slchi4- Pichia-R	*GCGGCCGCTTACATAGTATCGACTAAGA*

Bolded sequences indicate restriction sites and underlined sequences encode His- and Flag-tags.

Table S5. Primers used to construct deletion cassettes for *FoMep1, FoSep1* and *FoSep2.*

Gene	Primer sequence (5'-3')*
FoMep-P4-F	*GGGGACAACTTTGTATAGAAAAGTTGACCCCAGATAGCAATGTCGCAT*
FoMep1-P4-R	*GGGGACTGCTTTTTTGTACAAACTTGCGCAATTTTGTTGCTGCCAAC*
FoMep1-P2-F	*GGGGACAGCTTTCTTGTACAAAGTGGAGACGCCATCATCGATGCCGA*
FoMep1-P2-R	*GGGGACAACTTTGTATAATAAAGTTGGGTTCAAAGAATGGAGCGAC*
FoSep1- P4-F	*GGGGACAACTTTGTATAGAAAAGTTGGTGGACTAGCCAGGTTTGGG*
FoSep1- P4-R	*GGGGACTGCTTTTTTGTACAAACTTGTACAAGAGGAGATGAGGCTGTG*
FoSep1- P2-F	*GGGGACAGCTTTCTTGTACAAAGTGGGGTTACCTCTGAATAGACCCA*
FoSep1- P2-R	*GGGGACAACTTTGTATAATAAAGTTGGTTAGCCCCACTGAAGAGCC*
FoSep2- P4-F	*GGGACAACTTTGTATAGAAAAGTTGAATTCACCAAGCCTCTGACGATA*
FoSep2- P4-R	*GGGGACTGCTTTTTTGTACAAACTTGAGAGACGATTGGGGATGCTTGT*
FoSep2- P2-F	*GGGGACAGCTTTCTTGTACAAAGTGGTATATGAGATTGAGAATGACTACAAGATTA*
FoSep2- P2-R	*GGGGACAACTTTGTATAATAAAGTTGGTCCACTGTATACGTTCCAGGAT*
Gen-GRFP-F1	*TCCTATTCCGAAGTTCCTATTCTCTCAGTTAACGTCGACGGTATCGATT*
Gen-R1	*AGAGAATAGGAACTTCGGAATAGGA*
GW-Gen-F1	*GGGGACAAGTTTGTACAAAAAAGCAGGCTACGCTTACAATTTCCATTCGCCAT*
GW-GRFP-R1	*GGGGACCACTTTGTACAAGAAAGCTGGGTCGCGCAATTAACCCTCACTAAAG*

* Underlined sequences are the Gateway AttB flanking sequences.

Table S6. Primers used to screen transformants *Fusarium oxysporum* f. sp. *lycopersici*.

Gene	Primer sequence (5'-3')
FoMep1-F	*AGAATGATGCGGATGAACCCA*
FoMep1-R	*GGTTCGGCTGGAAGCACTAT*
FoSep1-F	*AAGCGTGACACTTCCGGTG*
FoSep1-R	*TGGAGTCGATGGCAGCAAC*
FoSep2-F	*TATGGCTTCCACGGCTATGC*
FoSep2-R	*ACCTTGACAGCCTGGATGGT*
FoSep1- US-F	*ACCCTGGACCCCTTTCTGT*
FoSep1- DS-R	*GCAAGGCTTCCTGCCCTAG*
FoSep2- US-F	*GGCTTCGAGACATAGAATCATG*
FoSep2- DS-R	*GCAATGCGGACCTTGCTATG*
FoMep1- US-F	*AAACCCAGCGCTTTGAGGTGA*
FoMep1- DS-R	*TTCAATGATGAGGCGCCAGA*
GFP-F	*GATCACTCACGGCATGGAC*
Hygromycin- R	*GTCCGAGGGCAAAGGAATAG*
Geneticin-R	*GAAGAACTCGTCAAGAAGGCGATA*
FoTub-F	*GTCTCACGAGCCAAGTCTACC*
FoTub-F	*TTGTCGGGACGGAAGAGCTGA*

Chapter 5

In search for interactors of the putative carboxypeptidase inhibitor Avr9 of *Cladosporium fulvum*

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ABSTRACT

Avr9 is an apoplastic effector secreted by the fungal tomato pathogen *Cladosporium fulvum* and is recognized by the tomato receptor-like protein Cf-9. So far, there is no experimental proof for direct interaction between Avr9 and Cf-9. According to the guard hypothesis, Cf-9 might monitor the target(s) of Avr9. As Avr9 shares significant structural similarity with carboxypeptidase inhibitors, its target(s) might be apoplastic proteases. Here, we aimed at identifying apoplastic interactors of Avr9 that might be virulence targets and guarded by Cf-9. Using biotinylated Avr9, we performed pull-down and far-western blotting assays with apoplastic fluids from a susceptible tomato cultivar inoculated with a *C. fulvum* race lacking the *Avr9* gene. However, no specific Avr9-interacting proteins could be identified. We then hypothesized that glycosylation of Avr9 might be crucial for interaction with host target(s). Mass spectrometry analysis revealed that Avr9 is N-glycosylated when secreted by *C. fulvum*, containing at least two N-acetylglucosamine (GlcNAc) and six mannose residues. The necrosis-inducing activity of glycosylated and non-glycosylated Avr9 was determined and it was found that both caused a comparable Cf-9-mediated hypersensitive response. The bottlenecks, challenges and alternative approaches to identify the intrinsic biological function(s) of Avr9 are discussed.

INTRODUCTION

Cladosporium fulvum is a non-obligate biotrophic fungus causing leaf mould of tomato (*Solanum lycopersicum*) (de Wit et al., 2012). During infection, *C. fulvum* secretes many effectors into the apoplast of tomato including Avr2, Avr4, Avr4E, Avr5 and Avr9; facilitating infection and colonization of tomato leaves (Stergiopoulos and de Wit, 2009; Mesarich et al., 2014). Avr2 and Avr4 provide protection against basal defence enzymes deployed by the host. Avr2 is an offensive virulence factor that inhibits apoplastic tomato cysteine proteases (Rooney et al., 2005; van Esse et al., 2008), while Avr4 is a defensive virulence factor, that specifically binds to chitin present in fungal cell walls, thereby providing protection against hydrolytic activity of plant chitinases (van den Burg et al., 2006; Van Esse et al., 2007). The function of Avr4E and Avr5 are unknown. Although Avr9 is the first effector cloned from *C. fulvum* (van Kan et al., 1991), its intrinsic function is not known yet. Expression of the *Avr9* gene is highly induced during plant colonization (van Kan et al., 1991), but could hardly be detected during mycelial growth *in vitro. Avr9* encodes a 63 amino acid pre-pro-protein that is processed by fungal and/or host proteases into a 28 amino acid mature peptide that accumulates in the apoplast (van den Ackerveken et al., 1993).

Nuclear Magnetic Resonance (NMR) studies revealed that Avr9 consists of three anti-parallel strands forming a rigid region of β-sheet with six cysteine residues that form disulfide bridges, resulting in a cystine knot structure (Vervoort et al., 1997; van den Hooven et al., 2001). Such structure is commonly found in carboxypeptidase inhibitors, ion-channel blockers and growth factors (Pallaghy *et al.*, 1994) and it was therefore suggested that Avr9 might inhibit tomato carboxypeptidases (Vervoort et al., 1997). However, such an activity has not been demonstrated, but this hypothesis has so far only been tested with commercially available carboxypeptidase (van den Hooven et al, 2001). The tomato resistance protein Cf-9 belongs to the Receptor-Like Protein (RLP) family and recognizes Avr9 triggering a hypersensitive response (HR) (van der Hoorn et al., 2005). This interaction likely requires additional interactors because no evidence for direct interaction between Avr9 and Cf-9 could be found when the Cf-9 protein was produced in insect cells, COS cells or in *Arabidopsis thaliana* (Luderer et al., 2001).

Using the I^{125} labelled Avr9 as a ligand, a high affinity binding site (HABS) was detected in plasma membranes of solanaceous and some non-solanaceous plant species, irrespective of the presence or absence of the *Cf-9* gene (Kooman-Gersmann et al., 1996). Introduction of *Cf-9* gene in tomato, potato and tobacco (containing the HABS) triggered an HR upon Avr9 infiltration. In contrast, *Cf-9*-transgenic *A. thaliana*, lacking the HABS, failed to induce HR after Avr9 infiltration (Kooman-Gersmann et al., 1998), suggesting that the HABS is essential for Avr9-triggered Cf-9-mediated HR. In addition, mutational analysis of the Avr9 peptide showed a positive correlation between binding affinity to the HABS and its ability to trigger a Cf-9-mediated HR (Kooman-Gersmann et al., 1998). However, it is not known whether this HABS is a target of Avr9 or a co-receptor required for its recognition. The HABS may function similarly to the Receptor-Like Kinases (RLK) *Sl*SOBIR1 or BAK1 to facilitate Avr9-triggered Cf-9-mediated defense signaling (Liebrand et al., 2014). These two RLKs were shown to interact with Cf-4, Cf-9 and Ve1 *in planta* and are required for the Cf-4-, Cf-9, and Ve1-mediated HR and immunity to the fungal tomato pathogens *C. fulvum* and *Verticillium dahliae*, respectively (Liebrand et al., 2013). Avr9 contains a potential N-glycosylation site (NSS signature). Most of the Av9 peptides purified from tobacco or tomato plants expressing the *Avr9* gene using the PVX expression system contained one N-acetyl glucosamine (GlcNAc) residue attached to the asparagine residue of the NSS glycosylation signature (Kooman-Gersmann et al., 1998). It is likely that Avr9 is also N-glycosylated when it is secreted by *C. fulvum*. The Avr9 peptide carrying one GlcNAc residue showed a lower affinity for the HABS than non-glycosylated Avr9.

However, it is not certain that the Cf-9 protein is correctly folded when expressed in heterologous hosts and these experiments could not exclude that correctly folded Cf-9 still binds to Avr9 (Luderer et al., 2001).

Based on its homology to carboxypeptidase inhibitors (van den Hooven et al., 2001), we envisaged that Avr9 might act like Avr2 as a protease inhibitor in the tomato apoplast (Rooney et al., 2005). Biotinylated ligands have frequently been used to find their interactors (de Jonge et al., 2010; Shinya et al., 2010). In the present study, we set out to isolate possible targets of Avr9 from the apoplast of *C. fulvum*-infected tomato using synthetic biotinylated Avr9 as a probe. In addition, we envisaged that glycosylation of Avr9 might be important for binding to the HABS, the Cf-9 protein or possible apoplastic targets. The glycosylation status of Avr9 produced by *C. fulvum in vitro* under the control of a constitutive promoter was determined by mass spectrometry. We also performed necrosis-inducing activity of glycosylated and non-glycosylated Avr9 on Cf-9 plants. We found that both glycosylated and non-glycosylated Avr9 caused a comparable Cf-9-mediated HR. However we could not produce sufficient amounts of glycosylated Avr9 proteins for pull down assays to detect their potential apoplastic host targets.

MATERIAL AND METHODS

Plant and fungal materials

Tomato cultivars MoneyMaker-Cf-4 (MM-Cf-4), -Cf-9 (MM-Cf-9) and the sequenced cultivar Heinz were used for all *in planta* studies, including assays for Cf-mediated hypersensitive response (HR) and virulence assays. Tomato plants were grown in a greenhouse at 70% relative humidity, 23-25 °C during daytime and 19-21 °C at night, with a light/dark regime of 16/8 hours and 100 W m^{-2} supplemental light when the sunlight influx intensity was less than 150 W m^{-2}. *C. fulvum* race 0 (the sequenced strain) (de Wit et al., 2012), and strain IPO2559 (this strain lacks the *Avr9* gene (race 2.4.4E.9)) and a *C. fulvum* transformant (race 5) that over-expresses the *Avr9* gene under the control of *Aspergillus nidulans gpd* promoter (*GPD*::*Avr9)* (van den Ackerveken et al., 1993) were used in this study.

Preparation of fungal inoculum

C. fulvum strains were grown on 1% Oxoid™ Potato Dextrose Agar (PDA) plates containing 100 µg/mL of streptomycin sulfate salt (Sigma-Aldrich) for 12 days at 20 °C. Spores were harvested from PDA plates by flooding the sporulating plates with sterile water. Fungal mycelia were removed using Miracloth (Calbiochem) and spores

were collected by centrifugation at 3,700xg for 15 min, re-suspended in sterile water and the concentration was adjusted to 10^6 spores/mL.

Isolation of apoplastic fluid from *Cladosporium fulvum*-infected tomato leaves

Inoculation of tomato plants with *C. fulvum* was performed as previously described (Ökmen et al., 2013). F*u*ngal spore suspensions were prepared from *C. fulvum* strains 0 (sequenced strain carrying all functional Avr genes)) and IPO2559 (carrying non-functional *Avr4* and *Avr9* genes), adjusted to 10^6 spores/mL and sprayed on the abaxial side of leaves from 4-week-old tomato plants (cv. Heinz). Leaf samples were collected at 0, 2, 4, 6, 8 and 10 days post inoculation (dpi) for apoplastic fluid (AF) isolation using a previously described method (de Wit and Spikman, 1982). AF samples were cleared by centrifugation at 12,000xg for 20 min at 4 $^{\circ}$C.

Biosynthesis and folding of biotinylated Avr9

Linear biotinylated Avr9 (B-Avr9) was synthesized (GenScript, USA). B-Avr9 contained one biotin molecule at the N-terminus (at the Tyr residue) of mature Avr9 (28 amino acids) without any linker. The purity of synthetic linear B-Avr9 was tested using High-Performance Liquid Chromatography (HPLC) before folding by incubation in reduced and oxidized glutathione (GSSG/GSH) as previously described (van den Hooven et al., 1999). Each folding reaction consisted of 15 mg purified linear B-Avr9, 0.2 M MOPS (pH=7.3), 0.4 M KCl, 2 mM EDTA, 1 mM GSH, 0.5 mM GSSG, and H_2O up to a total volume of 200 mL. The reaction mixture was incubated overnight at 4 °C and the proteins were separated by HPLC using a 300A DeltaPak C18 column (Waters). The proteins were eluted from the column by a gradient of acetonitrile (from 5% to 60%) and collected fractions were vacuum-dried and dissolved in H_2O. Three concentrations of folded B-Avr9 and native Avr9 (0.3 µM, 1 µM and 3 µM) were infiltrated into MM-Cf-9 plants for determining their HR-inducing activity.

Pull-down assays

As described above, AF from tomato leaves inoculated with strain IPO2559 was isolated. All AFs samples collected at different dpi were combined in an equal ratio. The mixture was calibrated to pH=6 using extraction buffer (100 mM KH_2PO_4 pH=6, 100 mM NaCl, 2 mM EDTA, protease inhibitor mix (Roche 1 complete tablet), 0.1% NP40, and 5% non-soluble PVPP (Polyvinylpolypyrrolidone)) and incubated while rotating for 2 h at 4 °C, followed by centrifugation for 20 min at 12000xg to remove PVPP and insoluble residues. The supernatant was passed through 0.2 µm filters. B-

Avr9 bound to streptavidin magnet beads was prepared as follows. For each reaction, 50 µL of streptavidin magnet beads (50% slurry, Promega) were washed using PBS buffer (100 mM KH_2PO_4 pH=6), and re-suspended in 400 µL PBS buffer. One hundred µg of B-Avr9 was incubated with streptavidin magnet beads in a reaction volume of 500 µL. Similarly, as a control, streptavidin magnet beads were incubated with the same amount (molarity) of biotin as B-Avr9 or with PBS buffer. Mixtures were then incubated for 2 h at 4 °C and washed three times to remove unbound B-Avr9. The streptavidin magnet beads incubated with biotin alone or B-Avr9 were subsequently incubated with 2 mL of AF while rotating for 2 h at 4 °C. Samples were then washed five times with 1 mL PBS buffer using the magnetic stand. Putative interactors were dissolved in 20 µL of SDS loading buffer, heated for 10 min at 95 °C and centrifuged at 14000xg. Supernatants were separated on a 16% tricine SDS polyacrylamide gel (SDS-PAGE) that was subsequently stained with coomassie brilliant blue.

Far-western blotting

To find putative Avr9 interactors in AF of tomato, far-western blotting was performed using B-Avr9 as a probe and biotin as a control. Samples included apoplastic proteins harvested from cv. Heinz inoculated with *C. fulvum* strain IPO2559 at 0, 2, 4, 6, 8 and 10 dpi. These samples were mixed with SDS loading buffer and incubated for 30 min without heating for 10 min at 95 °C. Samples were loaded and run on a 16% tricine SDS-PAGE gel. Proteins were then transferred from the gel to an Immuno-Blot® PVDF membrane (Bio-Rad) using electro-blotting at 200 mA for 2 h. The PVDF membrane was incubated with blocking solution containing 3% skimmed milk for 2 h at 20 °C. To detect proteins with affinity for Avr9, blots were incubated in 1 µM B-Avr9 or biotin as control for 12 h at 4 °C. Blots were subsequently incubated with 1:2,000 diluted streptavidin-HRP solution (streptavidin conjugated to Horse Radish Peroxidase, Sigma). Blots were subsequently developed using a mixture of Pico and Femto peroxidase substrates solutions (1:1). Signals were detected using the BioRad Chemi Doc sensitive chemiluminescent system.

Isolation and purification of glycosylated Avr9 from culture filtrates of a *Cladosporium fulvum Avr9* overexpressing strain grown *in vitro*

To identify the sugar moiety present at the N-glycosylation site of Avr9, spores of transgenic *C. fulvum* overexpressing *Avr9* were inoculated (1×10^5 spores/mL) in 10 sterile baffled flasks, each containing 100 mL of sterile Difco™ Potato Dextrose Broth (PDB). Flasks were incubated in an orbital shaker incubator at 22 °C and 200 rpm, and

cultures from five flasks were harvested at 6 and 12 dpi, respectively. Mycelia were removed using Miracloth and culture filtrates were cleared by a two-step centrifugation at 3,700xg for 15 min and subsequently at 12,000xg for 20 min at 4 °C. To purify secreted Avr9, culture filtrates were passed through Amicon filters to remove proteins above 30 kDa in size. The flow-through was washed three times with water and concentrated 100-fold by passing over a 1kDa Amicon filter.

To demonstrate the presence of Avr9, 15 µL of concentrated culture filtrates were loaded on a 15% low pH-PAGE gel as previously described (van den Ackerveken et al., 1993) and run under non-denaturing conditions using pyronine Y as a front marker. Electrophoresis was carried out at 200 V for 90 min. A part of gel was cut and subsequently stained with coomassie brilliant blue. The stained part was used as a guide to cut the gel bands and to purify Avr9 peptide from the non-stained parts of the gel. To test their capacity to induce an HR on Cf-9 tomato plants, bands were cut from low pH-PAGE were washed 3 times with water, and then gel pieces were incubated in water to elute proteins from the gel. Concentration of eluted glycosylated and non-glycosylated Avr9 proteins were measured and infiltrated into MM-Cf-9 tomato at 0.3, 1 and 3 µM to determine their ability to induce Cf-9-mediated HR. To identify sugar residues present at the N-glycosylation site of Avr9, the masses of the eluted Avr9 proteins were analysed by LC-ESI-MS. Spectral peaks in the chromatogram were de-convoluted and de-isotoped. The de-convoluted and de-isotoped masses of selected spectra were used for calculating the number of hexose (presumably mannose), and GlcNAc residues.

RESULTS

Chemically synthesized and folded biotinylated Avr9 triggers a Cf-9-mediated hypersensitive response

Processing of pre-pro-protein Avr9 from 40 to 32 amino acids by fungal and plant proteases to a mature 28 amino acid peptide has been previously shown (van den Ackerveken et al., 1993). Mature Avr9 is stable and no further processing by fungal and plant proteases has ever been reported. Thus, we chose to synthesize the 28-amino acid Avr9 peptide fused to biotin, a small molecule (244.3 Dalton) that strongly and specifically binds to avidin and streptavidin (Shinya et al., 2010). Linear B-Avr9 was folded under conditions as previously described (van den Hooven et al., 1999). HPLC analysis showed that linear B-Avr9 eluted at 29.13 and folded B-Avr9 at 23.52 min (Fig. 1).

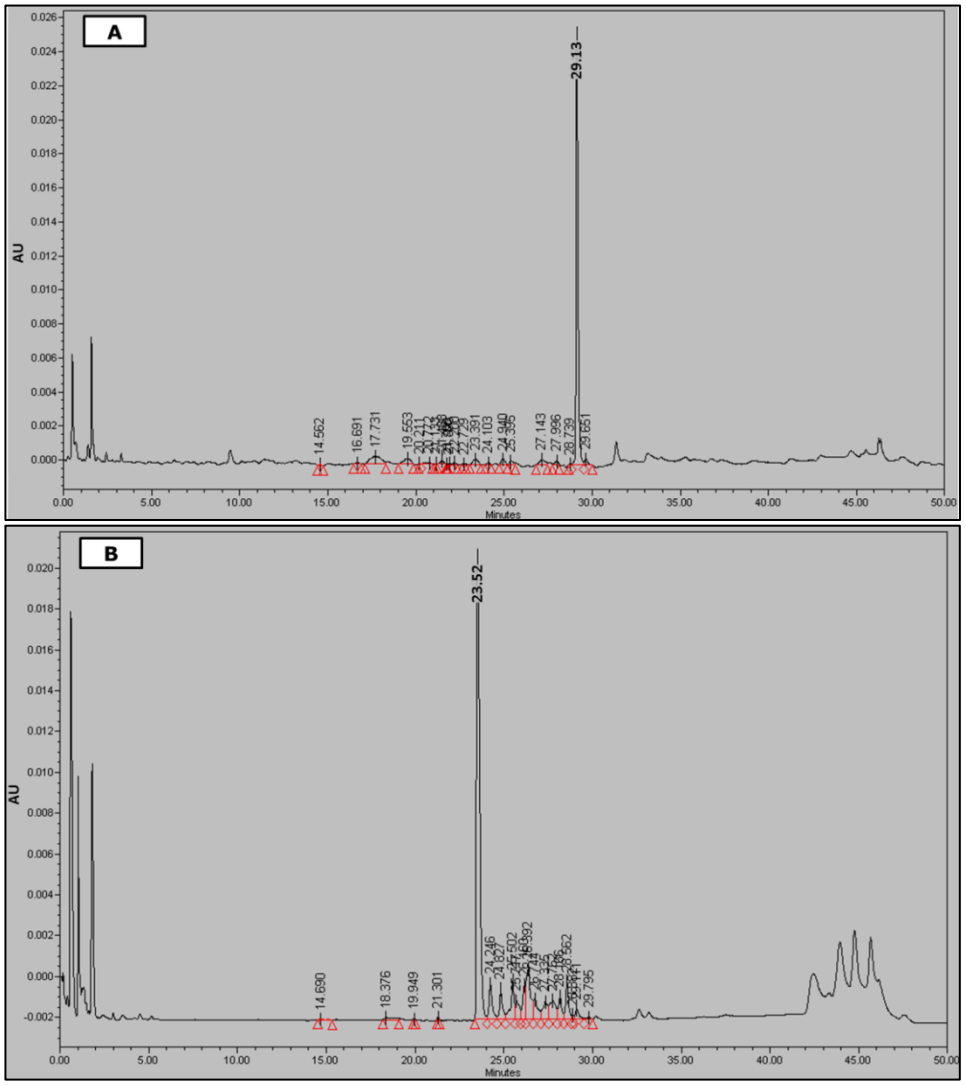

Figure 1. HPLC chromatogram of elution for linear and folded biotinylated Avr9 (B-Avr9). To isolate correctly folded B-Avr9 from reaction mixtures, samples of linear and folded B-Avr9 were analysed by HPLC. **(A)** Linear B-Avr9 eluted at 29.13 min after injection. **(B)** Correctly folded B-Avr9 eluted at 23.53 min after injection as reported previously (van den Hooven et al, 1999). Small peaks eluting between 23.52 and 29,13 min correspond to incorrectly folded or partially reduced linear B-Avr9. Y-axis shows the absorption at 214 nm represented by arbitrary unit (AU). Red triangles are signs for baseline calculation.

Small peaks eluting between 23.6 and 30 min likely correspond to incorrectly folded and partially reduced B-Avr9. We tested the HR-inducing activity and specificity of folded B-Avr9 by infiltrating protein fractions into leaves of MM-Cf-4 and MM-Cf-9 tomato plants. As expected, native Avr9 triggered an HR only on MM-Cf-9 plants (Fig.

2). Similar to native Avr9, correctly folded B-Avr9 induced an HR on MM-Cf-9 plants, but not on MM-Cf-4 plants. This result indicates that folded B-Avr9 is recognized by Cf-9 plants as specifically and efficiently as native Avr9.

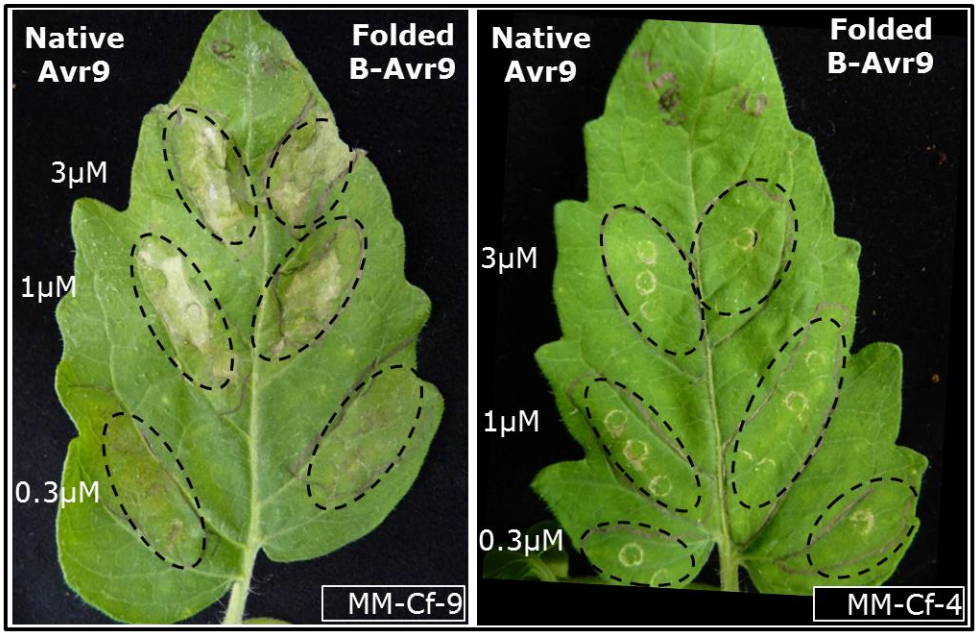

Figure 2. Native Avr9 and correctly folded biotinylated Avr9 induce a specific Cf-9-mediated hypersensitive response. Folded biotinylated Avr9 (B-Avr9), and native Avr9 were infiltrated into MM-Cf-9 (left panel) and MM-Cf-4 (right panel) leaves at 3 different concentrations (0.3 µM, 1 µM and 3 µM). The hypersensitive responses are induced by correctly folded B-Avr9 and native Avr9 on MM-Cf-9, but not on MM-Cf-4 plants, indicating that both proteins are specifically recognized by Cf-9 plants.

Pull-down assays and far-western blotting did not result in identification of Avr9 interactors

We hypothesized that Avr9 might target tomato proteins, likely proteases, present in the apoplast of tomato leaves. To identify such interacting proteins, we used the folded B-Avr9 peptide to perform pull-down assays with apoplastic fluid harvested from cv. Heinz inoculated with strain IPO2559. This strain lacks the *Avr9* gene, which avoids occupation of target proteins with endogenous non-labelled Avr9 proteins produced by the fungus during infection. The absence of Avr9 in the collected AF from IPO2559-inoculated Heinz plants was confirmed by the absence of any HR-inducing activity on MM-Cf-9 plants after infiltration with AF obtained from these plants. In contrast, AF from race 0-inoculated Heinz plants showed clear HR-inducing activity on MM-Cf-9 plants (Fig. 3).

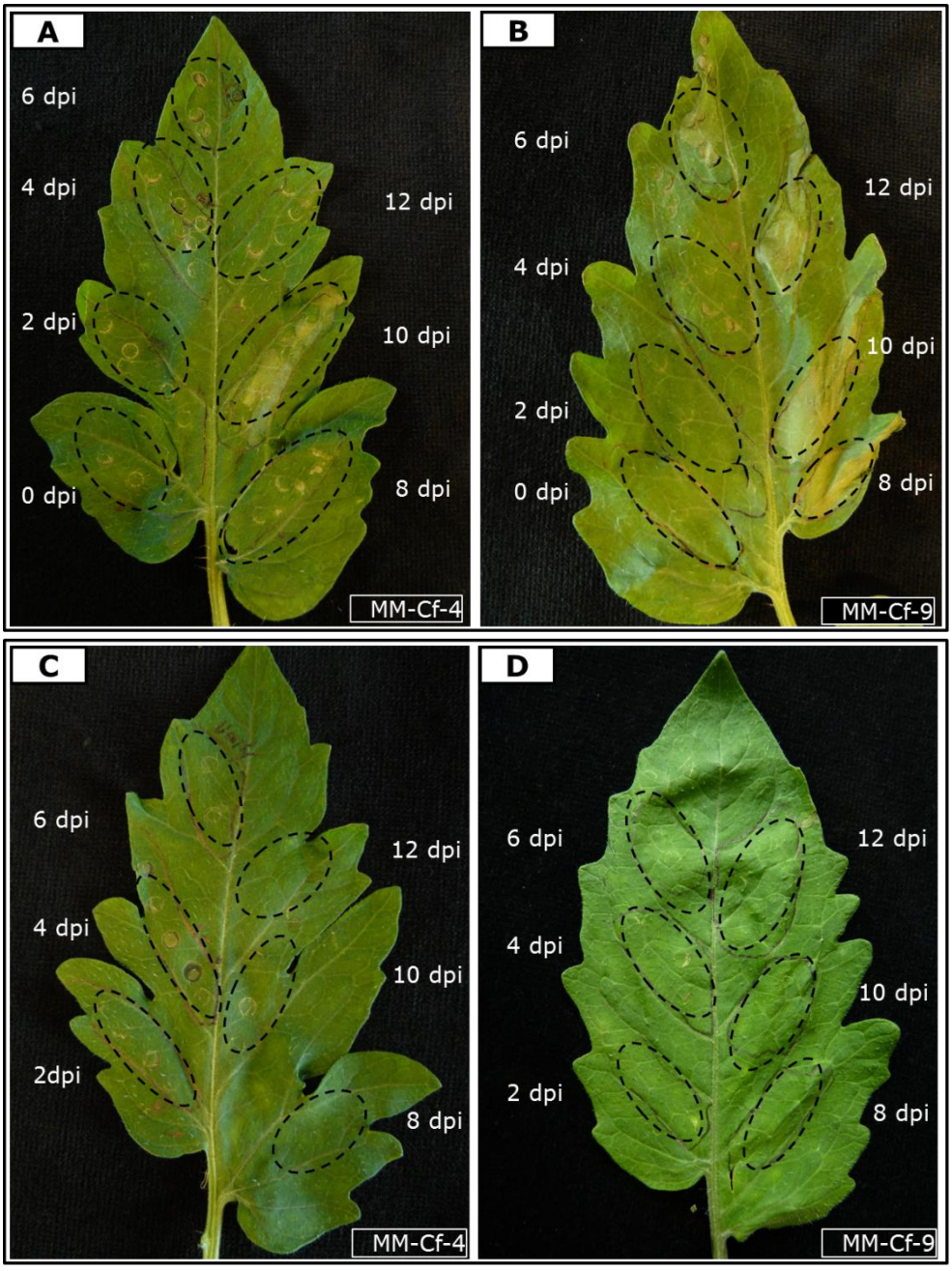

Figure 3. HR-inducing activity of apoplastic fluid isolated from tomato cultivar Heinz inoculated with either race 0 (sequenced strain) or strain IPO2559 (lacking the *Avr9* gene) of *Cladosporium fulvum*. MM-Cf-4 and MM-Cf-9 plants were infiltrated with apoplastic fluid isolated at 0, 2, 4, 6, 8, 10 or 12 days post inoculation (dpi) from tomato cultivar Heinz inoculated with race 0 **(A)** and **(B)** or strain IPO2559 of *C. fulvum* **(C)** and **(D)**.

Folded B-Avr9 bound to streptavidin magnetic beads served as bait for pull-down assays. B-Avr9-bound streptavidin magnet beads were incubated with AF and, after several washing steps, proteins putatively interacting with B-Avr9 were eluted and loaded on a 16% tricine SDS-PAGE gel. Only one major band (approximately 14 kDa in size) and a few faint bands were observed for all samples, including controls, after coomassie blue staining (Fig. 4). This indicates that no specific protein was captured by B-Avr9 and the observed major band originates from beads.

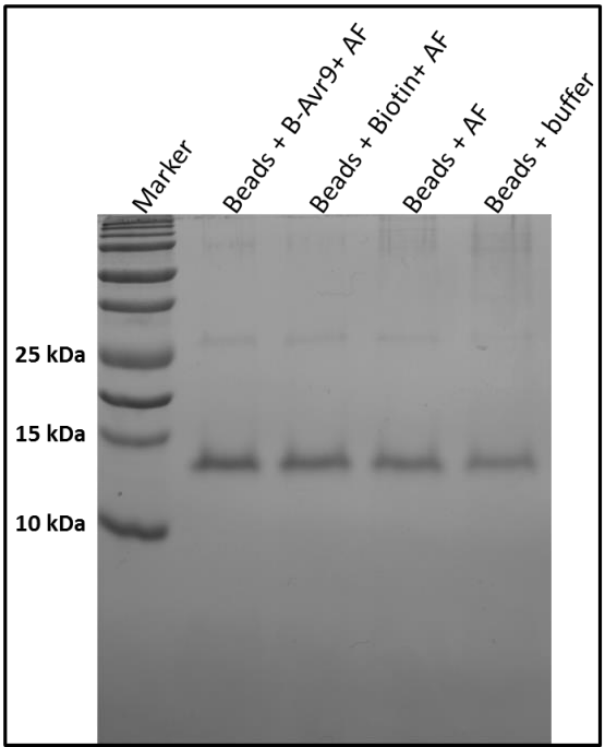

Figure 4. Pull-down assay using folded B-Avr9 bound to streptavidin magnetic beads as bait. Streptavidin magnet beads pre-incubated with B-Avr9, biotin, or buffer were used in pull-down assays using apoplastic fluid (AF) isolated from tomato cv. Heinz inoculated with strain IPO2559. After pull-down and repeated washing steps, the three samples as well as control magnetic beads were boiled in SDS sample buffer and eluents were loaded and run on a 16% tricine SDS-PAGE gel and subsequently stained with coomassie blue. Numbers on the left show the size of protein markers (kDa).

In addition, far-western blotting was performed using AF isolated from plants inoculated with strain IPO2559 at 0, 2, 4, 6, 8, and 10 dpi. Samples were incubated at room temperature with sample buffer and subsequently run on a 16% tricine SDS-PAGE gel. Proteins were blotted on a PMVDF membrane, which was subsequently incubated with folded B-Avr9 as a first probe. The presence of B-Avr9 was detected

after incubation with the streptavidin-HRP probe followed by treatment with peroxidase substrates. A signal was observed at a size of about 37 kDa, which increased in concentration in AF isolated at later stages of infection (Fig. 5A). However, when control membranes incubated with biotin alone or with buffer alone and treated with peroxidase membrane, the same signal was observed (Fig. 5B). This suggests that this signal is likely generated by an endogenous plant peroxidase present in AF that reveals its activity on the substrates used for signal detection on the PVDF membrane. This indicates that B-Avr9 did not bind to any protein transferred to the membrane. Only a signal was observed at 32 kDa likely generated by endogenous plant peroxidase present in AF, and did not correspond to a potential interactor of Avr9 or biotin. The induction of peroxidases upon infection of tomato by *C. fulvum* (de Wit and Bakker, 1980) and infection of maize and spruce by *Ustilago maydis* and *Ceratocystis polonica,* respectively (Hemetsberger et al., 2012; Nagy and Fossdal, 2013) has been reported. Overall, pull-down and far-western assays did not result in detection of any interactor of B-Avr9.

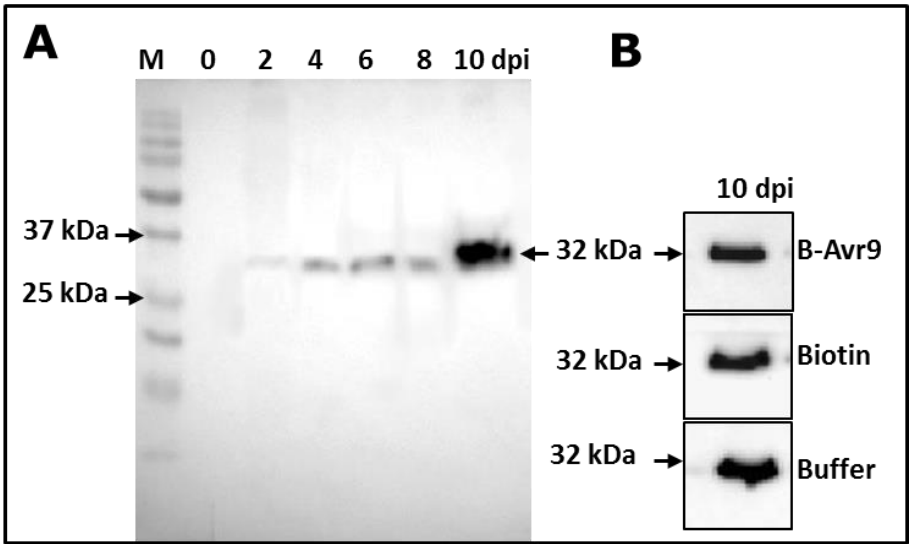

Figure 5. Far-western blotting using folded biotinylated Avr9 (B-Avr9) and apoplastic fluid obtained from tomato cv. Heinz inoculated with *Cladosporium fulvum*. (A) Apoplastic fluid (AF) isolated from Heinz tomato cv. inoculated with strain IPO2559 (lacking the *Avr9* gene) at 0, 2, 4, 6, 8, and 10 days post inoculation (dpi) were run on a 16% tricine SDS-PAGE gel. Proteins were transferred onto PVDF membrane and incubated with B-Avr9 as a first probe and streptavidin-HRP (horse radish peroxidase) as a second probe. **(B)** Far-western was performed under the same condition with AF isolated at 10dpi; B-Avr9 or biotin was used as first probe and streptavidin-HRP as second probe. For the buffer control, no probe was used and immobilized proteins on the immuno-blot were directly incubated with the peroxidase substrates.

Avr9 peptide produced by *Cladosporium fulvum* is glycosylated

It was previously reported that Avr9 is glycosylated by the plant glycosylation machinery when transiently expressed using the PVX expression system in plants (Kooman-Gersmann et al., 1998). To determine whether *C. fulvum*-secreted Avr9 is also glycosylated, a *C. fulvum* transformant constitutively expressing *Avr9 in vitro* (*GPD*::*Avr9*) (van den Ackerveken et al., 1993) was grown in liquid culture for either 6 or 12 days. Secreted proteins smaller than 30 kDa and larger than 1kDa were collected using columns with a cut-off of 30 and 1 kDa, respectively. Collected and concentrated Avr9 proteins were separated on low pH-PAGE gel (Fig. 6A). Staining with coomassie brilliant blue revealed two major bands that might represent differently processed and glycosylated Avr9 peptides. Proteins present in these bands were isolated and analysed by ESI-MS. Based on the calculated masses of amino acids present in the different Avr9 peptides, the masses and numbers of sugar residues could be calculated (Table 1). Band 1 contained Avr9 molecules consisting of 31 or 32 amino acids. These Avr9 carried no sugar residue at 6 days post incubation, but they contained one GlcNAc residue at 12 days post incubation. Band 2 also contained Avr9 consisting of 31 and 32 amino acids at both 6 and 12 days post incubation, but Avr9 consisting of 34 amino acids was only found at 12 days post incubation. All Avr9 peptides present in band 2 carried two GlcNAc and six mannose residues at 6 and 12 days post incubation (Table 2). These results indicate that the majority of Avr9 produced *in vitro* by *C. fulvum* is glycosylated and partially processed (Fig. 7). To test their Cf-9-mediated HR-inducing activity, Avr9 peptides extracted from gel pieces corresponding to these two bands were infiltrated into MM-Cf-9 as well as MM-Cf-4 plants at three different concentrations (Fig. 6B). No clear difference in HR inducing activity on Cf-9 plants between Avr9 peptides extracted from band 1 and 2 was observed, suggesting no clear effect of N-glycosylation of Avr9 on its HR-inducing activity.

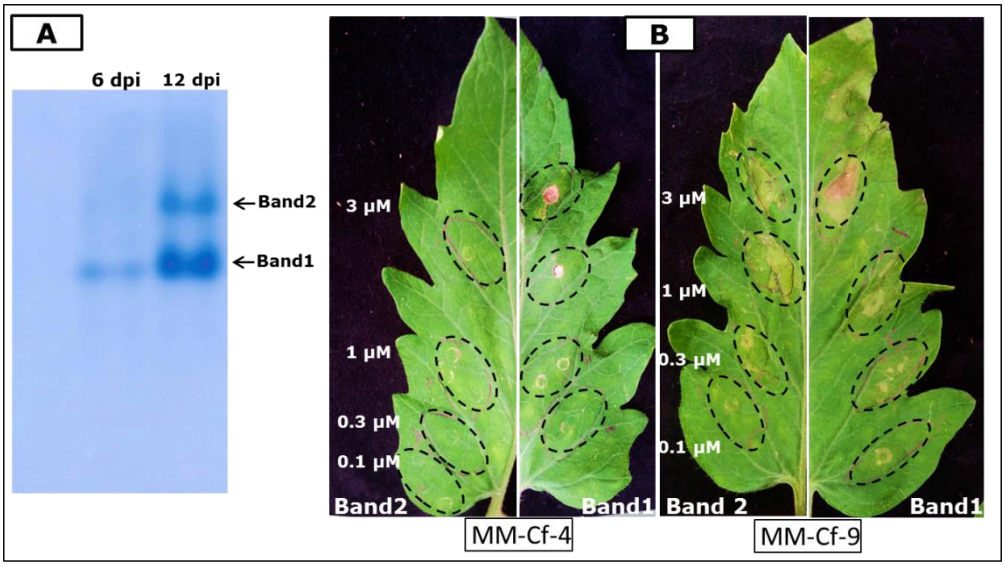

Figure 6. Cf-9-mediated HR activity of (non-) glycosylated Avr9 peptides secreted by transgenic *Cladosporium fulvum* constitutively producing Avr9. Cultures of *C. fulvum* overexpressing Avr9 were harvested after 6 or 12 days post incubation (dpi) on potato dextrose broth and proteins below 30 kDa were collected using an Amicon filter with 1kDa cut-off. **(A)** 15 µl of 100x concentrated culture filtrate harvested at 6 or 12 dpi were loaded on a 15% low pH-PAGE gel. Bands indicated by arrows were excised for mass spectrometry analysis. **(B)** glycosylated- (band 2) and less/non-glycosylated Avr9 (band 1) belonging to 12 dpi were eluted from band of non-stained low pH gel and their HR-inducing activity was tested on MM-Cf-4 and MM-Cf-9 at four different concentrations.

Table 1. Expected masses of Avr9 containing different numbers of amino acid and sugar residues.

Avr9 # amino acids	MW (Da) of Avr9	MW(Da) of Avr9+1 GlcNAc	MW(Da) of Avr9+2GlcNAc	MW (Da) of Avr9+ 2GlcNAc plus 6 mannoses
28	3415.39	3618.47	3821.55	4793.87
29	3475.41	3678.49	3881.57	4853.89
30	3571.48	3774.56	3977.64	4949.96
31	3628.50	3831.58	4034.66	5006.98
32	3741.58	3944.66	4147.74	5120.06
33	3798.60	4001.68	4204.76	5177.08
34	3897.67	4100.75	4303.83	5276.15

Table 2. Measured masses of the Avr9 peptides and their sugar composition secreted in culture filtrate obtained from *in vitro*- grown Avr9-transgenic *Cladosporium fulvum*.

Origin of sample [a]	peak retention time (min)	Highest monoisotopic m/z, z[c]	Determined monoisotopic (Da)[d]	# of amino acids in peptide[e]	glycosylation pattern[f]
Band1-6dpi	20.89	726.71, 5	3628.50	31	-
Band1-6dpi	23.0	749.32, 5	3741.58	32	-
Band2-6dpi	19.5	1002.40, 5	5006.97	31	2GlcNAc6Man
Band2-6dpi	22.0	1025.02, 5	5120.06	32	2GlcNAc6Man
Band1-12dpi	22.0	767.32, 5	3831.58	31	GlcNAc
Band1-12dpi	24.5	789.94, 5	3944.66	32	GlcNAc
Band1-12dpi	26.0	749.32, 5	3741.58	32	-
Band2-12dpi	18.5	1002.40, 5	5006.98	31	2GlcNAc6Man
Band2-12dpi	22.0	1025.02, 5	5120.07	32	2GlcNAc6Man
Band2-12dpi	24.1	1056.24, 5	5276.16	34	2GlcNAc6Man

[a] indicates the origin of samples for mass spectrometry excised form gel (Figure 6A).
[b] indicates the number of minutes that a solute spends in a column (time elapsed from injection to elution).
[c] indicates the mass (m) and charge(z) of Avr9 peptide. m/z is the ratio of mass to charge.
[d] indicates the whole size of monoisotopic Avr9 peptide carrying N-glycan in Dalton.
[e] indicates the number of amino acid residues found in the Avr9 peptide
[f] indicates the number of N-acetyl-glucosamine (GLCNAc) and Mannose (Man) residues present on the asparagine residue of the Avr9 peptide.

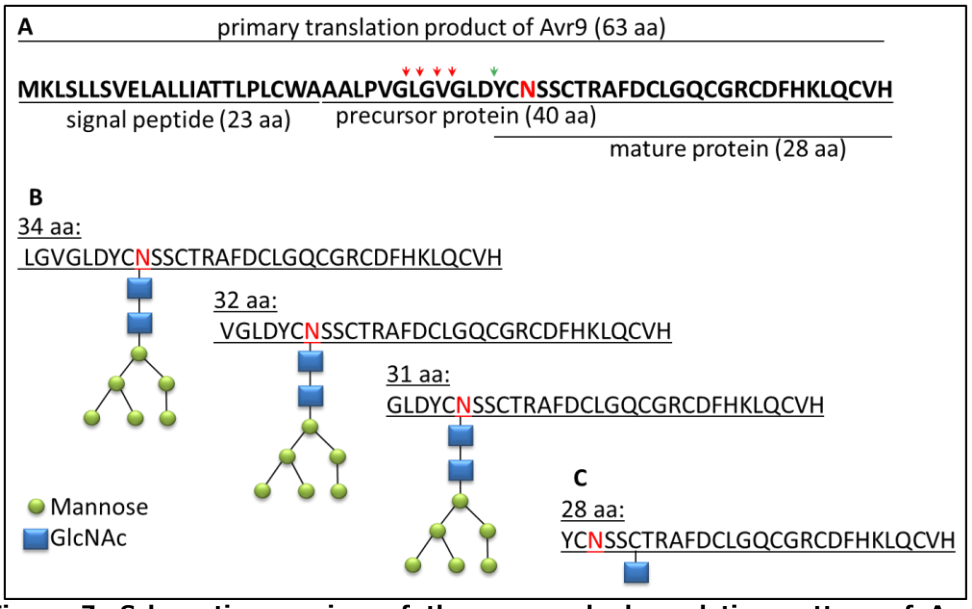

Figure 7. Schematic overview of the proposed glycosylation pattern of Avr9 isolated from culture filtrates of transgenic *Cladosporium fulvum* constitutively producing Avr9 as determined by ESI-MS. (**A**) The complete Avr9 peptide (63 amino acids) encoded by the *Avr9* gene. The glycosylation site is highlighted in red. The precursor Avr9 secreted by a *C. fulvum* strain that overexpresses Avr9 is processed by fungal proteases from 40 amino acids to 34, 32 or 31 amino acids by fungal proteases (red arrows) and by plant proteases (green arrow) as shown previously (van den Ackerveken et al., 1993). (**B**) The glycosylation site is predicted to carry two GlcNAc and six Mannose residues as measured by ESI-MS from Avr9 secreted by *C. fulvum* during *in vitro* and one HexNAc residue during *in planta* (**C**). The schematic representation of the proximal N-linked glycan structure is based on observations in yeast and filamentous fungi (Maras et al., 1999).

DISCUSSION

Avr9 is an effector of *C. fulvum* that in its mature form contains 28 amino acids and three disulphide bridges. Although it was the first cloned fungal effector (van Kan et al., 1991), its intrinsic function is still unknown. Avr9 was discovered in AF of *C. fulvum*-infected tomato plants by its strong HR-inducing activity on tomato plants carrying the extracellular leucine-rich repeat-containing Cf-9 receptor-like protein (van der Hoorn et al., 2005). In previous attempts no direct interaction between Avr9 and Cf-9 was observed (Luderer et al., 2001). However, it was suggested that Cf-9 likely requires an additional membrane protein, HABS, which function as a co-receptor and consequently activates downstream defense signaling (Kooman-Gersmann et al, 1996).

Based on structural homology of Avr9 with carboxypeptidase inhibitors, it was proposed that its intrinsic biological function might be to target host serine carboxypeptidases (SCPs) or SCP-like (SCPL) enzymes (Pallaghy *et al.*, 1994; van den Hooven *et al.*, 2001). Plants have undergone a significant expansion of SCPs (van der Hoorn, 2008) and SCPLs (Milkowski and Strack, 2004; Fraser et al., 2005) that display antimicrobial activity against pathogens (van der Hoorn and Jones, 2004; Zhou and Li, 2005; Mugford et al., 2009). For successful colonization, pathogens need to overcome these antimicrobial activities by secreting corresponding inhibitors (Misas-Villamil and van der Hoorn, 2008; Shabab et al., 2008; Mueller et al., 2013; Karimi Jashni et al., 2015). Such an inhibitory action was shown for Avr2 against the apoplastic plant cystine protease Rcr3 (Rooney et al., 2005) and additional cysteine proteases (Shabab et al., 2008; van Esse et al., 2008). In the present study, we hypothesized that Avr9 might target proteins from the apoplast of tomato. To induce the expression and accumulation of Avr9 targets, and to avoid occupation of target proteins by endogenous non-labelled Avr9 produced by the fungus, susceptible tomato plants were inoculated with a *C. fulvum* strain lacking the *Avr9* gene. With B-Avr9 a pull-down assay was performed at pH=6, which is comparable to the pH in the apoplast of tomato. However analysis of potentially interacting proteins on SDS PAGE gels did not reveal any specific protein released from B-Avr9. We may not exclude that binding of Avr9 to interactor(s) requires conditions different from those that we have employed. It is also possible that the concentration of the target(s) is lower than the detection level on SDS PAGE gels stained with coomassie brilliant blue. To optimize interacting conditions and/or increase the detection level, we also performed a far-western blotting assay. To this aim we separated and immobilized apoplastic proteins onto PVDF membranes and performed far-western blotting after separation of apoplastic proteins on native SDS-PAGE gels using B-Avr9 and streptavidin-HRP as the first and second probes, respectively. We identified a band with peroxidase activity at all the time points, including the controls that were not incubated with B-Avr9 or with biotin alone, suggesting that the observed peroxidase activity was derived from endogenous plant peroxidases separated on the gel rather than from the HRP of the second probe. In conclusion, pull-down assays and far-western blotting failed to identify potential Avr9 target(s) in tomato AF. Considering all different experiments that we have performed with Avr9, we envisage a few more possibilities that might be considered to find the biological function of Avr9 in the future.

The first possibility assumes that the interaction of Avr9 with its targets requires a specific condition that differs from those that we have employed in our assays. We

performed the pull down assays at pH 6 that is close to pH of the apoplast. We also used cocktails of protease inhibiotrs to avoid proteolytic activity of apoplstic proteases on cleavage of Avr9. However, some of Avr9 peptides still might be removed from the biotin tags as reported priviously for various other tags linked to *C. fulvum* effector molecules (van Esse et al., 2006). Although adding protease inhibiotrs might somewhat prevent cleavage of biotin tag, it might also lead to occupation of potential target proteases of Avr9. Thus, optimazation of various parameters might result in a more successful pulldown. The second possibility assumes that Avr9 acts as ion-channel blocker (Pallaghy *et al.*, 1994) or growth factor as previously proposed (van den Hooven et al., 2001). In this case, processed Avr9 would block anion channels as well as Ca^{2+} and K^{+} channels that along with intracellular signaling proteins and second messengers (Ligterink et al., 1997) are critical components of signal transduction in higher plants (Aducci et al., 1997). However, in this case we envisage a host target in the plasma membrane of the plant and not in the apoplast that we used in this study. The third possibility assumes that glycosylated forms of Avr9 are required to interact with its target(s), whereas the mature 28 amino acid peptide observed *in planta* might be the result of processing after its initial interaction with a host target. Mass spectrometry analysis of isolated Avr9 from culture filtrates of *C. fulvum* overexpressing Avr9 showed that *C. fulvum*-secreted Avr9 is glycosylated. The role of glycan complexes in signalling and in biological functions of proteins has been previously described (Paulson, 1989; Arnold et al., 2007).

Fungi have a conserved N-glycosylation pathway (Deshpande et al., 2008), that generates glycoproteins with two GlcNAc and a low or high mannose content as observed in filamentous fungi, (Maras et al., 1999) or yeasts (Herscovics, 1999). In filamentous fungi, the type of N-glycan structures varies between different glycoproteins and glycoprotein enzymes. For example, a glucose oxidase from *Aspergillus niger* contains maximally seven mannose residues, while an acid carboxypeptidase from *A. saitoi* contains 11 mannose residues attached to the GlcNAc residue (Maras et al., 1999; Fig. 8). N-Glycosylation of effectors was reported to be important for their function. For example, the rice blast fungus *Magnaporthe oryzae*, secretes the effector Slp1 (Secreted LysM Protein1) that binds to chitin and competes with the plant CEBiP (Mentlak et al., 2012). Slp1 has three glycosylation sites and is N-glycosylated by an α-1,3-mannosyltransferase encoded by *Alg3* (Chen et al., 2014). Pathogenicity assay with the mutants of *Alg3* showed that glycosylation of this effector is required for its stability and chitin binding activity and allows the pathogen to evade the plant immune response (Mach, 2014). We found that *C. fulvum*-secreted Avr9

contains maximally 2 GlcNAc and 6 mannose residues. It is likely that the structure of the sugar moiety of Avr9 shares homology with that of N-linked oligosaccharides present on glucose oxidase from *A. niger* (Fig. 8). However, it does not exclude that *C. fulvum* produces glycosylated Avr9 with a higher number of mannose residues similar in structure to for the N-linked oligosaccharides present on acid carboxypeptidase from *Aspergillus saitoi* (Fig. 8).

Mass spectrometry data of isolated Avr9 from culture filtrates of *C. fulvum* revealed Avr9 peptides with no or only one GlcNAc residue. This pattern is similar to the plant-produced Avr9 that contains no or only one GlcNAc residue. These Avr9 molecules with fewer sugar residues have likely undergone processing by different types of secreted fungal glycosidades. In plants glycoproteins contain different types of N-glycan structures compared glycoproteins in fungi. Plant glycoproteins can be decorated by xylose, galactose and fucose residues (Bosch et al., 2013). Glycosylation of Avr9 is not required for HR induction in Cf-9 plants, but it might be required for binding to its host target. Almost all experiments presented in this study to identify targets of Avr9 have been performed using non-glycosylated Avr9. Production of sufficient amounts of glycosylated Avr9 for biochemical studies is a challenge.

A genetic approach might be more efficient to determine whether glycosylation of Avr9 might be important for interacting with its virulence target or the RLP Cf9. In the future *C. fulvum* mutants that are unable to produce glycosylated Avr9 should be generated to study the requirement of N-glycosylation of Avr9 to function as a virulence or avirulence factor.

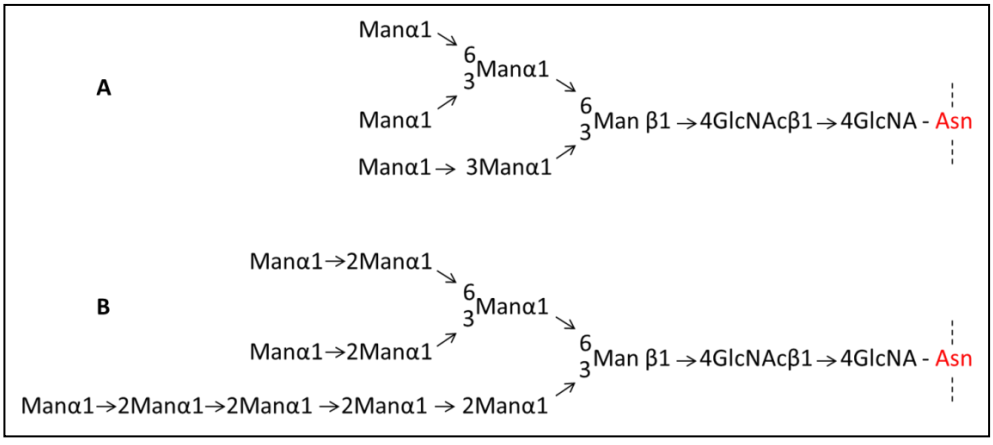

Figure 8. N-glycan linkages as observed in glucose oxidase of *Aspergillus niger* (A) and in acid carboxypeptidase of *Aspergillus saitoi*. Figure is depicted and modified from (Maras et al., 1999).

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Chapter 6

The battle in the apoplast: further insights into the roles of proteases and their inhibitors in plant-pathogen interactions

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Abstract

Upon host penetration, fungal pathogens secrete a plethora of effectors to promote disease, including proteases that degrade plant antimicrobial proteins, and protease inhibitors (PIs) that inhibit plant proteases with antimicrobial activity. Conversely, plants secrete proteases and PIs to protect themselves against pathogens or to mediate recognition of pathogen proteases and PIs, which leads to induction of defense responses. Many examples of proteases and PIs mediating effector-triggered immunity in host plants have been reported in the literature, but little is known about their role in compromising basal defense responses induced by microbe-associated molecular patterns. Recently, several reports appeared in literature on secreted fungal proteases that modify or degrade pathogenesis-related proteins, including plant chitinases or PIs that compromise their activities. This prompted us to review the recent advances on proteases and PIs involved in fungal virulence and plant defense. Proteases and PIs from plants and their fungal pathogens play an important role in the arms race between plants and pathogens, which has resulted in co-evolutionary diversification and adaptation shaping pathogen lifestyles.

Introduction

For successful infection of host plants and establishment of disease, fungal pathogens need weaponry to facilitate penetration, host colonization and uptake of nutrients for growth and reproduction, and at the same time to protect themselves against host defense responses. On the other hand, plants have developed surveillance systems to recognize and defend themselves against invading pathogens. Plant immune receptors recognize conserved microbe-associated molecular patterns (MAMPs) like chitin oligomers released from fungal cell walls during infection. This recognition leads to MAMP-triggered immunity (MTI) and initiates basal defense responses including the activation of structural and (bio)chemical barriers (Jones and Dangl, 2006; Spoel and Dong, 2012). However, adapted plant pathogens have gained the ability to overcome MTI by producing effector molecules that suppress or compromise MTI responses, thereby facilitating effector-triggered susceptibility (ETS) (Stergiopoulos and de Wit, 2009). In response, plants have developed an additional layer of defense that enables them to recognize pathogen effectors or effector-modified host targets leading to effector-triggered immunity (ETI) (Jones and Dangl, 2006).

Proteases and protease inhibitors (PIs) secreted by pathogens or their host plants have been extensively studied and have been demonstrated to play an important role in ETS and ETI (van der Hoorn, 2008). However, little is known about their role in MTI and related plant basal defense responses. Plant basal defense responses include the induction of pathogenesis-related proteins (PRs) such as antimicrobial chitinases, *β*-1,3-glucanases and proteases that hydrolyse the fungal cell wall components chitin, glucans, and polypeptides, respectively. The induction of these PR proteins upon plant infection, their antifungal activity, as well as their exploitation in engineering resistance in transgenic plants are very well documented (Wubben et al., 1996; Sels et al., 2008; Balasubramanian et al., 2012; Cletus et al., 2013). An early report in the literature suggested that pathogens might overcome the deleterious effects of plant chitinases by secreting proteases that modified them (Sela-Buurlage, 1996; Lange et al., 1996). This was further supported by recent studies, which indicate that chitinases are targeted by pathogen proteases and protected by PIs (Naumann et al., 2011; Slavokhotova et al., 2014). This encouraged us to review the recent advances on proteases and PIs that play a role in the arms race between plants and their fungal and oomycete pathogens.

Plant proteases and protease inhibitors involved in basal defense

Most PR proteins exhibit direct antimicrobial activities, such as chitinases that degrade chitin present in fungal cell walls. PR proteins play a role in both constitutive and induced basal defense responses (Avrova et al., 2004; Shabab et al., 2008; van Esse et al., 2008). For example, tomato and potato contain basal levels of proteases in their apoplast, including serine proteases like P69, and papain-like cysteine proteases (PLCPs) like Rcr3, which are required for resistance of tomato against *Cladosporium fulvum* (Song et al., 2009), as well as Pip1 (*Phytophthora* inhibited protease 1) (Tian et al., 2007; Shabab et al., 2008) and C14, which play a role in the resistance of potato against *Phytophthora infestans* (Kaschani et al., 2010; Bozkurt et al., 2011). After being challenged by pathogens, proteases are induced both locally (Tian et al., 2005) and systemically in the apoplast (Tian et al., 2007; Shabab et al., 2008; Song et al., 2009), which suggests that their activity affects pathogen growth directly or indirectly. Deletion or silencing of genes encoding these proteases enhanced the susceptibility of plants to pathogens, supporting their role in defense responses. Deletion of *Rcr3* increased the susceptibility of tomato to the late blight pathogen *P. infestans* (Song et al., 2009), to the leaf mould pathogen *C. fulvum* (Dixon et al., 2000), and also to the potato cyst nematode *Globodera rostochiensis* (Lozano-Torres

et al., 2012). Likewise, silencing of *C14* in *Nicotiana benthamiana* significantly increased their susceptibility to *P. infestans* (Kaschani et al., 2010). These findings suggest that proteases have a determinative role in the execution of defense against plant pathogens.

Plant PIs have also been reported to play a role in plant immunity, through the inhibition of pathogen proteases, or the regulation of endogenous plant proteases (Ryan, 1990; Mosolov et al., 2001; Valueva and Mosolov, 2004; Kim et al., 2009). This has been shown for PIs from barley (*Hordeum vulgare*) against proteases from *Fusarium culmorum* (Pekkarinen et al., 2007), as well as for PIs from broad bean (*Vicia faba*), which inhibited the mycelial growth of several pathogens (Ye et al., 2001). The *A. thaliana* unusual serine protease inhibitor (UPI) was shown to play a role in defense against the necrotrophic fungi *Botrytis cinerea* and *Alternaria brassicicola* (Laluk and Mengiste, 2011). The UPI protein strongly inhibited the serine protease chymotrypsin but also affected the cysteine protease papain (Laluk and Mengiste, 2011). Plants harbouring a loss-of-function *UPI* allele displayed enhanced susceptibility to *B. cinerea* and *A. brassicicola,* but not to the bacterium *Pseudomonas syringae*. Also, hevein-like antimicrobial peptides from wheat (WAMPs) were shown to inhibit class IV chitinase degradation by fungalysin, a metalloprotease secreted by *Fusarium verticillioides* (Slavokhotova et al., 2014). WAMPs bind to fungalysin, but are not cleaved by the enzyme due to the presence of a Ser residue between the Gly and Cys residues where cleavage of class IV chitinase by fungalysin normally takes place (Naumann et al., 2011; Slavokhotova et al., 2014). Adding equal molar quantities of WAMP and chitinase to fungalysin was sufficient to completely inhibit fungalysin activity suggesting a higher affinity of the protease to the WAMP than to the chitinase.

Interestingly, some pathogens can also manipulate the transcription of plant PIs to inhibit deleterious effects of plant proteases in their favour. For example, production of maize cysteine proteases is induced during infection by *Ustilago maydis*, but at the same time the fungus induces the production of maize cystatin CC9 that inhibits cysteine proteases to facilitate infection (van der Linde et al., 2012b; Mueller et al., 2013). This suggests an evolutionary arms race in which the infection strategy of the pathogen benefits from the host's antimicrobial defense to suppress its defense responses.

Fungal proteases targeting host defense proteins

The arms race between pathogens and their hosts is often explained by recognition of MAMPs or effectors through pattern recognition receptors or resistance

proteins, which results in MTI or ETI (Jones and Dangl, 2006). However, several components of basal defense are both constitutive and induced after interaction between MAMPs/effectors and immune receptors. PR proteins provide an excellent example of this. PR proteins are generally stable proteins that often exhibit a basal level of expression, but are also strongly induced after infection (Sels et al., 2008). PR proteins and their antifungal activity have been exploited to improve broad-spectrum resistance in plants. Plants such as tobacco, tomato, potato, peanut and cacao have been engineered to over-express chitinases alone (Schickler and Chet, 1997; de las Mercedes Dana et al., 2006; Maximova et al., 2006; Iqbal et al., 2012; Cletus et al., 2013) or in combination with other PR proteins in pea and rice (Sridevi et al., 2008; Amian et al., 2011), and showed enhanced resistance to fungal pathogens.

Plant chitinases and especially chitin-binding domain (CBD)-containing chitinases play an important role in defense against pathogenic fungi (Iseli et al., 1993; Suarez et al., 2001). Some fungal pathogens such as *C. fulvum* secrete chitin-binding effector proteins like CfAvr4 into the colonized extracellular space of tomato leaves to protect themselves against the antifungal activity of apoplastic plant chitinases (van den Burg et al., 2006). Indeed, CfAvr4 binds to chitin of fungal cell walls, making chitin inaccessible to plant chitinases, thereby preventing hydrolysis by these enzymes (van den Burg et al., 2006). Functional homologs of CfAvr4 have been identified in other Dothideomycete plant pathogens, in which they likely also protect the fungal cell wall against plant chitinases (de Wit et al., 2012; Mesarich et al., 2015; Stergiopoulos et al., 2010). However, many fungal pathogens do not carry homologs of the *CfAvr4* gene in their genome. It appears that some fungi secrete proteases that cleave CBD-chitinases. For example, *F. solani* f. sp. *phaseoli* is able to modify chitinases during infection of bean to facilitate host colonization (Lange et al., 1996). Also an extracellular subtilisin protease from *F. solani* f. sp. *eumartii* was reported to modify chitinases and *β*-1, 3-glucanases present in intercellular washing fluids of potato (Olivieri et al., 2002). More recently, it was shown that *F. verticillioides* and other maize pathogens, including *Bipolaris zeicola* and *Stenocarpella maydis*, secrete two types of proteases that truncate maize class IV CBD-chitinases (Naumann, 2011). A fungalysin metalloprotease of *F. verticillioides* was found to cleave within the CBD domain between conserved Gly and Cys residues (Naumann et al., 2011), while a novel polyglycine hydrolase present in many fungi belonging to the family of *Pleosporineae* cleaved within the polyglycine linker present in the hinge domain of class IV chitinases (Naumann et al., 2014; Naumann et al., 2015). In another recent study it was shown that the fungal tomato pathogens *B. cinerea, V. dahliae* and *F.*

oxysporum f. sp. *lycopersici* secrete proteases that modify tomato CBD-chitinases (Karimi Jashni et al., 2015). For *F. oxysporum* f. sp. *lycopersici*, the synergistic action of a serine protease, FoSep1, and a metalloprotease, FoMep1 (the ortholog of fungalysin from *F. verticillioides*), was required for cleavage and removal of the CBD from two tomato CBD-chitinases (Karimi Jashni et al, 2015). Removal of the CBD from two tomato CBD-chitinases by these two enzymes led to a reduction of their chitinase and antifungal activity. In addition, mutants of *F. oxysporum* f. sp. *lycopersici* lacking both *FoSep1* and *FoMep1* exhibited reduced virulence on tomato, confirming that secreted fungal proteases are important virulence factors by targeting CDB-chitinases to compromise an important component of plant basal defense (Karimi Jashni et al., 2015).

Collectively, the activity of fungal proteases might explain why overexpression of plant chitinases in transgenic plants has not become an effective strategy to obtain durable resistance against fungal pathogens. Secretion of proteases and PIs by pathogens to modify, degrade or inhibit basal defense proteins might have played an important role during co-evolution with their host plants (Hörger and van der Hoorn, 2013). Therefore, overexpression of chitinases from a heterologous source in transgenic plants might be a more efficient approach to obtain durable resistance against pathogens, as they have not co-evolved with these "foreign" defense proteins.

Fungal protease inhibitors targeting host proteases

Plant pathogens also secrete PI effectors to inhibit plant defense proteases and promote disease development. These effectors are targeted to various host compartments (Tian et al., 2009). One such effector, Avr2, secreted by *C. fulvum* during infection, is required for full virulence of this fungus on tomato (Rooney et al., 2005). Avr2 inhibits the tomato apoplastic PLCPs Rcr3 and Pip1 to support growth of *C. fulvum* in the apoplast. Also, plants expressing Avr2 showed increased susceptibility to other pathogenic fungi, including *B. cinerea* and *V. dahliae* (van Esse et al., 2008). Moreover, *A. thaliana* plants expressing Avr2 triggered global transcriptional reprogramming, reflecting a typical host response to pathogen attack (van Esse et al., 2008). Two other PI effectors are the cystatin-like proteins EPIC1 (extracellular proteinase inhibitor C1) and EPIC2B (extracellular proteinase inhibitor C2B), whose expression is strongly induced in the oomycete *P. infestans* during biotrophic growth on tomato leaves (Tian et al., 2007; Song et al., 2009). These PIs selectively target the plant PLCPs Rcr3, Pip1, and C14 in the apoplast of potato and tomato. The EPICs inhibit C14 and possibly other PLCPs over a wider pH range than that observed for

Avr2, which only inhibits Pip1 and Rcr3 at pH values occurring in the apoplast where the pathogen grows. In addition, *P. infestans* secretes two serine PIs (EPI1 and EPI10) that target and inhibit the major apoplastic serine protease P69B, likely to decrease its role in defense (Tian et al., 2004; Tian et al., 2005). It was proposed that EPI1 protects EPIC1 and EPIC2B proteins from degradation by P69B (Tian, 2005). Furthermore, the maize pathogen *U. maydis* secretes the cysteine protease inhibitor Pit2 that strongly inhibits three abundant defense-related maize cysteine proteases (CP2 and its two isoforms CP1A and CP1B) (van der Linde et al., 2012b; van der Linde et al., 2012a; Mueller et al., 2013). These findings indicate that cysteine and serine PIs secreted by different groups of filamentous fungal and oomycete pathogens, as well as their activity against plant proteases, can compromise plant basal defense responses. A schematic overview of different types of interactions between pathogen and host proteases and PIs at the plant-pathogen interface is presented in Figure 1.

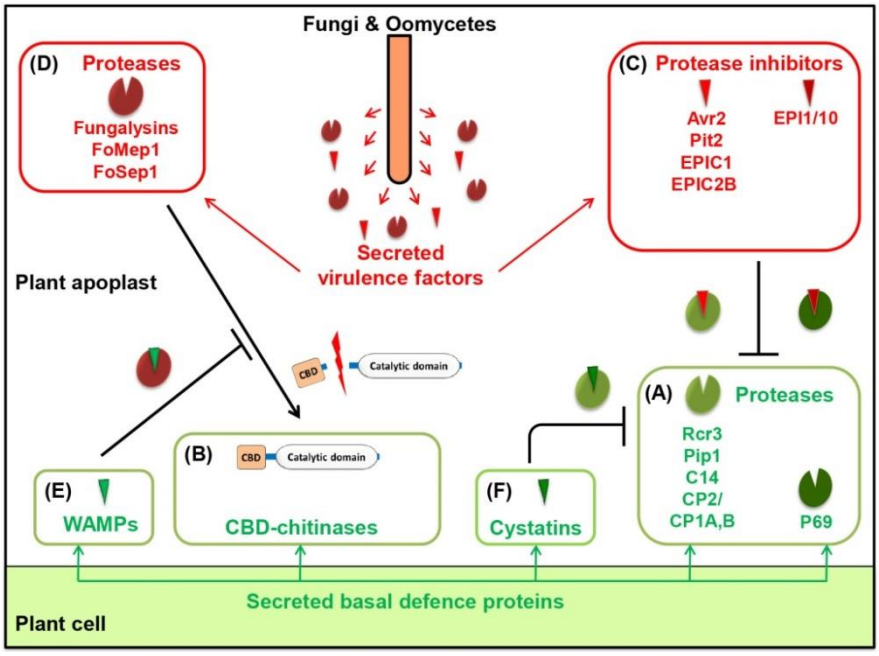

FIGURE 1, Proteases and protease inhibitors at the plant–pathogen interface. As part of their basal defense response, plants secrete deleterious enzymes such as proteases **(A)** and chitin-binding domain (CBD)-containing chitinases **(B)** that target pathogen components. In response, filamentous pathogens secrete protease inhibitors **(C)** that inhibit plant cysteine or serine proteases. Filamentous pathogens also secrete fungalysin metallo- or serine proteases **(D)** that process antifungal CBD-chitinases of plants. In response, plants secrete antimicrobial peptides such as hevein-like antimicrobial peptides from wheat (WAMPs) **(E)** that inhibit fungalysin metalloproteases or cystatins **(F)** that inhibit endogenous plant cysteine proteases. Examples shown in this figure are discussed in the text.

Proteases, PI effectors, and their role in receptor-mediated host defense responses

The plant immune system is able to recognize pathogen effectors to mount receptor-mediated defense responses. Although the intrinsic function of protease and PI effectors secreted by some pathogenic fungi promote disease through manipulation of host defense, proteases and PI effectors can also be recognized by host immune receptors mediating defense responses. This adaptation and counter-adaptation reflects the arms race between pathogens and their host plants. A clear example of such an evolutionary arms race are the cysteine PIs Avr2 from *C. fulvum* and Gr-VAP1 (*Globodera rostochiensis* Venom Allergen-like Protein) from *G. rostochiensis* that bind and inhibit the tomato cysteine protease $Rcr3^{pim}$. The tomato immune receptor protein Cf-2 senses this interaction and mediates the induction of defense responses (Song et al., 2009; Lozano-Torres et al., 2012). Most likely, the interaction causes a conformational change in Rcr3, which is recognized by the Cf-2 receptor (Krüger et al., 2002; Rooney et al., 2005). This hypothesis is supported by the finding that a natural variant of Rcr3 is recognized by Cf-2 in an Avr2-independent manner (Dixon et al., 2000). Moreover, in tomato plants lacking the Cf-2 receptor, targeting of Rcr3 is not sensed and plants are more susceptible to *G. rostochiensis* (Lozano-Torres et al., 2012).

Co-evolution between plants and their pathogens is reflected by the numerous variant proteases and PIs in the genomes of both organisms

The genomes of fungal plant pathogens encode predicted proteases belonging to various subfamilies that vary in number between pathogens with different lifestyles. Generally, hemi-biotrophs and saprotrophs contain higher numbers of secreted proteases than biotrophs (Ohm et al., 2012). However, these predictions are based on gene numbers and may not be supported by their transcription and translation profiles. For example, *C. fulvum*, which is a biotrophic fungus, has numbers of proteases that are comparable to the phylogenetically closely related hemi-biotroph *Dothistroma septosporum* (de Wit et al., 2012). However, likely due to its adaptation to a different host and lifestyle, many *C. fulvum* protease genes are not expressed *in planta* and some have undergone pseudogenization (van der Burgt et al., 2014). Deletion and duplication of protease genes were reported to occur in the genome of the grass endophytic fungus *Epichloë festucae* (Bryant et al., 2009) but their biological implications have not yet been studied.

Adaptation of PI effectors from pathogens to inhibit different host proteases has been observed in several cases. The Avr2 PI of *C. fulvum*, for example, has a high affinity for the host proteases Rcr3 and Pip1 and a low affinity for C14 (Shabab et al., 2008; Hörger et al., 2012). *P. infestans* EPICs have a high affinity for C14 and a low affinity for Rcr3 and Pip1 (Kaschani et al., 2010). Furthermore, *U. maydis* Pit2 inhibits the maize cysteine proteases CP1, CP2, and XCP2, but does not inhibit cathepsin CatB (Mueller et al., 2013). Different types of selection pressure may lead to the circumvention of protease inhibition by PIs. For example, purifying or diversifying selection has been reported for the proteases Rcr3, C14, and Pip1, and has been shown to act at their PI binding sites. Sequencing of the tomato proteases Rcr3 and Pip1 across different wild tomato species has shown that these proteins are under strong diversifying selection imposed by Avr2. For instance, one of the variant residues in the binding site of Rcr3 prevented inhibition by Avr2, indicating selection for evasion from recognition by this inhibitor (Shabab et al., 2008). C14 from solanaceous plants is also the target of EPICs secreted by *P. infestans* and is under diversifying selection in potato and under conservative selection in tomato. This demonstrates that C14 plays an active role in host immunity against this pathogen and variations in the sequence of C14 in natural hosts of *P. infestans* highlight the co-evolutionary arms race at the plant-pathogen interface (Kaschani et al., 2010).

Evolutionary diversification may vary from point mutation to gene deletion or insertion. EPIC1 and EPIC2 are PIs present in *P. infestans,* however their orthologs were lost in *P. sojae* and *P. ramorum* (Tian et al., 2007). *P. mirabilis,* a species closely related to *P. infestans*, is a pathogen of *Mirabilis jalapa*, and secretes the protease inhibitor PmEPIC1, an ortholog of EPIC1 that inhibits C14 but not Rcr3 (Dong et al., 2014). However, *M. jalapa* secretes MRP2, a PLCP homolog of Rcr3, that is more effectively inhibited by PmEPIC1 than by EPIC1 (Dong et al., 2014). Substitution of one amino acid residue in PmEPIC1 and EPIC1 restored the inhibitory function of PmEPIC1 on Rcr3 and of EPIC1 on MRP2, respectively. These results show that proteases and PIs have played important roles in adaptation of the two *Phytophthora* species to their respective host plants, although the two species diverged only a thousand years ago (Dong et al., 2014). This is an excellent example for a role of a protease and PI in the arms race between a plant and its pathogen and exemplifies how diversification and adaptation of a protease-PI complex may work at the molecular level.

Conclusion and perspective

The recent advances reviewed here exemplify determinative roles of proteases and PIs in shaping plant-pathogen interactions. Analyses of genome databases of both plants and pathogens show that these organisms encode numerous proteases and PIs, of which we are just beginning to understand some of their roles. Advanced transcriptome and proteome tools such as RNA sequencing and protease profiling will facilitate identification of important proteases and PIs for further functional analysis. The redundancy of proteases in pathogens is a technical challenge that has so far hampered defining their biological functions. Targeted deletion of one or even two protease genes failed to change virulence of the plant pathogenic fungi *Glomerella cingulata* (Plummer et al., 2004) and *B. cinerea* (ten Have et al., 2010), respectively. Karimi Jashni and colleagues (2015) could only show decreased virulence of a double protease mutant of the tomato pathogen *F. oxysporum* by a combined biochemical and genetic approach, and using a defined plant enzyme (CBD-chitinase) as a substrate that was presumed to be involved in plant defense. This indicates that multi-gene targeting of protease and PI genes to identify their role in virulence or avirulence remains a challenge in filamentous fungi. Targeting multiple protease and PI genes might also be hampered by lack of sufficient numbers of selection markers for targeted gene replacement. In the latter case multiple protease and PI genes might be targeted by targeted gene silencing.

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Chapter 7

General discussion

1. Proteases; versatile weaponry employed by many parasites

All types of plant-associated organisms including viruses, bacteria, fungi, oomycetes and nematodes, produce proteases. In chapter 6, the various roles of secreted proteases in plant-pathogen interactions for plant pathogenic fungi and oomycetes were extensively reviewed. Non-plant pathogenic fungi also employ proteases to compete with other microbes in their ecological niches (Leger et al., 1997). For example, *Trichoderma virens* secretes an extracellular serine protease TVSP1 that enhances its parasitic ability against soilborne fungal pathogens such as *Rhizoctonia solani* (Markovich and Kononova, 2003). Nematophagous, entomopathogenic and nematode-trapping fungi also secrete proteases along with other enzymes like collagenases and chitinases to penetrate their host's cuticle. For example, the endoparasitic fungus *Hirsutella rhossiliensis* secretes an alkaline serine protease associated with virulence that is able to degrade cuticle proteins of juveniles of the soybean-cyst nematode *Heterodera glycines* (Wang et al., 2009). The nematode-trapping fungus *Arthrobotrys oligospora* is able to immobilize the free-living nematode *Panagrellus redivivus* by secretion of a neutral serine protease (Aoz1) that causes structural changes in the nematode cuticle likely by targeting host proteins (Minglian et al., 2004). Similar enzymes have also been purified and characterized from the entomopathogenic fungi *Purpureocillium lilacinum* and *Beauveria bassiana* (Fan et al., 2010; Castillo Lopez et al., 2014). Saprophytic fungi (In et al., 2014) and also some pathogenic fungi like *Sclerotinia sclerotiorum* during its saprophytic growth on sunflower cotyledons, secrete aspartic proteases (Billon-Grand et al., 2002). Similar to fungi, bacteria, nematodes and viruses also employ proteases to invade their hosts. Plant pathogenic bacteria secrete several type III effectors such as AvrPphB, AvrRpt2, YopT and XopD that show proteolytic activity and target host defense signalling protein kinase PBS1 (Shao et al., 2003), plant-specific RIN4 (Kim et al., 2005), host Rho-like GTPases (Shao et al., 2002) and desumoylate ethylene responsive transcription factor SlERF4 (Kim et al., 2013), respectively. Bacteria may secrete proteases to target defense-related proteins. An excellent example is a metalloprotease secreted by *Erwinia carotovora* ssp. *carotovora* that degrades the potato lectin, that is implicated in disease resistance (Heilbronn et al., 1995). To compete with their neighbours for space and resources, bacteria may secrete virulence factors that also function against other microorganisms. For example, serine proteases Bace16 and Bae16 secreted by *Bacillus nematocida* (strain B16) exhibit nematotoxic activities against the free-living nematode *Panagrellus redivius* and the plant parasitic nematode *Bursaphelenchus xylophilus* (Niu et al., 2012). Plant-pathogenic nematodes employ an integration of

behavioural and physiological strategies to facilitate and sustain plant colonization (Mbega and Nzogela, 2012). This includes secretion of proteases likely for destruction of plant defense proteins or nutritional pre-digestion (Bellafiore et al., 2008) as shown for *Meloidogyne incognita*, that secretes cysteine protease MiCpl1 (*Meloidogyne incognita* cathepsin L-like protease 1) (Neveu et al., 2003; Shingles et al., 2007) and aspartyl protease-like protein (Mi-ASP2) (Perry et al., 1992; Vieira et al., 2011). Similarly, entomoparasitic nematodes such as the virulent strains of *Steinernema carpocapsae* secrete aspartic protease (Sc-ASP113) (Balasubramanian et al., 2012) and astacin metalloprotease, Sc-AST (Jing et al., 2010), during their parasitic phase. Also viruses employ proteases. This is evident in the case of potyvirus turnip mosaic virus that encodes a helper-component proteinase (HC-Pro) involved in polyprotein processing, aphid transmission, and suppression of antiviral RNA silencing (Guo et al., 2011). Altogether, these data indicate that proteases are important weaponry employed by various types of microbes and invertebrates.

2. Pseudogenization and strict regulation of proteases as hallmarks of fungal biotrophy?

Initially, it was believed that fungi secrete proteases to function as digestive enzymes, for nutrient acquisition (Rao et al., 1998). However, recent studies indicate that fungi secrete proteases with a significant role in immune evasion during interactions with their hosts (chapter **6**). Non-biotrophic fungi secrete large quantities of enzymes, including proteases, to thrive in very different ecological niches (van Kan, 2006). In these fungi, the regulation and secretion of proteases might be affected by the availability of different substrates (Rolland and Bruel, 2008; Bergmann et al., 2009; Zou et al., 2010). In contrast, biotrophs have to manipulate their hosts in a subtle manner to keep host cells alive for a prolonged time (Ökmen and Doehlemann, 2014). This requirement also applies for proteases and might occur at different levels. First, during co-evolution with their hosts, fungi have differentiated their genome content whilst retaining genetic signatures of a common ancestry (de Wit, 2012). Previous studies and results provided in chapter **2** suggest that protease gene family contraction might be a type of adaptation to a biotrophic lifestyle through mechanisms such as gene loss or pseudogenization, a mechanism that led to non- or partially functional proteins (van der Burgt et al., 2014). Pseudogenization events have been described in chapter **3** for two phylogenetically closely related fungi, where protease pseudogenes are more abundant in the biotroph *C. fulvum* compared to the

hemibiotroph *Dothistroma septosporum,* suggesting adaptation to a different host (tomato *versus* pine) and lifestyle. Second, results presented in chapter **2** clearly showed that many protease genes are not expressed during host colonization by *C. fulvum*. This regulation apparently occurs also at post-transcriptional level, as out of 59 putatively secreted proteases, only three were identified by mass spectrometry in the apoplast of *C. fulvum*-infected tomato. Third, once proteases are translated as inactive zymogens, their secretion and post-translation modification might be regulated in appropriate conditions. Activation of proteases is irreversible (Gál et al., 2005) and, once activated, they can only be inhibited by protease inhibitors.

3. Bottleneck hampering functional analysis of fungal proteases

During recent years, rapid accumulation of data resulting from genome sequencing and genome mining have provided basic information to address the repertoire of protease functions within an organism (Ohm et al., 2012). Despite significant progress in studies about proteases associated with fungal human pathogens (Yike, 2011), this field is less studied for fungal proteases associated with plants. Results of chapter **2** as well as previous studies (Ohm et al., 2012) have shown that genomes of fungal pathogens contain a considerable number of predicted genes encoding proteases, of which the secreted ones might play a role in plant-fungus interactions. However, identifying their role requires extensive studies at transcriptional and post-transcriptional level due to their complex regulation. Many independent studies have shown that fungal proteases were induced or secreted during host colonization. For example, various phytopathogenic fungi such as *Botrytis cinerea* (ten Have et al., 2010), *Pyrenopeziza brassicae* (Ball et al., 1991), *S. sclerotiorum* (Billon-Grand et al., 2002), *Magnaporthe poae* (Sreedhar et al., 1999), *Stagonospora nodorum* (Carlile et al., 2000) and *Alternaria solani* (Chandrasekaran et al., 2014) secrete proteases during host colonization. It is important to know whether these secreted proteases indeed play role in the interaction of these fungi with their hosts. There are a few issues that have hampered the functional analysis of secreted proteases:

The first issue concerns the redundancy of proteases. Attempts to determine the virulence function of protease genes using a single gene knock-out approach can fail (Robertsen, 1984; Murphy and Walton, 1996; Di Pietro et al., 2001) because of functional redundancy present across protease multigene families. Indeed, inactivation of one protease encoding gene may result in up-regulation of related proteases with a similar function (Bindschedler et al., 2003; Plummer et al., 2004).

The second issue concerns the specificity of proteases. Although some proteases might have a board range of targets, the majority of proteases have very distinctive substrate specificities (Hedstrom, 2002; Monod *et al*., 2002; Yike, 2011). One approach to identify the function of a protease makes use of substrate libraries to determine protease specificity and discover optimal substrates on the basis of cleavage (Turk et al., 2001). However finding natural targets of proteases is the bottleneck in applied biological research. In this thesis, we showed that secreted serine protease (FoSep1) and metalloprotease (FoMep1) by *F. oxysporum* synergistically cleave CBD-chitinases. Double knock-out ΔΔ*fosep1:fomep1* still secretes high levels of FoSep2, but it is not responsible for cleavage of CBD-chitinases and might have other substrates. Without having these substrates available it would have been difficult to assign chitinase cleavage activity to these proteases.

The third issue concerns functional analysis of proteases with a minor role in pathogenicity. In chapter **4**, we showed that only a double gene knock-out of both a serine and a metalloprotease gene from *F. oxysporum* leads to a reduction in virulence of the fungus on tomato. It is fairly difficult to characterize the function of proteases with a minor role in virulence.

The last issue concerns the complexity of protease regulation. Results provided in chapter **2** revealed that analysis of the protease catalogue in a genome is only a starting point, as fungi might differently regulate their secreted protease genes at transcriptional, translational and post-transcriptional level. Expression analysis of *C. fulvum* proteases genes showed differential expression patterns for some at early or late stages of infection, suggesting timely regulation of protease genes. Individual proteases might be active in a short period of infection process (van der Hoorn et al., 2004).

4. Protease inhibitors another weaponry of plant pathogens

Protease inhibitors are also employed by plant pathogens likely as a counter-defense mechanism to defend themselves against host proteases. In chapter **6**, secreted protease inhibitors from various plant pathogenic fungi were extensively reviewed. Remarkably, many of the identified and characterized protease inhibitors of plant pathogens are cysteine protease inhibitors. This might be an adaptation of these fungal pathogens to prevent hydrolytic activity of plant cysteine proteases during host colonization (Shindo and van Der Hoorn, 2008; Stergiopoulos and de Wit, 2009; Hörger and van der Hoorn, 2013). Diversification at the interaction surfaces (Hörger et

al., 2012; Dong et al., 2014) and substrate adaptation (Hörger and van der Hoorn, 2013) reveal the battlefield between these proteases and protease inhibitors. Less frequently, serine proteases inhibitors also were described from plant pathogens (Tian et al., 2004; Tian et al., 2005; Sabotič and Kos, 2012). Avr9 is an apoplastic effector secreted by C. *fulvum* and shares significant structural similarity with carboxypeptidase inhibitors. Based on this similarity, it is expected that Avr9 exhibits a function similar to Avr2 and inhibits host serine proteases to promote the virulence of *C. fulvum* (Rooney et al., 2005; van Esse et al., 2008). However, all attempts aiming at identifying targets of Avr9 were not successful. Different hypotheses that might lead to the identification of its targets are discussed in chapter **5**. Another important question that needs to be answered is whether Avr9 is required for virulence of *C. fulvum* similar to Avr2? It is important to note that *Avr9* is one of the highest *in planta* expressed genes of *C. fulvum*, and in spite of its abundance there is yet no evidence of its role in virulence. Intriguingly, *C. fulvum* races lacking the *Avr9* gene are still fully virulent on susceptible tomato, suggesting other highly expressed effectors to compensate for the function of Avr9. This worthwile to mention that Avr9 was the first effector protein islolated from the apoplast of *C. fulvum*-infected tomatoin 1985 (de Wit et al., 1985), that its encoding gene Avr9 was cloned in 1991 (van Kan et al., 1985), while its role during host colonization is a mystery.

5. Proteolytic targeting of defense related proteins: another fungal counter-defense mechanism

In the zigzag model (Jones and Dangl, 2006) the innate immunity story always starts with recognition of PAMP molecules such as chitin oligomers leading to PAMP-triggered immunity (PTI). However an important question to be answered is how initially these chitin oligomers are released and which of fungal or plant chitinases are responsible for releasing them. It is very likely that after penetration into the plant apoplast, fungal penetrating hyphae encounter extracellular chitinases that are present at a basic level; this encounter likely leads to a release of chitin oligomers from fungal hyphae (Sánchez-Vallet et al., 2013). When chitin oligomers are perceived by plant receptors, plant basal defense responses including additional chitinase accumulation are induced that create an antifungal environment (Joosten and Wit, 1989). One mechanism employed by *C. fulvum* to prevent recognition of chitin oligomers by the chitin receptors of plants, is the secretion of Ecp6, which is a chitin oligomer scavenger (de Jonge et al., 2010; Sánchez-Vallet et al., 2013)

It is shown that plant pathogenic fungi employ different mechanisms to defend themselves against deleterious effects of CBD-chitinases. They either secrete a chitin-binding protein, Avr4, that interferes with chitin-binding of CBD-chitinases to chitin of fungal hyphae (van den Burg *et al.*, 2006; van Esse *et al.*, 2007) or secrete proteases that cleave the CBD of CBD-chitinases as shown in this thesis and previous studies (Naumann and Wicklow, 2013). In the current thesis, we showed that full cleavage CBD-chitinases requires synergistic action of two proteases that are required for full virulence of *F. oxysporum* on tomato. Cleavage of the CBD from extracellular CBD-chitinases and reduction of their antifungal activity towards *F. oxysporum* indirectly demonstrated the importance of CBD-chitinases in basal defence of tomato against this fungus. This also shows that during co-evolution with their hosts, fungi have developed efficient counter-defense mechanisms to protect themselves against detrimental effects of plant chitinases.

Overall, this thesis provided evidence for the role of proteases in pathogenicity of plant pathogenic fungi. It also demonstrated the importance of plant chitinase cleavage in fungal virulence. In addition, we showed that plant pathogenic fungi are enriched in protease genes of which additional ones might function as virulence factors. Future studies should address to what extent additional fungal proteases play role in plant-microbe interactions. It is essential to identify the targets of proteases in natural conditions and explain the specificity of their hydrolytic action when secreted into plants. The major challenge with functional analysis of fungal proteases is their redundancy. It is important to find and apply sophisticated approaches for functional analysis of proteases involved in plant-fungus interactions.

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SUMMARY

Pathogens cause disease on both animal and plant hosts. For successful infection and establishment of disease, pathogens need proper weaponry to protect themselves against host defenses and to promote host colonization to facilitate uptake of nutrients for growth and reproduction. Indeed, plant pathogens secrete various types of effector molecules (proteins and secondary metabolites) to manipulate host responses for their own needs. Secreted proteases and protease inhibitors (PIs) are such effector molecules. Proteases can hydrolyze plant defense proteins and PIs can inhibit plant proteases that are part of the host surveillance system. Despite the importance of proteases and PIs secreted by fungal pathogens, little information about their role in virulence is available. The recent advances in genomics, bioinformatics, transcriptomics and proteomics have facilitated identification and functional analysis of proteases and PIs relevant to plant-fungus interactions.

Chapter 1 is an introduction to the thesis outlining the general concept of plant-microbe interactions. It briefly describes the current knowledge of pathogenicity mechanisms employed by fungal plant pathogens and defense mechanisms employed by their host plants. It further introduces proteases and PIs and their potential role in modifying pathogenesis-related (PR) proteins to facilitate fungal virulence. It completes with an outline of the PhD research project.

In **chapter 2**, we analyzed and compared the number of putatively secreted proteases present in the genomes of 30 fungi with different lifestyles. The analysis showed that fungi with a saprotrophic and hemibiotrophic lifestyle contain more secreted protease genes than biotrophs. Surprisingly, the number of protease genes present in the genome of *Cladosporium fulvum*, a biotrophic tomato pathogen, is comparable with that of hemibiotrophs and saprotrophs. We analyzed all *C. fulvum* protease genes both at the transcriptome and proteome level by means of RNA-Seq/RT-qrtPCR and mass spectrometry analyses, respectively. Results showed that many proteases of *C. fulvum* are not expressed during growth *in planta*, likely sustaining the biotrophic growth pattern of this fungus.

In **chapter 3,** using an alignment-based gene prediction tool, we identified pseudogenes containing disruptive mutations (DMs) that likely lead to the production of nonfunctional proteins, including a group of putatively secreted proteases from *C. fulvum.* Fewer DMs were observed in other fungi including *Dothistroma septosporum*, a hemibiotrophic pine needle pathogen and close relative of *C. fulvum,* and suggested that the difference in pseudogenization of proteases between these two pathogens might in part explain their different lifestyle.

In **chapter 4,** we analyzed the tomato genome and identified 30 candidate chitinases genes, of which six encoded chitin binding domain (CBD)-containing chitinases. Transcriptome and proteome data were collected after inoculation of tomato with several fungal pathogens and allowed the identification of two CBD-chitinases (SlChi2 and SlChi13) with a putative role in protecting tomato against *C. fulvum* and *F. oxysporum* f. sp. *lycopersici* (*F. oxysporum*), respectively. Purified CBD-chitinases SlChi1, SlChi2, SlChi4 and SlChi13 were incubated with secreted protein extracts (SPEs) from seven fungal tomato pathogens and we could show that SPEs from *F. oxysporum*, *Verticillium dahliae*, and *Botrytis cinerea* modified SlChi1 and SlChi13. LC-MS/MS analysis revealed that incubation with SPE from *F. oxysporum* removed the N-terminal 37 and 49 amino acids, comprising part and complete CBD domain from SlChi1 and SlChi13, respectively. Removal of the CBD of SlChi1 and SlChi13 by SPE of *F. oxysporum* reduced the antifungal activity of the two chitinases. We identified a fungal metalloprotease (FoMep1) and a subtilisin serine protease (FoSep1) that synergistically cleaved both SlChi1 and SlChi13. Transgenic *F. oxysporum* in which the genes encoding these two proteases were knocked out by homologous recombination lost the ability to cleave the two chitinases and were compromised in virulence on tomato compared to the parental wild type. These results suggest an important role of the two chitinases in defense of tomato against this pathogen.

In **chapter 5,** we searched for host target(s) of the apoplastic effector Avr9 secreted by *C. fulvum* during infection of tomato. Based on the structural homology of Avr9 with carboxy peptidase inhibitors, we hypothesized that the host target of Avr9 might be apoplastic proteases. To isolate and identify Avr9 targets in apoplastic fluids, we used synthetic biotinylated Avr9, and performed pull-down and far-western blotting assays with apoplastic fluids from tomato inoculated with a *C. fulvum* race lacking the *Avr9* gene. However, we found no specific Avr9-interacting proteins from pull-down complexes analyzed by mass spectrometry or by far-western blotting. Then, we hypothesized that glycosylation of Avr9 might be required for its biological function. The results of mass spectrometry analysis revealed that Avr9 is N-glycosylated when secreted by *C. fulvum*, containing at least two GlcNac and six mannose residues. The necrosis-inducing activity of glycosylated and non-glycosylated Avr9 was assayed but appeared not significantly different; however, we could not produce sufficient amounts of (biotinylated)-glycosylated Avr9 to perform pull-down assays for identification of potential glycosylated Arv9-interacting proteins by mass spectrometry.

Previous studies as well as the results present in this PhD thesis showed that fungal pathogens secrete a plethora of effectors including proteases and PIs. Many of identified proteases and PIs mediate effector-triggered immunity in host plants. In **chapter 6,** we reviewed the recent advances on the various roles of proteases and PIs in compromising basal defense responses induced by microbe-associated molecular patterns.

Chapter 7 is a summarizing discussion of the PhD thesis. We showed determinative roles of proteases and PIs in shaping plant-pathogen interactions. The expression and pseudogenization studies on proteases of *C. fulvum* showed that the genome content does not necessarily reflect the lifestyle of this fungus. This is true for many classes of fungal genes, including proteases. Fungi contain many different types of proteases whose functions may partly overlap. This hampers the discovery of their biological functions. We could demonstrate that two different types of proteases (metalloprotease (FoMep1) and subtilisin serine protease (FoSep1)) of *F. oxysporum* act synergistically to modify and reduce antifungal activity of two plant CBD-chitinases. Identifying additional proteases is achievable by a targeted proteomics approach using known targets as we did in chapter 4. However, identification of biological functions of proteases is a technical challenge when targets are not known. Multi-gene targeting of protease and PI genes is required to reveal their function in plant-pathogen interactions, which can only be addressed by using advanced genetic tools in future research.

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Certainly, this research has been achieved through collaborations with national and international groups. I want to extend my gratitude to Dr Sjef Boeren from the Laboratory of Biochemistry. Sjef, you initially did help me with running of the HPLC system for folding of biotinylated Avr9 and later with all mass spectrometry analysis. Sjef, so far, our collaborations have resulted into the MPMI paper and I hope more papers are to come in future! Thank you very much for that! I would like also to express my sincere appreciation to Prof. Reinier van der Hoorn for hosting me at the time I was in his group at the Max-Planck -Institute (Cologne, Germany) to carry out activity-based protease profiling experiments. Renier, you were impressed by the report of the experiments that I did in your lab, but unfortunately those data did not fit into this PhD thesis. Reinier, I never forget your positive thoughts, your unconditional support and encouragement. I wish you all the best☺. I am also grateful to Todd Naumann, at USDA-ARS-NCAUR, Peoria, USA for letting us share recombinant maize chitinases to initiate and set up the chitinase cleavage assays in our laboratory. Thank you Todd!

Certainly, the lovely small city of Wageningen and its memorable experiences has become part of my life. I never forget these beautiful years inside and outside the lab. Of those, I spent the major part of my time in the phytopathology lab and I had the opportunity to have many joyous moments with many people during social gatherings, coffee and lunch breaks, drinks, and behind the computer. It was great to be part of such an international community to learn from your thoughts and ideas and cultures in formal and informal meetings and during our international travels. I wish you all colleagues at phytopathology much success in your personal and scientific lives! Outside the lab, I had a great time with my dear Iranian and Dutch friends and also

friends from other parts of world! I am very grateful and proud of all you. Our community had several ups and downs regarding the number of members, but always has been happy and strong and created many memorable moments. Our gatherings for different events, parties, invitations, BBQing, and especially weekly football playing were very wonderful and made me feel being with my family. I will never forget this lively social environment and appreciate your unconditional help from beginning till now! Thank you very much and hope to be able to visit you and the city of life science in the near future.

The best regards,
Wageningen, September 2015,
Mansoor Karimi Jashni

Curriculum Vitae

Mansoor Karimi Jashni was born on September 11, 1978, in Arsanjan, Fars province, Iran. He did the primary and secondary school in his hometown and was awarded a scholarship to do his high school in one of excellent schools in the beautiful city of Shiraz in 1992. After he obtained his high school diploma in 1996, he successfully passed the national exam for universities and enrolled at Shiraz University to study plant protection in the College of Agriculture in 1997. He obtained his BSc with specialization in "the role of micronutrients in tolerance and resistance of plants to disease" in 2001. Subsequently, he passed the national exam and started to study phytopathology at Tehran University in 2002. He performed his MSc thesis on "Identification of the pathotypes of *Blumeria graminis* f. sp. *graminins* in Iran and histopathological studies of the disease resistance in some wheat cultivars" and finished his MSc in 2005. After his MSc degree, Mansoor did research for a few years in the plant protection institute. In 2008, he was awarded a scholarship to pursue his PhD studies in molecular plant-microbe interactions. He was accepted by the Laboratory of Phytopathology of Wageningen University and started his PhD August, 2009 in the group of Professor Pierre de Wit. He worked on "Identification and functional characterization of proteases and protease inhibitors involved in virulence of fungal tomato pathogens" which has become the main subject of his PhD thesis. He worked hard on his research topic and also contributed to other projects that resulted in several articles in peer-reviewed international journals, and various oral and poster presentations in national and international scientific meetings. A few interesting research questions remain to be addressed and he hopes to complete those when he will join the Department of Plant Pathology in Tarbiat Modares University in Tehran.

List of publications

1. **Karimi Jashni M.,** Mehrabi, R., Collemare, J., Mesarich, C. H., and de Wit, P.J.G.M. 2015. The battle in the apoplast: further insights into the roles of proteases and their inhibitors in plant-pathogen interactions. Frontiers in plant Science (in press).

2. **Karimi Jashni M.,** Dols, I., Iida, Y., Boeren, S. Mehrabi, R., Collemare, J., and de Wit P.J.G.M. 2015. Synergistic action of a metalloprotease and a serine protease from *Fusarium oxysporum* f. sp. *lycopersici* cleaves chitin-binding tomato chitinases, reduces their antifungal activity, and enhances fungal virulence. Molecular Plant Microbe interaction (in press).

3. Mesarich, C.H., Stergiopoulos, I., Beenen, H.G., Cordovez, V., Guo, Y., **Karimi Jashni, M.,** Bradshaw, R.E. and de Wit, P.J.G.M. 2015. A conserved proline residue within Dothideomycete Avr4 effector proteins is required to trigger a Cf-4–dependent hypersensitive response. Molecular Plant Pathology. DOI: 10.1111/mpp.12265

4. Collemare, J., Griffiths, S., Iida, Y., **Karimi Jashni, M.,** Battaglia, E., Cox, R.J. and de Wit, P.J.G.M. 2014. Secondary Metabolism and Biotrophic Lifestyle in the Tomato Pathogen *Cladosporium fulvum*. PLoS ONE 9, e85877.

5. van der Burgt, A., **Karimi Jashni, M**., Bahkali, A.H. and de Wit, P.J.G.M. 2014. Pseudogenization in pathogenic fungi with different host plants and lifestyles might reflect their evolutionary past. Molecular Plant Pathology 15, 133-144.

6. de Wit, P.J.G.M., van der Burgt, A., Ökmen, B., Stergiopoulos, I., Abd-Elsalam, K.A., Aerts, A.L., Bahkali, A.H., Beenen, H.G., Chettri, P., Cox, M.P., Datema, E., de Vries, R.P., Dhillon, B., Ganley, A.R., Griffiths, S.A., Guo, Y., Hamelin, R.C., Henrissat, B., Kabir, M.S**., Karimi Jashni, M.,** Kema, G., Klaubauf, S., Lapidus, A., Levasseur, A., Lindquist, E., Mehrabi, R., Ohm, R.A., Owen, T.J., Salamov, A., Schwelm, A., Schijlen, E., Sun, H., van den Burg, H.A., van Ham, R.C.H.J., Zhang, S., Goodwin, S.B., Grigoriev, I.V., Collemare, J. and Bradshaw, R.E. 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, e1003088.

7. Mehrabi, R., Bahkali, A.H., Abd-Elsalam, K.A., Moslem, M., Ben M'Barek, S., Gohari, A.M., **Karimi Jashni, M.**, Stergiopoulos, I., and Kema, G.H.J., de Wit P.J.G.M. 2011. Horizontal gene and chromosome transfer in plant pathogenic fungi affecting host range. FEMS Microbiology Reviews: 35:542-54.

8. Razavi, M., **Karimi Jashni, M**., Dehgan, M. A., Safavi, S. A. and Barari, H. 2010. Study on the variability for virulence in *Blumeria graminis* f. sp. *tritici* cause of

wheat powdery mildew using trap nursery in Iran. Applied Entomology and Phytopathology 78:97-106.

9. **Karimi Jashni, M.**, Torabi, M., Roustaie. A., Etebarian, H.R., Okhovat, S.M. 2009. Study on some components of resistance and development of *Blumeria graminis* f.sp. *tritici*, in six wheat Lines. Seed and Plant 25:245-262.

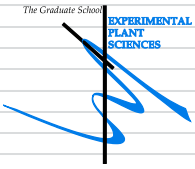
10. Razavi, M., Dehgan, M. A., Safavi, S. A., Barari, H., Torabi, M., **Karimi Jashni, M.,** Kazemi H. 2009. Evaluation of the field and seedling resistance of some advanced and elite lines of wheat to *Blumeria graminis* f. sp. *tritici* cause of powdery mildew of wheat in Iran. Applied Entomology and Phytopathology 77:133-150.

11. **Karimi Jashni, M.**, Torabi, M., Roustaie, A., Etebarian, H.R., Okhovat, S.M., Razavi, M., Yazdanpanah, F. 2006. Pathotypes of *Blumeria graminis* (Dc. Ex Merat) Speer f. sp. *tritici*, the causal agent of wheat powdery mildew from some regions of Iran. Seed and Plant 22:257-271.

12. **Karimi Jashni, M.**, Torabi, M., Rustaee, A., Etebarian, H.R., Okhovat, M., Razavi, M., Yazdanpanah, F. 2005. Evaluation of resistance of some wheat commercial cultivars and advanced lines to pathotypes of *Blumeria graminis* (Dc. Ex Mert) Speer f. sp. *tritici*, in greenhouse. Seed and Plant 21:411-423.

Education Statement of the Graduate School

Experimental Plant Sciences



Issued to:	**Mansoor Karimi Jashni**
Date:	**15 September 2015**
Group:	**Laboratory of Phytopathology**
University:	**Wageningen University & Research Centre**

1) Start-up phase	*date*
▸ **First presentation of your project**	
Molecular characterization of *Cladosporium fulvum* protease inhibitors targeting tomato	Sep 09,2009
▸ **Writing or rewriting a project proposal**	
"Molecular characterization of *Cladosporium fulvum* proteases and protease inhibitors directed against tomato proteases"	Feb 2010
▸ **Writing a review or book chapter**	
The battle in the apoplast: further insights into the roles of proteases and their inhibitors in plant-pathogen interactions, Frontiers in Plant Sciences	Jun 2015
▸ **MSc courses**	
Bioinformation technology	Oct-Sep 2009
▸ **Laboratory use of isotopes**	
Subtotal Start-up Phase	*10.5 credits**

2) Scientific Exposure	*date*
▸ **EPS PhD student days**	
EPS PhD student day, Utrecht University	Jun 01, 2010
EPS PhD student day, University of Amsterdam	Nov 30, 2012
▸ **EPS theme symposia**	
EPS Theme 2 'Interactions between Plants and Biotic Agents', Utrecht University	Jan 15, 2010
EPS theme 2 'Interactions between plants and biotic agents', University of Amsterdam	Feb 3, 2011
EPS theme 2 'Interactions between plants and biotic agents', Wageningen University	Feb 10, 2012
EPS theme 2 'Interactions between plants and biotic agents', Utrecht University	Jan 15, 2013
▸ **NWO Lunteren days and other National Platforms**	
ALW meeting 'Molecular Genetics Annual Meeting', Lunteren	Oct 15-16, 2009
ALW meeting 'Molecular Genetics Annual Meeting', Lunteren	Oct 14-15,2010
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 04-05, 2011
ALW meeting 'Molecular Genetics Annual Meeting' Lunteren	Oct 06-07, 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**	
Plant Science seminars Pierre de Wit, Fred van Euwijk	Nov 10, 2009
1st join meting of WUR-Marburg	Sep 2010
Mini symposium on writing of world-class paper	Oct 26, 2010
Mini symposium on plant breeding	Nov 25,2011
Invited seminar Rosie Bradshaw	Aug 04, 2011
Invited seminar Brian Staskawicz	May 21, 2013
EPS Flying Seminar Dr. David M. Weller	Sep 25, 2013
Seminar Gabino Sanchez Perez	Mar 12, 2013
WEES Seminar, Jaap de Roode (USA)	Mar 15, 2012
EPS Symposium 'Omics Advances for Academia and Industry - Towards True Molecular Plant	Dec 11, 2014
Invited seminar Sophien kamoun	May 28, 2014
▸ **Seminar plus**	
▸ **International symposia and congresses**	
10thEuropean Conference of Fungal Genetics (ECFG10)	Mar 29-Apr 01, 2010
International conference of Plant Proteases, Sweden	Apr 10-14, 2011
3rd European Retreat of PhD students in Plant Sciences, Orsay, France,	Jul 05-08, 2011
5rd European Retreat of PhD students in Plant Sciences, Gent, Belgium	Jul 23-26, 2013
Molecular Plant-Microbe interaction (MPMI), Rhodos, Greece	Jul 06-10, 2014
Workshop on structure-guided investigation of effector function, action and recognition, Bucharest, Romania	Sep 10-12, 2014
▸ **Presentations**	
ECFG10 (Poster)	Mar 29-Apr 01, 2010
PhD EPS day (Poster)	Jun 01, 2010
PhD retreat, Orsay, France (Poster)	Jul 05-08, 2011
PhD retreat, Gent, Belgium (Talk)	Jul 23-26, 2013
MPMI, Greece (Talk)	Jul 06-10, 2014
Plant Protease conference, Sweden (Poster)	Apr 10-14,2011
Workshop structure-guided investigation, Bucharest, Romania (Poster)	Sep 10-12-2014

►	**IAB interview**	
	Meeting with a member of the International Advisory Board of EPS	Nov 14, 2012
►	**Excursions**	
	Subtotal Scientific Exposure	*20.0 credits**
3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	PhD course: Spring School RNAi Scilencing	Apr 14-16, 2010
	Advanced PhD Course: Comparative Proteomics	Apr 21-23, 2010
	Bioinformatics: A Users Approach (a practical course)	Aug 30- Sep 03, 2010
	PhD course: Molecular phylogeny	Oct 18-22, 2010
	Autumn school: Host-Microbe interactomics	Nov 01-03, 2011
	PhD Course: The power of RNA seq	Jun 05-07, 2013
►	**Journal club**	
	Member of literature discussion group at Phytopathology	2009-2014
►	**Individual research training**	
	Plant Chemetics Group, Max Planck Institute, Clogne Germany	Jan 15-Feb 04, 2012
	Laboratory of Biochemistry, Wageningen University	Jun 14-27, 2010
	Subtotal In-Depth Studies	*12.6 credits**
4) Personal development		*date*
►	**Skill training courses**	
	ExPectationS (EPS career day), Wageningen	Nov 18, 2011
	ExPectationS (EPS career day), Wageningen	Feb 01, 2013
	Techniques for writing and presenting a scientific paper	Feb 14-17, 2012
	Time and project management	May 15, 28, Jun 25, 2013
►	**Organisation of PhD students day, course or conference**	
	Lab-outing organization for phytopatology	Jun 05, 2012
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*3.0 credits**
	TOTAL NUMBER OF CREDIT POINTS*	**46.1**
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		
** A credit represents a normative study load of 28 hours of study.*		

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