Secretomics identifies *Fusarium graminearum* proteins involved in the interaction with barley and wheat

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SUMMARY

Fusarium graminearum is a phytopathogenic fungus primarily infecting small grain cereals, including barley and wheat. Secreted enzymes play important roles in the pathogenicity of many fungi. In order to access the secretome of *F. graminearum*, the fungus was grown in liquid culture with barley or wheat flour as the sole nutrient source to mimic the host–pathogen interaction. A gel-based proteomics approach was employed to identify the proteins secreted into the culture medium. Sixty-nine unique fungal proteins were identified in 154 protein spots, including enzymes involved in the degradation of cell walls, starch and proteins. Of these proteins, 35% had not been identified in previous *in planta* or *in vitro* studies, 70% were predicted to contain signal peptides and a further 16% may be secreted in a nonclassical manner. Proteins identified in the 72 spots showing differential appearance between wheat and barley flour medium were mainly involved in fungal cell wall remodelling and the degradation of plant cell walls, starch and proteins. The *in planta* expression of corresponding *F. graminearum* genes was confirmed by quantitative reverse transcriptase-polymerase chain reaction in barley and wheat spikelets harvested at 2–6 days after inoculation. In addition, a clear difference in the accumulation of fungal biomass and the extent of fungal-induced proteolysis of plant β -amylase was observed in barley and wheat. The present study considerably expands the current database of *F. graminearum* secreted proteins which may be involved in Fusarium head blight.

INTRODUCTION

Fusarium graminearum is a pathogenic filamentous fungus which can infect barley, wheat and other small grain cereals, causing a very destructive disease, namely Fusarium head blight (FHB), resulting in reduced grain yields and quality (Bushnell *et al.*, 2003). More important, however, is the contamination by mycotoxins, including trichothecenes, such as deoxynivalenol (DON) and derivatives, as well as the oestrogenic polyketide zearalenone,

which pose a critical hazard to food safety and human health (Becher *et al.*, 2011; Desjardins, 2007). The biological roles of the mycotoxins and secondary metabolites produced by *F. graminearum* are not clear in all cases. Although some mycotoxins, including DON, which inhibits protein synthesis, are considered to be virulence factors in pathogenesis (Boddu *et al.*, 2006; Maier *et al.*, 2006), others, e.g. zearalenone, are not (Lysøe *et al.*, 2006). Disease control is a major focus area and includes management practices and breeding for resistant cultivars (Bai and Shaner, 2004).

The development of FHB in barley and wheat is a complex process. In barley, the infection is usually initiated after the spikes emerge from the flag leaf sheath in the late-milk to soft-dough stages of seed development (Bushnell *et al.*, 2003). The preferred infection site is the extruded ovary epithelial hairs on the kernel tip and *F. graminearum* colonizes the pericarp within 2 days after inoculation (dai) (Boddu *et al.*, 2006). Disease symptoms do not spread in the spikes of most barley cultivars, indicating type II resistance which operates against fungal spread (Jansen *et al.*, 2005). In contrast, wheat spikes are most vulnerable to *F. graminearum* during anthesis. Fungal hyphae often attack the host via the stomatal openings and through the inner surfaces of the glumes, lemma and palea, and then colonize the ovary and floral bract (Boddu *et al.*, 2006). Wheat mainly displays type I resistance which operates against the initial infection. Thus, after infection, the fungus can spread through the vascular tissues in the rachilla and rachis. However, some wheat cultivars show type II resistance to pathogen spread (Boddu *et al.*, 2006; Jansen *et al.*, 2005). During seed development, the fungus invades the aleurone layer and grows into the endosperm. This is associated with the disintegration of cell walls, disappearance of protein bodies and alteration of the starch granule structure (Jackowiak *et al.*, 2005; Jansen *et al.*, 2005).

The pathogen must obtain nutrients from the plant, requiring the secretion of an arsenal of hydrolases (Carapito *et al.*, 2009). Proteomics techniques have revealed that *F. graminearum* produces extracellular enzymes, such as lipases, xylanases, pectinases, cellulases and proteases, when grown in synthetic medium supplemented with different carbon sources including plant cell walls (Paper *et al.*, 2007; Phalip *et al.*, 2005). Alkaline proteases have been detected by Western blotting in protein extracts of

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barley seeds infected with *Fusarium culmorum*, but not in uninfected barley grain (Pekkarinen *et al.*, 2003). *Fusarium* can also produce other proteins, such as hydrophobins, small cysteine-rich proteins which may act as pathogenicity factors in plant-microbe interactions (Kleemola *et al.*, 2001). Some *F. graminearum* extracellular proteins, including pectin-degrading oligogalacturonases, can act as elicitors of defence reactions (Paper *et al.*, 2007).

The availability of the complete genome sequence for *F. graminearum* (Cuomo *et al.*, 2007) has facilitated functional genomics, transcriptomics and proteomics approaches to understand fungal pathogenicity, including the role of secreted proteins. An *F. graminearum* GeneChip has been used to detect differential transcript accumulation when the fungus is cultivated in medium or infected barley plants (Güldener *et al.*, 2006). It has been shown by two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) that the *in vitro* exoproteome of *F. graminearum* grown on medium containing either glucose or hop cell walls as a carbon source contains 23 or 84 unique proteins, respectively, which are mainly involved in cell wall polysaccharide degradation (Phalip *et al.*, 2005). Using liquid chromatography (LC)-MS/MS, 120 and 229 fungal proteins, mostly glycoside hydrolases and proteases, were identified in the secretome of *F. graminearum* during the infection of wheat heads or during growth on 13 synthetic media containing various polysaccharide supplements in the form of plant cell walls or maize bran and stover, respectively (Paper *et al.*, 2007). Recent proteomics studies of *F. graminearum*-infected barley grains at maturity (Yang *et al.*, 2010a) and 2 days after infection (Yang *et al.*, 2010b) have resulted in the identification of one and nine fungal proteins, respectively. This low number is explained by the high ratio of host to pathogen biomass, resulting in the dominance of host proteins, and emphasizes one of the major challenges of studying fungal secreted proteins *in planta*.

Until now, most studies of the fungal secretome have used complex nutrient media that may not closely mimic the nutritional situation of the fungus *in planta*, and may not induce the same proteins as produced during infection. To address this shortcoming, in this study, *F. graminearum* was grown with wheat or barley flour as the sole nutrient source to induce the production of secreted proteins which would most probably be important during interaction with the two hosts.

RESULTS

Identification of proteins in *F. graminearum* culture supernatants

Approximately 170 protein spots were resolved on two-dimensional gels of culture supernatants from *F. graminearum*

grown in the presence of either wheat or barley flour (Fig. 1). The overall protein patterns were quite similar for growth in the two different flours (Fig. 1). Using matrix-assisted laser desorption/ionization-time of flight-time of flight (MALDI-TOF-TOF) MS, proteins in 158 spots were identified, representing 71 unique protein accessions (Table S1, see Supporting Information). Two plant proteins, serpin and fasciclin-like protein, were found in five and one spot, respectively; all other identified proteins originated from the fungus. Almost 35% (or 24) of these identified fungal gene products had not been reported previously in proteomics studies. Some of the identified proteins (FG05292, FG00806, FG03975, FG11008 and FG01956) were present on two-dimensional gels as proteolytic fragments, as the migration of the spots was faster than predicted for the full-length protein (Table S1). Most identified fungal proteins were present in multiple spots with distinct pI values, indicating either post-translational modifications of the same gene product or the existence of closely related gene products. The identified fungal proteins could be assigned according to predicted biological functions, including glycoside hydrolases (38%), proteases (31%), esterases (16%), oxidoreductases (21%), nucleases (7%), housekeeping enzymes (11%), proteins with other functions (20%), such as involvement in cell wall organization and biogenesis, and proteins with unknown function (11%) (Fig. 2). In total, 48 fungal proteins found in 119 spots had an N-terminal signal peptide sequence (Table S1) predicted by SignalP (<http://www.cbs.dtu.dk/services/SignalP/>; Bendtsen *et al.*, 2004b), indicating that they are secreted proteins. Moreover, SecretomeP (<http://www.cbs.dtu.dk/services/SecretomeP/>; Bendtsen *et al.*, 2004a) predictions for 11 of the remaining proteins observed in 16 spots suggested that these may be secreted in a nonclassical way (Table S1).

Genes encoding pathogenicity factors with putative secreted functions, e.g. of cell wall-degrading enzymes, have been reported to cluster in 'pathogenicity islands' in the genome (Paper *et al.*, 2007) which are often highly enriched in regions of high single nucleotide polymorphism (SNP) density (Cuomo *et al.*, 2007). An analysis of the chromosomal locations of genes encoding the identified proteins revealed that about 34% of the genes encoding secreted enzymes predicted to function in the penetration and maceration of plant tissues for nutrient acquisition were in regions of high SNP density. Over 80% of these were located on chromosomes 2 and 3 (Table S1). Paper *et al.* (2007) have also shown that the genes for 120 *in planta* secreted proteins are over-represented in two regions located on chromosomes 2 and 3.

A comparison of *F. graminearum* secreted protein patterns after growth in barley and wheat flour medium revealed 72 spots with significantly different intensity. Forty-eight spots exhibited greater intensity in wheat medium (Table S1). Four of these contained wheat proteins and four contained probable proteolytic fragments. The proteins identified in the remaining 38 spots were 1,3- β -glucosidase (FG06616), glucoamylase (FG06728), pepsin-

Fig. 1 Two-dimensional electrophoresis Sypro Ruby-stained gels from the secretome of *Fusarium graminearum* grown in medium containing barley grain flour (A) and wheat grain flour (B). Molecular size markers and pI ranges are indicated. The protein spots which are identified and numbered refer to Table S1 (see Supporting Information).

Fig. 2 Classification of identified *Fusarium graminearum* proteins into functional categories.

retropepsin-like family protein (FG03975), several esterase-lipase family proteins (FG02616, FG03687, FG11386), FMN reductase (FG01403), FAD-binding protein (FG04732), peroxidase/catalase (FG12369), metallo-dependent hydrolase (FG05738), pyruvate kinase (FG07528), transaldolase (FG08723), chitin deacetylase

(FG06549), two hypothetical cell wall proteins (FG07074, FG10089), cyclophilin (FG00777), Rossmann superfamily protein (FG10433) and several hypothetical proteins of unknown function (FG07644, FG02560, FG04741, FG08833). Twenty-four spots were more intense in barley medium (Table S1), one of which contained a probable fragment. The remaining spots contained xylosidase/arabinosidase (FG11468), cellulase family protein (FG01596), alkaline protease (FG00806), intradiol-dioxygenase family protein (FG08037), flavin reductase (FG02013), putative bacterial-type deoxyribonuclease (FG06993), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (FG06257), cysteine synthase (FG02465) and L-xylulose reductase/D-mannitol dehydrogenase (FG04826). In three cases, spots with different pI, but containing the same gene product, exhibited different intensity in the two media [endoglucanase-5 (FG02658), hypothetical protein (FG11097) and carboxypeptidase (FG04527); Table S1], suggesting a difference in post-translational modification of these proteins or the differential expression of closely related gene products.

Fungal infection and disease development in barley and wheat plants

In order to determine whether genes encoding the secreted proteins identified in liquid media were also expressed *in planta*, barley and wheat plants were point inoculated in parallel with the fungus. At 2 dai, brown discoloration was visible in inoculated barley spikelets, but not in wheat. At 4–6 dai, clear symptoms were observed in both plant species (Fig. S1, see Supporting Information), with more rapid development in barley.

For the accurate quantification of infection levels (Yang *et al.*, 2010b), fungal biomass was determined. In agreement with the observed disease symptoms, the concentration of *F. graminearum* DNA was very low at 2 dai in infected spikelets and increased significantly at 4–6 dai in both barley and wheat, with higher concentrations in barley than in wheat at 4 and 6 dai (Fig. 3A). However, no significant difference was observed in the concentration of DON relative to fungal biomass between infected wheat and barley spikes at 6 dai, despite the higher infection level in barley (Fig. 3C).

Previously, it has been shown that discrete proteolytic fragments of β -amylase are induced by *F. graminearum* infection of barley at 3 dai, and that these can serve as a proteome-level indicator of fungal infection (Yang *et al.*, 2010b). These observations were confirmed in the present study using antibodies raised against barley β -amylase, revealing the appearance of β -amylase fragments at 4 and 6 dai in barley (Fig. 3B). Notably, in wheat, only full-length β -amylase was observed.

Gene expression *in planta*

The expression patterns of 10 fungal genes encoding selected secreted proteins were analysed in infected wheat and barley grains at 2, 4 and 6 dai using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) (Fig. 4). The genes were chosen to represent a variety of functions, i.e. oxidative stress (peroxidase, FG12369), modification of the fungal cell wall (chitin deacetylase, FG06549), degradation of host cell walls (1,3- β -glucosidase, FG06616 and xylosidase, FG11468), degradation of starch (glucoamylase, FG06278) and proteins (alkaline protease, FG00806 and endothiapepsin, FG07775), as well as proteins with predicted esterase/lipase function (FG02616), FAD-binding function (FG04732) and a putative secreted protein (FG06993). Several of these genes correspond to proteins produced in increased amounts in wheat (FG06616, FG06278, FG02616, FG04732, FG12369, FG06549) or barley (FG11468, FG06993) flour-containing cultures. In addition, the *Tri5* gene (FG03537), encoding trichodiene synthase, which catalyses the formation of farnesyl pyrophosphate to trichodiene as the first specific step in the biosynthesis of all trichothecene mycotoxins (Niessen *et al.*, 2004), was included for expression analysis. Expression of all selected genes was detected in infected plants (Fig. 4), but not in uninfected controls (data not shown). All examined transcripts were down-regulated between 4 and 6 dai, except for FG12369 in barley and FG11468 in wheat, which exhibited stable expression. Expression profiles were more variable between 2 and 4 dai, with FG12369, FG07775 and FG03537 down-regulated, FG04732, FG06616 and FG11468 up-regulated and FG00806 stably expressed in both barley and wheat. FG02616 and FG06993 were

Fig. 3 The development of *Fusarium graminearum* infection of barley and wheat spikelets at 2, 4 and 6 days after inoculation (dai). (A) Fungal biomass determined in triplicate and expressed as the content of fungal DNA. (B) Western blotting using antibodies recognizing β -amylase in protein extracts from control (left) and *F. graminearum*-infected (right) spikelets. Bands corresponding to the full-length forms of β -amylase are indicated. Three biological replications are shown for each time point. (C) Concentration of the mycotoxin deoxynivalenol (DON) related to the fungal biomass at 6 dai, determined in triplicate.

Fig. 4 Fungal gene expression analysis in infected barley and wheat spikelets by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) at 2–6 days after inoculation (dai) (2-log scale). Asterisks indicate significant differences ($P < 0.05$) in relative gene expression after normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) between barley and wheat plants at each time point.

down-regulated in barley, but stably expressed in wheat. FG06278 was down-regulated in wheat, but stably expressed in barley.

Although the overall expression profiles of most genes were similar in barley and wheat, despite the different infection levels in the two plants, seven of the genes showed significantly different expression levels in barley and wheat for at least one time point (Fig. 4, indicated by asterisks). Notably, there was an increased relative expression level of the *Tri5* gene in barley relative to wheat at 2–6 dai (Fig. 4), despite a similar amount of DON produced per fungal biomass in the two hosts (Fig. 3C).

DISCUSSION

Given the great losses in the yield and quality of cereal grain caused by *F. graminearum*, it is important to investigate the mechanisms underlying the pathogenic processes of this fungus. In this respect, degradative secreted enzymes, in particular, which

play an important role in nutrient acquisition, host colonization and ecological interactions, have attracted attention (Kang and Buchenauer, 2000). Because of the small amount of fungal biomass in infected plants, it is very challenging to identify fungal proteins *in planta*. This motivated the present profiling of the secreted proteome of *F. graminearum* cultured with barley or wheat flour as the only nutrient source. The flour closely represents the natural hosts of the fungus, in contrast with previous studies of the *F. graminearum* *in vitro* secretome (Paper *et al.*, 2007; Phalip *et al.*, 2005). This approach, combined with protein separation by 2-DE, provides an ideal model to understand plant-pathogen interactions, as a large number of fungal secreted proteins can be identified, and information about the mass and pI of multiple protein forms, resulting from either post-translational modification or closely related gene products, can be obtained.

Among the proteins identified, putative cell wall-, starch- and protein-degrading enzymes were predominant in the fungal

secretome (over 50% of the identifications). These enzymes appeared in multiple forms on the two-dimensional map. Several proteins involved in fungal cell wall modification were identified, including cell wall endoglucanases (FG03017 and FG05292) and putative cell-wall proteins with unknown functions (FG04074 and FG10089). Chitin deacetylase (FG06549) acts on chitin which is the main component of the fungal cell wall, and β -1,6-glucanase (FG08265) and β -1,6-galactanase (FG11184) act on polysaccharides present in fungal, but not plant, cell walls. Similar proteins have been found in other fungi, including *Saccharomyces cerevisiae*, *Ustilago maydis*, *Trichoderma reesei* and ectomycorrhizal fungi, and are also assumed to be involved in modification of the fungal cell walls (Mueller *et al.*, 2008; Nagendran *et al.*, 2009). The identification of these proteins suggests that cell wall remodelling is likely to be important during vegetative growth of the fungus. However, few reports have studied the changes in fungal cell wall structure during growth or infection (Nagendran *et al.*, 2009).

Among the identified fungal proteins, 21, including five proteins annotated as putative housekeeping enzymes, lacked the predicted N-terminal signal peptides. In a previous report, an even higher proportion of sequences (around 56%) lacking signal peptides was observed in extracellular extracts obtained by vacuum infiltration of *F. graminearum*-infected wheat heads (Paper *et al.*, 2007). This may reflect the lysis of fungal cells during growth or sample extraction. Alternatively, the proteins may be secreted by a nonclassical pathway, as demonstrated in other fungi, e.g. *S. cerevisiae*, *Candida albicans*, *Aspergillus fumigatus* and *Claviceps purpurea* (Nombela *et al.*, 2006). Indeed, 11 of the proteins identified in the present study, including GAPDH, cysteine synthase and ubiquitin-like protein, were predicted to be secreted in a nonclassical way using SecretomeP. Pancholi and Chhatwal (2003) have suggested that several housekeeping enzymes, including GAPDH, are present on the surface of pathogens and serve as virulence factors. Some fungal proteins, such as β -xylosidase and superoxide dismutase, neither of which possesses signal peptides, have been suggested to be secreted, as their activity can be detected in fungal culture filtrates (Moore *et al.*, 2002; Ransom and Walton, 1997). Notably, some housekeeping proteins were identified in the *in planta* secretome and in the present study using liquid medium containing wheat or barley flour, but not in other *in vitro* secretomes (Paper *et al.*, 2007; Phalip *et al.*, 2005), suggesting that these proteins may be secreted only in response to a host-derived factor. Seventeen of the 69 fungal proteins identified in the current study were found in the secretome of *F. graminearum* in infected wheat heads, 40 were also identified in liquid cultures using synthetic media with supplements of plant cell walls or polysaccharides, and 31 were identified in liquid cultures using synthetic media containing glucose or other sugar (Paper *et al.*, 2007; Phalip *et al.*, 2005). Twenty-four of the proteins identified in the current study have not been observed previously (Table S1).

When comparing secretome profiles in barley and wheat flour medium, several proteins with a putative role in the degradation of plant cell walls, starch and proteins showed a differential appearance (Table S1). Barley and wheat differ noticeably in cell wall and grain composition. Arabinoxylans (70% w/w) are the main polysaccharides of cell walls of the mature wheat endosperm, whereas β -(1 $\rightarrow$ 3)-(1 $\rightarrow$ 4) glucans (20%–29% w/w) are present in a higher proportion in the aleurone layer with minor amounts of glucomannans (2%–7% w/w) and cellulose (2%–4% w/w) (Philippe *et al.*, 2006). In contrast, barley endosperm cell walls contain a high level (71%) of β -(1 $\rightarrow$ 3)-(1 $\rightarrow$ 4) glucans (Noots *et al.*, 2003), whereas aleurone cell walls consist almost entirely of arabinoxylans (80%) with cellulose (8%) and protein (6%) (McNeil *et al.*, 1975). As the endosperm constitutes a much greater proportion of the grain than the aleurone layer, the composition of the endosperm cell walls may be expected to have a greater influence on the induction of cell wall-degrading enzymes. However, the induced enzymes are in fact more suitable for the degradation of the respective aleurone cell walls, as a very noticeable increase in the abundance of glucan 1,3- β -glucosidase (FG06616) was observed in wheat medium and of xylosidase/arabinosidase (FG11468) in barley medium. Several putative esterase–lipase superfamily proteins were up-regulated in wheat medium, which may be involved in the degradation of plant cell walls. Wheat grain has a higher starch content than barley grain (Andersson *et al.*, 2001), which may explain the greater abundance of fungal glucoamylase (FG06278) observed in wheat than in barley medium. Notably, chitin deacetylase and two putative cell wall proteins were found with greater abundance in cultures grown with wheat flour than with barley, suggesting that fungal cell wall remodelling is induced by a factor in wheat flour.

The detection of all the selected transcripts *in planta* confirms the relevance of the approach for the identification of fungal proteins involved in the interaction with the plant. The differences observed between extracellular protein accumulation in barley and wheat medium, however, were not clearly reflected in the gene expression profiles. This is to be expected because of the difference between the *in planta* and *in vitro* growth conditions, combined with the fact that protein abundance is determined by a variety of factors in addition to mRNA abundance, for example the protein turnover rate. In particular, extracytoplasmic proteins rely on processing and targeting events before reaching their location. For all genes tested, relative expression levels determined by qRT-PCR decreased or did not change significantly from 4 to 6 dai, but different profiles were observed from 2 to 4 dai, suggesting that this is an important stage of the infection process. For example, *F. graminearum* has been proposed to switch from a biotrophic to a necrotrophic phase at approximately 3 dai (Kang and Buchenauer, 1999) and *F. graminearum*-induced proteolysis of barley β -amylase is observed from 3 dai (Yang *et al.*, 2010b).

Although the expression profiles of the analysed fungal genes were similar in infected wheat and barley (Fig. 4), clear differences in the accumulation of fungal biomass and fungal-induced proteolysis of wheat and barley β -amylase were observed (Fig. 3A,B). This may reflect the lower infection level of wheat plants, a difference in timing, type or amount of protease produced during fungal infection in barley and wheat, or a difference in susceptibility of barley and wheat β -amylases to fungal proteases, despite 82% sequence identity of the β -amylase sequences.

The production of DON has been clearly implicated as a virulence factor in the pathogenesis of FHB (Boddu *et al.*, 2006), and higher disease severity in wheat was observed after inoculation with a DON-producing *F. graminearum* strain than with a non-producing strain (Nicholson *et al.*, 1998). As the fungus produced the same amount of DON relative to biomass in both barley and wheat in the present study, the observed difference in disease severity in wheat and barley was not caused by different DON production levels. In addition, there was a poor correlation between the level of *Tri5* gene expression and DON production in wheat and barley, possibly because trichodiene synthase is not the sole critical enzyme involved in DON biosynthesis (Niessen *et al.*, 2004).

In conclusion, 2-DE-based proteome analysis of the *F. graminearum* secretome in barley or wheat flour-containing medium identified new proteins likely to be involved in fungal nutrition and FHB, which are difficult to obtain from *in planta* experiments. A high abundance of cell wall-, starch- and protein-degrading enzymes was produced, which showed differences between wheat and barley flour-containing medium. Clear differences were observed that highlight the nutrition-dependent secretion, the influence of the aleurone cell wall composition and the probable importance for growth of fungal cell wall modification. Justifying the approach, 35% of the fungal proteins identified were experimentally observed for the first time in the present study. Although not a precise mimic, the *in vitro* system used in the present study more strongly resembles *in vivo* conditions than do synthetic or complex media, and can provide information regarding nutritional differences between the two hosts. The approach was further validated by confirming that the genes encoding the identified proteins were indeed expressed during the infection of barley and wheat plants. This combined proteomics and gene expression analysis is a first and highly efficient strategy for the elucidation of the molecular interactions between *F. graminearum* and its cereal hosts.

EXPERIMENTAL PROCEDURES

Preparation of fungal culture

Macroconidial suspensions of *F. graminearum* strain PH1 (Agricultural Research Service Culture Collection, Northern Regional Research Labora-

tory, US Department of Agriculture, Peoria, IL, USA; NRRL 31084) were prepared as described by Yang *et al.* (2010b) and used for *in vitro* growth and plant inoculation studies. Barley (cv. Hydrogen) and wheat (cv. Vinjett) grains were ground finely to flour in a coffee mill and the growth medium was prepared by mixing 50 g of flour with 1 L of water prior to autoclaving (Schmidt-Heydt and Geisen, 2007). Erlenmeyer flasks containing 100 mL of liquid medium were inoculated with 1 mL of macroconidial suspension (10^6 spores/mL). The flasks were gently shaken by hand and incubated in the dark at 20 °C. Three independent biological replications were carried out in each medium. After 7 days, the supernatant was collected by filtration through cheesecloth and centrifugation (14 000 g for 30 min, 4 °C).

Isolation of secreted proteins and 2-DE

The supernatant was subjected to ultrafiltration (4000 g, 4 °C) through Centriprep® centrifugal filters (molecular mass cut-off, 3000 Da). The protein concentration was determined by the Bradford assay (Bradford, 1976) using bovine serum albumin as a standard. Twenty micrograms of protein were precipitated by four volumes of 10% (w/v) trichloroacetic acid in acetone at -20 °C overnight. Protein pellets were washed sequentially three times with 80% (v/v) acetone before dissolving in 200 μ L of reswelling buffer, as described by Yang *et al.* (2010a). Samples were applied to 11-cm, pI 3–10 IPG strips and isoelectric focusing (IEF) was run on an Ettan IPGphor (GE Healthcare, Chalfont St Giles, UK) for 6 h at 30 V, 6 h at 60 V, 1 h at 500 V, 1 h at 1000 V, 30-min gradient to 8000 V and 4 h at 8000 V. IPG strips were trimmed to a range covering pH 4–8.5 and applied to the second dimension. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (NuPAGE® Novex 4%–12% Bis-Tris ZOOM® Gel; Invitrogen) was performed in the XCell SureLock™ Mini-Cell system (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Duplicate gels were run for each biological replication. Gels were stained by Sypro Ruby (Invitrogen).

Image analysis

Images of the Sypro Ruby-stained gels were captured in a Typhoon® scanner (GE Healthcare) with the following settings: filter, 580 BP30; laser, green (532 nm); sensitivity, normal; photomultiplier, 520 V; pixel size, 100 μ m. In total, six images for each growth condition were imported to Progenesis SameSpots (Nonlinear Dynamics, Newcastle-upon-Tyne, UK) for the quantification of spot volume and statistical analysis. After warping, matching and aligning to a chosen reference gel, six gels for each growth condition were grouped to calculate the average volume of each spot. Protein spots showing ≥ 1.5 -fold change in normalized average volume and analysis of variance (ANOVA) ($P < 0.05$) were considered to show differential appearance in wheat and barley medium.

In-gel digestion and protein identification

Spots were excised from Sypro Ruby-stained gels after post-staining with colloidal Coomassie brilliant blue (Rabilloud and Charmont, 2000), and subjected to in-gel digestion followed by MALDI-TOF-TOF analysis using an Ultraflex II mass spectrometer (Bruker-Daltonics, Bremen, Germany), as described by Yang *et al.* (2010b). Proteins were identified by searching

the Broad Institute gene index for *F. graminearum* (http://www.broad.mit.edu/annotation/genome/fusarium_graminearum), the NCBI nr at the National Center for Biotechnology Information (NCBI) and the wheat gene index (release 11.0) (<http://compbio.dfci.harvard.edu/tgi>). The following parameters were set for searching: allowed global modification, carbamidomethyl cysteine; variable modification, oxidation of methionine; missed cleavages, 1; peptide tolerance, 80 ppm; MS/MS tolerance, ± 0.5 Da. In some cases, MS/MS was used to confirm identifications. To be considered as a positive identification, the following criteria had to be fulfilled: a significant score calculated by the Mowse scoring algorithm in MASCOT and at least four matched independent peptides in MS analysis or one peptide in MS/MS analysis. Hypothetical proteins with unknown function were annotated by BLAST search in NCBI. Protein identifications are documented in Table S1. All identified proteins were assessed for signal peptides using SignalP (<http://www.cbs.dtu.dk/services/SignalP>) and for nonclassical secretion using SecretomeP (mammalian) (<http://www.cbs.dtu.dk/services/SecretomeP>). The chromosome location and SNP identification of the genes encoding the identified proteins were determined by searching in databases from the Broad Institute for *F. graminearum*.

Plant growth and inoculation

The susceptible barley cv. Hydrogen and wheat cv. Vinjett were grown in a glasshouse as described by Yang *et al.* (2010b). Spikelets of wheat and barley were point inoculated at anthesis (stage 65; Zadoks *et al.*, 1974) by micropipetting 5 μ L of a macroconidial suspension (10^5 spores/mL) into the flower, leaving the bottom and top two spikelets uninoculated. Separate control plants were mock inoculated with water. The inoculated spikes were covered in sealed water-sprayed plastic bags in the dark for 72 h. Three spikes collected from different plants served as one biological replication. Three biological replications were harvested separately at 2, 4 and 6 dai and stored at -80°C until use. Inoculated spikelets were lyophilized and ground into a fine powder in liquid nitrogen.

Determination of fungal biomass and DON

Total DNA was extracted from 20 mg of ground plant material of each sample, and the determination of the amounts of *Fusarium* and plant DNA by qPCR was carried out as described by Yang *et al.* (2010b). *Fusarium graminearum* biomass was expressed as *Fusarium* DNA/plant DNA (ng/ μ g). The DON concentration from 100 mg of ground plant sample at 6 dai was determined by the RIDASCREEN® DON kit (R-Biopharm AG, Darmstadt, Germany) following the manufacturer's protocol.

Protein extraction and Western blotting

Water-soluble proteins were extracted from 20 mg of ground plant material of each sample with 400 μ L of buffer containing 5 mM Tris-HCl, pH 7.5, 1 mM CaCl_2 and the protease inhibitor cocktail 'complete' (Roche, Basel, Switzerland), as described by Yang *et al.* (2010a). The protein concentration was determined by the Bradford assay (Bradford, 1976) with bovine serum albumin as a standard. Western blotting with β -amylase antibodies (kindly supplied by Dr Evan Evans, University of Tasmania, Australia) was carried out as described by Yang *et al.* (2010b).

qRT-PCR analysis

RNA was extracted from 30 mg of ground plant material of each sample prior to the removal of genomic DNA, as well as cDNA synthesis, as described by Yang *et al.* (2010b). Primers for *F. graminearum* genes (Table S2, see Supporting Information) were designed and specificity was tested as before (Yang *et al.*, 2010b). *F. graminearum* GAPDH served as a reference gene for the quantification of gene expression. Both GAPDH and elongation factor 1 α (EF1 α) have been used previously as reference genes and found to be stably expressed in *F. graminearum*-infected barley (Yang *et al.*, 2010b). Real-time PCR was set up with 40 cycles of amplification and carried out in technical duplicates with 10-fold dilution of cDNA templates (Yang *et al.*, 2010b). All relative expression values of genes were reported as means $\pm$ SD on a 2-log scale. An analysis of significant differences in gene expression in barley versus wheat at each time point was performed by Student's *t*-test.

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REFERENCES

- Andersson, A.A.M., Andersson, R. and Åman, P. (2001) Starch and by-products from a laboratory-scale barley starch isolation procedure. *Cereal Chem.* **78**, 507–513.
- Bai, G.H. and Shaner, G. (2004) Management and resistance in wheat and barley to Fusarium head blight. *Annu. Rev. Phytopathol.* **42**, 135–161.
- Becher, R., Weihmann, F., Deising, H.B. and Wirsig, S.G.R. (2011) Development of a novel multiplex DNA microarray for *Fusarium graminearum* and analysis of azole fungicide responses. *BMC Genomics*, **12**, 52–68.
- Bendtsen, J.D., Jensen, L.J., Blom, N., von Heijne, G. and Brunak, S. (2004a) Feature based prediction of non-classical and leaderless protein secretion. *Protein Eng. Des. Sel.* **17**, 349–356.
- Bendtsen, J.D., Nielsen, H., von Heijne, G. and Brunak, S. (2004b) Improved prediction of signal peptides: SignalP 3.0. *J. Mol. Biol.* **340**, 783–795.
- Boddu, J., Cho, S., Kruger, W.M. and Muehlbauer, G.J. (2006) Transcriptome analysis of the barley–*Fusarium graminearum* interaction. *Mol. Plant–Microbe Interact.* **19**, 407–417.
- Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* **72**, 248–254.
- Bushnell, W.R., Hazen, B.E. and Pritsch, C. (2003) Histology and physiology of Fusarium head blight. In: *Fusarium Head Blight of Wheat and Barley* (Leonard, K.J. and Bushnell, W.R., eds), pp. 44–83. St Paul, MN: APS Press.
- Carapito, R., Imberty, A., Jeltsch, J.M., Byrns, S.C., Tam, P.H., Lowary, T.L., Varrot, A. and Phalip, V. (2009) Molecular basis of arabinobio-hydrolase activity in phytopathogenic fungi: crystal structure and catalytic mechanism of *Fusarium graminearum* GH93 exo- α -L-arabinanase. *J. Biol. Chem.* **284**, 12 285–12 296.
- Cuomo, C.A., Guldener, U., Xu, J.R., Trail, F., Turgeon, B.G., Di Pietro, A., Walton, J.D., Ma, L.J., Baker, S.E., Rep, M., Adam, G., Antoniw, J., Baldwin, T., Calvo, S.,

- Chang, Y.L., Decaprio, D., Gale, L.R., Gnerre, S., Goswami, R.S., Hammond-Kosack, K., Harris, L.J., Hilburn, K., Kennell, J.C., Kroken, S., Magnuson, J.K., Mannhaupt, G., Mauceli, E., Mewes, H.W., Mitterbauer, R., Muehlbauer, G., Münsterkötter, M., Nelson, D., O'donnell, K., Ouellet, T., Qi, W., Quesneville, H., Roncero, M.I., Seong, K.Y., Tetko, I.V., Urban, M., Waalwijk, C., Ward, T.J., Yao, J., Birren, B.W. and Kistler, H.C. (2007) The *Fusarium graminearum* genome reveals a link between localized polymorphism and pathogen specialization. *Science*, **317**, 1400–1402.
- Desjardins, A.E. (2007) *Fusarium Mycotoxins: Chemistry, Genetics, and Biology*. St Paul, MN: APS Press.
- Güldener, U., Seong, K.Y., Boddu, J., Cho, S., Trail, F., Xu, J.R., Adam, G., Mewes, H.W., Muehlbauer, G.J. and Kistler, H.C. (2006) Development of a *Fusarium graminearum* Affymetrix GeneChip for profiling fungal gene expression *in vitro* and *in planta*. *Fungal Genet. Biol.* **43**, 316–325.
- Jackowiak, H., Packa, D., Wiwart, M. and Perkowski, J. (2005) Scanning electron microscopy of *Fusarium* damaged kernels of spring wheat. *Int. J. Food Microbiol.* **98**, 113–123.
- Jansen, C., von Wettstein, D., Schäfer, W., Kogel, K.H., Felk, A. and Maier, F.J. (2005) Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. *Proc. Natl. Acad. Sci. USA*, **102**, 16 892–16 897.
- Kang, Z. and Buchenauer, H. (1999) Immunocytochemical localization of *Fusarium* toxins in infected wheat spikes by *Fusarium culmorum*. *Physiol. Mol. Plant Pathol.* **55**, 275–288.
- Kang, Z. and Buchenauer, H. (2000) Ultrastructural and cytochemical studies on cellulose, xylan and pectin degradation in wheat spikes infected by *Fusarium culmorum*. *J. Phytopathol.* **148**, 263–275.
- Kleemola, T., Nakari-Setälä, T., Linder, M., Penttilä, M., Kotaviita, E., Olkku, J. and Haikara, A. (2001) Characterisation and detection of the gushing factors produced by fungi. In: *Proceedings of the 28th European Brewery Convention Congress, Budapest*, Contribution no. 12, CD-ROM, ISBN 90-70143-21-6 (Astrup, S. ed.), pp. 129–138. Nürnberg: Fachverlag Hans Carl.
- Lysøe, E., Klemsdal, S.S., Bone, K.R., Frandsen, R.J.N., Johansen, T., Thrane, U. and Giese, H. (2006) The PKS4 gene of *Fusarium graminearum* is essential for zearealenone production. *Appl. Environ. Microbiol.* **72**, 3924–3932.
- Maier, F.J., Miedaner, T., Haderer, B., Felk, A., Salomon, S., Lemmens, M., Kassner, H. and Schäfer, W. (2006) Involvement of trichothecenes in fusarioses of wheat, barley and maize evaluated by gene disruption of the trichodiene synthase (*Tri5*) gene in three field isolates of different chemotype and virulence. *Mol. Plant Pathol.* **7**, 449–461.
- McNeil, M., Albersheim, P., Taiz, L. and Jones, R.L. (1975) The structure of plant cell walls. VII. Barley aleurone cells. *Plant Physiol.* **55**, 64–68.
- Moore, S., de Vries, O.M. and Tudzynski, P. (2002) The major Cu, Zn SOD of the phytopathogen *Claviceps purpurea* is not essential for pathogenicity. *Mol. Plant Pathol.* **3**, 9–22.
- Mueller, O., Kahmann, R., Aguilar, G., Trejo-Aguilar, B., Wu, A. and de Vries, R.P. (2008) The secretome of the maize pathogen *Ustilago maydis*. *Fungal Genet. Biol.* **45**, S63–S70.
- Nagendran, S., Hallen-Adams, H.E., Paper, J.M., Aslam, N. and Walton, J.D. (2009) Reduced genomic potential for secreted plant cell-wall-degrading enzymes in the ectomycorrhizal fungus *Amanita bisporigera*, based on the secretome of *Trichoderma reesei*. *Fungal Genet. Biol.* **46**, 427–435.
- Nicholson, P., Simpson, D.R., Weston, G., Rezanoor, H.N., Lees, A.K., Parry, D.W. and Joyce, D. (1998) Detection and quantification of *Fusarium culmorum* and *Fusarium graminearum* in cereals using PCR assays. *Physiol. Mol. Plant Pathol.* **53**, 17–37.
- Niessen, L., Schmidt, H. and Vogel, R.F. (2004) The use of *tri5* gene sequences for PCR detection and taxonomy of trichothecene-producing species in the *Fusarium* section *Sporotrichiella*. *Int. J. Food Microbiol.* **95**, 305–319.
- Nombela, C., Gil, C. and Chaffin, L.J. (2006) Non-conventional protein secretion in yeast. *Trends Microbiol.* **14**, 15–21.
- Noots, I., Derycke, V., Jensen, H.E., Michiels, C., Delcour, J.A. and Coppens, T. (2003) Studies on barley starchy endosperm cell wall degradation by *Rhizopus* VII. *J. Cereal Sci.* **37**, 81–90.
- Pancholi, V. and Chhatwal, G.S. (2003) Housekeeping enzymes as virulence factors for pathogens. *Int. J. Med. Microbiol.* **293**, 391–401.
- Paper, J.M., Scott-Craig, J.S., Adhikari, N.D., Cuomo, C.A. and Walton, J.D. (2007) Comparative proteomics of extracellular proteins *in vitro* and *in planta* from the pathogenic fungus *Fusarium graminearum*. *Proteomics*, **7**, 3171–3183.
- Pekkarinen, A.I., Sarlin, T.H., Laitila, A.T., Haikara, A.I. and Jones, B.L. (2003) *Fusarium* species synthesize alkaline proteinases in infested barley. *J. Cereal Sci.* **37**, 349–356.
- Phalip, V., Delalande, F., Carapito, C., Goubet, F., Hatsch, D., Leize-Wagner, E., Dupree, P., Van Dorsselaer, A. and Jeltsch, J.M. (2005) Diversity of the exoproteome of *Fusarium graminearum* grown on plant cell wall. *Curr. Genet.* **48**, 366–379.
- Philippe, S., Robert, P., Barron, C., Saulnier, L. and Guillon, F. (2006) Deposition of cell wall polysaccharides in wheat endosperm during grain development: Fourier transform-infrared microspectroscopy study. *J. Agric. Food Chem.* **54**, 2303–2308.
- Rabilloud, T. and Charmont, S. (2000) Detection of proteins on two-dimensional electrophoresis gels. Two-dimensional gel electrophoresis and identification methods. In: *Proteome Research* (Rabilloud, T., ed.), pp. 109–110. Berlin, Heidelberg: Springer-Verlag.
- Ransom, R.F. and Walton, J.D. (1997) Purification and characterization of extracellular β -xylosidase and α -arabinosidase from the plant pathogenic fungus *Cochliobolus carbonum*. *Carbohydr. Res.* **297**, 357–364.
- Schmidt-Heydt, M. and Geisen, R. (2007) A microarray for monitoring the production of mycotoxins in food. *Int. J. Food Microbiol.* **117**, 131–140.
- Yang, F., Jensen, J.D., Spliid, N.H., Svensson, B., Jacobsen, S., Jørgensen, L.N., Jørgensen, H.J., Collinge, D.B. and Finnie, C. (2010a) Investigation of the effect of nitrogen on severity of *Fusarium* head blight in barley. *J. Proteomics*, **73**, 743–752.
- Yang, F., Jensen, J.D., Svensson, B., Jørgensen, H.J., Collinge, D.B. and Finnie, C. (2010b) Analysis of early events in the interaction between *Fusarium graminearum* and the susceptible barley (*Hordeum vulgare*) cultivar Scarlett. *Proteomics*, **10**, 3748–3755.
- Zadoks, J.C., Chang, T.T. and Konzak, C.F. (1974) A decimal code for the growth stages of cereals. *Weed Res.* **14**, 415–421.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Disease symptoms on water (control)- and *Fusarium graminearum*-inoculated spikelets of barley (top panel) and wheat (bottom panel) at 6 days after inoculation (dai).

Table S1 Proteins identified in *Fusarium graminearum* culture supernatants after growth in wheat or barley flour-containing medium.

Table S2 Primer sequences for quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis of fungal genes.

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