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A conserved GH17 glycosyl hydrolase from plant pathogenic Dothideomycetes releases a DAMP causing cell death in tomato

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SUMMARY

To facilitate infection, pathogens deploy a plethora of effectors to suppress basal host immunity induced by exogenous microbe-associated or endogenous damage-associated molecular patterns (DAMPs). In this study, we have characterized family 17 glycosyl hydrolases of the tomato pathogen *Cladosporium fulvum* (CfGH17) and studied their role in infection. Heterologous expression of *CfGH17-1* to *5* by potato virus X in different tomato cultivars showed that CfGH17-1 and CfGH17-5 enzymes induce cell death in *Cf-0*, *Cf-1* and *Cf-5* but not in *Cf-Ecp3* tomato cultivars or tobacco. Moreover, CfGH17-1 orthologues from other phytopathogens, including *Dothistroma septosporum* and *Mycosphaerella fijiensis*, also trigger cell death in tomato. CfGH17-1 and CfGH17-5 are predicted to be β-1,3-glucanases and their enzymatic activity is required for the induction of cell death. CfGH17-1 hydrolyses laminarin, a linear 1,3-β-glucan with 1,6-β linkages. *CfGH17-1* expression is down-regulated during the biotrophic phase of infection and up-regulated during the necrotrophic phase. Deletion of *CfGH17-1* in *C. fulvum* did not reduce virulence on tomato, while constitutive expression of *CfGH17-1* decreased virulence, suggesting that abundant presence of CfGH17-1 during biotrophic growth may release a DAMP that activates plant defence responses. Under natural conditions CfGH17-1 is suggested to play a role during saprophytic growth when the fungus thrives on dead host tissue, which is in line with its high levels of expression at late stages of infection when host tissues have become necrotic. We suggest that CfGH17-1 releases a DAMP from the host cell wall that is recognized by a yet unknown host plant receptor.

Keywords: cell death-inducing, *Cladosporium fulvum*, DAMP, effectors, GH17.

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INTRODUCTION

Plants constantly encounter a plethora of diverse pathogens. Consequently, they have evolved several types of surveillance systems to recognize self- or non-self-danger signals (Dodds and Rathjen, 2010). Microbe-associated molecular patterns (MAMPs) are broadly conserved essential functional components of microorganisms, which are recognized by host pattern recognition receptors (PRRs) to promote signals associated with innate immunity (Boller and Felix, 2009; Couto and Zipfel, 2016). In addition to MAMPs, which are exogenous danger signals, host cells also recognize endogenous danger signals, known as damage-associated molecular patterns (DAMPs), to induce innate immunity (Boller and Felix, 2009; Brutus *et al*., 2010). Although MAMP- or DAMP-triggered immunity effectively wards off non-adapted pathogens, adapted pathogens have evolved strategies to avoid, attenuate or suppress the host immune system by secreting effectors (Jones and Dangl, 2006; Lanver *et al*., 2017; Ökmen and Doehlemann, 2014; Stergiopoulos and de Wit, 2009). Effectors show diverse modes of action and act at different locations in the host. Some effectors act in the apoplastic space, while others are translocated into host cells to compromise host immunity or interfere with host metabolism (Lanver *et al*., 2017; Win *et al*., 2012).

Cladosporium fulvum is a hemibiotrophic fungal pathogen that is the causal agent of the leaf mould disease of tomato and exclusively colonizes the apoplastic space of host leaves (de Wit, 2016). In early reports, non-host-specific elicitors such as glycoproteins derived from the fungal cell wall were detected in synthetic media on which *C. fulvum* was grown. Isolation and infiltration of these compounds into host and non-host plants revealed that they induced defence-related responses, including phytoalexin synthesis and cell death (de Wit and Kodde, 1981; de Wit and Roseboom, 1980). Later these non-specific fungus-related compounds were called MAMPs.

Successful establishment of *C. fulvum* in the host plant in the presence of these non-specific elicitors led to the hypothesis that the fungus must produce suppressors of host defences (Lu and Higgins, 1993). A search for suppressors of non-specific elicitors in apoplastic fluids isolated from *C. fulvum*-infected tomato leaves revealed the presence of race-specific elicitors that appeared to be the products of avirulence (*Avr*) genes (de Wit, 1992). Later, it was shown that *Avr* genes encoding effectors comprise dual functions: their primary function is to suppress plant defence responses elicited by non-specific elicitors/MAMPs, while the plant's *R* gene products recognize them as Avr proteins (Cook *et al.*, 2015; Joosten and de Wit, 1999; de Wit, 2016). Recently, we have screened 41 *C. fulvum* effector candidates on several wild and domesticated tomato accessions carrying the *Cf-1*, *Cf-3*, *Cf-6*, *Cf-9B*, *Cf-11* or *Cf-Ecp3* resistant traits to determine the matching Avr1, Avr3, Avr6, Avr9B, Avr11 and Ecp3 avirulence proteins (Mesarich *et al.*, 2018). This resulted in identification of nine novel tomato cultivar-specific *Avr* genes in *C. fulvum* (Mesarich *et al.*, 2018).

Genome analysis of *C. fulvum* revealed that, although traditionally this fungus is considered to be a biotrophic plant pathogen, its glycosyl hydrolase (cell wall-degrading enzymes) repertoire is more similar to hemibiotrophic and necrotrophic plant pathogens (Hane *et al.*, 2018; de Wit *et al.*, 2012). This finding may also explain the presence of necrotic lesions on *C. fulvum*-infected tomato leaves at late stages of infection (Thomma *et al.*, 2005). With the availability of many sequenced fungal genomes, we are now able to more specifically address the role of fungal glycosyl hydrolases in plant pathogens and defence (de Wit *et al.*, 2012). For example, genome analysis of *C. fulvum* revealed the presence of α-tomatinase, a member of the glycosyl hydrolase family 10 (GH10), which contributes to virulence by hydrolysing the antifungal α-tomatine into the non-toxic tomatidine and lycotetraose (Ökmen *et al.*, 2013).

Proteome analysis of apoplastic fluids of tomato leaves infected by *C. fulvum* resulted in identification of several additional glycosyl hydrolases of which one induced plant cell death when expressed in tomato with the potato virus X (PVX) expression system. *In silico* analysis revealed that this protein has similarity to glucan-degrading enzymes found in other fungi. It is grouped in the GH17 family and was named CfGH17-1. Members of the GH17 family have been identified in bacteria, fungi and plants, and play key roles in different aspects of life ranging from developmental processes to host–pathogen interactions (Henrissat and Davies, 1997). Since the cell wall of filamentous fungi is typically composed of chitin, 1,3-β- and 1,6-β-glucan, mannan and glycoproteins, fungal glucanases may have roles in fungal cell wall remodelling and maintaining cell wall plasticity during morphogenesis and sporulation. Here we functionally characterized a GH17 family member of *C. fulvum* for its cell death-inducing activity (CDIA) on several tomato accessions. Results from transient expression assays performed for both the wild-type and active-site mutant of CfGH17-1 indicate that the enzyme releases a DAMP(s) from tomato cell walls that is recognized by a yet unknown receptor to subsequently induce cell death. Thus, during biotrophic growth of *C. fulvum* the expression of the *CfGH17-1* gene needs to be suppressed so that no DAMPs are released. We suggest that CfGH17-1 is an enzyme required at late stages of infection and during saprotrophic growth to release (oligo)saccharides from host cell walls to support its massive rate of propagation.

RESULTS

Conserved glycosyl hydrolase family 17 members in *C. fulvum* induce cell death in tomato

For successful colonization of plants, pathogens secrete effector proteins to compromise the host immune system. In a recent screen to identify novel avirulence genes from *C. fulvum* (Mesarich *et al.*, 2018), we identified a cell death-inducing protein (JGI protein ID: 188986) with high similarity to a fungal glucan-degrading enzyme, which belongs to glycosyl hydrolase family 17 (GH17) (Fig. 1A). The encoding gene was named *CfGH17-1*. Whole genome mining of the *C. fulvum* genome using the amino acid sequence of the CfGH17-1 as the query revealed the presence of eight CfGH17 homologues (named CfGH17-1 to CfGH17-8).

For better prediction of the CfGH17-1 function, a phylogenetic tree analysis was performed with all eight CfGH17 members and GH17 members from other organisms, including bacteria, fungi and plants. GH55 members were used as an out-group for

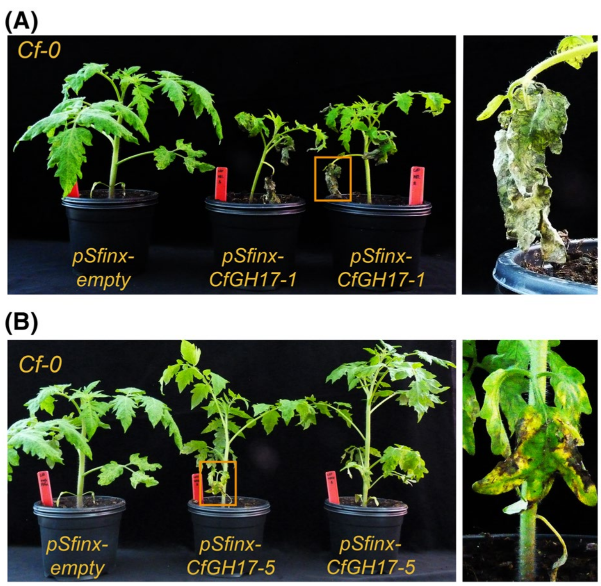

Fig. 1 Heterologous expression of *Cladosporium fulvum* glycosyl hydrolase family 17 (*CfGH17*) members in tomato. *CfGH17-1* to *CfGH17-5* genes were cloned into the PVX-based vector pSfinx and expressed in different tomato lines using the *A. tumefaciens* transient expression assay (A, B and Fig. S1). The empty vector (pSfinx-empty) was used as negative control. Heterologous expression of *CfGH17-1* and *CfGH17-5* resulted in necrosis in *Cf-0* tomato lines. Pictures were taken 21 days after infiltration. All pictures show representative plants of at least three biological replicates.

construction of this phylogenetic tree. Phylogenetic analysis of the eight CfGH17 family members revealed that they belong to two clades, one including CfGH17-1 to GH17-5 and the other including CfGH17-6 to CfGH17-8 (Fig. 2). CfGH17-6 to CfGH17-8 are considered not to be paralogues of CfGH17-1 to CfGH17-5 clade and were excluded from further investigation. In the cluster including CfGH17-1, so far only one GH17 protein from *Saccharomyces cerevisiae* has been characterized as an exo-1,3-β-glucanase (Klebl and Tanner, 1989). Furthermore, one GH17 from *Mycosphaerella fijiensis*, the causal agent of the black Sigatoka disease of banana, and one from *Dothistroma septosporum*, the causal agent of pine tree red band needle blight disease, clustered together with CfGH17-1 (Fig. 2). All CfGH17 proteins except CfGH17-6 are predicted to enter the secretory pathway based on the presence of a signal peptide (Table S1).

CfGH17-1 is up-regulated during late stages of infection of tomato

Quantitative RT-PCR (qRT-PCR) was performed to show the expression pattern of the *CfGH17* members at 4, 8, 12 and 15 days post-inoculation (dpi) of tomato with *C. fulvum*. The expression of *CfGH17-1* (12 dpi onwards) and *CfGH17-3* (8 dpi onwards) were significantly up-regulated as compared to growth on potato dextrose broth (PDB) (Fig. 3). However, differential expression was not observed for *CfGH17-2* and *CfGH17-5* genes

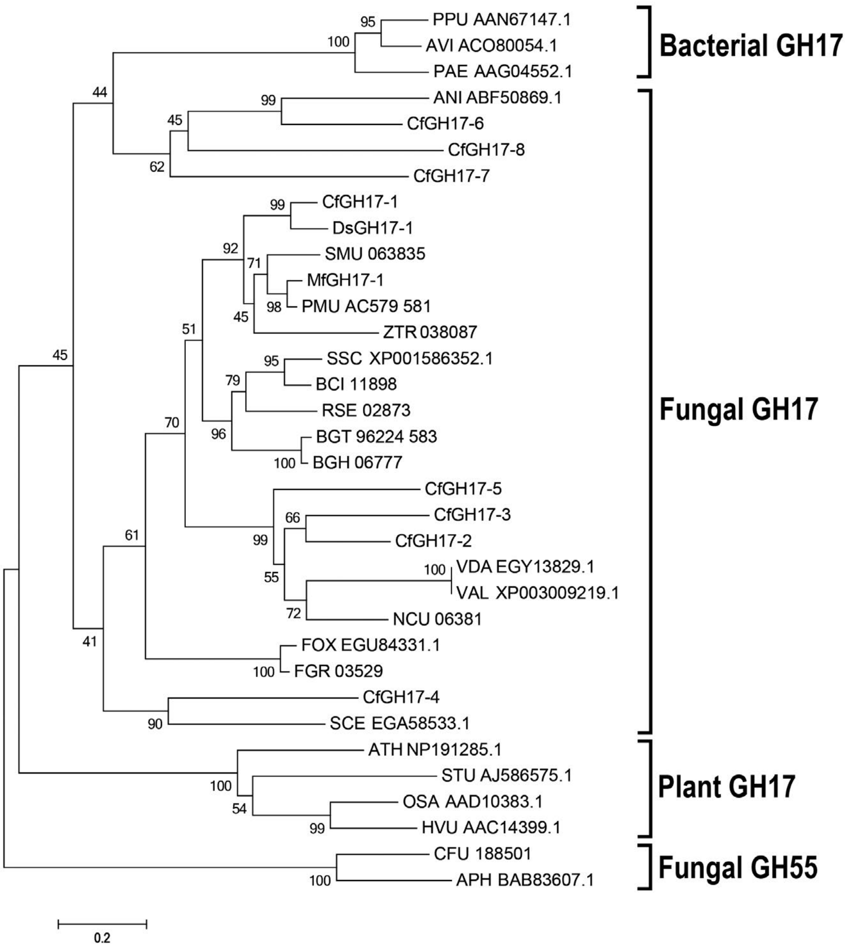

Fig. 2 Phylogenetic tree analysis of glycosyl hydrolase family 17 (GH17) members. A phylogenetic tree analysis was performed by using an alignment of the full-length amino acid sequence of GH17 homologues in different bacteria, fungi and plants by using ClustalOmega. The tree was built with the minimum evolution tree function using default settings and 1000 bootstrap replications in MEGA 7. The scale bar represents the number of substitutions per site. Numbers next to the species name correspond to the protein identifier or accession number at NCBI. Characterized glycosyl hydrolase family 55 (GH55) was used as an out-group. Alphabetical abbreviations: ANI, *Aspergillus nidulans*; APH, *Aspergillus phoenicis*; ATH, *Arabidopsis thaliana*; AVI, *Acetobacter vinlandii*; BCI, *Botrytis cinerea;* BGH, *Blumeria graminis* f. sp. *hordei;* BGT, *Blumeria graminis* f. sp. *tritici;* CFU/Cf, *Cladosporium fulvum*; Ds, *Dothistroma septosporum*; FGR, *Fusarium graminearum;* FOX, *Fusarium oxysporum*; HVU, *Hordeum vulgare*; Mf, *Mycosphaerella fijiensis*; NCU, *Neurospora crassa;* ZTR, *Zymoseptoria tritici*; OSA, *Oryza sativum*; PAE, *Pseudomonas auruginosa*; PMU, *Pseudocercospora musae;* PPU, *Pseudomonas putida*; RSE, *Rhynchosporium secalis;* SCE, *Saccharomyces cerevisiae*; Sm, *Septoria musiva*; SSC, *Sclerotinia sclerotiorum*; STU, *Solanum tuberosum*; VAL, *Verticillium albo-atrum*; VDA, *Verticillium dahliae.*

during colonization of tomato. While the highest *in vitro* expression was observed for *CfGH17-4* and *CfGH17*-5, the highest *in planta* expression was observed for *CfGH17-1* among the five *CfGH17* genes (Fig. 3). The *CfGH17-1* gene is suppressed at the biotrophic phase (3–8 dpi) and up-regulated only at the later necrotrophic phase (12–15 dpi).

CfGH17-1 and CfGH17-5 specifically induce cell death in *Cf-0*, *Cf-1* and *Cf-5* but not in *Cf-Ecp3* tomato and tobacco plants

To investigate whether the five selected CfGH17 proteins induce cell death in tomato, they were all heterologously expressed in different near-isogenic tomato lines (*Cf-0*, *Cf-1*, *Cf-5* and *Cf-Ecp3*) by using the PVX expression system (pSfinx) via the *Agrobacterium*-mediated transient transformation assay (ATTA) (Stergiopoulos *et al*., 2010). Empty pSfinx vector was used as a negative control. While heterologous expression of pSfinx-*CfGH17-1* and pSfinx-*CfGH17-5* in *Cf-0*, *Cf-1* and *Cf-5* tomato lines caused cell death in leaves at 3 weeks after infiltration, they did not in *Cf-Ecp3* plants (only mosaic symptoms caused by PVX were observed) (Figs 4 and S1A). The cell death-inducing activity (CDIA) of CfGH17-5 was weaker compared to CfGH17-1 and the symptoms observed for the pSfinx-*CfGH17-5*-infiltrated plants were restricted to the lower composite leaves only (Fig. 1B). None of the other three CfGH17 members induced cell death in any of the tomato lines (Fig. S1A). To analyse whether the CDIA of CfGH17-1 and CfGH17-5 is restricted to tomato, all *CfGH17* members were cloned into a binary vector pK2GW7 for heterologous expression in *Nicotiana tabacum* and *N. benthamiana* by using ATTA. The *Phytophthora infestans* necrosis and ethylene-inducing-like protein (PiNLP1) was used as a positive control for induction of cell death (Fellbrich *et al*., 2002). While the *PiNLP1* construct induced cell death in both *N. tabacum* and *N. benthamiana* at 7 dpi, none of the tested CfGH17 members induced cell death (Fig. S1B,C).

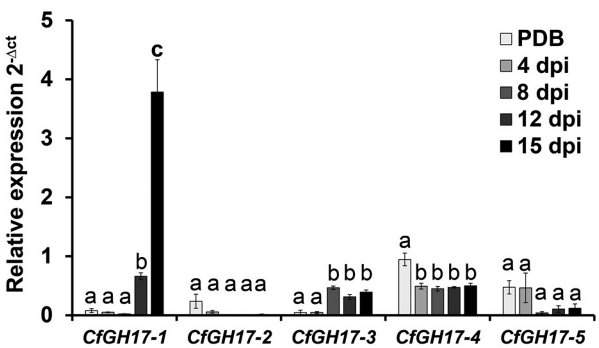

Fig. 3 Relative expression profile of *Cladosporium fulvum* glycosyl hydrolase family 17 (*CfGH17)* members. qRT-PCR was performed to assess the expression profile of *CfGH17-1* to *5* during *C. fulvum* infection on tomato at 4, 8, 12 and 15 days post-inoculation (dpi) and in potato dextrose broth (PDB). Expression levels were normalized using the *C. fulvum actin* gene. Error bars indicate standard errors of three biological replicates. Different letters represent significant differences within the time series of each *CfGH17* gene ($P < 0.05$; Fisher's LSD test).

Enzymatic activity of CfGH17 is required for induction of cell death

To determine whether the enzymatic activity of CfGH17-1 and CfGH17-5 is required for their CDIA, the active sites of CfGH17-1 and CfGH17-5 were mutated. The predicted proton donor and the nucleophile sites for CfGH17-1 [$Glu^{122}Asp^{123}$ (M1) and $Glu^{222}Asp^{223}$ (M2)] and CfGH17-5 [$Glu^{128}Asp^{129}$ (M1) and $Glu^{239}Thr^{240}$ (M2)] were replaced by Ala (A). Two single-site (M1 or M2) and one double-site mutant (DM) for CfGH17-1 and CfGH17-5 were tested on tomato accessions to monitor their CDIA by using the PVX expression system. While the wild-type pSfinx-*GH17-1* and pSfinx-*GH17-5* constructs showed CDIA in tomato, none of the active-site mutant constructs induced cell death (Fig. S2A,B). Moreover, replacement of only the nucleophile (Glu^{222}) site of CfGH17-1 by Gln (Q) also resulted in loss of CDIA (Fig. 5A). Mosaic symptoms caused by PVX were visible in all infiltrated tomato plants, confirming effective transient expression. In addition to the heterologous PVX experiments, both wild-type and active-site mutant CfGH17-1 proteins were produced in the *Pichia pastoris* expression system to determine their CDIA in

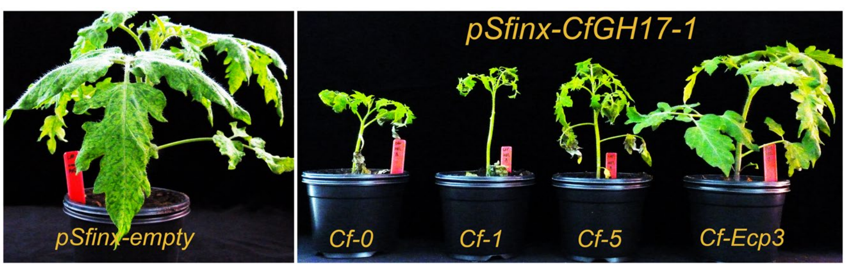

Fig. 4 Heterologous expression of *Cladosporium fulvum CfGH17-1* in tomato. *CfGH17-1* was cloned into the PVX-based vector pSfinx and expressed in different tomato lines, including *Cf-0*, *Cf-1*, *Cf-5* and *Cf-Ecp3*, by using the *Agrobacterium tumefaciens* transient expression assay. The empty pSfinx vector was used as negative control. Heterologous expression of *CfGH17-1* resulted in cell death in all tomato lines, except *Cf-Ecp3* plants. Pictures were taken 21 days after infiltration. All pictures show representative plants of at least three biological replicates.

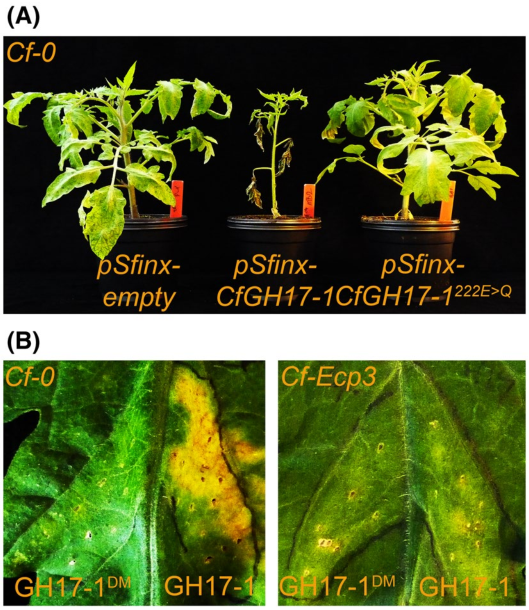

Fig. 5 Active site mutation of CfGH17-1. Mutations in the predicted active sites of CfGH17-1 were achieved by substitution of the glutamic acid residues serving as proton donor and nucleophile with glutamate. (A) Both wild-type and active-site mutant of *CfGH17-1* (substitution at the proton donor 222E > Q) were heterologously expressed in tomato plants. While wild-type CfGH17-1 induced cell death, the active-site mutated version resulted in loss of cell death-inducing activity. Empty pSfinx was used as a negative control. All pictures show representative plants of at least three biological replicates. The pictures were taken 21 days post-infiltration (dpi). (B) Representative tomato *Cf-0* and *Cf-Ecp3* leaves, infiltrated with 1 mg/mL purified recombinant wild-type and active-site double mutant CfGH17-1^{DM}. Pictures were taken at 7 dpi and the experiment was repeated four times with consistent results.

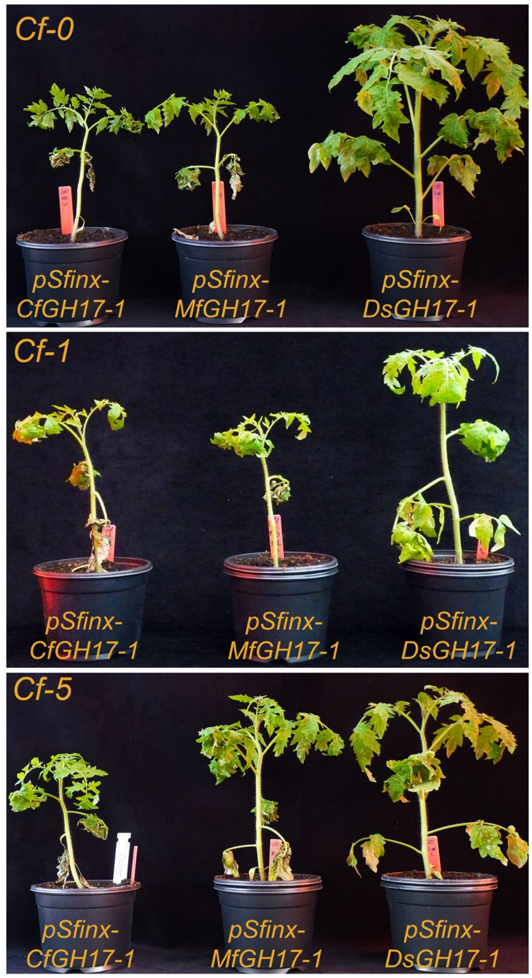

Fig. 6 Heterologous expression of *GH17*-1 orthologues in tomato. The *CfGH17-1* orthologues from *Mycosphaerella fijiensis* (*MfGH17-1*) and *Dothistroma septosporum* (*DsGH17-1*) were cloned into the PVX-based vector pSfinx and heterologously expressed in different tomato lines, including *Cf-0*, *Cf-1* and *Cf-5*, by using the *Agrobacterium tumefaciens*-mediated transient expression assay. Both *MfGH17-1* and *DsGH17-1* induced necrosis in all tested tomato lines. All pictures show representative plants of at least three biological replicates. The pictures were taken 21 days after infiltration.

tomato leaves (Fig. S3A). Infiltration of affinity-purified wild-type CfGH17-1 protein showed weak CDIA on *Cf-0* tomato leaves, but not in *Cf-Ecp3* leaves; however, the active-site mutant protein did not show any CDIA, indicating that an active CfGH17-1 enzyme is required for CDIA on tomato (Fig. 5B). No necrotic or chlorotic symptoms were observed in tomato leaves infiltrated with phosphate-buffered saline (PBS) (Fig. S3B).

GH17-1 orthologues from other members of Dothideomycetes fungi are functional homologues

Our phylogenetic analysis revealed that *M. fijiensis* and *D. septosporum*, close relatives of *C. fulvum*, also contain CfGH17-1 orthologues with high amino acid sequence similarity (Fig. 2). In this study, they were named MfGH17-1 and DsGH17-1, respectively (Table S1). To test whether they also showed CDIA on tomato, both *MfGH17-1* and *DsGH17-1* were cloned into the PVX-expression system and expressed in the same tomato lines as described for *CfGH17-1*. Heterologous expression of pSfinx-*MfGH17-1* and pSfinx-*DsGH17-1* resulted in CDIA on *Cf-0*, *Cf-1* and *Cf-5* tomato lines (Fig. 6). While pSfinx-*MfGH17-1* showed a comparable CDIA when compared to pSfinx-*CfGH17-1*, pSfinx-*DsGH17* consistently showed a weaker CDIA after 3 weeks (Fig. 6).

CfGH17-1 hydrolyses laminarin, a linear 1,3-β-glucan with 1,6-β branches

Characterized GH17 members act as glucan endo- or exo-1,3-β-glucanase, licheninase or 1,3-β-glucanosyltransglycosylase (the carbohydrate-active enzymes database; http://www.cazy.org). To assign an enzymatic function, a series of biochemical assays was performed with purified recombinant CfGH17-1 protein and its potential substrate, laminarin (1,3-β-glucan). In order to show that CfGH17-1 can hydrolyse 1,3-β-glucans, laminarin

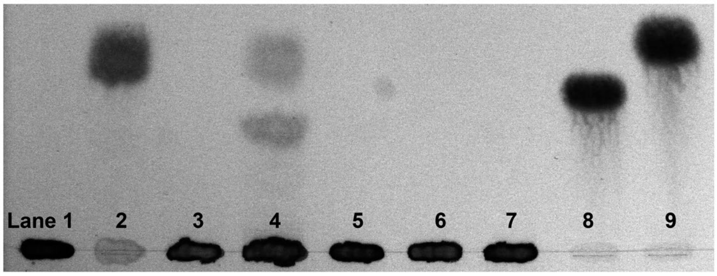

Fig. 7 Thin layer chromatography (TLC) assay. TLC silica gel 60 plate was used to show the activity of CfGH17-1 on laminarin. Laminarin was incubated with purified CfGH17-1 protein overnight at 30 °C and at pH 4.7. A mixture of isopropanol:*n*-buthanol:water (12:3:4 v/v/v) was used as mobile phase. Laminarin and its hydrolysis products were visualized by spraying the TLC plate with 5% H_2SO_4 (v/v, in ethanol) and subsequent drying at 105 °C for approximately 20 min. Lane 1, laminarin; lane 2, laminarin + endo-1,3-β-glucanase from *Helix pomatia* (Sigma-Aldrich); lane 3, laminarin + heat-inactivated endo-1,3-β-glucanase from *H. pomatia*; lane 4, laminarin + CfGH17-1; lane 5, laminarin + heat-inactivated CfGH17-1; lane 6, laminarin + active-site mutant CfGH17-1; lane 7, laminarin + heat-inactivated active site mutant CfGH17-1; lane 8, sucrose; lane 9, glucose.

was incubated overnight at 30 °C in the presence or absence of the CfGH17-1 protein (1 mg/mL). Heat-inactivated CfGH17-1 and active-site mutants of CfGH17-1^{DM} proteins were used as negative controls, while commercial endo-1,3-β-glucanase from *Helix pomatia* was used as a positive control. Glucose and sucrose were used as size markers. Thin layer chromatography (TLC) was performed to visualize the hydrolysis product of laminarin (Fig. 7). TLC analysis revealed that CfGH17-1 hydrolyses laminarin (Fig. 7, lane 4). While incubation of CfGH17-1 with laminarin showed hydrolysis products with a size similar to glucose and sucrose (Fig. 7, lanes 4, 8 and 9), no hydrolysis products were detected after incubation with heat-inactivated enzyme or the active-site mutant of CfGH17-1 (Fig. 7, lanes 5–7). Only one hydrolysis product was observed for laminarin incubated with commercial endo-1,3-β-glucanase (Fig. 7, lane 2). No hydrolysis of laminarin was observed when the commercial endo-1,3-β-glucanase was heat-inactivated before incubation with laminarin, showing that there was no autodegradation of the substrate.

To test whether CfGH17-1 has licheninase activity, the recombinant protein was also incubated with lichenin. However, no hydrolysis products were observed after TLC analysis (data not shown).

Deletion and constitutive expression of *CfGH17-1* in *C. fulvum*

Gene expression assays showed that *CfGH17-1* is up-regulated in *C. fulvum* at late stages of infection (Fig. 3). To show a possible role of the CfGH17-1 in virulence, gene deletion mutants of *CfGH17-1* were created in *C. fulvum* by homologous recombination using *A. tumefaciens*-mediated fungal transformation (Fig. S4A). Two Δ*cfgh17-1* mutants were obtained out of 150 transformants. While PCR analysis showed absence of the deleted gene in the genome of the *C. fulvum* mutant, quantitative PCR analysis confirmed a single insertion event for each of the two transformants (Fig. S4B,C). The Δ*cfgh17-1* mutants did not show a phenotype different from the parental wild-type *C. fulvum* isolate when grown *in vitro* on potato dextrose agar (PDA) . The effect of constitutive expression of *CfGH17-1* on virulence was also analysed. To this end, *CfGH17-1* was expressed in *C. fulvum* under the control of the constitutive *ToxA* promoter. Three transformants were obtained and qRT-PCR analysis showed that expression of *CfGH17-1* in these constitutive expression mutants (OE-*CfGH17-1*) was significantly higher than in wild-type *C. fulvum* (Fig. S4D).

To determine the effects of *CfGH17-1* expression on virulence, wild-type, Δ*cfgh17-1* mutants, ectopic insertion mutant and OE-*CfGH17-1* mutants of *C. fulvum* were inoculated onto susceptible tomato plants and symptom developments were monitored for 2 weeks. Although there were no significant differences in symptom development of wild-type, Δ*cfgh17-1* and ectopic insertion mutants, fewer disease symptoms were observed for OE-*CfGH17-1* mutants compared to wild-type (Fig. 8). These results were consistently observed for the all three OE-*CfGH17-1* mutants (results for only one OE-*CfGH17-1* mutant are shown in Fig. 8).

DISCUSSION

In the search for Avr proteins, *C. fulvum* genes encoding apoplastic effector candidates were heterologously expressed in wild-type and several domesticated tomato accessions, carrying *Cf-1*, *Cf-3*, *Cf-5* and *Cf-Ecp3* resistance traits (Mesarich *et al.*, 2018). By screening with the PVX-based expression system, we identified nine novel proteins that caused cell death in an accession-specific manner, including *Cf-1* and *Cf-3* (Mesarich *et al.*, 2018). In addition to nine novel avirulence proteins, we identified some that caused non-specific cell death reminiscent of non-specific elicitors or MAMPs.

Heterologous expression of *CfGH17-1* and *CfGH17-5* induced cell death in *Cf-0*, *Cf-1* and *Cf-5*, but not in *Cf-Ecp3* tomato cultivars, indicating that they act as non-specific elicitors. Although heterologous expression of *CfGH17* shows non-specific CDIA on tomato, none of the CfGH17 family members induced cell death in

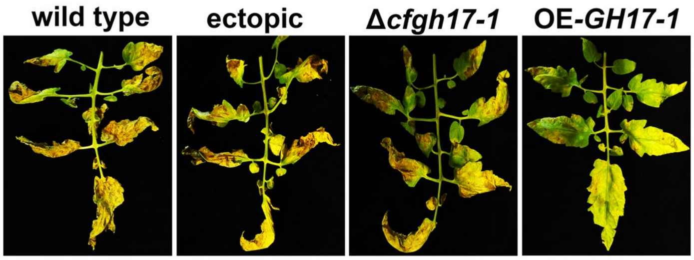

Fig. 8 Gene deletion and constitutive expression of *CfGH17-1* in *Cladosporium fulvum*. Five-week-old tomato plants (*Cf-0*) were inoculated with conidial suspensions of wild-type *C. fulvum* race 0WU, one ectopic insertion mutant, one Δ*cfgh17-1* mutant and one *CfGH17-1* constitutive expression mutant (OE-*CfGH17-1*). Pictures were taken at 21 days post-inoculation. Pictures are from representative leaves of two biological replicates.

N. tabacum and *N. benthamiana*. Differences in cell wall composition or absence of a receptor that mediates CDIA in tobacco plants may explain these results. Furthermore, active-site mutant proteins lacking enzymatic activity did not induce cell death in any tomato lines, suggesting that CfGH17-1 and CfGH17-5 proteins did not act as a MAMP. Thus, we suggest that CfGH17-1 and CfGH17-5 do not directly induce cell death, rather a yet unknown plant cell wall component (DAMP), released by the enzyme, is recognized by an unknown receptor, leading to induction of cell death, while *Cf-Ecp3* plants lack such a receptor and are blind to this DAMP.

CfGH17-1 showed high similarity to 1,3-β-glucanase. The CfGH17-1 released a monosaccharide and some oligosaccharides when incubated with laminarin, a 1,3-β-glucan, indicating it indeed has 1,3-β-glucanase activity. GH17 family members are involved in the modification of cell wall glucan, which is essential for the growth and development of both fungal and plant cells (Aspeborg *et al.*, 2005; Beauvais and Latgé, 2018). The expression of *CfGH17-1* is almost completely silent during *in vitro* growth, but significantly up-regulated during infection at the switch from the biotrophic growth to the necrotrophic growth stage (12 dpi). Similarly, the *CfGH17-1* orthologue from *D. septosporum* (hemibiotrophic pine tree pathogen) is also up-regulated 2.6-fold during infection when necrotic lesions occur (8 weeks post-infection) (Bradshaw *et al.*, 2016). Furthermore, neither deletion nor constitutive expression of the *CfGH17-1* gene in *C. fulvum* affected *in vitro* growth or sporulation of the fungus. Although one cannot fully rule out the possibility that CfGH17-1 is also involved in *C. fulvum* cell wall remodelling at the end of the infection cycle, when an explosive increase of fungal biomass occurs, our results suggest that *C. fulvum* secretes CfGH17-1 to target host cell walls to remove sugar molecules to support fungal growth and reproduction.

A conserved GH17 family member in Dothideomycetes induces cell death in tomato

Previously, it has been shown that *C. fulvum* Avr4 homologues from *M. fijiensis* and *D. septosporum* can be also recognized by *Cf-4* tomato lines (Stergiopoulos *et al.*, 2010; de Wit *et al.*, 2012). Moreover, GrVap1 from *Globodera rostochiensis* (nematode), which is a functional homologue of *C. fulvum* Avr2 (although they do not share any amino acid homology), induces a hypersensitive response (HR) upon inhibiting tomato Rcr3 in the presence of the *Cf-2* resistance gene (Lozano-Torres *et al.*, 2012). Consistently, heterologous expression of a CfGH17-1 homologue from *M. fijiensis* (banana pathogen) and *D. septosporum* (pine tree pathogen) also induces non-specific CDIA on different tomato cultivars. As CfGH17-1, MfGH17-1 and DsGH17-1 do not show CDIA on the *Cf-Ecp3* tomato line, their CDIA is likely mediated by a receptor that is lacking in *Cf-Ecp3* plants. These results indicate functional conservation of this protein in these two additional hemibiotrophic phytopathogens. Although *C. fulvum* is considered to be a biotrophic tomato pathogen, the presence of high numbers of plant cell wall-degrading enzymes similar to hemibiotrophic and necrotrophic phytopathogens is an indication of a hemibiotrophic lifestyle under natural conditions (de Wit *et al.*, 2012), where a short biotrophic phase is quickly followed by a necrotrophic phase when infected leaves become completely necrotic (Thomma *et al.*, 2005). However, whether CfGH17-1 contributes to formation of those necrotic spots on tomato leaves needs further investigation. Deletion of *CfGH17-1* affected neither *in vitro* nor *in planta* growth (virulence) of *C. fulvum* mutants compared to the wild-type strain. As *C. fulvum* has seven additional genes encoding GH17 members, they might functionally compensate for the deleted gene.

CONCLUSION

CfGH17-1 likely functions as a glucanase that supports fungal growth during late stages of infection and during the necrotrophic phase when the host no longer responds to released DAMP(s). Expression of *CfGH17-1* at early stages of infection is suppressed, as released DAMP(s) would lead to induction of host defence responses and partial restriction of fungal growth. This conclusion is supported by the finding that constitutive expression of *CfGH17-1* in OE-*CfGH17-1* mutants results in reduced fungal growth on tomato. DAMPs induce cell death by a yet to be identified PRR that is present in most tomato cultivars but is lacking in *Cf-Ecp3* and tobacco.

EXPERIMENTAL PROCEDURES

Bacterial, fungal and plant material

Cladosporium fulvum race 0WU (CBS131901), *M. fijiensis* and *D. septosporum* (CBS128783) were grown on half-strength PDA at 20 °C for 2–3 weeks for conidia production. For fungal liquid cultures, 10^6 conidia/mL were incubated in flasks containing 75 mL PDB. The cultures were incubated for 1 week at 22 °C with 200 rpm shaking before harvesting fungal mycelium by filtration through a mira-cloth. *Pichia pastoris* strain GS115 was used for protein production at 30 °C. *Agrobacterium tumefaciens* strain GV3101 was used for transient gene expression in tobacco and tomato.

Heinz tomato cultivars, lacking any *Cf* resistance gene, were used for *C. fulvum* virulence assay, and near-isogenic Moneymaker line carrying *Cf-0*, *Cf-1*, *Cf-5* or *Cf-Ecp3* genes for resistance were used for heterologous gene expression experiments. All plants were grown in the greenhouse at 70% relative humidity, at 23–25 °C during daytime and at 19–21 °C at night, with a light/dark regime of 16/8 h and 100 W/m^2 supplemental light when light intensity was less than 150 W/m^2. *Nicotiana tabacum* and *N. benthamiana* plants were grown in a growth chamber at 20 °C and 70% relative humidity with a photoperiod of 12 h.

Phylogenetic tree analysis of GH17 family members

The amino acid sequence of CfGH17-1 (jgi ID 184408) was used in a BLAST search against the *C. fulvum* genome database (http://www.jgi.doe.gov/) to identify CfGH17-1 paralogues including *CfGH17-2* (jgi ID 192695), *CfGH17-3* (jgi ID 192173), *CfGH17-4* (jgi ID 190924) and *CfGH17-5* (jgi ID 197171). CfGH17-1 homologues in other Dothideomycetes, such as DsGH17-1 (jgi ID 71501) from *Dothistroma septosporum* and MfGH17-1 (jgi ID 6274) from *Mycosphaerella fijiensis*, were identified using the BLASTP algorithm on the Joint Genome Institute (http://www.jgi.doe.gov/). CfGH17-1 homologues from other organisms were found by using BLASTP on the NCBI database. To construct a phylogenetic tree, the amino acid sequences of all selected CfGH17-1 homologues were aligned by using Clustal Omega (Sievers *et al.*, 2011) and edited in GeneDoc software (Nicholas *et al.*, 1997). Subsequently, a consensus phylogenetic tree was constructed by using the minimum evolution algorithm with default settings and 1000 bootstrap replications in MEGA 7 software (Tamura *et al.*, 2011). The glycosyl hydrolase family 55 (GH55) proteins were used as an out-group.

Nucleic acid methods

Cladosporium fulvum genomic DNA isolation was performed as described in Ökmen *et al.* (2013). Briefly, *C. fulvum* mycelia obtained from PDB cultures, or *C. fulvum*-infected tomato leaves were ground in liquid nitrogen using a mortar and pestle. Subsequently, the DNeasy plant mini kit (Qiagen Benelux BV, Venlo, Netherlands) was used to isolate genomic DNA according to the manufacturer's instructions. Total RNA isolation from fungal mycelia or *C. fulvum*-infected tomato leaves at 4, 8, 12 and 15 dpi was performed using a hybrid method described by van Esse *et al.* (2008). cDNA was synthesized from 5 μg isolated total RNA using a SuperScript III first-strand kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Quantitative PCR and qRT-PCR was performed using SensiMix SYBR Hi-ROX mastermix (Bioline, London, UK) according to the manufacturer's instructions. The thermal profile started with 95 °C initial denaturation for 10 min followed by 40 cycles of 15 s denaturation at 95 °C and annealing/extension for 45 s at 60 °C. Primer efficiency and specificity were tested with a dilution series of genomic *C. fulvum* DNA. The *C. fulvum actin* gene was used as a reference for expression. Results were analysed using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). Three biological replicates were used for relative gene expression analysis.

All PCRs were performed using GoTaq DNA polymerase (Promega, Madison, WI, USA) according to the manufacturer's instructions (100 ng genomic DNA or cDNA as template). All primers used in this study are listed in Table S2.

Cloning of *GH17* family members into heterologous expression systems

GH17 family members from *C. fulvum* (*CfGH17-1*, jgi ID 184408; *CfGH17-2*, jgi ID 192695; *CfGH17-3*, jgi ID 192173; *CfGH17-4*, jgi ID 190924; *CfGH17-5*, jgi ID 197171), *D. septosporum* (*DsGH17-1*; jgi ID 71501) and *M. fijiensis* (*MfGH17-1*; jgi ID 6274) were amplified from *C. fulvum*, *D. septosporum* and *M. fijiensis* cDNA using specific primer sets (Table S2). In cloning, the native fungal signal peptide sequences were replaced with *N. tabacum* signal peptide *PR1A* (NCBI accession BAA14220).

The amplified *PR1A-GH17* fragments were inserted into p207 donor vector (Invitrogen) using the Gateway cloning technique using Gateway BP Clonase II (Invitrogen) according to the manufacturer's instructions. After sequence confirmation of each construct via sequencing at Macrogen Inc. (Amsterdam, Netherlands), all genes were transferred to pK2GW7 destination vectors using Gateway LR Clonase II enzyme mix (Invitrogen) according the manufacturer's instructions. The binary PVX-based vector pSfinx (Takken *et al.*, 2000) was used for heterologous expression of the *GH17* members in tomato plants. Correct inserts were cut from the p207 vector using *Asc*I and *Not*I restriction enzymes (Promega) according the manufacturer's instructions. Ligation of the isolated *PR1A-GH17* genes and pSfinx digested with *Asc*I and *Not*I was performed using T4 DNA ligase (Promega) also according to the manufacturer's instructions. Active-site mutant versions of *CfGH17-1* and *CfGH17-5* were obtained by designing primers encoding for the amino acid substitutions $Glu^{122}Asp^{123} > Ala^{122}Ala^{123}$ (proton donor) (M1) and/or $Glu^{222}Asp^{223} > Ala^{222}Asp^{223}$ (nucleophile) (M2) in CfGH17-1; $Glu^{128}Asp^{129} > Ala^{128}Ala^{129}$ (proton donor) (M1) and/or $Glu^{239}Asp^{123} > Ala^{239}Ala^{240}$ (nucleophile) (M2) in CfGH17-5 (Table S2). All mutated versions were cloned in the pSfinx vector.

For transformation, chemically competent *Escherichia coli* DH5α cells were used according to a standard heat shock protocol (Sambrook and Russell, 2001). Transformants were selected on LB agar plates supplemented with appropriate antibiotics. Plasmids were isolated by using the QIAprep Spin MiniPrep Kit (Qiagen) according to the manufacturer's instructions. All constructed plasmids, primer pairs and sites that were used for cloning procedures are indicated in Table S2. The obtained binary pK2GW7- and pSfinx-*GH17* constructs were then introduced into *A. tumefaciens* GV3101 for plant expression via electroporation using 2.4 V and 400 Ω as settings. *Agrobacterium tumefaciens* cells were grown on LB agar plates supplemented with 50 µg/mL kanamycin and 25 µg/mL rifampicin for 2 days at 28 °C.

Heterologous expression of *GH17* members in tomato and tobacco plants

Wild-type and active-site mutated *GH17* members were heterologously expressed in tomato, *N. benthamiana* and *N. tabacum* plants via ATTA using a classical protocol as reported (Van der Hoorn *et al.*, 2000). *Agrobacterium* strains containing the pSfinx-*GH17* constructs were infiltrated with an $OD_{600} = 1.0$ into cotyledons of 10-day-old tomato seedlings (three plants per experiment). Different near-isogenic Moneymaker lines (*Cf-0*, *Cf-1*, *Cf-5* and *Cf-Ecp3*) were used for this experiment. *Agrobacterium* clones containing the pK2GW7-*CfGH17* constructs were also infiltrated with an $OD_{600} = 1.0$ into *N. benthamiana* and *N. tabacum* leaves (approximately 5–6 weeks old). As a positive control, a pK2GW7 construct containing the *P. infestans* necrosis and ethylene-inducing-like protein *(PiNLP1)* gene was used. Tomato and *N. tabacum* plants were grown in the greenhouse for 3–4 weeks and analysed 5 days after agroinfiltration. Cell death induction was monitored and a representative plant or leaf from at least three biological replicates was photographed with a digital camera.

Pichia pastoris-mediated CfGH17-1 protein production

Cloning and protein production of CfGH17-1 protein and an active site mutant (double site mutant; DM) were performed according to a protocol described by Kombrink (2012) based on the *Pichia* Expression Kit Version F (Invitrogen). All plasmids and primer pairs used for cloning procedures are indicated in Table S2.

The supernatant obtained from the fermenter was concentrated to a volume of 1/10 of the starting volume using a Vivaflow 200 protein concentrator (Fisher Scientific, Hampton, NH, USA) with an exclusion size of 10 kDa. Subsequently, the proteins were purified by using Ni-NTA Superflow slurry (Qiagen) according to the manufacturer's instructions. Aliquots containing more than 4 mg protein mL^{-1} were pooled and dialysed against 50 mM PBS (pH 6). For dialysis a Spectra/Por molecular porous membrane (Spectrum Laboratories, Rancho Dominguez, CA, USA) with a size exclusion of >12–14 kDa was used according to the manufacturer's instructions.

Protein activity test *in vitro* and *in planta*

CDIA of CfGH17-1 and the active-site mutant protein was determined by infiltration of the protein to 3–4-week-old Moneymaker *Cf-0* leaves using a syringe without a needle. A dilution series was prepared in water (2, 1, 0.4, 0.2, 0.02 mg/mL). As negative control 50 mM PBS was used. CDIA was monitored after 7 days and a representative leaf from at least three biological replicates was photographed.

1,3-β-glucanase activity was tested using the method described by Morohashi and Matsushima (2000). In this assay, laminarin from *Laminaria digitata* (Sigma-Aldrich) was used as a substrate. The reaction mixture contained 3 mg/mL laminarin dissolved in 50 mM PBS (pH 6) and 1 mg/mL purified CfGH17-1 protein stored in 50 mM PBS in a total volume of 100 µL. As a positive control, 1 mg/mL endo-1,3-β-glucanase from *H. pomatia* (Sigma-Aldrich, St. Louis, MO, USA) in PBS was used. As negative control, the protein solutions were inactivated by heating at 95 °C for 15 min and cooled on ice for 2 min before adding to the reaction mixture. Solutions containing only laminarin, glucose or sucrose were used as markers. The pH in the final reaction mixture was set to 4.7 by adding 20 µL citric acid buffer (10 mM). The reactions were incubated overnight at 60, 50, 37, 30, 25 and 20 °C, respectively.

The released products from the reactions were analysed by TLC using a TLC silica gel 60 plate (Merck; 20 × 20 cm). The TLC plates were loaded with 50 µL of the reaction mixtures. A mixture of isopropanol:*n*-buthanol:water (12:3:4 v/v/v) was used as the mobile phase. The TLC plate was developed for approximately 7 h. Laminarin and its hydrolysis products were visualized on the TLC plate after spraying with 5% H_2SO_4 (v/v, in ethanol) and subsequent drying at 105 °C for approximately 20 min.

The 1,3-1,4-β-glucanase activity was also analysed by TLC with lichenin from *Cetraria islandica* (Sigma-Aldrich) as a substrate. Lichenin was dissolved overnight in 50 mM PBS and shaking at 200 rpm. The reaction mixture was set up as for the laminarin assay with the same concentrations and pH. However, the mobile phase used in the TLC experiment consisted of a mixture of chloroform:acetic acid:water (6:7:1 v/v/v).

Gene knockouts of *C. fulvum GH17-1* and *-5*

Gene replacements constructs for *CfGH17-1* and *CfGH17-5* were created using the MultiSite Gateway Three-Fragment Vector Construction Kit (Invitrogen) according to manufacturer's instructions. The upstream (US) region of *CfGH17-1* (1.1 kb) and *CfGH17-5* (0.9 kb), and the downstream (DS) region of *CfGH17-1* (1.0 kb) and *CfGH17-5* (2.0 kb) were amplified from *C. fulvum* genomic DNA using specific primer pairs (Table S2) with overhangs homologous to the *Att*B4 and *Att*B1r, and *Att*B2r and *Att*B3 recombination sites, respectively. Purified US and DS amplicons were cloned in the vectors pDONRP4-P1R and pDONRP2R-P3, respectively, by using Gateway BP Clonase II (Invitrogen) as described above. Furthermore, a pDONR221 entry vector containing a hygromycin resistance (*HYG*) and green fluorescent protein (*GFP*) cassette was used as a replacement construct. For assembly of the final replacement vector, all three entry clones were combined into destination vector pDESTR4-R3 (Invitrogen) using Gateway BP Clonase II as described above. Subsequently, the gene replacement constructs were transferred into *A. tumefaciens* strain AGL1 by electroporation.

Cladosporium fulvum transformation was performed according to a protocol described by Ökmen *et al.* (2013) (Zwiers and De Waard, 2001). To confirm gene replacement in the selected transformants, genomic DNA was isolated from mycelium grown in PDB medium. The absence of the genes of interest and the upstream and downstream insertion location of the gene replacement construct was tested in a standard PCR using the isolated genomic DNA. Furthermore, a single insertion event of the replacement constructs opposed to multiple insertion events was tested via quantitative PCR using the primers specific for *HYG* (Table S2). Genomic DNA was used for that quantitative PCR and *C. fulvum actin* was used as a reference gene. A single insertion was characterized by a Ct value ratio of *HYG*:*actin* genes of approximately 1:1.

Constitutive expression of *CfGH17* in *C. fulvum*

Cladosporium fulvum mutants constitutively expressing *CfGH17-1* and *CfGH17-5* were created (OE-*CfGH17-1* and OE-*CfGH17-5* mutants) via *Agrobacterium*-mediated transformation. In order to construct constitutive expression vectors, *CfGH17-1* and *CfGH17-5* were amplified from genomic DNA of *C. fulvum* using specific primer sets (Table S2). The inserts were ligated behind the constitutive *ToxA* promoter region of vector pFBT029 (kindly provided by Dr Bart Thomma). The resulting pFBT029-*CfGH17-1* and pFBT029-*CfGH17-5* constructs were transferred into *A. tumefaciens* strain AGL1 by electroporation. Transformation of *C. fulvum* race 0 conidia with the pFBT029-*CfGH17-1* and pFBT029-*CfGH17-5* constructs was performed in the same way as described above. To confirm constitutive expression of OE-*CfGH17-1* and OE-*CfGH17-5*, qRT-PCR was performed for the mutants, which were grown in PDB cultures.

Virulence assays

Five-week-old tomato Heinz plants were inoculated with conidial suspensions of wild-type *C. fulvum*, two Δ*CfGH17-1* mutants, three Δ*CfGH17-5* mutants, ectopic mutant strains (two and three strains, respectively), and three OE-*CfGH17-1* and *OE-CfGH17-5* mutants as described by Ökmen *et al.* (2013). The abaxial side of tomato leaves were spray-inoculated with 1×10^6 conidia/mL of each strain. After spraying, plants were kept in a transparent plastic cabinet to maintain a relative humidity of 100% for optimum conidial germination for 2 days. Infected leaves were photographed at 21 dpi.

Online tools

The presence of a signal peptide in the candidate proteins was predicted using the SignalP 4.1 server (Petersen *et al.*, 2011). Amino acid sequence alignments were performed using Clustal Omega software (Sievers *et al.*, 2011). Primer pairs for quantitative PCR were designed using Primer3Plus software (Untergasser *et al.*, 2007).

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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:

Fig. S1 Heterologous expression of *Cladosporium fulvum CfGH17-1* paralogues in tomato and tobacco. Four *CfGH17-1* paralogues *(CfGH17-2* to *5)* were cloned into the PVX-based vector pSfinx and the binary pK2GW7 vector. (A) The pSfinx constructs containing *CfGH17-2* to *-5* were heterologously expressed in different tomato lines, including *Cf-0*, *Cf-1*, *Cf-5* and *Cf-Ecp3*, by using the *Agrobacterium tumefaciens*-mediated transient expression assay. Heterologous expression of only *CfGH17-5* resulted in necrosis in all tomato lines, except *Cf-Ecp3* plants. Pictures were taken 21 days after infiltration. All pictures show representative plants of at least three biological replicates. (B) and (C) The pK2GW7 constructs containing *CfGH17-1* to *-5* genes were heterologously expressed in *Nicotiana tabacum* (B) and *N. benthamiana* (C) leaves. The *Phytophthora infestans necrosis and ethylene-inducing-like protein* (*PiNLP1*) gene was used as a positive control for necrosis induction. The pictures were taken at 7 days post-infiltration. All pictures show representative leaves from at least three biological replicates.

Fig. S2 Heterologous expression of *Cladosporium fulvum CfGH17-1 and CfGH17-5* active-site mutants in tomato. Active-site mutant versions of *CfGH17-1* and *CfGH17-5* were obtained by substitutions of the glutamic acid residues serving as proton donor and nucleophile with alanine. *CfGH17-1* (A) and *CfGH17-5* (B) were heterologously expressed as wild-type (wt), with substitution at the proton donor (M1), with substitution at the nucleophile (M2), or with substitution at both active sites (DM). Heterologous expression of all active-site mutated versions resulted in loss of necrosis-inducing activity in all tested tomato lines, as shown for *Cf-1* plants. All pictures show representative plants of at least three biological replicates.

Fig. S3 Production and purification of recombinant CfGH17-1 and active-site mutant protein. Wild-type *CfGH17-1* and *CfGH17-1* mutated in the predicted active sites (*CfGH17-1* DM) were cloned into the *Pichia pastoris*-compatible pPic9 vector and recombinant His- and FLAG-tagged proteins were produced by growing transformed *P. pastoris* cells under inducing conditions. Culture medium was collected and proteins were purified using a Ni-NTA column. (A) Western blot analysis of samples taken from *P. pastoris* culture filtrates of wild-type and active-site mutant CfGH17-1. Western blot assay performed with anti-FLAG antibody results in protein bands at the expected molecular mass of CfGH17-1 (31.7 kDa). (B) Representative tomato *Cf-0* leaf injected with a dilution series of purified wild-type CfGH17-1. As negative control, the protein storage buffer (50 mM phosphate-buffered saline, PBS, pH 6.0) with the same dilution ratio was used. Pictures were taken 2 weeks after infiltration, and the experiment was repeated four times with consistent results.

Fig. S4 Creation of *Cladosporium fulvum* deletion mutant for *CfGH17-1* gene. (A) Schematic representation of the *CfGH17-1* locus in the wild-type and deletion mutant after homologous recombination. The *CfGH17-1* gene is replaced by hygromycin (*HYG*) and *GFP* genes. (B) Confirmation of gene deletion of *CfGH17-1* via PCR. Lanes show products for a positive control (1, α-tomatinase), upstream (US) region (2), downstream (DS) region (3) and the gene to be deleted (4). (C) Single insertion event was confirmed by quantitative PCR by using genomic DNA of each mutant strain. The *HYG* gene was used as a measure for number of insertion events, together with *tubulin* gene for normalization and *actin* gene as a single copy reference gene, according to the $2^{-\Delta\Delta Ct}$ method. (D) Constitutive expression of *CfGH17-1* in *C. fulvum*. qRT-PCR was performed to check expression level of *CfGH17-1* in *OE-CfGH17-1* mutant strains compared to wild-type in PDB liquid medium. Expression levels were normalized using *C. fulvum actin* gene. Error bars indicate standard errors of three biological replicates.

Table S1 Glycosyl hydrolase family 17 (GH17) members in *Cladosporium fulvum* and CfGH17-1 orthologues in other Dothideomycetes. Cf, *Cladosporium fulvum*; Ds, *Dothistroma septosporum*; Mf, *Mycosphaerella fijiensis.*

Table S2 All oligonucleotides and plasmids used in this study

