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

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As part of their defense 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 defense 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 were 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, 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.

Upon recognition of general elicitors, plants induce basal defenses that confer resistance to a wide range of pathogens. An important component of these defenses 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 De 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 overexpress 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 a primary amino acid sequence of the catalytic domain, chitinases are grouped into two glycosyl hydrolase (GH) families, GH18 or 19 GH19. The GH19 family is largely composed of 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 harbor 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 mold 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*

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induces the synthesis of tomato chitinases in the apoplast (Joosten and De 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, which 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 overexpressing 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 *F. 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.

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; Supplementary Fig. S1; Supplementary 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 nonfunctional genes (Supplementary Fig. S2). This

Fig. 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. An 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.

observation suggests frequent gene duplication events, which might indicate a role in coevolution with fungal pathogens. Twenty-six predicted tomato chitinases were grouped into the six described classes, based on sequence similarity, protein structure, and phylogenetic analysis (Supplementary Figs. S3 through S12). 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). 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. In addition, two chitinases from *Picea* species that were reported to belong to class VII (Kolossova et al. 2014) lack two conserved regions compared with class VII chitinases and share high sequence similarity to class IV chitinases throughout the entire chitinase domain. A phylogenetic analysis of GH19 chitinases also showed that *Picea* chitinases belong to a monophyletic clade that comprises class IV chitinases only (Supplementary 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.

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 defense of tomato against fungal pathogens and were selected for further analyses.

Induction of CBD chitinase genes in tomato after inoculation with *C. fulvum* and *F. oxysporum* suggests a role in plant defense.

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 De 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*. Expression profiles of the six genes encoding CBD chitinases were analyzed using real time quantitative reverse transcription-polymerase chain reaction. All six genes exhibited a low basal expression level at 0 days postinoculation (dpi) in both leaves and roots (Fig. 2). None of them were induced during early infection (0 to 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

Fig. 2. Expression of tomato genes encoding chitin-binding domain (CBD)-containing chitinases is induced upon fungal infection. **A**, Susceptible tomato cultivar Money-Maker Cf-0 was inoculated with *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 postinoculation (dpi) for RNA extraction and subsequent real time quantitative reverse transcription-polymerase chain reaction. Expression of CBD-chitinase genes is normalized to that of the tomato β -tubulin gene (*LeTub*) using the E^{-ΔCt} method (Livak and Schmittgen 2001). The bars show the average of three biological replications with standard deviation. A two-way analysis of variance with a posthoc Tukey's multiple comparisons test was performed. Black asterisks indicate significant differential expression compared with the previous time point. When not applicable, gray asterisks indicate significant differential expression compared with 0 dpi (* indicates P value < 0.5; **, P value < 0.01; ***, P value < 0.001; and ****, P value < 0.0001).

presence of the CBD chitinase proteins SIChi1, SIChi2, SIChi3, and SIChi13. In contrast, in roots inoculated with *F. oxysporum*, the predicted extracellular chitinase *SIChi13* was highly induced from 4 dpi onwards (Fig. 2B) and, thereafter, its expression decreased gradually to reach, at 20 dpi, the same expression level as at 0 dpi. In addition, the vacuolar chitinases *SIChi3* and *SIChi4* were transiently up-regulated at 8 dpi only and to a lower extent than *SIChi13*. Altogether, these results showed that *SIChi3* and *SIChi4* were slightly and transiently up-regulated in both leaves and roots in response to fungal infection, suggesting a minor role in tomato defense. *SIChi2* and *SIChi13* 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*, *Alternaria solani*, *Alternaria 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 SIChi1, SIChi2, SIChi4, and SIChi13 could be produced and purified in sufficient amounts. These four CBD chitinases were incubated for 20 h with secreted protein extracts from the seven fungal tomato pathogens, and their possible cleavage was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). None of the secreted protein extracts could cleave the vacuolar CBD chitinases SIChi2 and SIChi4, including extracts from *C. fulvum* (Fig. 3A). However, a slight increase in electrophoretic mobility was observed for SIChi4 after incubation with extract from *B. cinerea*, suggesting that this fungus

Fig. 3. Secreted protein extracts from fungal pathogens remove the chitin-binding domain (CBD) of extracellular tomato chitinases. **A**, The four tomato CBD chitinases SIChi1, SIChi2, SIChi4, and SIChi13 were produced by *Pichia pastoris*. Pure chitinases were incubated for 20 h 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 sodium dodecyl sulfate-polyacrylamide electrophoresis gels. Control is pure chitinase incubated in 10 mM sodium acetate buffer (pH 5.2) for 20 h. The white asterisks indicate cleaved products of SIChi1 and SIChi13 that were isolated for mass spectrometry analysis. Chitinase modifying assays were performed at least three times. **B**, Cleaved products of SIChi1 and SIChi13 after incubation with protein extract from *F. oxysporum* were sequenced by mass spectrometry to determine the cleavage sites. The cleavage site for SIChi1 is located within the CBD and the cleavage site for SIChi13 is located at the beginning of the hinge between the CBD and the 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.

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 SIChi1 and SIChi13 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 SIChi13, 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 SIChi1 and SIChi13 products cleaved by the *F. oxysporum* extract, they were excised from the SDS-PAGE gel and were analyzed by liquid chromatography/tandem mass spectrometry (LC-MS/MS). Mass spectrometric analysis showed full coverage of the C-termini of both cleaved SIChi1 and SIChi13. No peptide covered the first 37 amino acids at the N-terminus of SIChi1, while native SIChi1 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; Supplementary Fig. S14). Such cleavage likely results in a nonfunctional CBD. Mass spectrometry analysis of the cleaved product of SIChi13 showed that no peptide covered the first 49 amino acids at the N-terminus (Fig. 3B; Supplementary 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.

Removal of the CBD from SIChi1 and SIChi13 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 SIChi1 and SIChi13 significantly reduced the chitinase activity, using insoluble chitin azure as a substrate (Supplementary Fig. S16). Chitinase activity of SIChi2, of which the CBD 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 SIChi1 and SIChi13 was also significantly reduced after cleavage of the CBD by secreted protein extracts from *F. oxysporum*. Native SIChi1, SIChi2, and SIChi13 significantly inhibited the growth of germ tubes of *F. oxysporum* when compared with the controls (buffer and secreted protein extracts) (Fig. 4A). The average length of germ tubes was reduced by 32 to 44% after incubation with SIChi1, SIChi2, and SIChi13 (Fig. 4B). Mixing SIChi1 and SIChi13 CBD chitinases with secreted protein extracts from *F. oxysporum* prior to incubation with fungal spores resulted in significant reduction of growth inhibition as shown by longer germ tubes (Fig. 4). These results demonstrate that removal of the CBD from SIChi1 and SIChi13 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 SIChi2 that was either treated or not treated with secreted protein extract from *F. oxysporum*.

Fig. 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 h 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 SIChi1, SIChi2, and SIChi13 chitinases. Scale bars indicate 50 μm in size. **B**, Average length and standard deviation of germ tubes after treatments shown in A. Number of measured germ tubes is indicated on parentheses. A one-way analysis of variance with a posthoc Tukey's multiple comparisons test was performed. Black asterisks indicate significant differences in length of germ tubes compared with control, and gray asterisks indicate significant differences between intact and cleaved chitinases. (* indicates P value < 0.5; ****, P value < 0.0001).

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

The fungalsin 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 (Supplementary Fig. S17). Therefore, we hypothesized that this metalloprotease might be responsible for cleavage of SiChi1 or SiChi13 or both. Three targeted deletion mutants were obtained in which a cassette carrying the hygromycin-resistance and green fluorescent protein (GFP) genes replaced *FoMep1*. All three $\Delta fomep1$ 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 $\Delta fomep1$ deletion mutants, wild type, and the ectopic transformant strain for incubation with SiChi1 and SiChi13. The chitinase-modifying assay showed that all protein extracts exhibited the same chitinase cleavage activity on SiChi1 but lost part of their activity on SiChi13 (Fig. 5A). This result indicates that the fungalsin metalloprotease encoded by *FoMep1* is required for partial modification of class IV CBD chitinase SiChi13 but not for class I CBD chitinase SiChi1.

In order to determine the type of protease that is responsible for additional chitinase-modifying activity in the $\Delta fomep1$ 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 (phenylmethylsulfonyl fluoride [PMSF]), metalloproteases (EDTA), and aspartic proteases (PepA). The chitinase-modifying assay showed that cleavage of SiChi1 and SiChi13 is inhibited by PMSF only at a concentration of 1 mM and higher (Fig. 5B; Supplementary Fig. S18). This result indicates that serine proteases are likely responsible for additional cleavage of tomato CBD chitinases.

Deletion of the serine protease *FoSep1* reduces chitinase cleavage activity of *F. oxysporum* $\Delta fomep1$ deletion mutants.

Identification of serine protease candidates was performed by fractionating secreted protein extracts from culture filtrates of the $\Delta fomep1$ deletion mutant and testing each fraction for chitinase-modifying activity. The culture filtrates were size-fractionated on a Superdex G-75 column, 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 were analyzed by mass spectrometry. Two subtilisin serine proteases (*FOXG_09801* and *FOXG_01145*) were identified in fractions 3 and 4 with chitinase-modifying activity (Supplementary Fig. S19, bands marked with asterisk). 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 $\Delta fomep1$ deletion mutant (double mutants) strains (Supplementary 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 with the wild type and ectopic transformants. As previously described, secreted proteins were isolated from each single and double mutant and were tested in chitinase-modifying assays. The results showed that $\Delta fosep1$ and $\Delta fosep2$ single deletion mutants retained full activity against both chitinases SiChi1 and SiChi13 (Fig. 5C). However, protein extracts from the $\Delta \Delta fomep1::fosep1$ double mutant appeared to have lost chitinase cleavage activity toward both SiChi1 and SiChi13 almost completely (Fig. 5C).

The $\Delta \Delta fomep1::fosep2$ and $\Delta \Delta fosep1::fosep2$ double mutants showed the same activity as the $\Delta fomep1$ 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 SiChi1 and SiChi13 chitinases is achieved by synergistic action of the fungalsin metalloprotease *FoMep1* and serine protease *FoSep1*. Furthermore, we could show that chitinase activity of SiChi1 and SiChi13 treated with secreted protein extract of $\Delta \Delta fosep1::fosep2$ mutant of *F. oxysporum* was not reduced and its antifungal activity was not affected.

A chitinase-modifying metalloprotease and a serine protease are required for full virulence of *F. oxysporum* on tomato.

To determine the role of *FoMep1* and *FoSep1* in virulence, roots of MM-Cf-0 tomato seedling were inoculated with independent

Fig. 5. Synergistic action of a serine protease and metalloprotease from *Fusarium oxysporum* f. sp. *lycopersici* (*F. oxysporum*) cleaves tomato chitin-binding domain-containing chitinases. **A**, SiChi1 and SiChi13 are cleaved after incubation with secreted protein extracts (SPE) isolated from three independent *F. oxysporum* fungalsin metalloprotease $\Delta fomep1$ deletion mutants. Control is 10 mM sodium acetate buffer (pH 5.2). **B**, The serine protease inhibitor phenylmethylsulfonyl fluoride inhibits the cleavage of SiChi1 and SiChi13 by SPE from the *F. oxysporum* $\Delta fomep1$ deletion mutants in a concentration-dependent manner. **C**, Only SPE from *F. oxysporum* double-mutant $\Delta \Delta fomep1::fosep1$ was not able to cleave SiChi1 and SiChi13.

single and double deletion mutants of *FoMep1* and *FoSep1*. Virulence was quantified at 4 weeks postinoculation 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 $\Delta fomep1$ and $\Delta fosep1$ performed slightly better growth than plants inoculated with the wild type and ectopic transformants (Fig. 6A and B), although this reduction of virulence is not correlated with a lower disease index (Fig. 6C). Plants inoculated with the double mutants $\Delta\Delta fomep1::fosep1$ were as high as mock-inoculated plants (Fig. 6A) and were significantly less diseased compared with those inoculated with the wild type and ectopic transformants (Fig. 6B and C). Altogether, these results indicate that synergistic action of *FoMep1* and *FoSep1* is required for full virulence of *F. oxysporum* on tomato.

DISCUSSION

Induction and accumulation of PR proteins constitute an important component of basal defense 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. CBD-containing chitinases have been reported to show high antifungal activity (Iseli et al. 1993; Suarez et al. 2001; Truong et al. 2003). We analyzed 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*. 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

Fig. 6. Synergistic action of a serine and metalloprotease from *Fusarium oxysporum* f. sp. *lycopersici* is required for full virulence on tomato. **A**, A representative picture showing MM-Cf-0 tomato plants 4 weeks after inoculation with *F. oxysporum* f. sp. *lycopersici* (*F. oxysporum*) wild type, ectopic transformants, single-, and double-deletion mutants of *FoMep1* and *FoSep1* ($\Delta fomep1$ #1, $\Delta fosep1$ #1, $\Delta\Delta fomep1::fosep1$ #2). Mock-inoculated plants serve as negative control. **B**, Fresh weight of upper parts and the disease index of inoculated plants is presented as the average (and standard deviation) of three different plants from two to three independent strains. Results for $\Delta fomep1$ -ectopic and $\Delta fosep1$ -ectopic transformants are presented as ectopic transformants. A two-way analysis of variance with a posthoc Dunnett's multiple comparisons test was performed. Black asterisks indicate significant differences compared with wild type (* indicates P value < 0.5; ****, P value < 0.0001).

SlChi13 is strongly induced at early stages of infection when the fungus colonizes 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 Fosdal 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*, all lacking 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 *FoMep1* and *FoSep1* 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 fungalyisin metalloprotease of *F. verticillioide*s, which infects maize (Naumann et al. 2011). Fungalyisin from *F. verticillioide*s 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. verticillioide*s, 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 action 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. verticillioide*s 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. verticillioide*s cleavages SlChi1 and SlChi13 chitinases (results not presented), suggesting a similar mechanism involved in both fungi. However, the effects of fungalyisin of *F. verticillioide*s 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 favor of coevolution 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 $\Delta\Delta\text{fo}mep1::\text{fo}sep1$ 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 *FoMep1*, *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, 2004). Here we show, for the first time, that full cleavage of class I and class IV tomato 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 toward *F. oxysporum* demonstrated the importance of CBD chitinases in basal defense 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.

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 to 25°C during daytime and 19 to 21°C at night, with a 16-h-light and 8-h-dark regime, and 100 W m⁻² of 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, The Netherlands. Strains of *Cladosporium fulvum* (R97, race 0, WU-CBS131901), *Verticillium dahliae* (VdLs17, race 1), *Botrytis cinerea* (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 10⁶ spores per milliliter were sprayed on the abaxial side of tomato leaves. Leaf samples were collected at different dpi 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 10⁷ spores per milliliter) for 1 min and were then planted into 13-cm² pots. Plant height, disease index, and fresh weight of upper part of plants were analyzed 4 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 National Center for Biotechnology Information website and were used as query sequences for tblastn searches against the tomato genome (Solgenomics Network). Furthermore, InterPro domains corresponding to different classes of plant chitinases (Supplementary 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 phylogenetic 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 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*, were properly washed in water, and were immediately frozen in liquid nitrogen. Total RNA was isolated from fully ground samples, using the Quick-RNA miniprep kit (Zymo Research) as provided by the company. RNA concentration and integrity was checked with

a NanoDrop 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 (Supplementary 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 polymerase chain reaction (qPCR) was performed using the 7300 system (Applied Biosystems). Each reaction consisted of 25 µl, containing 12.5 µl of Sensimix (Bioline), 1 µl of each forward and reverse primer (7.5 µM), 1 µl of 10× 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 s at 95°C and annealing and extension for 45 s 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 us to normalize the expression of other genes. Results were analyzed using the E^{-ΔCt} 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 (12,000 × g, 20 min) at 4°C and were concentrated three times using 1-kDa-cutoff Amicon filters. For xylem sap isolation, 10 plants of MM-Cf-0 were inoculated with *F. oxysporum*, and 5 weeks later, stems were cut off at 2 cm above ground level. Xylem sap was harvested using a previously described method (Rep et al. 2002). Xylem sap (50 ml) was cleared by centrifugation (12,000 × g, 20 min) at 4°C and was dialyzed against 10 mM sodium acetate pH 5.2 buffer, using standard dialysis membrane (Spectrum Labs) cutoff of 3.5 kDa. The sample was then freeze-dried and dissolved in 0.5 ml of 10 mM sodium acetate pH 5.2 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 Supplementary 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 the *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 (4,000 × g, 20 min). Supernatants were retrieved and centrifuged again (12,000 × g, 20 min) at 4°C. Cell-free supernatants were concentrated using a 10kDa cutoff filter (Pall Nanosep) to 50 ml and were run on a 10-ml Ni-NTA column (Qiagen) at 4°C. The column was washed with 20 ml of washing buffer (50 mM NaH₂PO₄, pH 8, 300 mM NaCl, and 10 mM imidazole), and proteins were eluted with 20 ml of elution buffer (50 mM NaH₂PO₄, pH 8, 300 mM NaCl, and 250 mM imidazole). Collected fractions containing the protein of interest were combined

and dialyzed against 10 mM sodium acetate pH 5.2 buffer, were concentrated using 3-kDa Amicon filters, and were 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 of streptomycin sulfate salt per milliliter (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 of streptomycin sulfate salt per milliliter (Sigma-Aldrich) was inoculated with a mycelium plug taken from PDA plates and was 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 $3,700 \times g$ for 15 min, were resuspended in sterile water, and were 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), were inoculated with 3 ml of calibrated spore suspensions, and were 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 $4,000 \times g$ for 5 min at 4°C, and supernatants were centrifuged again at $12,000 \times g$ 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 $\Delta fomep1$ deletion mutant was concentrated using a 3-kDa cutoff 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 h 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 analyzed 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 SIChi1 was incubated with 1 µl of each fraction in 50 mM sodium acetate buffer, pH 5.2, and was analyzed as described above.

Mass spectrometry analysis.

To determine the cleavage site in the targeted extracellular tomato CBD chitinases, SIChi1 and SIChi13 were incubated with protein extracts of *F. oxysporum*, as described in the chitinase modifying assay section. Extract (10 µl) with 3.5 µl of SDS gel loading buffer was boiled for 5 min and was run on a 16% tricine SDS-PAGE gel. Intact and processed SIChi1 and SIChi13 were excised from the tricine SDS-PAGE gel and were cut into cubes of 1 mm³. Proteins were reduced with 50 mM dithiothreitol dissolved into 50 mM ammonium

bicarbonate, were carboxamidomethylated with 100 mM iodoacetamide in 50 mM ammonium bicarbonate, and were digested by adding 25 µl of 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 analyzed by nano-scale (n)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 analyzed 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 deamidation of N and Q. The *F. oxysporum* database (4287-FO2) was downloaded from the Broad Institute and the tomato database ITAG2.3 was downloaded from the Solgenomics website. 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, porcine), 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 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 of potassium-phosphate buffer (100 mM, pH 6.0) by brief centrifugation. The washed chitin azure pellet was resuspended in 0.9 ml of the same buffer and was then mixed with purified proteins SIChi1, SIChi2, or SIChi13 at a final concentration of 0.1 mg/ml and incubated for 20 h at 37°C. Reactions were centrifuged at $12,000 \times g$ for 10 min at room temperature, and the optical density of 0.750 ml of supernatants was measured at 560 nm. Similar chitinase activity assays were performed with SIChi1, SIChi2, and SIChi13 chitinases treated with protein extracts of wild type and protease mutant ($\Delta\Delta fomep1::fjosep1$) of *F. oxysporum*.

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

Fungal spores of *F. oxysporum* were harvested in sterile Milli-Q water as described above, and spore concentration was adjusted to 10^5 spores/ml. Spore suspension (10 µl) was mounted on a microscopy slide and, first, was incubated for 4 h at room temperature inside a moist covered box. Then, 20 µl of non-cleaved SIChi1 or SIChi13 chitinase (in 10 mM sodium

acetate, pH 5.2) was added to the spore suspension at a final concentration of 1 µg/µl. After 16 h, samples were observed, using a Nikon eclipse 90i microscope controlled by the imaging software NIS-Element AR 2.30. The lengths of about 100 germ tubes were measured. Similar antifungal assays were performed with cleaved SICh1 and SICh13 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*.

Gene replacement vectors pR4R3Δfomep1, pR4R3Δfosep1, and pR4R3Δfosep2 were constructed, using the Multisite Gateway three-fragment vector construction kit (Invitrogen) according to the manufacturer's instructions. Genomic DNA was extracted from *F. oxysporum* mycelium that was grown in PDB for 3 days and frozen in liquid nitrogen, using the DNeasy plant mini kit (Qiagen Benelux bv.) according to the manufacturer's instructions. The upstream (US) 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*. The PCR reactions were performed using GoTaq DNA polymerase (Promega) and oligonucleotides (Supplementary 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 gene were introduced into pDONR P4-P1R and pDONR P2R-P3 vectors, respectively. A purified US or DS fragment (70 ng), 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 were 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) 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, vector pRM254 (Mehrabi et al. 2015), containing GFP and the 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. *Agrobacterium 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 of hygromycin or 150 µg of geneticin per milliliter. Stable transformants were selected by growing them on selective PDA plates, followed by a new culture on nonselective plates. Deletion mutants were screened and characterized by PCR and qrtPCR (Supplementary Table S6).

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