Application of proteomics to investigate barley-Fusarium graminearum interaction

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Application of proteomics to investigate barley-*Fusarium graminearum* interaction

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Preface and Acknowledgements

This thesis is based on the work I have done during Ph.D. study which was carried out in Enzyme and Protein Chemistry, Department of Systems Biology, Technical University of Denmark from 15th of March 2008 to 14th of March 2011, under the supervision of Christine Finnie (supervisor) and Susanne Jacobsen (co-supervisor). The project includes three-month study in Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Copenhagen, two-week study in Faculty of Agricultural Sciences, Research Centre Flakkebjerg, University of Aarhus and two-week study with Prof. H.C. Kistler in Cereal Disease Laboratory, Minnesota, USA. The project is funded by the Directorate for Food, Fisheries and Agri Business (DFFE) grant "Fusarium disease resistance – toxins and feed quality", Plant Biotech Denmark, the Centre for Advanced Food Studies (LMC) and a PhD stipend from the Technical University of Denmark.

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Lyngby, March, 2011

Fen Yang

Summary

Due to the great loss of barley grain yield and quality in addition to mycotoxins contamination caused by Fusarium head blight (FHB), it is essential to understand the molecular interaction between barley and *Fusarium graminearum*, one of the primary *Fusarium* species causing FHB, in order to control the disease. Due to the advantages of gel-based proteomics that differentially expressed proteins involved in the interaction can be directly detected by comparing protein profiles displayed on 2-D gels, it is used as a tool for studying the barley-*Fusarium graminearum* interaction form three different aspects in this thesis shown in Chapter 2, 3 and 4.

In Chapter 2, the effect of nitrogen on FHB in a susceptible barley cultivar was investigated with using two levels of nitrogen fertilizers (15 and 100 kg ha^{-1}). Albumin proteome analysis of the infected and control kernels under two N levels showed that i) spots increasing in intensity in the infected plants included fungal proteins and proteolytic fragments of plant proteins, ii) spots decreasing in intensity contained plant proteins possibly degraded by fungal proteases, iii) greater spot volume changes in response to the fungus were observed in plants under low N and iv) proteomes of uninfected plants were similar under two N levels. Correlation of level of proteolysis induced by the fungus with measurement of *Fusarium*-damaged kernels, fungal biomass and mycotoxin levels indicated that FHB was more severe in barley with low N.

In Chapter 3, the molecular mechanisms of barley defense to *Fusarium graminearum* at the early infection stage were studied. Antibodies against barley β-amylases were shown to be the markers for infection at proteome level and for selection of the time for proteome analysis before extensive degradation caused by the fungus. Pathogenesis-related (PR) proteins and proteins involved in energy metabolism were induced and protein involved in the secondary metabolism and protein synthesis changed in abundance in the infected barley. qRT-PCR analysis showed the upregulation of several PR genes and expression of two fungal genes encoding proteases which could be responsible for proteolysis of β-amylases in the infected barley.

In Chapter 4, the *in vitro* secretome of *F. graminearum* on the 2-D gels in the presence of substrates of barley or wheat grain was studied. Totally 69 unique fungal proteins identified were mainly cell-wall-degrading enzymes and proteases. Besides *Tri5* gene, ten selected genes encoding protein expressed *in vitro* were also expressed in the *F. graminearum*-infected wheat and barley from 2-6 day after inoculation (dai), suggesting the *in vitro* proteome approach may be an ideal strategy to discover pathogenicity factors. In addition, sharper increase in fungal biomass was observed in barley than in wheat and fungal induced proteolytic fragments of β-amylases were only observed in barley not in wheat.

Furthermore, a barley PR17 protein and a fungal hypothetical protein were expressed in *E. coli* and purified in Chapter 5. The functional characterization of two proteins is undergoing. In Chapter 6, microarray data of *F.*

graminearum during interaction with barley and wheat was analysed. The expression patterns of 11fungal genes in microarray analysis were different from qRT-PCR results in Chapter 4.

Overall, our results will give some insights into the cellular activities during the interaction between barley and *Fusarium graminearum* for designing new efficient strategies for the control of FHB disease.

Dansk Resumé

Patogene svampearter af slægten *Fusarium* udgør et stigende problem i kornproduktionen verden over af flere grunde. På grund af et stort tab af bygkerner såvel i udbytte som af kvalitet samt en kontaminering af mykotoksiner forårsaget af svampen *Fusarium* er det vigtigt at forstå de molekylære interaktioner mellem byg og svampen *Fusarium graminearum* for derved at kunne nedsætte antallet af *Fusarium*-angreb i byg. Til at finde frem til hvilke proteiner, der har betydning for samspillet mellem svamp og plante, og samtidigt studere effekten af to forskellige niveauer (15 og 100 kg ha^{-1})af kvælstoftilførsel blev der udført proteomanalyse af albuminfraktionen fra såvel inficerede som kontrol bygkerner. Resultaterne viste at der var ændringer i mængden af proteinerne i *Fusarium*-inficerede bygkerner i forhold til kontrolplanter. I de *Fusarium*-inficerede bygkerner var der desuden svampeproteiner samt spaltede bygproteiner. I forhold til kontrolplanter havde bygplanter, der havde groet ved lav nitrogentilførsel, de største proteinpletforskelle som svar på svampeinfektion. Proteomet af bygkerner fra ikke-inficerede planter groet ved lav og høj nitrogentilførsel var sammenlignelige.

En nærmere analyse af de molekylære mekanismer af bygs forsvar mod *Fusarium graminearum* ved et tidligt angreb og ved brug af byg beta-amylase antistoffer, som markører for infektion på proteomniveau, blev udført. Patogenese relaterede (PR) proteiner og proteiner i energistofskiftet blev induceret. I inficerede bygplanter blev indholdet af proteiner med funktion i sekundær stofskifte og proteinsyntesen ændret. Sammenhørende qRT-PCR analyse viste opregulering af adskillige PR-gener og udtryk af to svampegener kodende for proteaser som kunne bevirke en spaltning af β-amylaser i inficeret byg.

Ved et studium vha to-dimensional gelelektroforese af *in vitro* secretomet af *F. graminearum* sammen med byg og hvedekernesubstrater blev i alt 69 unikke svampeproteiner identificeret og som hovedsagligt var cellevægsnedbrydende enzymer og proteaser. Foruden *Tri5*-gen blev ti gener, som koder for proteiner, udtrykt *in vitro* og som også var udtrykt i *F. graminearum*–inficerede hvede- og byg-kerner fra 2-6- dage efter infektion. Resultaterne viser at data fra *in vitro* proteomanalyse kan være et godt udgangspunkt mhp at klarlægge patogenicitetsfaktorer. Resultaterne viste også, at i forhold til hvede sås i byg en større stigning i svampebiomasse, samt at svampeinduceret proteolytiske fragmenter af β-amylaser kun sås i byg.

I *E. coli* er et byg PR17-protein og et hypotetisk svampeprotein blevet udtrykt og oprenset med henblik på at karakterisere begge proteiner. Microarray-data af *F. graminearum* under interaktion med byg og hvede er analyseret. Ekspressionsmønstre af 11 svampegener var forskellige fra qRT-PCR-analysen.

Sammenlagt vil vores resultater give indsigt i cellens aktiviteter under vekselvirkningen mellem byg og *Fusarium graminearum* som kan bruges i udvikling af effektive nye strategier i kontrollen af FHB sygdommen.

Abbreviations

2-DE, two-dimensional gel electrophoresis

CWDEs, cell-wall-degrading enzymes

dai, days after inoculation

DIGE, differential gel electrophoresis

DON, deoxynivalenol

DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid)

DTT, dithiothreitol

EDTA, ethylenediaminetetraacetic acid

ESI, electrospray ionization

EST, expressed sequence tags

FDK, *Fusarium*-damaged kernels

FHB, Fusarium head blight

hai, hours after inoculation

HR, hypersensitive response

ICAT, isotope-code affinity tag

IEF, isoelectric focusing

IPTG, isopropyl β-D-1-thiogalactopyranoside

iTRAQ, isobaric tags for relative and absolute quantitation

LB, lysogeny broth

LC, liquid chromatography

MALDI, matrix assisted laser desorption/ionization

MS, mass spectrometry

NCBI, National Center for Biotechnology Information

NIV, nivalenol

PAGE, polyacrylamide gel electrophoresis

PAMPs, microbial /pathogen-associated molecular patterns

PCA, principal component analysis

PR, pathogenesis-related

PTM, post-translational modification

qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction

QTL, quantitative trait loci

ROS, reactive oxygen species

SDS, sodium dodecyl sulphate

SILAC, stable isotope labeling with amino acids win cell culture

SNP, single nucleotide polymorphism

TOF, time-of-flight

ZEA, zearalenone

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Chapter 1
Introduction

1.1 Fusarium head blight in barley and wheat

Fusarium head blight (FHB) is a devastating disease of cereals including barley and wheat in humid and semi-humid climates worldwide (Walter *et al*., 2009). FHB was first described in 1884 in England and has increased in Asia, Canada, Europe and South America since then (Stack, 1999). The disease has reached epidemic levels in several years and causes significant losses of grain yield and quality to be millions of dollars per annum in the USA alone (Nganje *et al.*, 2004). FHB has been identified by International Maize and Wheat Improvement Center as a major factor limiting wheat production in many parts of world (Stack, 1999). The more important is the contamination of the mycotoxins produced by the fungus, which adversely affect grain quality and is an enormous challenge for cereal breeders and the food or milling industry.

Several *Fusarium* species including *F. sporotrichioides*, *F. culmorum*, *F. avenaceum*, *F. poae* and the most prevalent *F. graminearum* (teleomorph: Gibberella zeae (Schwein) Petch) are the causal agents of FHB worldwide (Parry *et al.*, 1995). As a result, *F. graminearum* quickly become one of the most intensively studied fungal plant pathogen. The genome sequence released by Broad Institute in 2003 has greatly stimulated the research activity on *F. graminearum*. The fungus has a genome size (36.1Mb) and contains genes encoding 13937 predicted proteins distributed over four chromosomes with few repeat sequences (Cuomo *et al*., 2007; Trail, 2009). There are 2001 genes not similar to those of any other sequenced organisms and 5812 genes having homology to genes encoding proteins of unknown function (Trail, 2009). The availability of whole genome sequence provides the opportunity to study transcription and proteome profiling for identifying essential elements in pathogenesis and lead to development of new targets for fungal control.

Fusarium graminearum colonizes living host tissue at specific stages and establishes itself in senescent tissue and debris as saprophytic mycelia due to a brief biotrophic relationship with its host before switching to the necrotrophic phase (Bai and Shaner, 1994; Goswami and Kistler, 2004). The necrotrophic stage is associated with an increase in vigour of colonization and eventually plant death leads to thorough colonization of the host substrate (Goswami and Kistler, 2004). The warm moist weather is favourable for the development and maturation of conidia and perithecia on the crop debris which produce ascospores (Goswami and Kistler, 2004). The rain and wind spread the soil-born inoculums, mainly ascospores to the plants (Bai and Shaner, 1994). The

abundance of the primary inoculums and weather conditions, mainly moisture and temperature, during and after anthesis determine the severity of FHB (Bai and Shaner, 1994).

The spikelets of barley and wheat are most susceptible at anthesis or during the early dough stage of grain development. The preferred infection site of *Fusarium graminearum* is the tip of the kernel. Fig. 1.1 shows *Fusarium graminearum*-infected barley and wheat. However, the development of FHB in wheat and barley is different. In wheat, the fungal hyphae develop on the exterior surfaces of florets and glumes, possible leading to direct penetration of the epidermal cell (Bushnell *et al*., 2003). Alternately, the fungus directly enters the stomata and underlying parenchyma, exposed anthers and openings between the lemma and palea of the spikelet (Bushnell *et al*., 2003). Spread of the fungus among florets is through the vascular bundles in the rachis and rachilla (Ribichich *et al*., 2000). It is mainly type I resistance to initial infection rather than type II resistance to spread of infection within a spike in wheat. By contrast, in barley, the fungus penetrates directly stomata and grows from abaxial to adaxial side of floral bracts. The internal spread through the rachis is more limited, indicating the type II resistance (Bushnell *et al*., 2003). It was reported that infection-related structure development and other morphological changes were observed around 12 to 24 h earlier in wheat than in barley (Boddu *et al*., 2006). The fungus can invade the aleurone layer and grow into the starchy endosperm. The light and electron microscopy studies have shown during infection of the endosperm the cell walls were macerated, the protein matrix disappeared and the starch granule structures were changed (Nightingale *et al*., 1999).

Fig. 1.1. Disease symptoms on *F. graminearum*-infected spikelets of barley (left, photographed by Jens Due Jensen, University of Copenhagen) and wheat (right, Goswami and Kistler, 2004). The fourth barley spikelet from the bottom up to six spikelets shows premature necrosis and brown/grey discoloration. The third wheat spikelet from the bottom shows a darkened necrotic lesion whereas the second and fifth spikelets demonstrate tissue beaching (Goswami and Kistler, 2004).

During the penetration of cell wall and access to the plant nutrient for the growth, *F. graminearum* can produce an arsenal of hydrolases such as lipases, xylanases, pectinases, cellulases and proteases as well as the secondary metabolites mycotoxins such as the major trichothecene and zearalenone which are toxic to human or livestock consumption (Kang and Buchenauer, 2000; Maier *et al.*, 2006). These secreted enzymes are known to have an important role in pathogenicity of *F. graminearum* (Kang and Buchenauer, 2000). The toxin trichothecene is involved in blocking peptidyl transferase activity at the 60S ribosomal subunit in eukaryotes. Blocking ribosomal activity can inhibit nucleic acid synthesis and mitochondrial function and interfere initiation, elongation or termination of protein synthesis as well as have negative effect on cell division and membrane integrity (Khachatourians, 1990). The most prevalent trichothecene derivatives deoxynivalenol (DON) levels are regulated in food supplies of many countries. For example, the European Community limits DON levels to 0.5 μg g^{-1} for cereals and the United States limits DON levels to 1 μg g^{-1} for finished products for human consumption (Council for Agricultural Science and Technology, 2003) (Trail, 2009). Although zearalenone which causes estrogenic effects in animals and humans is of concern to the U.S. Food and Drug Administration, there are currently no regulatory standards limiting its levels in grain (Council for Agricultural Science and Technology, 2003) (Trail, 2009). DON is the only mycotoxin shown to be a virulence factor (Trail, 2009) and mediates partially the shift from the biotrophic to the necrotrophic stage of the fungus (Bushnell *et al.*, 2003). The accumulation of DON depends on the complicated interactions between the host and fungal genotypes as well as environmental conditions (Mesterhazy *et al*., 1999). Generally speaking, there is a correlation between FHB severity and DON concentration in the infected grain (Bushnell *et al.*, 2003). *F. graminearum* expresses genes for DON biosynthesis immediately following the infection of wheat (Jansen *et al*., 2005). DON causing tissue necrosis allows *F. graminearum* to spread into the rachis from florets but is not necessary for the initial infection in wheat (Jansen *et al*., 2005; Bluhm *et al*., 2007). It has been reported that trichothecene non-producing *F. graminearum* strain was pathogenic but produced a reduced incidence and severity of infection, less bleaching heads and less spread in spikelets in wheat comparing to the trichothecene-producing strain (Proctor *et al*., 1995; Maier *et al*., 2006). However, no significant difference in virulence was observed in barley between these *F. graminearum* strains (Maier *et al*., 2006). Spread of the disease is limited and virulence does not appear to be due to the presence of the toxin in barley (Jansen *et al*., 2005).

The main approaches for controlling FHB are management practices, fungicide application and development of resistant varieties. Management practices include crop rotation and tillage practice which can reduce the fungal survival on the residue and staggering planting of small grain crops (Stack, 1999). FHB severity can be reduced 50 to 60 % by application of the fungicides at early flowering stage in wheat and at the early heading stage in barley (Stack, 1999). However, results with application of fungicides can be variable due to the environmental effects and relationship between the cost and return is the limit factor (McMullen *et al*.,

1997). Using genetic approach to control FHB is the most desirable option. However, since the resistance in wheat and barley to FHB is a complex and quantitative trait involving the interactions among pathogen, trichothecenes, environments and genotypes, the development of resistant cultivars is very challenging. Current breeding strategies focus on combination of desired agronomic traits and type I and type II resistance in addition to selection for low DON in the kernels (Bai and Shaner, 2004). Quantitative trait loci (QTL) mapping, the statistical study of the alleles occurring at a locus and the phenotypes that they produce using molecular markers is a powerful tool to select resistant plants in the breeding program. So far most of the FHB resistant QTLs have been mapped to the same locations as those associated with morphological traits such as heading date, plant height, lateral floret size, spike angle and kernel plumpness (Bai and Shaner, 2004). QTL analysis is often associated with global gene expression profiling to identify the key gene markers involved in plant defense against infection, providing insights into defense mechanism (Bai and Shaner, 2004). Furthermore, as growth environmental factors such as temperature, humidity and fertilization can affect the disease severity, adjusting time and places of planting or changing the amount and type of fertilizers can be the option to control the disease (Yang *et al*., 2010a). Application of biological control such as microorganisms *Bacillus* spp., yeasts and *Trichoderma harzianum* is an additional strategy in management of FHB in cereals (Corio da Luz *et al*., 2003). Alternatively, transgenic expression of genes for antifungal proteins, genes involved in defense reaction and genes involved in reduction of DON in wheat and barley is an approach against *Fusarium* infection (Dahleen *et al*., 2001).

1.2 Molecular plant-pathogen interactions

Plant-pathogen interactions have been studies for several years in order to understand how plants and pathogens recognize each other and differentiate to establish either a successful or an unsuccessful relationship (Mehta *et al*., 2008). The pathogens may use the following strategies to attack and colonize the hosts: they produce hydrolytic enzymes to degrade the cell wall and break down the protein for nutrients, synthesize molecules that can induce the production of enzyme that degrades cell walls, starch and protein and produce some secondary metabolites like mycotoxins to interfere host metabolism (Bluhm *et al*., 2007). Unlike many phytopathogenic fungi, *F. graminearum* does not produce specialized infection structures such as appressoria or haustoria which are invaginated into the host cell plasma membrane forming an intimate interface during interaction (Jones and Dongl, 2006; Bluhm *et al*., 2007). Instead, colonization of tissues is facilitated primarily by the production of cell-wall-degrading enzymes (CWDEs) such as cellulases, pectinases and xylanases as well as proteases (Bluhm *et al*., 2007).

In plant, the response of extracellular signals form pathogen must be rapid, reliable and specific. The pathogen infection can initiate very complex chains of reactions in plants that lead to various defense responses. The recognition of CWDEs or molecules from pathogens which is called microbial /pathogen-associated molecular patterns (PAMPs) can trigger the plant basal defense including thickening plant cell wall, papilla deposition, transduction of signals such as phytohormones salicylic acid, jasmonates and ethylene to the other parts of the plants and synthesis of antimicrobial compounds like phytoalexins and biosynthesis of pathogenesis-related (PR) proteins and defense-related proteins in both compatible and incompatible interactions (Mehta *et al*., 2008; Pieterse *et al*., 2009). In the incompatible interaction superimposed on the basal defense plants can express disease resistance (R) proteins recognizing the virulence effectors and induce hypersensitive response (HR), which is the second response or gene to gene specific resistance to prevent pathogen invasion and disease development (Jones and Dongl, 2006; Mehta *et al*., 2008) (Fig. 1.2). A series of biochemical perturbations such as ion flues, lipid hyperperoxidation, protein phosphorylation, nitric oxide generation, a burst of reactive oxygen species (ROS) and biosynthesis of antimicrobial compounds are stimulated in HR which keep the pathogen isolated from the rest of the plant and prevent further damage (Mehta *et al*., 2008; Pieterse *et al*., 2009). Moreover, pathogen may also express some proteins such as superoxide dismutase and catalases to overcome the plant defense or to inactivate ROS for protecting themselves. Therefore, the interaction between host plant and pathogen is in a complicated and dynamic manner.

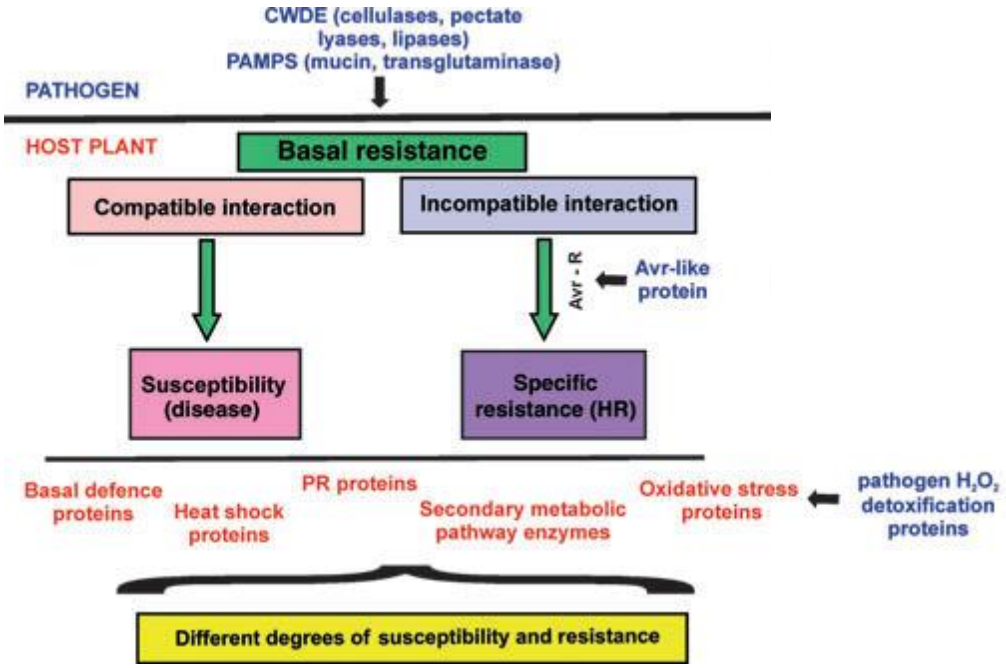

Fig. 1.2. Overview of plant–pathogen interactions. Plants process receptors that can activate basal resistance, mediated by pathogen-associated molecular patterns (PAMPs) or cell-wall-degrading enzymes (CWDEs), which may result in a compatible or incompatible interaction. In both interactions, several defense-related and biotic stress-responsive proteins are induced. Suppression of plant defenses by pathogen effectors leads to susceptibility in host plants. Some host plants express resistance (R) proteins, which guard against this interference and trigger a specific resistance, referred to as the hypersensitive response (HR) (Mehta *et al*., 2008).

Given the essential role in plant defense, PR proteins have been studied for several years from sequence to the biological function properties. PR proteins are usually defined as host-specific proteins that are induced in several plant species during pathological or related situations such as pathogen attack, wounding and abiotic stress (van Loon *et al*., 2006). However, it does not state clearly that they have functional roles in defense (van Loon *et al*., 2006). PR proteins are often low molecular weight proteins (10 to 40 kDa) which can survive and remain soluble in harsh environments such as extreme pH and be resistant to proteolytic cleavage due to their biochemical properties. There are 17 PR families based on amino acid sequences, serological relationship and biological activity (Table 1) (van Loon *et al*., 2006). The families were originally identified from tobacco and also other plant species including barley, wheat, rice and maize and are numbered by the order in which they were discovered. In each family there can be several different isoforms.

Table 1. *Recognized families of pathogenesis-related proteins (modified from van Loon* et al., *2006)*

Family	Type member	Properties
PR-1	Tobacco PR-1a	unknown
PR-2	Tobacco PR-2	β-1,3-glucanase
PR-3	Tobacco P, Q	Chitinase class I, II, IV-VII
PR-4	Tobacco R	Chitinase class I, II
PR-5	Tobacco S	Thaumatin-like
PR-6	Tomato inhibitor I	Proteinase-inhibitor
PR-7	Tomato P_{69}	Endoproteinase
PR-8	Cucumber chitinase	Chitinase class III
PR-9	Tobacco lignin-forming peroxidase	Peroxidase
PR-10	Parsley "PR-1"	Ribonuclease-like
PR-11	Tobacco "class V"chitinase	Chitinase, type I
PR-12	Radish Rs-AFP3	Defensin
PR-13	Arabidopsis THI2.1	Thionin
PR-14	Barley LTP4	Lipid-transfer protein
PR-15	Barley OxOa (germin)	Oxalate oxidase
PR-16	Barley OxOLP	Oxalate-oxidase-like
PR-17	Tobacco PRp27	Unknown

.

The specific functions of PR proteins are not fully understood. Various PR proteins have potential antimicrobial activity and are involved in defense mechanisms against pathogens (van Loon *et al*., 2006). Chitinase and β-1,3-glucanase have functions involved in the hydrolysis of fungal cell walls. Peroxidase is an antioxidant and can function in plant cell wall rigidification. Oxalate oxidase is involved in signal transduction (Christensen *et al*., 2002). PR proteins can also be detected during plant development and senescence (van Loon *et al*., 2006), which may indicate a more physically protective role of the cellular structures in order to stabilize sensitive membranes or macromolecules (van Loon and van Strien, 1999). Genetic engineering of plants for introduction of PR genes by transformation or manipulation of the signals that trigger the expression of PR proteins may be the approaches to improve plant resistance against pathogen infection. Transgenic wheat and barley expressing genes encoding chitinase, *α*-thionin, thaumatin-like protein or β-1,3-glucanase have shown the enhanced resistance against *Fusarium graminearum* (Dahleen *et al*., 2001; Mackintosh *et al*., 2007; Shin *et al*., 2008).

1.3 Technologies in proteomics

Genome only represents the first step in the complexity of understanding biological function. Transcripts can not give complete information on cellular regulations as gene expression is regulated post-transcriptionally and proteins which are responsible for the cell biological functions are expressed in a highly dynamic and interacted manner (Dhingra *et al*., 2005). Thus, it is necessary to determine the protein levels directly. Proteomics is the systematic study of all the proteins expressed by a genome or by a cell or tissue, particularly their interactions, modification, localization and functions (Coiras *et al*., 2008). Currently, proteomics has established itself as an indispensable technology to interpret the information from genomics and has been most successfully applied in protein sequencing, protein quantification, post translational modifications (PTMs) and protein interactions (Aebersold and Mann, 2003).

1.3.1 Proteomics workflow-protein preparation, separation and identification

Proteomics workflow mainly consists of protein preparation, protein separation and protein identification by mass spectrometry (MS). Protein preparation includes tissue and cell homogenization, protein solubilisation and denaturation with use of detergents such as 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, sodium dodecyl sulphate (SDS), urea and thiourea and removal of impurity in samples such as carbohydrates, lipids, salts, nucleic acids, *etc* which can interfere protein separation process. It is impossible to obtain the entire proteome at once since cellular protein populations have enormous diversity due to function, sequence, physical properties and relative abundance (Hurkman and Tanaka, 2007a). Proteins extracted strongly depend on the extraction protocols. The extraction of protein from plant samples is in particular challenging, because plants cells generally contain i) low amounts of proteins protected by cell walls that require extreme measures to disrupt, ii) proteases that remain active in the extraction buffer, reducing and altering protein populations and iii) various non-protein components such as cell wall, storage polysaccharides, lipids, phenolics, salts, nucleic acids and a broad array of secondary metabolites, which cause streaking and smearing of 2-DE patterns in the following separation procedure (Hurkman and Tanaka, 2007b). Therefore, the extraction methods should minimize protein degradation and eliminate non-protein components, which are the key steps for proteomic analysis, especially when 2-DE is used. In our study cases, the majority of barley seeds proteome is non-metabolic storage protein hordein which is alcohol-soluble. In order to access to metabolic albumins, protein was extracted at low salt and neutral pH followed by acetone precipitation for concentration and cleanup of samples (Østergaard *et al*., 2004)

The traditional approach for separating proteins or peptides is two-dimensional gel electrophoresis (2-DE) separating hundreds of proteins according to their pI and mass (gel-based approach). Although 2-DE technique is quite clear and reproducible to show a full picture of protein pattern, it has some limitations such as solubilisation of membrane proteins, invisibility of very low abundant proteins and segregation of extreme pI and mass (Ong and Mann, 2005). Poor separation of basic proteins due to streaking of spots is also a limiting factor in 2-DE (Bae *et al*., 2003). Currently the most powerful strategy is mono- or multi-dimensional liquid chromatography (LC) which allows high throughput separation of complex protein or peptides mixtures (gel-free approach) (Coiras *et al*., 2008). LC separates proteins and peptides according to their affinity for a stationary phase when a mobile phase is forced through a fine capillary.

Mass spectrometry consists of an ion source, a mass analyser that measures the mass to charge ratio (*m/z*) of the ionized analytes and a detector that registers the number of ions at each *m/z* value (Aebersold and Mann, 2003). The most common techniques for ionizing samples are matrix assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). The mass analysers include time-of-flight (TOF), ion trap, quadruple and fourier transform ion cyclotron (Aebersold and Mann, 2003). They can be used alone, called MS, or put together in tandem, namely MS/MS. In single mass analyser peptide ions are generated. In MS/MS specific precursor ions produced in the initial mass analyser are chosen and fragmentized through collision resulting in fragment ion spectra.

In general, 2-DE is often followed by MALDI-TOF or TOF-TOF mass spectrometry for analysis of relatively simple protein samples which are crystallized with matrix before ionization via laser pulses in MS. This instrument has high sensitivity, resolution and mass accuracy (Aebersold and Mann, 2003). LC is often coupled to ESI-tandem mass spectrometry (MS/MS) for analysis of samples in solution. This method can detect low abundant proteins and analyse proteolysed peptides without fractionation of protein samples comparing to 2-DE-based approach. The strategy in MALDI MS for protein identification is peptide mass fingerprinting which requires purified protein samples. A list of peptide masses of protein generated by MS is searched against a database which supplies theoretical peptide masses of proteins. Additionally, the fragment ion masses from selected precursors can be used to match against protein sequence database when MS/MS instrument is used. The identification is based on matching score calculated by software algorithms. The MS/MS data enables *de novo* sequencing and PTM analysis (Coiras *et al*., 2008).

1.3.2 Quantitative proteomics

In the term of relative quantification of protein, there are two major approaches. The first one is gel-based quantitative studies relying on protein labelling for later image comparison (Fig.1.3). The second one is gel-free

techniques making use of isotopic or isobaric labelling of proteins or peptides for LC-MS/MS analysis (Gstaiger and Aebersold, 2009) (Fig.1.3).

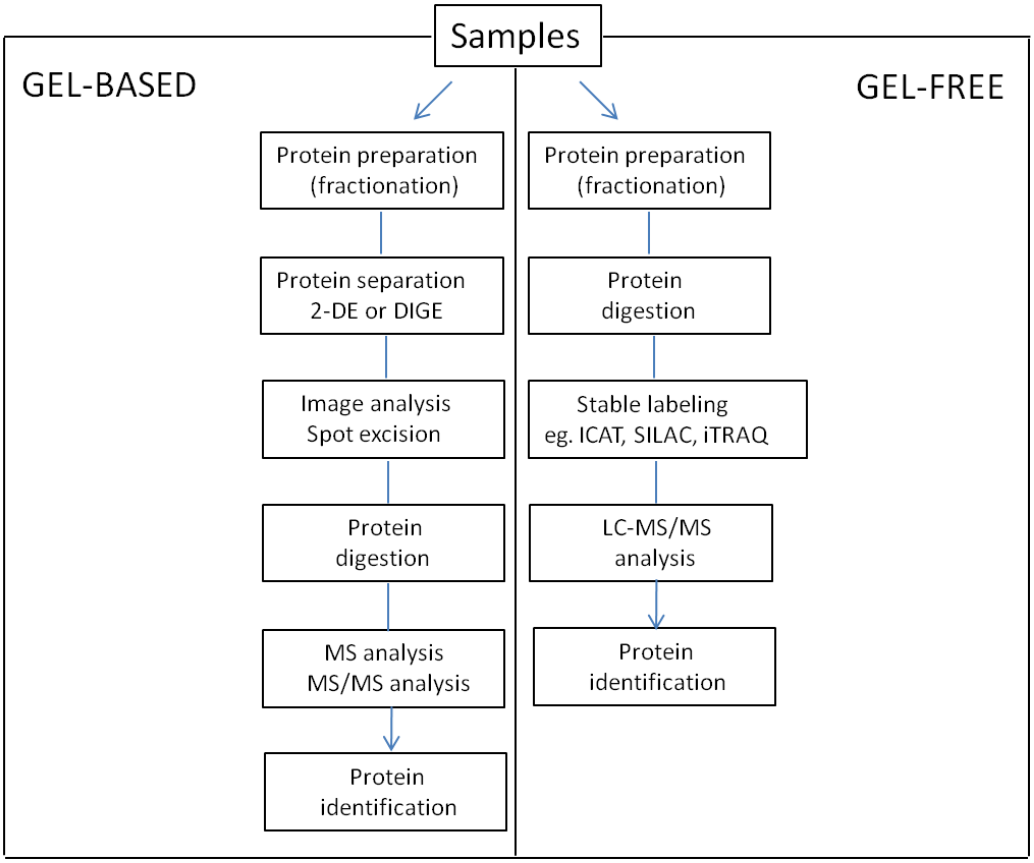

Fig. 1.3. Overview of quantitative proteomics workflow. Gel-based approach is based on image analysis of protein spot intensity. Gel-free approaches such as ICAT, SILAC and iTRAQ are based on isotope or isobaric labelling. The mass shift among the labelled peptides form different samples will be revealed in MS or MS/MS which allows identification and quantification of proteins.

Briefly, in gel-based approach, proteins are separated by 2-DE and stained by dye. Besides Coomassie Brilliant Blue staining, there are several fluorescent staining methods that have been developed for the visualization of 2-DE patterns and detection of less abundant proteins, including sypro ruby staining, silver staining and Cy-dyes. Sypro ruby staining has a comparable sensitivity with silver staining and allows much higher reproducibility, wider dynamic range, less false-positive staining (Berggren *et al*., 2000). Alternately, protein samples are labelled with different fluorescence dyes (Cy2, Cy3 or Cy5) reacting with lysine in the protein and separated in one 2-DE gel, which will be fluorescent according to the wavelengths. This technique

called differential gel electrophoresis (DIGE) is quite sensitive to reveal the protein amounts, has dramatically improved the reproducibility and accuracy of quantification, and avoids the gel-to-gel variation in the traditional 2-DE which analyses multiple samples in one gel (Unlu *et al*., 1997; Coiras *et al*., 2008). The 2-D gels are scanned and gel images will be imported into gel analysis software for spot detection, pattern matching, spot quantification and statistic analysis. The spots significantly changing in intensity among samples will be shown in this software automatically.

Given the requirement of good resolution of 2-D patterns and poor correlation between spots intensity and protein abundance in gel-based approach, MS-based quantification techniques by introduction of stable isotope labelling to peptides have emerged. The isotope-code affinity tag (ICAT) is one of the most employed chemical isotope labelling methods. Each ICAT reagent consists of a thiol reactive group that reacts with cysteine residues, an isotope-coded light or heavy linker and a biotin segment for affinity purification. In this system, two samples are labelled with light and heavy reagents on cycteine thiols, respectively. Then both samples are mixed, digested by trypsin and separated through chromatography. The relative level of protein in the two samples is determined by the ratios of signal intensities of the isotopically labelled peptide pairs revealed in MS analysis (Sethuraman *et al*., 2004). Different software programs such as proICAT are developed to analyse ICAT labelled MS data. ICAT is very useful to detect low abundant proteins but has major limitation in only selecting protein of high cycteine content and low sensitivity to acidic proteins (Gygi and Aebersold, 2000). Another strategy for determining the differentially expressed proteins from different cellular populations is the stable isotope labelling with amino acids in cell culture (SILAC) (Ong and Mann, 2005). The cells from different biological conditions are cultured with media supplemented with isotopically labelled amino acids. After metabolic incorporation of isotopes during protein synthesis, proteins from each sample are isolated, mixed, digested and analysed by LC-MS. The ratio of speak intensities of the isotopically labelled peptides in the mass spectrum reflects the ratio of the protein abundance. This technique is *in vivo* coding without chemical manipulation and allows comparison of expression levels of tissue proteome at different physiological states (Gruhler *et al*., 2005). Recently, a new approach called isobaric tags for relative and absolute quantitation (iTRAQ) has developed (Ross *et al*., 2004). The technique is based on chemically tagging the N-terminus of the digested peptides. The labelled samples are combined, fractionated by LC and analysed by MS/MS. Fragmentation of the tags attached to the peptides generates the isotope-encoded reporter ions which provide relative quantitative information on protein. This technology offers several advantages including the ability to analyse multiplex samples and increased analytical precision and accuracy, but it need a powerful multidimensional fractionation method for peptides before MS/MS (Aggarwal *et al*., 2006). Another method is ^{18}O stable isotope labelling where proteins are isotopically tagged by means of enzyme-catalysed incorporation of ^{18}O from ^{18}O water during proteolysis (Aebersold and Mann, 2003). Each peptide generated by the enzymatic reaction carried out in heavy water is

labelled at the carboxyl terminal. The labelled peptides from each sample are combined and analysed by MS. The resulting mass shift between differentially labelled peptide ions permits identification and quantitation of protein. Furthermore, there is label-free quantification strategy present which is based on spectral counting or peptide precursors ion intensities obtained in the first MS in a tandem MS. Spectral counting is based on the assumption that the rate at which a peptide precursor ion is selected for fragmentation in a mass spectrometer is correlated to its abundance. The spectral counts are then averaged into a protein abundance index for relative protein quantification. This method works for large and abundant proteins, the number of peptides from small protein and low abundant proteins is often insufficient for accurate quantification (Gstaiger and Aebersold, 2009).

1.3.3 Analysis of PTMs

Covalent modification of protein such as phosphorylation, glycosylation, acetylation and ubiquitin plays important roles in the control of the activity, localization and stability of proteins and their interactions with other macromolecules (Gstaiger and Aebersold, 2009). Functional genomics can not provide experimental evidence for protein modifications other than protein sequence information for the *in silico* prediction of candidate sites of modification (Gstaiger and Aebersold, 2009). MS is shown to be a useful tool for global and targeted analysis of PTMs of proteins. The principle is that the addition of a chemical moiety to an amino acid will lead to the mass shift of that residue, which will be revealed in MS. This will allow assignment of modifications to the peptide in MS or amino acid in MS/MS (Jensen, 2006). Computer programmes are continuously improved for the systematic scanning and annotation of PTMs from MS and MS/MS data. However, identification of PTM is quite challenging since the peptides bearing a particular modification can be a small fraction of the total amount of peptides in the samples (Dhingra *et al*., 2005; Gstaiger and Aebersold, 2009). Therefore, modification-specific enrichment is usually integrated prior to MS and MS/MS analysis (Dhingra *et al*., 2005). Some PTMs can be enriched by derivatization of protein modifications to make them accessible to chemical solid-phase capture techniques, and other PTMs can be purified using affinity chromatography or antibodies that are specific for a given modification (Gstaiger and Aebersold, 2009). For instance, phosphopeptides can be enriched by immunoprecipitation and TiO_2 column or immobilized metal-affinity chromatography which is using Fe (III) or Ga (III) for affinity of the phosphate moiety (Jensen, 2006). Hydrophilic-interaction liquid chromatography and lectin-mediated affinity are useful methods for purification of glycopeptides (Jensen, 2006).

1.3.4 Analysis of protein-protein interaction

Almost all the proteins function in the context of specific interactions with other proteins. In MS-based protein interaction experiments, there are three essential components including bait presentation, affinity purification of the complex and analysis of the bound proteins by LC-MS/MS (Aebersold and Mann, 2003). The workflow is that protein complexes are purified either using antibodies that recognize the endogenously expressed protein or using an affinity tag that is fused to the protein of interest, digested and analysed by LC-MS/MS. Compared with yeast two-hybrid and protein chip-based approaches, this strategy has the advantages that interactions take place in the native environment and cellular location, and that multicomponent complexes can be isolated and analysed in a single operation. However, this method can only detect a subset of protein interactions that actually occur since the biological interactions are of low affinity, transient and dependent on the specific cellular environment (Aebersold and Mann, 2003).

1.3.5 Limitations and expectations in proteomics

The proteomics research today is severely hampered by the lack of publicly available sequence information for the not completely sequenced organisms since protein identification is based on availability of the gene or protein sequences in the public databases (Grossmann *et al*., 2007). Peptide mass fingerprint is not well suited for protein identification for these organisms. In order to circumvent this limitation, two different approaches can lead to an increase in protein identifications. The first one is that MS or MS/MS data is searched against Expressed Sequence Tags (EST) database or a protein database of an evolutionarily closed related organism, although EST databases can have only partial coding sequences for the gene and many genes may not be represented in these databases due to the choice of tissue used for library construction or low mRNA abundance (Quirino *et al*., 2010). The second one is amino acid sequence of a peptide extracted from the MS/MS spectrum for *de novo* sequencing with aid of software tools. Peptide *de novo* sequencing can be combined with BLAST searches to identify peptides on the basis of their homology to peptides in the database (Grossmann *et al*., 2007). Other major bottlenecks in proteomics are related to analysis of huge amounts of MS data. Accurate, consistent and transparent data processing and analysis are integral and critical parts of proteomics workflows (Domon and Aebersold, 2006). Therefore, advanced bioinformatics tools are required for protein identification and validation and data repositories, which are challenging and ongoing tasks in proteomics.

1.4 Application of proteomics in plant-pathogen interaction

The proteomics techniques are mainly utilised in the following aspects for plant-pathogen interactions including plant-virus, bacteria, fungi and nematodes interactions: a) detection of plant pathogens; b) comparison of proteomes and detection of differential protein expression at quantitative and qualitative terms in both plant and pathogen; c) analysis of PTMs like phosphorylation or modification of proteins induced by the infection. In this thesis, we will mainly focus on and give the examples of researches on the plant-fungus interactions.

Traditionally, detection of plant pathogens may involve the use of time-consuming cultivation with a subsequent morphological or biochemical analysis of growth or biochemical characterization, bioassays, isolation and microscopy (Lopez *et al*., 2003). However, these techniques cause problems when different organisms produce similar symptoms in hosts or exhibit similar morphology. Lately, enzyme-linked immunosorbent assays, polymerase chain reaction, DNA sequencing, fluorescence *in situ* hybridization and DNA microarrays are the main techniques for phytopathogen detection (Lopez *et al*., 2003; Padliya and Cooper, 2006). There are limitations to molecular biology-based or antibody-based techniques as many of these protocols require reagents that are highly specific for individual pathogens (Kav *et al.*, 2007). The recent proteomics-based technology has been shown to be able to detect or identify phytopathogens accurately and efficiently which do not require pathogen-specific reagents (Kav *et al.*, 2007). The technique is based on the identification of phytopathogen protein in MS analysis which typically is using various publicly available protein databases with information on phytopathogenic organisms including virus, fungi, oomycete and bacteria (Padliya and Cooper, 2006). Thus, a relative paucity of data in genomic or protein databases pertaining to many pathogens is a significant obstacle in MS-based studies, but it can be solved partially by cross-species identification (Padliya and Cooper, 2006). With regard to pathogenic fungi, there are three other complications with identification by MS comparing to virus and bacteria (Padliya and Cooper, 2006). First, unpredictable PTM can confound the protein identifications. Second, pathogenic fungus can encode several thousands of proteins but no single protein will accumulate to high levels for good resolution in MS, especially with a complex plant protein background. Third, fungi can have several life cycle stages, indicating that the presence of some proteins used in identification can be in flux. So far, MS-based techniques have been successfully applied in the detection of phytopathogenic fungi such as *Ustilago maydis*, *Trichoderma harzianum*, *Uromyces appendiculatus*, *Phytophthora palmivora* and *Phytophthora infestans* (Shepherd *et al*., 2003; Ebstrup *et al*., 2005; Cooper *et al*., 2006; Padliya and Cooper, 2006).

In the plant-pathogen interaction system, it is still very challenging to study proteome of pathogen *in planta* since the biomass of the pathogen is a small portion of the total in the infected plant resulting in the dominance

of plant proteins. However, there is some achievement in the study of morphogenesis-, host- and signaling-responsive protein and proteome mapping in fungal phytopathogens by proteomic techniques. *M. grisea* causing rice blast disease, one of the most damaging diseases of rice, forms an appressorium from a germinating conidium allowing the infection peg to penetrate the rice cuticle (Kim *et al*., 2004b). Due to the vital function of the appressorium in disease initiation, gel-based proteomics was used to identify proteins during formation of the appressorium, revealing five proteins including 2 α-subunits of the 20S proteasome, serine carboxypeptidase Y and scytalone dehydratase (Kim *et al*., 2004b). The changes of the extracellular and intracellular proteomes of *M. grisea* were examined when exposed to extracts from resistant and susceptible rice cultivars (Kachroo *et al*., 1997). Protein spots induced by susceptible cultivar extracts were observed but not identified due to the limited availability of gene sequences at the time of study. *U. maydis* is the causal agent of smut in corn which undergoes a dimorphic transition from budding to form infective filamentous hypea (Bohmer *et al*., 2007). By using 2-DE, 250 different proteins were identified from cells of the fungus *U. maydis* cultured *in vitro*. In addition, in this study it was observed that 13 proteins involved in energy and general metabolism were upregulated during the filamentous growth stage and signaling pathway involving a small GTP binding protein is responsible for the generation of the filament during pathogenic development (Bohmer *et al*., 2007). The role of signal transduction in the pathogenicity of *S. nodorum* which causes glume blotch in wheat is well established (Tan *et al*., 2008). The Gna1 protein of cAMP pathway is of particular interest, as mutants displayed multiple phenotypic impairments such as reduced virulence, reduced extracellular depolymerase activities and abolished asexual sporulation (Tan *et al*., 2009). In order to identify the Gna1-regulated proteins, comparison of 2D proteome patterns of wildtype and *gna1* mutant coupled with LC-MS/MS was conducted, resulting in identification of positively regulated short-chain dehydrogenase which has critical roles in asexual sporulation and mycotoxin production (Tan *et al*., 2008, 2009). Cooper *et al*. (2006, 2007) has identified 468 and 461 proteins from uredospores and germlings, respectively, of *U. appendiculatus*, the rust fungus occurring on beans by LC-MS/MS. Both proteomes contained proteins involved in protein biosynthesis and folding, suggesting that spores and germlings become metabolically active primed by protein accumulation during infection. The proteome maps of *B. cinerea* mainly infecting wine grapes and *S. sclerotiorum* causing a disease called white mold in many flowers and vegetables were established (Fernandez-Acero *et al*., 2006; Yajima and Kav, 2006). A comparison of the mycelial protein profiles of *B. cinerea* strains differing in toxin production revealed differentially expressed malate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase between stains (Fernandez-Acero *et al*., 2006). The mycelial proteome and the secretome of *S. sclerotiorum* were analysed, resulting in identification of approximately 100 mycelial protein and 18 secreted proteins including cell-wall-degrading enzymes (Yajima and Kav, 2006). There are some proteomic studies on *F. graminearum*. It was shown by 2-DE and MS that the *in vitro* exoproteome of *F. graminearum* grown on glucose and on hop cell

walls contained 23 and 84 unique proteins, respectively, mainly involved in cell wall polysaccharide degradation (Phalip *et al*., 2005). By high-throughput LC-MS/MS, 229 and 120 fungal proteins, mainly including glycoside hydrolases and proteases, were identified in the secretome of *F. graminearum* during growth on 13 synthetic media with carbon supplements and during infection of wheat heads, respectively (Paper *et al*., 2007). A gel-based proteomic approach was employed to identify *F. graminearum* proteins secreted to culture medium containing barley or wheat grain flour, revealing 155 fungal protein identifications in 69 unique proteins in either medium which mainly included enzymes involved in degradation of cell walls, starch and proteins (Yang, unpublished data, see Chapter 4).

With regard to the plant response to pathogens, it has been found that proteins involved in diverse biological processes including defense and stress response, signal transduction, photosynthesis, protein folding and degradation and energy metabolism are regulated (Thurston *et al*., 2005; Mehta *et al*., 2008). Some examples reporting these proteins are mentioned here. The *M. grisea*-rice interaction has been well studied because of its great economic importance and availability of both genome sequences (Mehta *et al*., 2008). Gel-based proteomic analysis of rice leaves including the resistant and susceptible lines infected by *M. grisea* showed the induction of two receptor-like protein kinases, two β-1,3-glucanases, thaumatin-like protein, peroxidase, probenazole-inducible protein and rice PR10 protein in both lines (Kim *et al*., 2004a). Callose deposition and hypersensitive response was clearly visible in incompatible interactions but excessive invading hypha with branches was evident only in compatible interactions in this study (Kim *et al*., 2004a). It was reported that susceptibility of rice to rice blast disease increased with the excessive application of nitrogen nutrients (Long *et al*., 2000). Therefore, a study about effect of nitrogen nutrients on rice blast disease by proteomic approach was conducted, suggesting that proteins involved in photosynthesis was affected in the interaction and twelve proteins changed in response to different levels of nitrogen nutrient. Among these proteins, level of ribulose-1,5-bisphosphate carboxylase/oxygenase was increased with higher level of N (Konishi *et al*., 2001). Protein profiles of blackleg-resistant and susceptible canola cultivars after inoculation with *Leptosphaeria maculans* were investigated using 2-DE and tandem MS. Several antioxidant enzymes, including dehydroascorbate reductase and peroxiredoxin along with proteins involved in photosynthetic and nitrogen metabolism were found to be upregulated in the resistant cultivar compared to the susceptible cultivar (Subramanian *et al*., 2005). Different *Fusarium* species can cause different diseases in a diversity of plant hosts. Proteome analysis of the xylem sap of tomato in response to *Fusarium oxysporum* infection revealed accumulation of PR proteins such as glucanases, peroxidases and chitinases, polygalacturonase and a subtilisin-like protease, which were involved in defense, antioxidant protection and cell structure, as well as seven fungal proteins including arabinanase, oxidoreductase and serine protease (Rep *et al*., 2002; Houterman *et al*., 2007). Gel-based proteomics was performed to study the changes in the protein profiles of germinating maize embryos following infection by *Fusarium verticillioides*,

leading to the identification of PR proteins, antioxidant enzymes and protein involved in protein synthesis, folding and stabilization (Campo *et al*., 2004). Several proteome analysis of barley and wheat in response to *Fusarium graminearum* infection showed the induction of plant proteins associated with oxidative stress or pathogenesis-related responses and changes of abundance of the proteins involved in primary metabolism and protein synthesis (Zhou *et al*., 2006; Geddes *et al*., 2008; Yang *et al*., 2010b). In addition, transcriptome and metabolome analysis have been performed in *Fusarium graminearum*–barley or wheat interaction to gain more insights into the plant defense response to this pathogen. Microarray analysis of *Fusarium graminearum*-infected barley showed induction of plant genes encoding defense response proteins, oxidative burst-associated enzymes, phenylpropanoid pathway enzymes, and trichothecene and tryptophan catabolic enzymes (Boddu *et al*., 2006). Metabolome analysis of wheat infected by *Fusarium graminearum* indicated the higher abundance of several fatty acids and aromatic compounds in both susceptible and relatively resistant cultivars whereas coumaric acids, myo-inositol, certain sugars and malonic acid were only found in the relatively resistant cultivar (Hamzehzarghani *et al*., 2005).

Protein phosphorylation is considered as one of the most important PTMs because protein phosphorylation controls many basic cellular processes such as cell growth, differentiation, migration, metabolism, and cell death, and activates signal transduction pathways in cells in response to different stimuli such as growth factor stimulation or exposure to biotic and abiotic stress (Thingholm *et al*., 2009). The phosphorylated plant proteins in response to infection could be antioxidative enzymes or be involved in the early step of signal transduction pathways and located in the plasma membrane for the perception of a variety of microbial elicitors (Thurston *et al*., 2005). Particularly, the phosphorylation of plasma membrane proteins has wide-ranging implications for research in signal transduction, cell-cell communication and membrane transport processes, all of which are intimately in plant-microbe interactions (Thurston *et al*., 2005). For example, by using ^{32}P pulse-labelling in conjunction with 2DE and MS several phosphorylated proteins were identified including AtPhos43 protein in suspension-cultured cells of *Arabidopsis* in response to bacterial and fungal elicitors (Peck *et al*., 2001). The phosphorylation of AtPhos43 is dependent on FLS2, a receptor-like kinase involved in signalling pathway for the perception of microbial elicitors. It has been reported that Ca^{2+}-ATPase on the endoplasmic reticulum is involved in race-specific defense responses in tomato-*Cladosporium fulvum* interactions (Lam *et al*., 1998). However, PTMs which are very fast acting and dynamic mechanisms upon pathogen attack make it challenging to follow the early *in vivo* events (Thurston *et al*., 2005).

1.5 Objectives of the project

The aim of the project is to gain some insights into control of FHB in barley with the application of gel-based proteomic techniques. In order to achieve the goal, we conducted the experiments from three different aspects: i) It has been reported that type and amount of nitrogen fertilizer can affect the incidence and severity of FHB with different results. Therefore, we have investigated the severity of FHB in the susceptible barley using two different amounts of nitrogen fertilizer in the first study (Chapter 2); ii) In order to study the molecular mechanisms of barley defense response to *Fusarium graminearum*, in the second study a proteomic analysis was conducted during the initial stages of the interaction due to the extensive degradation of barley seed proteome caused by the pathogen at mature stage (Chapter 3); iii) We would like to investigate the proteome of the pathogen, especially secreted proteins known for a role in pathogenicity during interaction. However, it is very challenging to identify fungal proteins in the infected plants since fungal biomass is a small portion of the total. Therefore, in the third study we profiled the *in vitro* secreted proteome of *F. graminearum* on the 2-D gels in the presence of substrates of barley or wheat grains (Chapter 4). In addition, a PR17 protein was found to be upregulated in barley in response to *F. graminearum* in the second study and a fungal hypothetical protein (protein accession no: FG11033) was strongly upregulated in *YAP*- or *AP*-knockout *F. graminearum* mutant compared to wildtype (collaboration with Jens D. Jensen, see Appendix II), but the biological function of these two proteins is still unknown. Thus, recombinant proteins were made and bioassays were performed to examine their biological activity (Chapter 5). In order to correlate the proteome to transcriptome of *Fusarium graminearum*, microarray data of *Fusarium graminearum* during infection of barley and wheat was obtained from Prof. Corby Kistler and analysed (Chapter 6).

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Chapter 2

Investigation of the effect of nitrogen on severity of Fusarium Head Blight in barley

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Investigation of the effect of nitrogen on severity of Fusarium Head Blight in barley

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ABSTRACT

The effect of nitrogen on Fusarium Head Blight (FHB) in a susceptible barley cultivar was investigated using gel-based proteomics. Barley grown with either 15 or 100 kg ha^{-1}N fertilizer was inoculated with *Fusarium graminearum* (*Fg*). The storage protein fraction did not change significantly in response either to N level or *Fg*, whereas eighty protein spots in the water-soluble albumin fraction increased and 108 spots decreased more than two-fold in intensity in response to *Fg*. Spots with greater intensity in infected plants contained fungal proteins (9 spots) and proteolytic fragments of plant proteins (65 spots). Identified fungal proteins included two superoxide dismutases, L-xylulose reductase in two spots, peptidyl prolyl cis–trans isomerase and triosephosphate isomerase, and proteins of unknown function. Spots decreasing in intensity in response to *Fg* contained plant proteins possibly degraded by fungal proteases. Greater spot volume changes occurred in response to *Fg* in plants grown with low nitrogen, although proteomes of uninfected plants were similar for both treatments. Correlation of proteome changes with measurement of *Fusarium*-damaged kernels, fungal biomass and mycotoxin levels indicated that increased *Fusarium* infection occurred in barley with low N and suggests control of N fertilization as a possible way to minimise FHB in barley.

1. Introduction

Fusarium Head Blight (FHB) or scab, caused by *Fusarium* species including *Fusarium graminearum* (*Fg*) Schwabe (teleomorph: *Gibberella zeae* (Schwein) Petch) in humid and semihumid climates, is a devastating disease in wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) and other cereals and has the capacity to destroy a potentially high yield [1–3]. The disease reduces the grain yield due to floret sterility as well as poor grain filling and reduced kernel size [4]. In addition to deceased yield and quality, the infected grains often contain mycotoxins like deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA) which are hazardous to animals and humans causing neurological disorders and immunosuppression due to inhibition of protein biosynthesis [5].

The pathogen *Fg* attacks the barley spikes after they emerge from the flag leaf sheath in the late-milk to soft-dough stages of seed development [6]. Disease symptoms

Abbreviations: BBBI, Barley Bowman-Birk inhibitor; DON, Deoxynivalenol; FDK, Fusarium-damaged kernels; Fg, Fusarium graminearum; FHB, Fusarium Head Blight; NIV, Nivalenol; PCA, Principal component analysis; ZEA, Zearalenone.

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include premature necrosis and a brown/grey discolouration of spike tissue. In contrast to wheat, where the fungus spreads between spikes of the ear through the rachis, the fungus does not spread in barley [2,7].

It is highly challenging to control FHB in barley and wheat due to the poor understanding of the mechanisms of plant resistance, and since no highly resistant barley cultivar is yet available [2]. Development of FHB-resistant cultivars is a high-priority for many barley breeding programs worldwide. Agronomic and crop management strategies aimed at controlling FHB in wheat and barley include foliar fungicide application, crop rotation and tillage practices, however these are in general not highly effective [8–10]. Environmental factors play an important role in pathogenesis. High temperature and humidity levels (e.g. heavy dew) favour fungal attack and disease development [11,12].

Several studies, all carried out in Canada, suggest that the type and amount of nitrogen fertilizer can affect the incidence and severity of FHB, however the observed effect on the disease differed. Thus, in wheat and barley grown with an initial application of 70 kg ha^{-1}N (ammonium nitrate) at seedling stage, a significant increase of FHB was observed on plants supplied with an additional 50 kg ha^{-1} at Zadoks' growth stages 30 and 45, in comparison with plants receiving no supplement [10]. On the other hand, wheat grown in fields with 90 kg ha^{-1}N (ammonium nitrate, urea or both) or less showed significantly higher levels of FHB than those with more N [13]. It was also reported that wheat grown in clay loam or sandy loam soil without application of N had a greater incidence of FHB than with 100 kg ha^{-1}N (ammonium nitrate) [4]. In maize, soil N amendment with 100 kg ha^{-1} ammonium nitrate decreased both disease severity and levels of DON [14]. It is clear from these results that more research is required to determine how the N status of the host plant may influence the interaction with *Fg*.

Transcriptome profiling using microarrays, metabolome profiling by aid of GC/MS and proteome profiling have been used to investigate plant responses during the infection by *Fg* [2,3,7,15,16]. Microarray analysis of the barley–*Fg* interaction showed induction of plant genes encoding defence response proteins, oxidative burst-associated enzymes, phenylpropanoid pathway enzymes, and trichothecene and tryptophan catabolic enzymes [7]. Metabolome analysis of wheat infected by *Fg* indicated the higher abundance of several fatty acids and aromatic compounds in both susceptible and relatively resistant cultivars after infection whereas coumaric acids, myo-inositol, certain sugars and malonic acid were only found in the relatively resistant cultivar [15]. Gel-based proteome analysis of barley and wheat in response to *Fg* infection indicated that plant proteins associated with oxidative stress or pathogenesis-related responses were induced whereas proteins involved in photosynthesis and carbon metabolism decreased in abundance [2,3,16]. Additionally, proteome analysis was applied to *Fg* grown *in vitro* on synthetic media and *in planta* during infection of wheat head. In that study, 120 fungal proteins including secreted proteins and housekeeping enzymes were identified by LC-MS/MS *in planta* [17].

Proteomics is a useful approach for studying plant–pathogen interactions as differentially expressed proteins directly involved in plant–pathogen responses can be detected by comparing protein profiles [2,16,18]. Barley seed proteomes and their genetic and developmental variations have been described in some detail [19] and include the identification of several hundred proteins by mass spectrometry. This knowledge provides a solid background on which to base an investigation of the combined effect of N fertilization and FHB on the barley seed proteome.

In the present study, 2-DE followed by MALDI-TOF mass spectrometry was employed to examine changes in the proteome of mature barley seeds caused by *Fg* infection under different levels of N fertilization, providing the first molecular insight into the effect of N on FHB infection of barley. The proteome changes were correlated with quantification of mycotoxins and fungal biomass and included identification of some fungal proteins not previously observed

The results suggest that, even though the difference in nitrogen level does not appear to greatly affect abundance or composition of grain albumins and storage proteins, the severity of *Fg* infection increases significantly in plants grown with low N.

2. Materials and methods

2.1. Plant growth

Barley (cv. Scarlett) from a single batch was grown in plastic pots containing soil:sand:peat 2:1:1 (w/w/w) and necessary micro- and macronutrients with or without addition of nitrogen fertilizer (ammonium nitrate) resulting in nitrogen levels of around 100 (high N) or 15 kg ha^{-1} (low N). After germination, excess seedlings were removed and twelve seedlings per 8-litre pot (25 cm diameter) were grown to maturity in an unheated greenhouse under natural light conditions at 15–22 °C during the day (12 h) and 10–15 °C during the night (12 h). Sufficient water was supplied using a drip watering system. The leaf water potential was determined using a pressure chamber and watering was adjusted to a level between 0 and −0.5 mPa. Three biological replicates, consisting of one pot each, were prepared for *Fg* and control inoculations under low and high nitrogen levels.

2.2. Inoculum preparation and spike inoculation

F. graminearum R-77550 was grown on potato-dextrose agar (PDA) for 2 weeks before inoculation of mung bean broth (MBB) for macroconidia production. MBB was made by mixing 1 L boiling water with 40 g of mung bean seeds for 10 min followed by filtration through two layers of cheesecloth to remove seeds before autoclaving. MBB was then inoculated with 10 plugs (0.5 cm) of the *Fg* PDA culture and incubated on a shaker (200 rpm) at 26 °C in darkness for 4 days. Macroconidia were harvested by filtering the culture through two layers of cheesecloth to remove mycelium. The concentration of macroconidia was adjusted to 5×10^4 spores/mL with deionised water containing 0.1% Tween 20. Inoculation of barley spikes was conducted by applying 5 mL of inoculum at the anthesis stage 65 [20] using a "handsprayer". Control plants were mock-inoculated with water. After inoculation, spikes were kept under sealed plastic for 72 h. At least five spikes

were harvested at maturity stage for each replicate. The percentage of *Fusarium*-damaged kernels (FDK) was determined based on kernel colour and degree of shrivelling for each grain [21].

2.3. *F. graminearum* biomass determination

Kernels were ground in a cooled mill (4 °C) for 30 s and the flour was used for fungal biomass determination, mycotoxin analysis and protein extraction. *Fg* DNA was extracted from 100 mg of flour using a CTAB protocol followed by partial purification of DNA on a Qiagen DNeasy plant mini kit as described [22]. Samples were diluted to a concentration of 10 ng/nL total DNA before PCR. Primers for *Fg* elongation factor 1α (FusEF14 forward: 5′-ccacgtcgactctggcaag and FusEF125 reverse: 5′-cgcactggtagatcaagtgacc) were obtained from M. Nicolaisen, University of Aarhus, DJF, Flakkebjerg, Denmark. Both primer pairs were initially tested in the Mx3000P real-time PCR machine (Stratagene) by making standard and dissociation curves on a *Fg* DNA dilution series ranging from 10 ng to 1 pg, giving a detection limit of about 25 nuclei. PCR was performed with 1 μL of template DNA, 10 pmol of each forward and reverse primer, 12.5 μL of 2× SYBR Green master mix (Applied Biosystems) and 0.4 μL of a 500× diluted reference dye (Applied Biosystems) in a final volume of 25 μL and using the following program 95 °C for 10 min, 40 cycles of 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Fluorescence was detected after each cycle. After the last amplification cycle, the specificity of the PCR was determined in a melting curve analysis by increasing the temperature from 60 °C to 95 °C while measuring the fluorescence for every 0.5 °C increase. A no-template control was run with the primer pair.

2.4. Mycotoxin analysis

The mycotoxin analysis was performed as described [23] with slight modifications. One gram of milled grain was extracted with 4 mL of acetonitrile:water [84:16 (v/v)], followed by centrifugation. Two hundred microlitres of the supernatant was mixed with 700 μL water and 100 μL internal standard (^{13}C-labelled DON in 25% acetonitrile/water). The samples were filtered through a 0.45 μm filter before analysis by LC-MS/MS. The chromatographic separation was performed with a Hewlett-Packard 1100 system with gradient elution. Forty microlitres was injected onto a 250×2.1 mm BDS Hypersil C 18.5 μm column (Thermo Electron Corporation, Waltham, Massachusetts, US). The A-eluent was 99% water and 1% methanol and the B-eluent was 10% water and 90% methanol. MS/MS detection was carried out using a Sciex API 2000 instrument (Applied Biosystems) in electrospray negative multiple reaction ionisation mode for DON, NIV and ZEA toxins. Detection limits were 10 μg kg^{-1} for DON and NIV, and 2 μg kg^{-1} for ZEA. Relative standard deviations were 10%.

2.5. Protein extraction and quantification

Water-soluble protein was extracted as described [24] with minor modifications. Flour (200 mg) was extracted with 1 mL 5 mM Tris–HCl, pH 7.5, 1 mM $CaCl_2$ containing the protease inhibitor cocktail "complete" (Roche) by shaking (Eppendorf Thermomixer, maximum speed) for 30 min at 4 °C [25]. Duplicate extractions were carried out for each biological replicate such that six protein extracts were prepared per treatment. After centrifugation (20,000×*g*×30 min, 4 °C), the supernatant was stored in aliquots at −80 °C until needed. The protein concentration was determined by the Popov Amido Black-based method [26] with bovine serum albumin as standard. About 8 mg protein was obtained per gram flour. After extraction of the soluble protein fraction, the flour pellets were re-extracted with 800 μL of 70% ethanol as described [27] to obtain the storage protein fraction. As the Popov method is not ideal for determination of the concentration of alcohol-soluble proteins, the protein concentration of storage protein fractions was determined using the Bradford assay [28]. Storage protein extractions yielded about 1.5 mg protein/g flour.

2.6. Two-dimensional gel electrophoresis

For 2-DE of water-soluble protein extracts, 200 μg protein was precipitated by 4 volumes of acetone at −20 °C overnight. Protein was dissolved in 350 μL of "reswelling" buffer containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5% (v/v) IPG ampholytes (pH 4–7), "Destreak reagent" 1.2% (v/v) (GE Healthcare) and a trace of bromophenol blue. The protease inhibitor cocktail "complete" (Roche) was added to avoid proteolysis during the isoelectric focusing step [25]. Samples were centrifuged before application to 18 cm p*I* 4–7 IPG strips. Isoelectric focusing (IEF) was run on an Ettan IPGphor (GE Healthcare) for a total of 60,000 Vh as described [24]. Second dimension SDS-PAGE (12–14%, 18 cm×24 cm, GE Healthcare) was performed on a Multiphor II (GE Healthcare) as described [24]. For 2-DE of storage protein extracts, 50 μg protein was vacuum-dried before dissolving in 350 μL of "reswelling" buffer as above. Gels were stained by colloidal Coomassie Brilliant Blue [29].

2.7. Image analysis

Scanned gel images (greyscale, 16 bit) were imported into the image analysis software Progenesis SameSpots (Nonlinear Dynamics, UK). All gel images were warped, matched and aligned to a chosen reference gel. Six or four images representing three or two biological and two technical replicates for each of the four treatments were grouped to gain the average volume of each spot. The protein spot volumes were automatically normalised in the software. A list of spots which changed in abundance among the four treatments was generated. A threshold of ANOVA (p)<0.05, q<0.05, power >0.8 and at least two-fold change in average spot volume was used to define the protein spots chosen for further analysis. Principal component analysis (PCA) in the image analysis software was applied to analyse the similarity of protein patterns among gels and the expression profiles of protein spots fulfilling the above criteria.

2.8. In-gel digestion

Spots were excised from gels and subjected to in-gel trypsin digestion as described [30] with minor modifications. Gel pieces

were washed with 40% ethanol, shrunk by 100% acetonitrile and soaked in 2 μL 11.1 ng/μL trypsin (Promega, porcine sequencing grade) in 25 mM NH_4HCO_3 on ice for 45 min. Six μL of 25 mM NH_4HCO_3 was added to gel pieces followed by incubation at 37 °C overnight. Samples were prepared for MALDI-TOF analysis on an Anchorchip™ Target (Bruker-Daltonics, Bremen, Germany) as described [30]. A tryptic digest of β-lactoglobulin was used for external calibration.

2.9. *Protein identification*

An Ultraflex II mass spectrometer (Bruker-Daltonics, Bremen, Germany) was used for peptide mass mapping or peptide fragment ion mapping. An in-house Mascot server (http://www.matrixscience.com) was used for database searches in the NCBInr at the National Center for Biotechnology Information, the HvGI barley gene index Release 10.0 (http://compbio.dfci.harvard.edu/tgi) and the Broad Institute for *F. graminearum* gene index (http://www.broad.mit.edu/annotation/genome/fusarium_graminearum). The following parameters were used for searching: allowed global modification, carbamidomethyl cysteine; variable modification, oxidation of methionine; missed cleavages, 1; peptide tolerance, 80 ppm and MS/MS tolerance ±0.5 Da. To be considered as a positive identification, a significant score calculated by the Mowse scoring algorithm in MASCOT (above 67, 69 and 54 respectively for the HvGI, NCBI and Broad Institute databases) was required with at least four matched independent peptides for peptide mass mapping or two matched peptides in MS/MS analysis. All identified fungal protein sequences were assessed for the presence of signal peptides using SignalP (http://www.cbs.dtu.dk/services/SignalP), and sequences encoding proteins of unknown function were subjected to BLAST search in NCBI.

3. Results

3.1. *Disease incidence*

Barley plants grown with low or high N were inoculated with *Fg* or water as control. The percentage of *Fusarium*-damaged kernels (FDK) was higher for inoculated plants grown with low N than with high N (Fig. 1A). Since it can be difficult to distinguish FDK from other types of kernel damage or discolouration, fungal biomass and mycotoxin levels in the samples were also measured to determine the degree of fungal infection more precisely.

In agreement with the FDK analysis, the concentration of *Fg* DNA was very low or undetectable in control plants and higher in *Fg*-infected plants grown with low N than with high N (Fig. 1B). DON is the main mycotoxin produced by *Fg*, and again there was a clear increase in DON in infected samples treated with low N with respect to high N (Fig. 1C). Only very low levels of NIV (Fig. 1D) and ZEA (Fig. 1E) could be detected. Low levels of *Fg* DNA and mycotoxins in some control samples indicated low contamination from the natural environment.

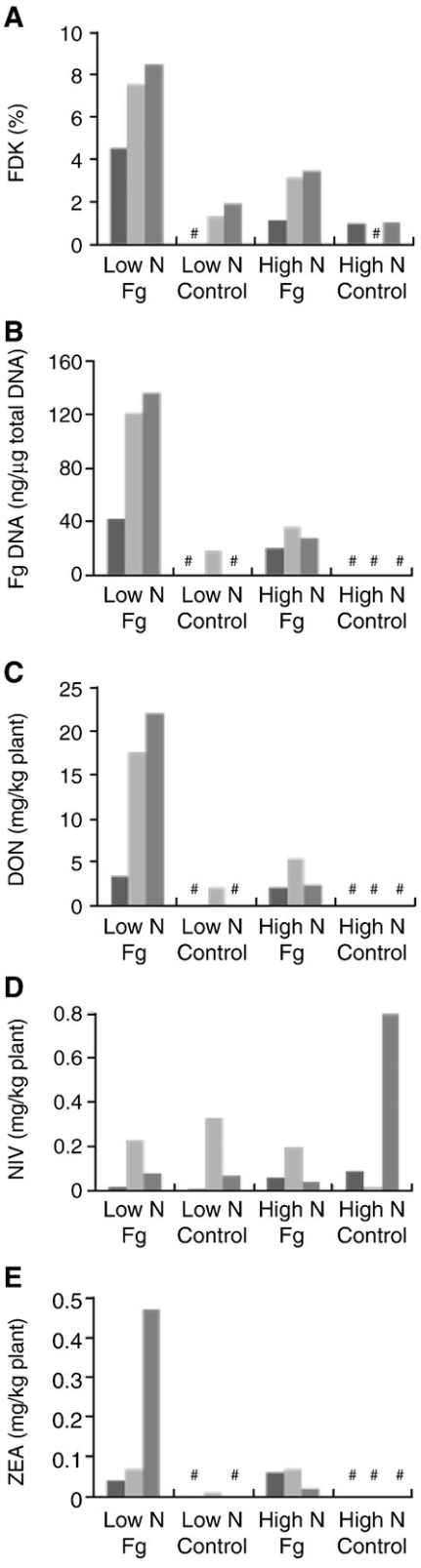

Fig. 1 – Incidence of *Fusarium* infection in barley cv. Scarlett grown under low and high N. A. Percentage of *Fusarium*-damaged kernels (FDK); B. Fungal biomass expressed as concentration of *Fg* DNA; C. Concentration of *Fg* mycotoxin DON; D. Concentration of *Fg* mycotoxin NIV; E. Concentration of *Fg* mycotoxin ZEA. The three biological replicates are shown for each treatment. In some cases, indicated by #, measurements were below the detection limit.

When comparing the three biological replicates, it was observed that both *Fg* DNA content and toxin concentration were much lower in biological replicate 1 from *Fg*-infected, low N than in replicates 2 and 3 (Fig. 1).

3.2. *Proteome analysis*

Two-dimensional gel electrophoresis (pH 4–7) was run using three biological replicates and two technical replicates from each of the four treatments. The 2-DE patterns of the non-infected samples grown with high and low N were highly similar to each other. Clear differences were apparent between *Fg*-infected and control samples grown under low N but were less apparent under high N (Fig. 2A). In particular, the intensity of several high molecular weight proteins decreased and many new spots with lower molecular weight appeared in the *Fg*-infected extracts (Fig. 2). In agreement with the FDK, fungal DNA and toxin analysis (Fig. 1), the 2-DE pattern of biological replicate 1 from *Fg*-infected, low N was intermediate between replicates 2 and 3 and the *Fg*-infected, high N samples when the gel images were subjected to principal component analysis (PCA; data not shown). This replicate was therefore removed from the subsequent analysis of 2-DE spot volumes. The statistical analysis was thus based on 22 gels. In total, 466 protein spots were detected on all gel images based on the criteria (ANOVA <0.05, $q<0.05$ and power >0.8). PCA was used to examine the relationship of the individual spots with the 2-DE patterns (Fig. 3A). The biological and technical replicates from each treatment clustered together, demonstrating the reproducibility of the 2-DE patterns. Sixty percent of the variance could be explained by principal component 1, which separated the gels according to the degree of *Fg* infection of the samples, whereas only about 6% of the variance was explained by principal component 2, separating gels based on the N level. The greatest difference was observed between the *Fg*-infected and control samples grown with low N in agreement with the FDK, *Fg* biomass and toxin data (Fig. 1).

The average volumes of 188 spots varied by at least two-fold among the four treatments. These spots were chosen for further analysis. The spots formed two clusters according to their appearance profiles (Fig. 3B). Protein spots in cluster A (80 spots)

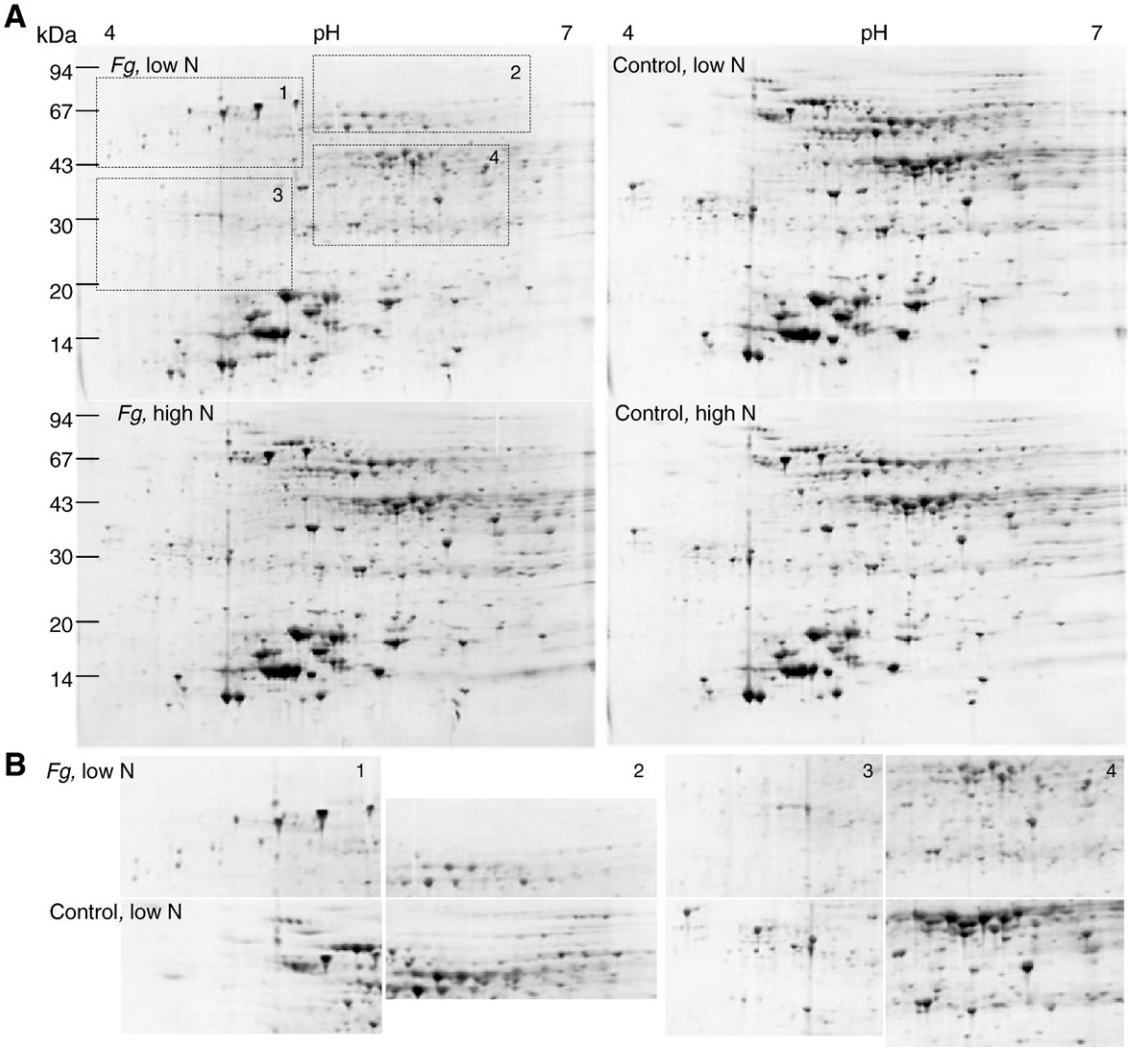

Fig. 2 – Water-soluble protein profiles of barley seeds. A. One representative colloidal Coomassie blue-stained gel covering the pH range 4–7 is shown for each treatment. Molecular size markers are indicated. Numbered boxes indicate selected regions of 2D-gels showing differences between infected and control treatments; B. Close-up views of boxes for infected and control low N samples.

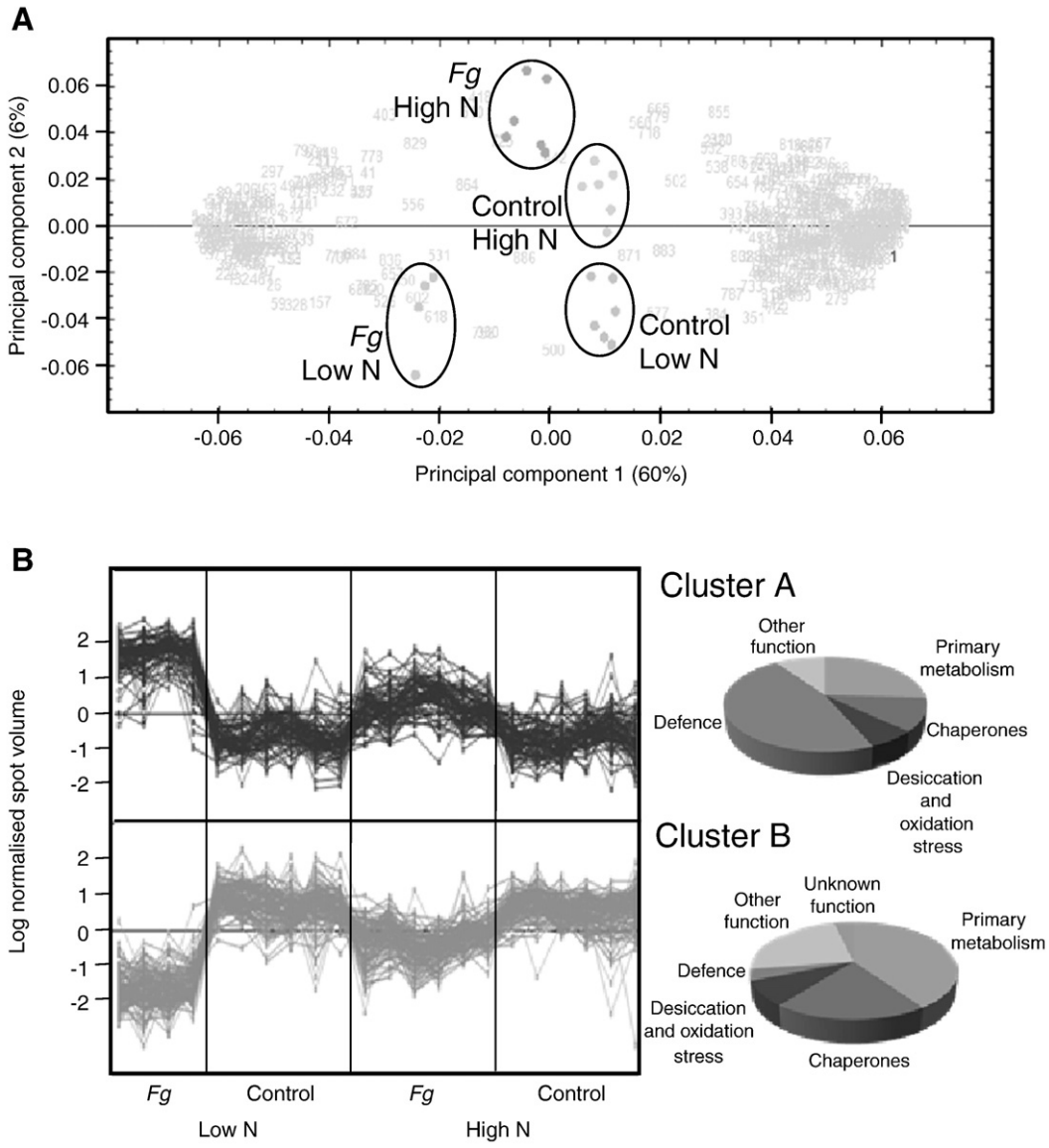

Fig. 3 – Protein spots varying in response to *Fg* infection and N level. A. Principal component analysis. Protein spots showing significant variation are indicated by numbers and gel images are indicated by circles. Biological and technical replicates are grouped in circles corresponding to the four treatments. B. Expression profiles of protein spots in cluster A (increased intensity with *Fg* infection) and cluster B (decreased intensity with *Fg* infection). Each trace corresponds to one 2-DE spot and each node corresponds to one replicate. Functional categories of the plant proteins identified in clusters A and B are indicated.

increased and spots in cluster B (108 spots) decreased in intensity in response to *Fg*. For each spot, a greater change in intensity in response to *Fg* was observed for low N than for high N except spots 119, 238 and 263 from cluster A which increased more in intensity under high N. No spots showed greater than 1.9-fold change when comparing the uninfected samples grown with low and high N (data not shown), supporting previous findings that altered levels of N fertilizer do not greatly affect the water-soluble seed proteome, although some individual proteins may be differentially expressed [31]. Overall, the results suggest that the observed changes in protein patterns are caused mainly by the *Fg* infection, and that these changes are greater in plants grown with lower levels of nitrogen.

In cluster A, containing the spots increasing in intensity in response to *Fg*, 9 spots were identified as fungal proteins, whereas 67 barley proteins were identified in 65 spots. Six spots could not be identified. Based on sequence coverage obtained in MS and lower observed molecular weight than expected (Supplementary Table S1), as well as previous identification of the same proteins in spots of expected molecular weight [32], most of the identified plant proteins were judged to be proteolytic fragments (Supplementary Table S1, Fig. S1). In cluster B, 109 barley proteins were identified in 107 spots (Supplementary Table S2, Fig. S2). A single spot could not be identified.

The identified plant proteins were assigned to functional categories (Fig. 3B). Plant proteins of known function which decreased in response to *Fg* infection were mainly chaperones, defence-related proteins, proteins involved in desiccation and oxidative stress, proteins involved in primary metabolism such

as glycolysis, starch metabolism, citric acid cycle and amino acid biosynthesis, the volume of which changed between 7.7-fold (Spot 1; protein similar to protamine P1; Supplementary Fig. S2) and 2-fold (Spots 350,379,395; serpin; Supplementary Fig. S2). The plant proteins in the spots increasing in intensity in response to *Fg* infection belonged to similar functional categories as the proteins that decreased in abundance, suggesting that plant proteins in cluster B were degraded to produce the fragments in cluster A. The main degradation products in cluster A were generated from major seed water-soluble proteins such as serpin, β-amylase and low molecular weight α-amylase and protease inhibitors like CI-1A, CI-1B, CI-2A, BMAI and BDAI. However, other spots previously identified as α-amylase/trypsin inhibitors CMa, CMb, CMd and trypsin/amylase inhibitor pUP 13 [32] were found with slightly increased or unchanged intensity (data not shown).

Nine fungal proteins were identified, suggesting that these were relatively abundant in the fungal *in planta* proteome (Table 1). Six of these spots were identified as proteins of annotated function. The remaining three fungal protein spots were annotated as hypothetical proteins. None of the fungal proteins were predicted to have signal peptides.

Analysis of the storage protein fractions was also carried out, but no spot volume change greater than 2-fold was observed in these fractions (data not shown).

4. Discussion

4.1. *The effect of nitrogen fertilization on the proteome of infected grain*

Analysis of FDK, fungal biomass and mycotoxin levels clearly showed that *Fg* infection was more severe in barley plants grown under low N compared to high N, suggesting that high FHB incidence correlates with low N status. Nitrogen can increase biomass of leaf and stalk tissue and enable higher grain yield [33] as well as affect plant responses to pathogens [14,33]. Furthermore, it has been reported that nitrogen depletion in the medium used for *in vitro* fungal growth could induce synthesis of trichothecene [34,35] and depletion of nitrogen or nutrients was required for *Fusarium* spores to germinate and infect plants [33,36]. However, in the present study, although high N plants displayed delayed flowering time and prolonged grain filling, no major difference was observed in the appearance of the control plants under two nitrogen levels at maturity stage. Since the ratio of DON to *Fg* DNA was similar under low and high N, it seems unlikely that the mycotoxin is produced as a response to low N status.

With respect to the analysis of the water-soluble proteome, we observed similar protein profiles of the two uninoculated groups and clear differences between infected and control gels, where considerable differences in response to fungal infection occurred with low N, indicating that incidence of FHB disease in barley grain was increased. The proteome results were in good agreement with analysis of FDK, fungal biomass and mycotoxin levels.

The group of proteins down-regulated in response to *Fg* infection include functions such as chaperones, defence-related proteins, proteins related to desiccation and oxidative stress and proteins involved in primary metabolism. Partial or complete disappearance of the protein matrix and starch granules has been reported in kernels of spring wheat damaged by *Fusarium* using scanning electron microscopy [37]. Protein bodies of wheat kernels were degraded to polypeptides of lower molecular weight during infection by *Fg* [38,39]. An increase in protease activity of diseased barley grain has also been shown [40]. *Fusarium* species were discovered to produce alkaline proteases associated with the degradation of certain water-soluble proteins in infested barley [41]. Several *Fg* peptidases and aspartyl proteases were also identified during infection of wheat head [17]. It is possible that in our study, the observed widespread proteolysis was caused by fungal proteases acting as pathogenicity factors, produced to obtain nutrition from the host [42,43]. While fragments of barley protease inhibitors such as CI-1A, CI-1B, CI-2A, BMAI and BDAI were observed, others such as α-amylase/trypsin inhibitors were not reduced in amount, suggesting differential sensitivity to proteases. Previous studies performed

Table 1 – *Fusarium graminearum* proteins identified in infected barley seed proteomes.

Spot number[a]	MASCOT score	Matched masses	Sequence coverage (%)	Theor./ Obs. MW (kDa)	Theor./ Obs. pI	Accession number[b]	Protein and conserved domains
29	159	11	56	28.6/30.7	5.6/5.8	FGSG_04826[c]	L-xylulose reductase
36	55	4	30	25.0/25.0	7.1/6.3	FGSG_04454	Superoxide dismutase, mitochondrial precursor
49[d]	91	6	58	11.9/14.6	5.3/5.3	gi|94711854[c,e]	Peptidyl-prolyl cis–trans isomerase (PPIase)
64	56	5	14	64.7/57.7	5.9/4.8	FGSG_02625	Hypothetical protein, COG3670, Lignostilbene-alpha, beta-dioxygenase
90	64	6	11	60.0/52.7	5.2/4.6	FGSG_06534	Hypothetical protein
102	87	6	29	27.2/28.7	5.2/5.2	FGSG_06702[c]	Triosephosphate isomerase
143	55	6	20	28.6/30.7	5.6/5.5	FGSG_04826[c]	L-xylulose reductase
157	73	8	26	40.2/49.3	9.2/4.3	FGSG_05460	Hypothetical protein
167[d]	127	8	61	16.0/17.8	5.8/6.2	FGSG_08721[c,e]	Cu–Zn Superoxide dismutase

[a] Spot numbers refer to Supplementary Fig. S1.
[b] FGSG: accession number in *Fusarium graminearum* gene index from Broad Institute; gi: accession number in NCBI.
[c] Protein also identified in [17].
[d] Two peptides additionally confirmed by MS/MS analysis.
[e] Protein also identified in [3].

on the barley protease inhibitors CI-1A, CI-1B, CI-2A, BASI and barley Bowman-Birk inhibitor (BBBI) have shown that they differ in their ability to inhibit *Fusarium* serine proteases [44,45]. The interplay between fungal proteases and plant protease inhibitors may thus influence the outcome of the host–pathogen interaction [45].

Alternatively, the observed degradation may be a consequence of endogenous proteases as a result of necrosis or defence responses. Exactly which proteases contribute to the degradation of albumins *in vivo* remains unclear since no proteases were identified in the infected seed proteomes. Further investigation of the proteases causing hydrolysis of the barley proteome *in vivo* is required.

4.2. *F. graminearum does not affect the storage protein fraction*

In contrast to the changes observed in the water-soluble seed proteome, no significant alteration in 2-DE patterns of the hordein storage protein fraction was observed in response to N and/or *Fg* infection although it has been reported previously that higher N can result in an increase in hordein, whereas the other protein fractions including the water-soluble albumin fraction are relatively unaffected [46]. Our results indicated that the low nitrogen conditions in this study caused very few changes in storage protein or albumin fractions and that *Fg*-induced proteolysis mainly occurred in the albumin fraction regardless of fertilizer level. *Fg* may preferentially proteolyse the more accessible albumin fraction to establish infection, rather than the proline-rich storage proteins in the starchy endosperm. In agreement with this hypothesis, it was shown that a major reduction of *Fg* and DON in diseased wheat kernels was achieved by pearling, a process in which most of the outer layers, including the aleurone layer, are removed [47] suggesting that the fungus has limited contact with the storage proteins of the starchy endosperm. Our results suggest that the observed effect of differential N levels on *Fg* infection is not caused by a change in overall protein composition or amount in the seeds.

4.3. *Proteins of fungal origin*

Nine proteins were identified that originated from *Fg*, the genome of which is predicted to contain 13,332 genes (www.broad.mit.edu). The *Fg* proteins were probably dominated by the host plant proteins given the high ratio of plant to fungal biomass [18]. In agreement with this, only 25 *Fg* transcripts were detected as accumulating 72 h after inoculation of barley [7] and only 8 fungal proteins were identified when the wheat–*Fg* interaction was examined using 2-DE [3]. In contrast, 120 *in planta Fg* proteins were identified in vacuum-infiltrated fluid from infected wheat heads [17], including five out of the 9 fungal proteins identified in the present study. However, the fact that four additional proteins were identified here strongly suggests that complementary approaches are required to analyse the *in planta* fungal proteome. The five proteins common to these studies were Cu–Zn superoxide dismutase, triose phosphate isomerase, peptidyl-prolyl cis–trans isomerase and L-xylulose reductase (Table 1). The Cu–Zn superoxide dismutase and peptidyl-prolyl cis–trans isomerase were also observed in the *Fg*-infected wheat grain proteome [3]. The identification of an additional form of superoxide dismutase in the present study strongly suggests that the pathogen is exposed to, and attempts to overcome plant defence-related reactive oxygen species. L-Xylulose reductase is involved in the assimilation of L-arabinose, derived from breakdown of plant cell walls, into the fungal pentose phosphate pathway [48] and was identified in two spots varying in p*I*, illustrating a strength of 2-DE based studies and suggesting that the protein may be post-translationally modified. It was however not possible on the basis of the MS data to determine the nature of the modification.

Three of the fungal proteins identified for the first time in this study had unknown functions. One protein shared 82% sequence identity with torulene oxygenase from *Fusarium fujikuroi*. Torulene oxygenase is involved in the carotenoid pigment biosynthetic pathway [49], the initial steps of which are common to biosynthesis of gibberellin, which is produced by *F. fujikuroi* in large amounts in low nitrogen medium [50]. Therefore, nitrogen depletion in our study possibly induces the higher expression of genes in this pathway and suggests that a role for gibberellins may be found in this interaction.

One of the identified *Fg* proteins (Spot 157) with unknown function appeared in the 2-DE pattern with a p*I* of 4.2 which is surprisingly lower than the predicted theoretical value of 9.2. (Table 1, Supplementary Fig. S1). The observed molecular mass (49 kDa) was also slightly higher than expected (40 kDa). Such large p*I* discrepancy suggests that the protein is post-translationally modified. The protein sequence contains a potential Asn-X-Thr N-glycosylation site and the regions of the protein not covered by peptide mass data are rich in Ser and Thr, suggesting that the protein could be glycosylated, however in-depth analysis by mass spectrometry will be required to confirm this.

5. Conclusion

Taken together, the positive correlation of FDK, fungal biomass, mycotoxins and proteome changes observed in this study strongly suggests that FHB is more severe in barley grown with low N than with high N. To our knowledge, this is the first report to clarify the effect of nitrogen on FHB in barley using proteomic approaches. The *Fg*-infected proteome patterns of barley seeds reveal degradation of the water-soluble albumin protein fraction and detection of fungal proteins with metabolic and antioxidative functions. Further work will confirm whether appropriate N amendments in the field can decrease both disease severity and mycotoxin accumulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jprot.2009.10.010.

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Chapter 3

Analysis of early events in the interaction between *Fusarium graminearum* and the susceptible barley (*Hordeum vulgare*) cultivar Scarlett

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RESEARCH ARTICLE

Analysis of early events in the interaction between *Fusarium graminearum* and the susceptible barley (*Hordeum vulgare*) cultivar Scarlett

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A proteomic analysis was conducted to map the events during the initial stages of the interaction between the fungal pathogen *Fusarium graminearum* and the susceptible barley cultivar Scarlett. Quantification of fungal DNA demonstrated a sharp increase in fungal biomass in barley spikelets at 3 days after inoculation. This coincided with the appearance of discrete *F. graminearum*-induced proteolytic fragments of β-amylase. Based on these results, analysis of grain proteome changes prior to extensive proteolysis enabled identification of barley proteins responding early to infection by the fungus. In total, the intensity of 51 protein spots was significantly changed in *F. graminearum*-infected spikelets and all but one were identified. These included pathogenesis-related proteins, proteins involved in energy metabolism, secondary metabolism and protein synthesis. A single fungal protein of unknown function was identified. Quantitative real-time RT-PCR analysis of selected genes showed a correlation between high gene expression and detection of the corresponding proteins. Fungal genes encoding alkaline protease and endothiapepsin were expressed during 1–3 days after inoculation, making them candidates for generation of the observed β-amylase fragments. These fragments have potential to be developed as proteome-level markers for fungal infection that are also informative about grain protein quality.

Keywords:
Cereal grain / Fusarium head blight / Gene expression / Pathogenesis-related proteins / Plant proteomics / Protease activity

1 Introduction

Fusarium head blight (FHB) of barley (*Hordeum vulgare*) and other cereals is a destructive disease worldwide due to yield loss, reduced grain quality and contamination by mycotoxins hazardous to animals and humans [1]. Especially in humid weather conditions, the disease can be caused by many *Fusarium* species including the prevalent *Fusarium graminearum* Schwabe (teleomorph: *Gibberella zeae* (Schwein) Petch) [1]. Controlling FHB is challenging due to (i) lack of completely resistant cultivars, (ii) ineffective fungicides and (iii) limited understanding of mechanisms of plant resistance at the molecular level.

Barley spikes are most susceptible to *F. graminearum* at anthesis or during the early dough stage of grain development [1, 2]. The preferred infection site is the extruded ovary epithelial hairs on the kernel tip and fungal growth proceeds along the epicarp at the space between the lemma and palea [2]. The pericarp may be colonised within 2 days after inoculation (dai) [2]. Fungal hyphal growth is delayed by the testa for some time before invasion of aleurone layer and endosperm occurs [3]. In contrast to wheat, symptoms do not spread along the spikes in many barley cultivars, including those susceptible to the fungus, indicating so-called type II resistance of barley. *Fusarium* produces cell wall degrading enzymes and proteases to obtain nutrients

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Abbreviations: dai, days after inoculation; **FHB,** Fusarium head blight; **PR,** pathogenesis-related; **qRT-PCR,** quantitative real-time RT-PCR

from the host plant, and mycotoxins for increased virulence [1]. Plant defence responses triggered upon pathogen attack can include cell wall fortification, production of antimicrobial secondary metabolites, accumulation of pathogenesis-related (PR) proteins and programmed cell death [4].

Several genomic, transcriptomic and proteomic studies have been performed to investigate responses of wheat and barley to *F. graminearum*. Northern blotting analysis indicated that genes encoding PR proteins PR-1, PR-2 (β-1,3-glucanases), PR-3 and PR-4 (chitinases), PR-5 (thaumatin-like protein) and PR-9 (peroxidase) were induced from 6 to 12 h with highest expression 36–48 h after inoculation [5, 6]. Microarray analysis showed limited fungal development and little change in barley transcript accumulation before 2 dai, increased fungal development and transcript accumulation between 2 and 4 dai with a majority of host transcripts accumulating at 3 dai and reduction of transcript levels between 4 and 6 dai [2]. Accumulating barley transcripts included genes encoding defence response proteins, oxidative burst-associated enzymes, phenylpropanoid pathway enzymes, and trichothecene and tryptophan catabolic enzymes [2]. Gel-based proteomic analysis of a susceptible wheat cultivar 1–3 dai with *F. graminearum* revealed accumulation of plant proteins involved in oxidative stress, PR responses and nitrogen metabolism [7]. Proteomic analysis of six barley cultivars with varying resistance to *F. graminearum* also revealed upregulation of PR proteins and proteins associated with oxidative stress at 3 dai [8]. It was recently demonstrated that the proteome of mature *F. graminearum*-infected barley seeds is considerably degraded, based on the identification of numerous plant protein fragments [9], which may be the result of fungal protease activity. The *F. graminearum* exoproteome from *F. graminearum*-infected mature wheat heads was also analysed by LC-MS/MS, resulting in identification of 120 fungal proteins including several secreted proteases [10]. The infection stage at which degradation of plant proteins occurs and the responsible fungal proteases are still unknown. In the present study, for the first time, plant defence gene expression and proteome changes including proteolysis in response to *F. graminearum* are correlated with fungal gene expression and quantification of fungal biomass. Furthermore, pathogen-dependent proteolytic fragments of β-amylase are identified as markers of *F. graminearum* infection at the proteomic level.

2 Materials and methods

2.1 Plant growth and spike inoculation

Barley cultivar Scarlett was grown in 2-litre plastic pots containing the soil mix Pindstrup Substrate 2 (Pindstrup Mosebrug A/S, Denmark) in a greenhouse (18°C, 60–70% relative humidity in the day/15°C, 80–90% relative humidity in the night, 16-h photoperiod) supplemented with light from fluorescent tubes (Philips Son-T Agro 400 W). After germination, excess seedlings were removed leaving three seedlings *per* pot. Plants were watered every second day and fertilised with a 0.5% standard nutrient solution (NPK 5-1-4+Mg and S) (Hornum, Brøste A/S, Copenhagen, Denmark) once a week from the tillering stage 21 [11] throughout the experiment.

F. graminearum strain PH1 (ARS Culture Collection, NRRL 31084) macroconidial suspension was prepared as described [12] and stored at −80°C in 10% glycerol until inoculation. The concentration of macroconidia was determined using a haemocytometer and adjusted to 10^5 spores/mL. Point inoculation was conducted at anthesis stage 65 [11] by micropipetting 5 μL into approximately 16 out of total 24 spikelets leaving the bottom and top three spikelets uninoculated. Separate control plants were mock-inoculated with water. After inoculation, spikes were covered in sealed water-sprayed plastic bags to maintain humidity and plants were sealed in dark for 72 h. Three biological replicates for each time-point (1, 2 and 3 dai) were prepared, consisting of 48 inoculated spikelets from three spikes collected from different plants for each biological replicate. Spikelets were lyophilised and ground into fine powder in liquid nitrogen using a mortar and pestle. The powder was used for DNA, RNA and protein extractions as described below.

2.2 *Fusarium graminearum* biomass determination

Total DNA was extracted from 25 mg ground samples using DNeasy plant mini kit (Qiagen, Venlo, Netherlands) following the manufacturer's protocol and diluted to 10 ng/μL for PCR. Determination of *Fusarium* DNA by quantitative PCR was carried out as described [9]. Plant DNA was determined by quantitative PCR using primers for the wheat elongation factor gene (Forward primer: 5′-ACCCTGA-CAAGGTTCCCTTC-3′; Reverse primer: 5′-ACCAGT-CAAGGTTGGTGGAC-3′). *F. graminearum* biomass was expressed as *Fusarium* DNA/plant DNA (ng/μg). Data represent a continuous variable and were analysed by analysis of variance assuming a normal distribution. Variances were stabilised by appropriate transformations when necessary. Data were analysed by PC-SAS (release 9.1; SAS Institute, Cary, NC, USA).

2.3 Protein extraction and western blot analysis

Water-soluble proteins were extracted from 200 mg ground samples with 1 mL of buffer containing 5 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$ and the protease inhibitor cocktail "complete" (Roche) as described [9]. Protein concentration was determined by Amido Black [13] with bovine serum albumin as standard. Five micrograms protein were separated on 4–12% BisTris NuPAGE gels (Invitrogen) followed by blotting to nitrocellulose membranes (Hybond-N, GE

Healthcare). Antibodies recognising β-amylase (kindly provided by Evan Evans, University of Tasmania) were used for Western blotting. Polyclonal goat anti-rabbit immunoglobulins conjugated with alkaline phosphatase (Dako A/S) were used as secondary antibody. The BCIP/NBT chloride system (Sigma) was used for detection.

2.4 Two-dimensional gel electrophoresis and protein identification

Two hundred micrograms protein was precipitated by 4 volumes of acetone overnight at −20°C. IPG gels (pH 4−7, GE Healthcare) and second dimension SDS-PAGE (12–14%, 18 cm × 24 cm, GE Healthcare) were run as described [9]. Duplicate gels for each biological replicate were run and stained by colloidal Coomassie Brilliant Blue [14]. Scanned gel images (300 dpi, 16-bit greyscale) were imported into Progenesis SameSpots (Nonlinear Dynamics, UK), warped, matched and aligned to a chosen reference gel. Quantification of spots was based on the average normalised volumes from six gels. A threshold of ANOVA (p) < 0.05 and at least 1.5-fold change in average spot volume in infected samples was used to define the protein spots chosen for further analysis. Protein spots were excised from gels and in-gel digested with trypsin followed by MALDI-TOF analysis (Ultraflex II, Bruker-Daltonics, Bremen, Germany) as described [9]. Protein identification by peptide mass mapping was done using an in-house Mascot server (http://www.matrixscience.com) to search the NCBInr at the National Center for Biotechnology Information, the HvGI barley gene index Release 10.0 (http://compbio.dfci.harvard.edu/tgi) and the Broad Institute for *Fusarium graminearum* gene index (http://www.broad.mit.edu/annotation/genome/fusarium_graminearum). Details of search criteria are given in Supporting Information Table S2.

2.5 RNA extraction and qRT-PCR analysis

Total RNA was extracted from 50 mg of ground sample using the RNeasy Plant Mini Kit (Qiagen) with addition of 50 μL Plant RNA Isolation Aid (AM9690, Ambion) following the manufacturer's protocol. Removal of genomic DNA and cDNA synthesis were carried out as described [15]. Primers (Supporting Information Table S1) were designed using primer3 (http://fokker.wi.mit.edu/primer3/input.htm). Primer specificity was tested by blasting primer sequences against the NCBI database. The barley 18S rRNA gene [16] and *F. graminearum* EF1α and GAPDH [17] served as reference genes for quantification of barley and fungal gene expression, respectively. Real-time PCR was carried out in technical duplicates, using 25-fold dilutions of reverse transcription mixtures as template [15]. After the last amplification cycle, a melting curve analysis for each primer pair verified amplification of a specific product. Difference in Ct value between technical replicates was under 0.5. Relative expression of genes was determined using the formula: relative expression $= 2^{-[\Delta Ct \text{ target gene} \Delta Ct \text{ reference gene}]}$ [18] where Ct refers to the threshold cycle. The detection limit for plant gene relative expression was around −25 (2-log scale) due to 40 cycles of setup amplification and a threshold cycle of approximately 15 for the 18S rRNA gene. Statistical analysis of plant gene expression in infected *versus* control samples was performed by Student's *t*-test. Regulation of plant genes during 1−3 days after anthesis was tested by ANOVA.

3 Results

3.1 Development of FHB

To investigate initial stages of infection of barley by *F. graminearum*, spikelets must be infected uniformly and at the same time. Since it is difficult to distinguish uninfected from infected spikelets at early stages and in our experience, use of the point inoculation method almost completely eliminates the FHB disease variation, point rather than spray inoculation was used, which enabled infected spikelets to be marked. Discoloration symptoms appeared on some spikelets at 2 dai, developing rapidly at 3 dai (Supporting Information Fig. S1). No symptoms were observed in uninoculated spikelets. Fungal biomass was determined to quantify the infection. No *F. graminearum* DNA was detected in control samples. The concentration of *F. graminearum* DNA in infected spikelets was very low at 1 dai, increased slightly at 2 dai and dramatically at 3 dai (Fig. 1A), indicating rapid colonisation by *F. graminearum* from 2 to 3 dai.

3.2 *F. graminearum*-induced proteolysis of β-amylase

The proteome of mature barley seeds infected by *F. graminearum* contained many proteolytic fragments [9]. To identify changes related to the early stages of infection, spikelets must be analysed prior to extensive proteolysis, since widespread breakdown of proteins will induce secondary effects on metabolism and numerous proteolytic fragments on 2-D gels will mask proteins of interest. To determine the stage at which proteolytic degradation occurred, and identify the ideal timepoint for proteome analysis, polyclonal antibodies were used to detect fragments of β-amylase, a major grain protein for which proteolytic fragments were observed in 2-D gel patterns of *F. graminearum*-infected grains [9]. Bands corresponding to full-length β-amylase were observed on SDS-PAGE in control and *F. graminearum*-infected samples at 1, 2 and 3 dai (Fig. 1B). The two β-amylase isoforms, β-amylase 1 (gi|29134855) and β-amylase 2 (gi|61006818) were identified from corresponding 2-D gels (data not shown). In agreement with these observa-

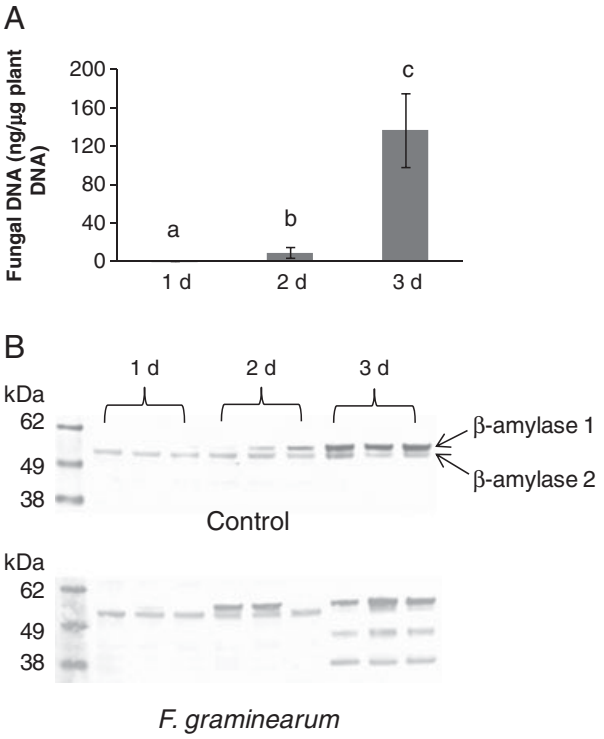

Figure 1. Development of *F. graminearum* infection in barley at 1, 2 and 3 dai. (A) Fungal biomass determined in triplicate expressed as content of *F. graminearum* DNA. Significant differences ($p<0.05$) by ANOVA in levels of fungal DNA in barley are indicated by different letters. (B) Western blotting using antibodies recognising β-amylase in protein extracts from control (upper panel) and *F. graminearum*-infected spikelets (lower panel). Bands corresponding to full-length forms of β-amylase 1 and β-amylase 2 are indicated. Three biological replicates are shown for each time point.

tions, the most abundant endosperm-specific β-amylase 1 accumulates during grain filling whereas the less abundant β-amylase 2 is highly expressed at 5 days after anthesis but is undetectable during later stages of seed development [19]. Discrete bands with lower molecular mass were observed in infected samples only at 3 dai (Fig. 1B), confirming *F. graminearum*-induced degradation of β-amylase. The degradation after 3 dai corresponds with the increase in *F. graminearum* biomass observed at this time-point. The generation of discrete fragments suggests the action of a specific endoprotease. Mass spectrometry resulted only in identification of β-amylase 1 in fragments separated on 2-D gels (data not shown), suggesting that β-amylases 1 and 2 differ in susceptibility to the proteases, despite sharing 82% sequence identity.

3.3 Proteome analysis

Based on fungal biomass and *F. graminearum*-induced proteolysis, fungal colonisation was significant at 2 dai, although degradation of the plant proteome was not yet severe. Samples at 2 dai were thus chosen for analysis. Approximately 500 spots were resolved on 2-D gels. The volume of 38 spots increased and 13 decreased in intensity in response to *F. graminearum* infection based on the criteria ($p<0.05$ and fold ≥ 1.5; Fig. 2). Fifty of the 51 differentially displayed protein spots were identified by MS (Fig. 2; Supporting Information Table S3). Ten spots with greater intensity in infected samples were judged to be proteolytic fragments based on sequence coverage obtained in MS and an observed molecular weight much lower than expected (Supporting Information Table S3).

3.4 PR-gene expression analysis

Proteins accumulating following *F. graminearum* infection included PR-3 (Supporting Information Table S3) and the basic PR-1 and PR-5 proteins, which were observed on gels covering p*I* range 6–11 (data not shown) and increased 1.5 and 1.6-fold ($p<0.05$), respectively. Expression profiles of the corresponding genes were analysed by qRT-PCR with

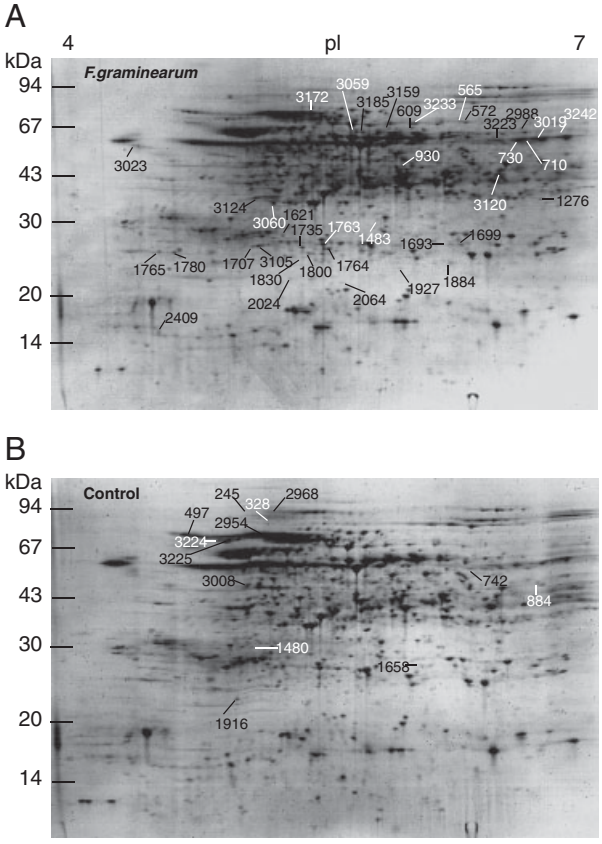

Figure 2. Water-soluble proteome of barley spikelets. Representative gels are shown for barley infected by *F. graminearum* (A) and control (B) at 2 dai. Molecular size markers are indicated. Protein spots changing in intensity between infected barley and control are numbered.

additional PR genes *PR-1b*, *PR-2*, *PR-9* and *PR-15*. No significant changes in expression level were observed in control plants. Comparison based on normalised gene expression levels indicated that the *PR-1*, *PR-2*, *PR-3* and *PR-5* genes were more highly expressed in control plants than the other PR genes examined. Expression of *PR-15* was not detected in control plants (Table 1). All except *PR-2* were significantly upregulated from 2 dai in response to *F. graminearum*. *PR*-1, *PR-3* and *PR-5* were strongly upregulated compared to the other PR genes in accordance with detection of the corresponding protein spots.

3.5 Fungal gene expression analysis

A single fungal protein (FGSG10974) was identified (Supporting Information Table S3), which was also found at 3 dai in *F. graminearum*-infected wheat [7]. This was also chosen for gene expression analysis, together with alkaline protease, a subtilisin-like serine protease (FGSG00806) and endothiapepsin, an aspartic protease (FGSG07775), which were identified in the secretome of *F. graminearum* grown in medium containing barley flour (F. Yang, unpublished data) and therefore candidates for the proteolysis of barley proteins observed after 3 dai.

All three fungal transcripts were detected in infected samples but not in controls (Fig. 3).The relative expression level of FGSG10974 did not change significantly during infection. In agreement with its detection in the grain proteome, its expression level was much higher than the two protease-encoding genes. The proteases showed contrasting expression profiles, the alkaline protease being upregulated whereas endothiapepsin was strongly downregulated during 1−3 dai (Fig. 3).

4 Discussion

Several proteomics studies have analysed the effect of *F. graminearum* on cereal seed proteomes [7–10]. However, due to different inoculation methods (spray inoculation *versus* point inoculation) and growth conditions between laboratories, infection levels in many studies may not be comparable. The aim of this study was to compare the proteome with gene expression profiles at clearly defined levels of fungal infection, determined by measurements of fungal biomass after point inoculation of barley spikelets.

One effect of *F. graminearum* on barley is widespread protein degradation [9]. To determine when this proteolysis occurs and to select the time-point for proteome analysis, we exploited antibodies against β-amylase, one of the proteins degraded in *F. graminearum*-infected seeds [9]. Discrete β-amylase fragments were observed at 3 dai, correlating with the increase in fungal biomass observed from 2 dai to 3 dai. This supports previous suggestions that *F. graminearum* operates as an endophyte or biotroph during the initial 2–3

Table 1. Gene expression analysis by qRT-PCR in control and *F.graminearum*-infected barley at 1–3 dai

	1 dai			2 dai			3 dai		
	Infected[a)]	Control[a)]	Fold change[b)]	Infected[a)]	Control[a)]	Fold change[b)]	Infected[a)]	Control[a)]	Fold change[b)]
Chitinase II (PR-3)	−12.6±1.1	−17±1.7	20.5	−10.2 ±2.0	−15.5±1.7	39.0[c)]	−8.7±1.2	−14.5±3.7	57.9[c)]
PR-1	−13.5±0.2	−15.3±0.2	3.6[c)]	−9.8±1.6	−14.5±0.8	26.8[c)]	−8.8±1.4	−14.2±1.8	42.9[c)]
TLP6 (PR-5)	−15.6±0.1	−18.5±0.4	7.6[c)]	−12.8±1.7	−18.9±0.9	71.5[c)]	−12.2±0.9	−17.4±1.4	37.3[c)]
PR-1b	−19.1±0.5	−20.2±0.9	2.1	−14.4±0.6	−19.9±0.7	44.4[c)]	−13.2±0.5	−18.9±0.7	51.2[c)]
Peroxidase (PR-9)	−18.8±0.8	−19.9±0.4	2.0	−16.3±0.5	−20.1±0.3	14.5[c)]	−16.3±0.3	−19.9±2.7	12.1[c)]
Oxalate oxidase (PR-15)	−21.2±0.1	−23.8±2.3	6.2	−16.3±1.4	n.d.[d)]	*[c)]	−15.6±0.5	n.d.[d)]	*[c)]
β-1,3-glucanase 2a (PR-2)	−19.7±1.0	−18.1±0.5	0.3	−16.9±2.7	−17.1±1.1	1.2	−17.2±1.2	−16.5±2.3	0.6

a) Relative expression±standard deviation after normalisation to 18S rRNA (2-log scale).
b) Fold-change in infected samples, calculated after normalisation to 18S rRNA.
c) Significant at $p < 0.05$.
d) Expression was below the detection level of −25 relative to 18S rRNA (2-log scale).

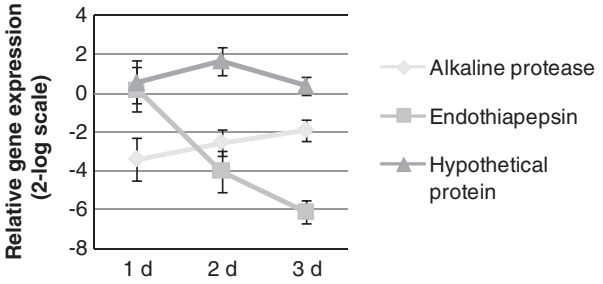

Figure 3. Fungal gene expression analysis in barley spikelets by qRT-PCR at 1–3 dai.

dai and switches to a necrotrophic phase at approximately 3 dai [2]. The discrete fragments of β-amylase can be developed as a proteome-level marker for *F. graminearum* infection. β-amylase is one of the most abundant water-soluble grain proteins and thus readily extractable and detectable. Since *F. graminearum*-induced proteolysis has a severe effect on grain proteins, such a proteome-level marker is a highly informative indicator of grain quality.

Alkaline protease activity in *Fusarium* has been associated with degradation of water-soluble proteins in infected barley [20]. For insight into which fungal proteases might contribute to proteolysis, qRT-PCR was used to monitor the *in planta* expression of two *F. graminearum* genes encoding proteases with different specificities. Differential regulation of the alkaline protease and endothiapepsin transcripts suggests that these proteases play different roles in fungal nutrition and development. The high transcript level of FGSG10974 compared to the protease-encoding genes, and its relative abundance allowing detection on 2-D gels, suggest that this protein may have an important structural or functional role in the initial colonisation of the plant by *F. graminearum*.

Many proteins affected by *F. graminearum* infection were involved in defence or stress responses such as aldehyde dehydrogenase. Aldehyde dehydrogenase detoxifies aldehydes formed by lipid peroxidation due to ROS generated during abiotic and biotic stress [21, 22]. A previous proteomics study on barley responding to *F. graminearum* at 3 dai showed that ascorbate peroxidases, peroxidase, glutathione transferase, malate dehydrogeneses and peroxiredoxins increased in abundance [8]. Aldehyde dehydrogenase gene expression was also upregulated during the incompatible interaction between barley and the fungus *Pyrenophora teres* [22]. Overexpression of the gene in *Arabidopsis* resulted in improved tolerance to oxidative stress [23]. Accumulation of this protein in the present study suggests an oxidative burst in the interaction between *F. graminearum* and a susceptible barley cultivar.

As expected, several PR proteins were upregulated in response to *F. graminearum* infection. Previous proteomic analysis of barley infected with *F. graminearum* revealed upregulation of one PR-3 and three PR-5 proteins [8]. In the present study, three spots containing PR-3 (increasing by 2.0, 2.1 and 2.5 fold, respectively), one containing basic PR-1 (increasing by 1.5-fold) and one containing PR-5 (increasing by 1.6-fold) accumulated at 2 dai. Good agreement was observed between protein and transcript levels for these PR genes and significant increases in transcript abundance were observed for PR -1b, -3, -5, -9 and -15 in barley from 2 to 3 dai (Table 1). However, in contrast to the strong accumulation of both PR-2 protein and transcript observed in *F. graminearum*-infected wheat [5–7], PR-2 transcripts did not change significantly 1–3 dai in the present study. Others showed barley PR-2 gene expression to increase after 3 dai using microarray analysis and Northern blotting [2]. The lack of PR-2 accumulation observed here may reflect different responses of wheat and barley, or cultivar-related differences to *F. graminearum* infection.

Other proteins affected by *F. graminearum* infection were involved in primary metabolism. Enzymes in glycolysis, pyruvate metabolism, citric acid cycle, as well as ribulose 1,5-bisphosphate carboxylase/oxygenase were upregulated, suggesting an increased energy requirement during initial stages of infection. Similar observations were made for a compatible interaction between cotton and the fungus *Thielaviopsis basicola* [24]. It was suggested previously that ribulose 1,5-bisphosphate carboxylase/oxygenase may be downregulated in wheat infected with *F. graminearum* at 3 dai; however, this was based on the appearance of proteolytic fragments on 2-D gels and not on a change in amount of full-length protein [7]. Enzymes in starch metabolism were also affected. The observed decrease in sucrose synthase may result in a reduction in starch synthesis in infected kernels. An increase in the amount of β-amylase 1 was also observed. The increase in abundance of ribosomal protein, translation initiation factor and isovaleryl-CoA dehydrogenase, and decrease in abundance of elongation factor and alanine aminotransferase suggest changes in protein synthesis and turnover. Combined with the potential reduction in starch synthesis this may indicate a shift in the balance of carbon–nitrogen metabolism in the infected grains. Nitrogen metabolism is important for several aspects of the *F. graminearum* interaction with cereals although the molecular mechanisms are not fully understood. The fungus is thought to be in a state of nitrogen starvation during interaction with the plant [25] and depletion of nitrogen or nutrients is required for *Fusarium* spores to germinate and infect plants [26]. Recently, proteome analysis of mature barley seeds from plants grown with low or high levels of nitrogen and infected with *F. graminearum* indicated that FHB and *F. graminearum*-induced proteolysis was more severe in plants grown with low N [9].

Several spots containing HSP 70 either increased or decreased in intensity in infected plants. Interestingly, most of the HSP70 spots decreasing in intensity in infected barley were predicted to be chloroplastic forms that were not the source of the proteolytic fragments which increased in intensity (Supporting Information Table S3). HSP70 has been shown to have a protective role during abiotic and biotic stresses [24]. One spot containing S-adenosyl-L-homocysteine hydrolase, an enzyme in the activated methyl

cycle, increased in response to *F. graminearum*. S-adenosyl-L-methionine, the major methyl-group donor in the cycle, is required for the biosynthesis of various phenylpropanoid derivatives and is also an intermediate in the biosynthesis of ethylene, both of which are associated with plant defence [2, 27]. The gene encoding S-adenosyl-L-homocysteine hydrolase was activated rapidly in cultured cells and leaves of parsley after treatment with fungal elicitors [27, 28]. An expansin-like protein was also upregulated, possibly resulting in cell wall alterations affecting susceptibility to pathogens [29]. Two spots with decreased intensity in *F. graminearum*-infected spikelets contained thiamine biosynthetic enzyme and progesterone 5-β-reductase, involved in biosynthesis of secondary metabolites thiamine and cardenolide, respectively, which may affect plant responses to pathogens [30, 31]. The decrease in abundance of cell division control protein in two spots in infected plants suggested that fungal attack may reduce cell division and thus interfere with grain development.

The proteins identified here are probably among the most abundant proteins responding to the early stages of *F. graminearum* infection. A deeper insight into less abundant proteins would be achieved by applying enrichment strategies combined with LC-MS/MS for protein identification. However, it is worth noting that the identification and analysis of proteolytic fragments necessitates a gel-based approach since information about protein mass is lost in gel-free approaches. In fact, in cases such as *F. graminearum* infection, where one of the biological effects is significant proteolysis, misleading quantification of proteins may result from gel-free analysis.

In conclusion, by characterising the degree of *F. graminearum* colonisation based on measurements of fungal biomass and fungal-induced proteolysis it was possible to carry out proteome analysis at a well-defined stage prior to extensive degradation of plant proteins. This enabled identification of barley proteins responding early to infection by the fungus. These changes were compared with the induction of PR-gene expression in the first days of infection. The results suggest that initially there is an increased energy metabolism in infected seeds that may aid the growth of the fungus. Protease genes expressed by the fungus are likely to cause the massive degradation of plant proteins previously observed. Proteolytic activity in infected grains causes the early appearance of discrete fragments of β-amylase, which can serve as a proteome-level indicator of infection and grain protein quality.

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Chapter 4

Secretome-based proteomics for uncovering pathogenicity factors in *Fusarium graminearum* during interaction with barley and wheat

Secretome-based proteomics for uncovering pathogenicity factors in *Fusarium graminearum* during interaction with barley and wheat

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SUMMARY

Fusarium graminearum is a phytopathogenic fungus infecting a number of small grain cereals including barley and wheat. Secreted enzymes are known to have an important role in pathogenicity of many fungi. In order to access the secretome of *F. graminearum*, a gel-based proteomic approach was employed to identify proteins secreted to culture medium containing barley or wheat grain flour, the most natural host, for the first time, revealing 155 fungal protein identifications in 69 unique proteins in either medium. The proteins identified mainly included enzymes involved in degradation of cell walls, starch and proteins. Seventy-two spots significantly changing in intensity between two media were identified to be proteins with diverse function. Subsequently, some genes encoding proteins expressed in the two media were examined by qRT-PCR analysis in *F. graminearum*–infected spikelets of barley and wheat from 2 to 6 days after inoculation (dai), showing expression of all selected genes and similar down-regulation profiles of most genes during infection in both hosts. The correlation between protein *in vitro* and the corresponding gene expression *in planta* indicates that the *in vitro* proteome approach may be an ideal strategy to discover proteins involved in pathogenicity during plant infection. Differences in host-*F. graminearum* interactions were noted between barley and wheat, *e.g.* an increase in fungal biomass in barley was correlated with the appearance of fungal induced proteolytic fragments of β-amylase in barley, but not in wheat.

INTRODUCTION

Fusarium graminearum is a pathogenic filamentous fungus, which can infect barley, wheat and other small grain cereals, causing a destructive disease, namely Fusarium head blight (FHB). The disease results in reduced grain yield and quality. However, more important is the contamination by mycotoxins including tricothecenes such as deoxynivalenol (DON) and derivatives, as well as the polyketide zearalenone which poses a significant hazard to food safety and human health (Desjardins, 2007). Control of the disease has attracted much attention including 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 dai (Skadsen *et al*., 2000; Boddu *et al*., 2006). Disease symptoms do not spread in the spikes of most barley cultivars, indicating type II resistance of barley (Jansen *et al*., 2005). In contrast, wheat spikes are vulnerable to *F*. *graminearum* mainly during anthesis. Fungal hyphae 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). The disease can spread by the way of the vascular tissues in the rachilla and rachis, although some resistant 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 disintegration of cell walls, disappearance of protein bodies and alteration of the starch granule structure (Jansen *et al*., 2005; Jackowiak *et al*., 2005).

The pathogen must penetrate the cell wall and consume plant nutrients for its growth, suggesting the secretion of an arsenal of hydrolases (Kang and Buchenauer, 2000). Proteomic techniques revealed that *F*. *graminearum* produced several extracellular proteins such as lipases, xylanases, pectinases, cellulases and proteases when grown in synthetic or hop cell wall medium (Paper *et al*., 2007; Phalip *et al*., 2005) and some of these proteins were also observed in infected wheat spikes by using enzyme labelling and LC-MS (Kang and Buchenauer, 2000; Paper *et al*., 2007). Alkaline proteases were detected by western blotting from protein

extracts of barley seeds infected with *F. culmorum*, but not in uninfected barley grain (Pekkarinen *et al*., 2003). *Fusarium* can also produce some 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 oligogalacturonases degrading pectin, can act as elicitors of defence reactions (Paper *et al*., 2007). Additionally, certain mycotoxins produced by *F. graminearum*, such as DON which inhibits protein synthesis, are often considered as virulence factors in pathogenesis (Boddu *et al*., 2006; Maier *et al.* 2006), others apparently do not, *e.g.*, the oestrogenic zearalenone (Lysøe *et al.* 2006). The biological role of the mycotoxins and other natural products (or secondary metabolites) is, in general, still unclear.,.

The availability of the complete genome sequence for *F. graminearum* provides increased opportunity to understand the pathogenicity of the fungus such as secreted proteins involved by using functional genomics, transcriptomics and proteomics analysis (Paper *et al*., 2007; Phalip *et al*., 2005; Güldener *et al*., 2006). Differential transcript accumulation was detected using a *F. graminearum* GeneChip when the fungus was cultivated in media or infected barley plants (Güldener *et al*., 2006). It was shown by 2-DE and MS that the *in vitro* exoproteome of *F. graminearum* grown on glucose and on hop cell walls contained 23 and 84 unique proteins, respectively, mainly involved in cell wall polysaccharide degradation (Phalip *et al*., 2005). By high-throughput LC-MS/MS, 229 and 120 fungal proteins, mainly including glycoside hydrolases and proteases, were identified in the secretome of *F. graminearum* during growth on 13 synthetic media with carbon supplements and during infection of wheat heads, respectively (Paper *et al*., 2007). However, half of the *in planta* proteins lacked signal peptides in that study. Overall, it is still highly challenging to study secreted proteins involved in pathogenicity *in planta*due to high ratio of biomass and genome size between host and pathogen resulting in the dominance of host proteins.

To obtain a better understanding of pathogenicity during the interaction between *F. graminearum* and its hosts barley and wheat, the fungal extracellular proteins were studied by 2-DE-based proteomics. To best of our knowledge, it is the first time that the approach of accessing fungal secreted proteins by growing *F. graminearum* in liquid medium only containing barley grain or wheat grain which is the most natural host have been applied. This system is easily manipulated. Subsequently, the *in vitro* secretome was examined in

a real biological plant–pathogen interaction system. We observed a high abundance of cell wall-, starch- and protein-degrading enzymes displayed in the secretome which was dependent on the medium composition and partially different from protein identifications in the previous secretomic studies and noticed a good correlation between secreted proteins *in vitro* and corresponding transcripts *in planta*.

RESULTS

Secretome *in vitro*

The 2D patterns of culture supernatants from *F. graminearum* grown in barley and wheat grain media were similar (Fig. 1). Approximately 170 protein spots were resolved on both representative 2D gels (Fig. 1). In total, proteins in 158 spots were identified in 71 unique protein accessions (Table S1). Of these, six spots were identified as belonging to two plant proteins, namely serpin and fasciclin-like protein. Based on much lower observed molecular weight than expected for the full-length protein, some identifications (*i.e.* FG05292, FG11097, FG00806, FG04527, FG03975, FG03687, FG11008 and FG01956) might represent proteolytic fragments (Table S1). Most identified fungal proteins were present in several spots exhibiting distinct pI values, indicating possible post-translational modifications (PTMs) of the same gene product or sequence-related isoforms. According to the predicted biological function, the identified fungal proteins were assigned to categories including glycoside hydrolases (38%), proteases (31%), esterases (16%), oxidoreductases (21%), nucleases (7%), lyases (1%), housekeeping enzymes (11%), proteins with other function (19%) such as involvement in cell wall organization and biogenesis and proteins with unknown function (11%) (Fig. 2). In total, 119 fungal identifications of 48 unique proteins had N-terminal signal peptide sequences predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/), indicating that they are extracellular proteins whereas 16 fungal identifications of 11 unique fungal proteins were predicted to represent proteins secreted in a non-classical way using SecretomeP (using algorithm for mammalian protein, http://www.cbs.dtu.dk/services/SecretomeP/) (Table S1). Given the previous findings that pathogenesis genes with putative secreted function such as genes encoding cell wall-degrading enzymes could be clustered into “pathogenicity islands” (Paper *et al*., 2007) and are often highly enriched in the high- single nucleotide polymorphism (SNP)-density regions (Cuomo *et al*., 2007), we examined the location of the corresponding

genes of identified proteins. This revealed that around 34% of the genes encoding putative secreted enzymes predicted to function in the penetration and maceration of plant tissues for the acquisition of nutrients were in high-SNP-density regions, of which around 82% were located in chromosome 2 and 3 (Table S1).

Seventy-two spots significantly changed in intensity when comparing *F. graminearum* growth in barley and wheat flour medium. Of these, 48 exhibited greater intensity in the wheat medium and 24 in the barley medium (Table S1). Sixty-eight of these spots identified as fungal proteins had diverse function such as glycoside hydrolases, proteases, esterases, oxidoreductases and housekeeping enzymes. Additionally, in some cases multiple spots with altered pI representing the same protein (e.g. FG04527 and FG02658) exhibited different expression patterns between two media.

Fungal infection and disease development in barley and wheat

The disease symptom of brown discolouration was visible in barley spikelets, but not in wheat at 2 dai. From 4 to 6 dai, clear symptoms were observed in both plant species with more rapid development in barley (Fig. S1). In order to examine the disease development and levels of infection more accurately, fungal biomass associated with proteome degradation patterns in plants induced by *F. graminearum* was determined. Consistent with disease symptoms, the concentration of *F. graminearum* DNA was very low at 2 dai in infected spikelets of both plants and increased more dramatically from 4 to 6 dai in barley than wheat (Fig. 3A). Furthermore, it was demonstrated previously by western blotting that proteolytic fragments of barley β-amylase were induced by *F. graminearum* in infected barley at 3 dai which could serve as proteome-level indicator of fungal infection (Yang *et al*., 2010b). In the present study, β-amylase antibodies were therefore used to detect proteolysis, revealing the appearance of discrete proteolytic fragments of β-amylase at 4 and 6 dai in barley, but not in wheat (Fig. 3C). This may be due to differences in the susceptibilities of the β-amylases in barley and wheat to fungal proteases despite their 82% sequence identity or the distinct type or amount of fungal proteases produced in the different host plants. The observation of sharper increases in fungal biomass in barley than in wheat from 2 to 6 dai might be connected with the appearance of β-amylase fragments in barley, but not in wheat. Furthermore, there was no significant difference in the concentration of DON relative to fungal biomass at 6 dai between infected wheat and barley despite of a higher level of

infection in barley (Fig. 3B). The production of DON is clearly implicated as a virulence factor in pathogenesis in FHB (Boddu *et al*., 2006) and greater disease severity in wheat grain was observed by inoculation with a DON-producing *F. graminearum* strain than a non-producing strain (Nicholson *et al*., 1998). Therefore, our results support that different severity of infection in wheat and barley is not due to the difference in DON production levels.

Gene expression *in planta*

The expression patterns of ten fungal genes encoding proteins identified *in vitro* including peroxidase, proteases, glycoside hydrolases, chitin deacetylase as well as proteins of unknown function were examined *in planta* by qRT-PCR at 2, 4 and 6 dai. In addition, the *Tri5* gene encoding trichodiene synthase, an enzyme which catalyses the folding of farnesyl pyrophosphate to trichodiene as the first specific step in the biosynthesis of all trichothecene mycotoxins (Niessen *et al*., 2004) was included in the analysis. The expression of all selected genes was detectable in infected plants, but not in the controls. Surprisingly, no substantial difference in relative expression profiles of fungal genes at 2-6 dai were observed between barley and wheat although some genes were significantly differentially expressed between barley and wheat at certain time points (Fig. 4). The examined transcripts were down-regulated at 2-6 dai, except three (FG04732, FG06616 and FG11468), with the highest relative expression at 4 dai. Five transcripts (FG00806, FG06278, FG02616, FG06993 and FG06549) showed a stable relative expression at 2-4 dai. Furthermore, there was an increased relative expression level of the *Tri5* gene in barley compared to wheat at 2-6 dai (Fig. 4), despite a similar amount of DON production per fungal biomass in two hosts. The reason of the poor correlation between *Tris5* gene expression and DON production may be that trichodiene synthase is not the sole enzyme involved in biosynthesis of DON.

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 particular, the fungal secreted degradative enzymes, which play an important role in nutrient acquisition, substrate colonization

and ecological interactions have attracted attention (Kang and Buchenauer, 2000), but the fact that the fungal biomass constitute a small portion of the total interaction in infected plants makes it very challenging to identify fungal proteins *in planta*. So far, only a few proteomic studies have been performed on this organism with host plant substrates. Although high-throughput LC-MS/MS has been applied to protein extracts from *F. graminearum*–infected wheat heads obtained by vacuum infiltration, resulting in identification of 120 fungal proteins, surprisingly half of protein identifications lacked signal peptides including several housekeeping enzymes (Paper *et al*., 2007). Possible explanations for this could be breakage of fungal cells by extraction, fungal cell lysis or that proteins without signal peptides are in fact secreted. Therefore, we profiled the secreted proteome of *F. graminearum* on 2D gels in the presence of substrates of barley and wheat grains flour which are the most natural hosts for the fungus, unlike the substrates used in the previous studies of *F. graminearum in vitro* secretome (Paper *et al*., 2007; Phalip *et al*., 2005). Approximately 80% of protein identifications were predicted to be extracellular. This approach provides an ideal model to understand plant–pathogen interactions and offers a major advance in identification of a large number of fungal secreted proteins displayed on a 2D map, especially providing valuable information on mass and pI of proteins, which are generally known to be present in multiple forms in the fungal secretome. This study considerably expands the current database of *F. graminearum* secreted proteins which can be involved in FHB.

Among the proteins indentified, putative cell wall-, starch- and protein-degrading enzymes were predominant in the fungal secretome (approximately 54% of the identifications) and these appeared in multiple forms, strongly suggesting the fungus may be able to disrupt host cell wall and access the internal protein and starch in the grains for growth. Some proteins were identified as cell wall proteins (FG03017, FG05292, FG04074 and FG10089), chitin deacetylase (FG06549) which converts chitin, the main component of the fungal cell wall, β-1,6-glucanase (FG08265) and β-1,6-galactanase (FG11184) which act on polysaccharides not found in plant cell walls. They may function in supporting vegetative growth by remodelling the fungal cell wall during growth and development (Nagendran *et al*., 2009). All these proteins or the corresponding genes, except FG05292, were also detected in previous proteome studies (Paper *et al*., 2007; Phalip *et al*., 2005) and microarray analysis of infected barley (Güldener *et al*., 2006). These types of secreted proteins or genes encoding putative secreted enzymes with similar function have been found in

several other fungi, including *Saccharomyces cerevisiae*, *Ustilago maydis*, *Trichoderma reesei* and ectomycorrhizal fungi, and are assumed to be involved in modification of fungal cell walls (Mueller *et al*., 2008; Nagendran *et al*., 2009). However, there are very few reports on changes of fungal cell wall structure during growth, especially in pathogenic fungi such as *Ustilago maydis* and *F. graminearum* during infection. Thirty-six fungal identifications including 11 annotated as putative housekeeping enzymes lacked N-terminal peptides. Of these, 16 including housekeeping enzymes glyceraldehyde-3-phosphate dehydrogenase, cysteine synthase and ubiquitin-like proteins were predicted to be secreted in a non-classical way using SecretomeP (using the algorithm for mammalian proteins, http://www.cbs.dtu.dk/services/SecretomeP/).The observation of identifications without predicted signal peptides might due to the lysis of fungal cells occurred during experimental operation or growth *in vitro*. Alternatively, these fungal proteins may in fact be secreted in the non-classical pathway which has been demonstrated in several fungi such as yeast, *Aspergillus fumigates* and *Claviceps purpurea* (Nombela *et al*., 2006) and in bacteria and mammals (http://www.cbs.dtu.dk/services/SecretomeP/). Pancholi *et al*. (2003) reported that several housekeeping enzymes without extracellular functions were present on the surface of pathogens serving as virulence factors such as glyceraldehyde-3-phosphate dehydrogenase, which was also identified in three spots in the present work. Some fungal proteins, such as β-xylosidase, lectin and superoxide dismutase, which do not possess signal peptides, have been found to be truly secreted by experimental characterization (Paper *et al*., 2007).

The proteins in the present study shared 17 and 41 fungal protein identifications from the secretome of *F. graminearum* in infected wheat heads and grown on 13 media consisting of synthetic medium with variable carbon supplements by LC-MS/MS (Paper *et al.*,2007), respectively. The proteins shared 8 and 15 protein identifications from secretome of *F. graminearum* grown on glucose and on hop cell walls by 2-DE/MS (Phalip *et al*., 2005), respectively (Table S1). Most common *in vitro* identifications were glycoside hydrolases, proteases and esterases whereas most common identifications included mainly glycoside hydrolases, housekeeping enzymes and cell wall proteins when comparing the present *in vitro* secretome to the *in planta* secretome. It is not surprising that a higher number of common identifications were obtained when the fungus was grown *in vitro* than *in planta* because different fungal regulatory mechanisms can be

triggered during the interaction with a plant compared to growth *in vitro* where enzymes for degradation of substrates were mainly produced. More interestingly, despite the presence of housekeeping proteins in the *in planta* secretome, but absent from the *in vitro* secretome with a synthetic medium (Paper *et al*., 2007; Phalip *et al*., 2005), several housekeeping proteins were identified from the *in vitro* secretome in this study with barley or wheat flour medium, suggesting they may be secreted in response to the plant milieu. Thus, our results demonstrate that an *in vitro* grain medium may more closely reflect infection conditions *in planta* than a chemical medium. Moreover, besides housekeeping enzymes, most of the proteins identified only in our study were glycoside hydrolases, oxidoreductases and proteins with unknown function which most likely reflect the different growth conditions.

A direct comparison between two fungal proteomic maps can lead to rapid identification of medium-specific secretion responses. Therefore, we conducted the comparative proteome analysis of fungal secretomes. As expected, some proteins, differentially expressed at transcript level or differentially modified at post-translational levels under the two growth conditions, were involved in degradation of plant cell walls, starch and protein (Table S1) since there sre notable differences in cell wall and grain composition between barley and wheat. Arabinoxylans (70% w/w) are the main non-starch polysaccharides of cell walls of the mature wheat endosperm. Other components are β-(1→3)-(1→4) glucans (20-29% w/w), which are in 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→3)-(1→4) glucans (Noots *et al*., 2003). Aleurone cell walls in barley consist almost entirely of arabinoxylans (80%) with lesser amounts of cellulose (8%) and protein (6%) (McNeil *et al*., 1975). The increased abundance of glucan 1,3-β-glucosidase (FG06616) in the wheat medium and xylosidase (FG11468) in the barley medium suggests that the cell walls of the aleurone layer may be the first access to nutrient acquisition during growth. Wheat grain has 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. Overall, our results suggest the *F. graminearum* secretome is medium-dependent and that the fungus has the capacity to adapt to the growth conditions.

One way to confirm that protein secreted *in vitro* may be produced *in planta* is to examine identified

proteins at the transcription level *in planta*. Despite the similar growth behaviour of the fungus in barley and wheat media, based on similar concentration and numbers of secreted proteins, there were significant differences in *F. graminearum* colonization between barley and wheat grain at 2-6 dai, based on measurements of the fungal biomass and fungal induced proteolysis. Thus, it was informative to profile the regulation of several fungal genes during infection of these two host plants. The detection of all the chosen transcripts for proteins, identified from the *in vitro* experiments, *in planta* further confirms that our approach is a promising strategy for studying fungal pathogenicity factors. Despite of similar alkaline protease and endothiapepsin gene expression in both plants, the appearance of plant β-amylase fragments only in barley, but not in wheat, at 4 and 6 dai, indicates that these proteases may not be the essential contributors for discrete proteolytic fragments of β-amylase in barley. For all the genes tested, the relative expression levels decreased or did not change significantly at 4-6 dai, but they had variable regulation profiles at 2-4 dai, which may be the important stage of fungal development. Up-regulation of xylosidase and β-1,3-glucosidase and consistently expressed glucoamylase, alkaline protease and hydrolase at 2-4 dai suggests that the pathogen attempts to degrade the cell walls and consume the grain protein for nutrition. Down-regulation of peroxidase at 2-4 dai suggests that the fungus is under decreased oxidative stress during colonization. It has been reported that increased fungal infection and barley transcript accumulation correlated to higher DON accumulation were observed at 2-4 dai, the majority of host gene transcripts detected at 3 dai as well as development of hyphal mats and high DON accumulation. Reduction of host transcripts accumulation was observed at 4-6 dai (Boddu *et al*., 2006). It has also been shown that proteolytic fragments of barley β-amylase were induced by *F. graminearum* in infected barley at 3 dai (Yang *et al*, 2010b). Approximately day 3 is proposed as the timeponit for switching from a biotroph to a necrotrophic phase in *F. graminearum* (Kang and Buchenauer, 1999). Taken together, it strongly suggests day 3 is an important time point for the interaction in barley and wheat. The reason for the observed gene regulation profiles at 2-6 dai may be that the fungus needs to secrete higher amount of enzymes involved in breakdown of complex substrates to establish the initial colonization in plants. After a certain time of invasion (around 3 dai), when the fungus becomes necrotrophic, decreased biosynthesis of some enzymes are required due to the massively damaged host tissues. On the other hand, *F. graminearum* microarray analysis of all the examined genes in infected

barley at 1-6 dai showed an increased accumulation of most of fungal transcripts up to 6 dai except FG02616 and FG07775 (Güldener *et al*., 2006). The reason for differences in fungal gene expression profiles compared to previous microarray studies may be that microarray analysis only reflects the absolute transcript abundance during the infection process rather than gene regulation since the increased fungal biomass is not taken in account (Güldener *et al*., 2006). Our results showing gene regulation patterns at 2-6 dai are highly complementary to microarray analysis. Further detailed studies are needed on fungal gene regulation profiling at a genome-wide scale during the host-pathogen interaction and responding to different infection stages and toxin accumulation.

In conclusion, 2-DE-based proteomics was successfully used to study the secretome of *F. graminearum* grown in medium containing barley or wheat grain flour, revealing nutrition-dependent secretion and the dominance of plant cell wall-, starch- and protein-degrading enzymes. Unlike similar growth in two media, the fungus exhibited faster development in barley than in wheat based on the measurements of fungal biomass and fungal induced proteolysis. All ten selected genes with diverse functions, encoding proteins found on 2D maps *in vitro* were similarly expressed in both host plants at 2-6 dai, generally with decreased transcript abundance. This suggests that our *in vitro* approach is promising to study pathogenicity factors during infection and colonization.

EXPERIMENTAL PROCEDURES

Preparation of fungal culture

Macro-conidial suspensions of *F. graminearum* strain PH1 (ARS Culture Collection, NRRL 31084) were prepared as described by Yang *et al*. (2010b) and used for *in vitro* growth and plant inoculation studies. The growth medium was prepared by mixing 50 g ground grain flour of barley (cv. Hydrogen) or wheat (cv. Vinjett) with 1 L water prior to autoclaving (Schmidt-Heydt and Geisen, 2007). Erlenmeyer flasks containing 100 mL of liquid medium were inoculated with 1 mL macro-conidial suspension (10^6 spores / mL). The flasks were gently shaken by hand and incubated in darkness at 20˚C. Three independent biological replications for each growth condition were carried out. After 7 days, the supernatant was collected by filtration through cheesecloth and centrifugation (14000 g x 30 min, 4°C).

Isolation of secreted proteins and two-dimensional gel electrophoresis

The supernatant was ultrafiltrated (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) with bovine serum albumin as standard. Twenty µg protein was precipitated by 4 volumes of 10% w/v TCA and acetone at -20°C overnight. Protein pellets were sequentially washed three times with 80% v/v acetone before they were dissolved in 200 µL of "reswelling" buffer as described by Yang *et al*. (2010a). Samples were applied to 11 cm pI 3–10 IPG strips. Isoelectric focusing (IEF) was run on an Ettan IPGphor (GE Healthcare) with the following programme: 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 8000V. After IPG strips were cut 1.5 cm at acidic end and 2.5 cm at basic end, resulting in a pH range from 4 to 8.5, the second dimension SDS-PAGE (NuPAGE® Novex 4-12% Bis-Tris ZOOM® Gel, Invitrogen) was performed in the XCell *SureLock*™ Mini-Cell system(Invitrogen) according to the manufacturer' 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 microns. In total, six images for each growth condition were imported to Progenesis SameSpots (Nonlinear Dynamics, UK) for quantification of spot volume and statistic 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. A minimum threshold of 1.5-fold change in normalized average volume of spots between two groups and ANOVA (p) < 0.05 were set to define more or less abundant proteins.

In-gel digestion and protein identification

Spots were excised from the 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 analysis in an Ultraflex II mass spectrometer (Bruker-Daltonics, Bremen, Germany) as described by Yang *et al*. (2010b). Positive protein identifications were determined as described by Yang *et al*. (2010a) by searching databases at the Broad Institute for *Fusarium graminearum* gene index (http://www.broad.mit.edu/annotation/genome/fusarium_graminearum), the NCBInr at the National Center for Biotechnology Information and the wheat gene index Release11.0 (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 and 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 were 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. All identified proteins were assessed for signal peptides using SignalP (http://www.cbs.dtu.dk/services/SignalP) and for non-classical secretion using SecretomeP (Mammalian) (http://www.cbs.dtu.dk/services/SecretomeP). The chromosome location and SNP identification of genes encoding the identified proteins were determined by searching in databases from Broad Institute for *Fusarium graminearum*.

Plant growth and inoculation

The susceptible barley cv. Hydrogen and wheat cv. Vinjett were grown in the greenhouse as described by Yang *et al*. (2010b). Spikelets of wheat and barley were point-inoculated at anthesis (stage 65, Zadoks *et al*., 1974) by micro-pipetting 5 µL of a macro-conidial 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 darkness for 72 h. Three spikes collected from different plants served as one biological replication. Three biological replications were harvested at 2, 4 and 6 dai and stored at -80˚C until use. Inoculated spikelets were lyophilized and ground into fine powder in liquid nitrogen.

Determination of fungal biomass and DON

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

Protein extraction and western blotting

Water-soluble proteins were extracted from 20 mg ground plant material of each sample with 400 µL of buffer containing 5 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$ and the protease inhibitor cocktail "complete" (Roche) as described (Yang *et al*., 2010a). Protein concentration was determined by the Bradford assay (Bradford, 1976) with bovine serum albumin as standard. Western blotting with β-amylase antibodies was carried out as described by Yang *et al*. (2010b).

qRT-PCR analysis

RNA was extracted from 30 mg ground plant material of each sample prior to removal of genomic DNA as well as cDNA synthesis took place as described by Yang *et al*. (2010b). Primers for *F. graminearum* genes (Table S2) were designed and specificity tested as before and *F. graminearum* GAPDH served as a reference gene for quantification of gene expression (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 ± S.D. in 2-log scale. Statistical analysis of gene expression in barley versus wheat at each time point was performed by Student's *t*-test.

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SUPPORTING INFORMATION

Fig. S1 Disease symptoms on water (control) or *F. graminearum*-inoculated spikelets of barley (upper panel) and wheat (bottom panel) at 6 dai.

Table S1 Identification of *F. graminearum* proteins from culture supernatants of the medium containing barley grain flour or wheat grain flour (in CD-ROM).

Table S2 Primer sequences for qRT-PCR analysis of fungal genes.

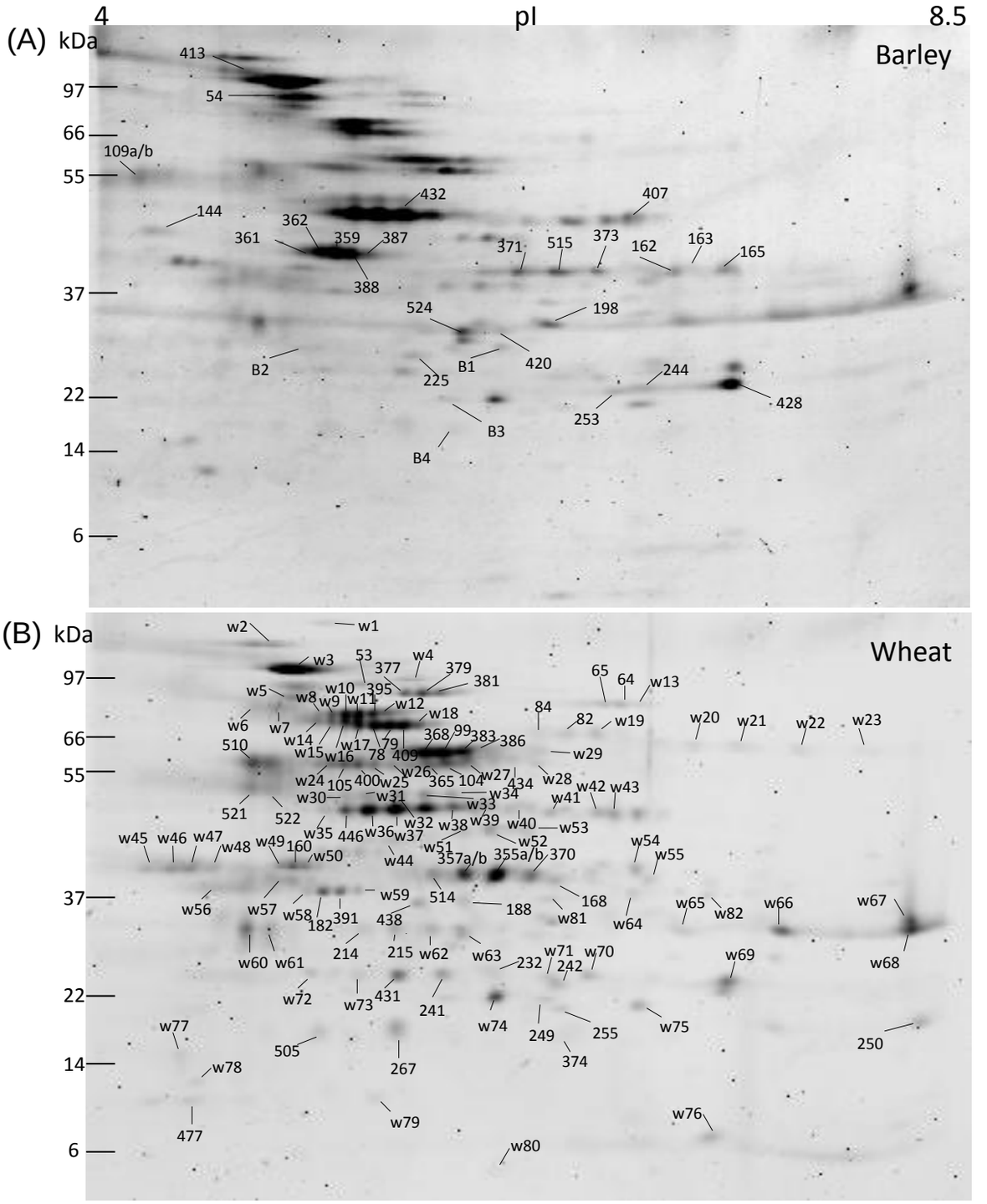
Figure legends

Fig. 1 2-DE Sypro-Ruby stained gels from the secretome of *F. graminearum* grown in medium containing (A) barley grain flour or (B) wheat grain flour. Molecular size markers and pI ranges are indicated. Protein spots which are identified are numbered.

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

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

Fig. 4 Fungal gene expression analysis in infected barley and wheat spikelets by qRT-PCR at 2-6 dai (2-log scale). Asterisks indicate significant differences ($p<0.05$) in relative gene expression after normalization to GAPDH between barley and wheat plants at each time point. The fold-changes is indicated by '+' or '-' (+: more in wheat; -: more in barley).


4
pI
8.5
(A) kDa
Barley
97
66
55
37
22
14
6
413
54
109a/b
144
362
361
359
387
432
407
371
515
373
162
163
165
388
524
198
B2
B1
420
225
244
428
253
B3
B4
(B) kDa
Wheat
w2
w1
w3
53
377
w4
379
381
65
64
w13
w5
w8
w9
w11
w10
395
w12
w18
84
82
w19
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w23
w6
w7
w14
w15
w16
w17
79
78
409
368
99
383
386
510
w29
w24
105
400
w25
w26
365
104
w27
434
w28
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521
522
w32
w38
w39
w40
w53
w45
w46
w47
w48
w49
160
w35
446
w36
w37
w51
w52
w54
w50
w44
357a/b
355a/b
370
w55
w59
514
168
w65
w82
w66
w67
w56
w57
w58
182
391
438
188
w81
w64
w60
w61
214
215
w62
w63
232
w71
242
w70
w69
w68
w72
w73
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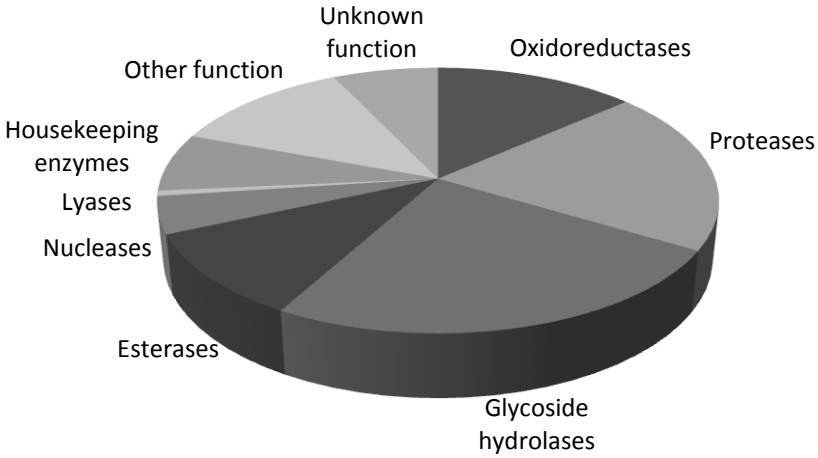
267
374
w78
w79
w76
477
w80

Fig. 1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Oxidoreductases
Other function
Proteases
Housekeeping enzymes
Lyases
Nucleases
Esterases
Glycoside hydrolases

Fig. 2

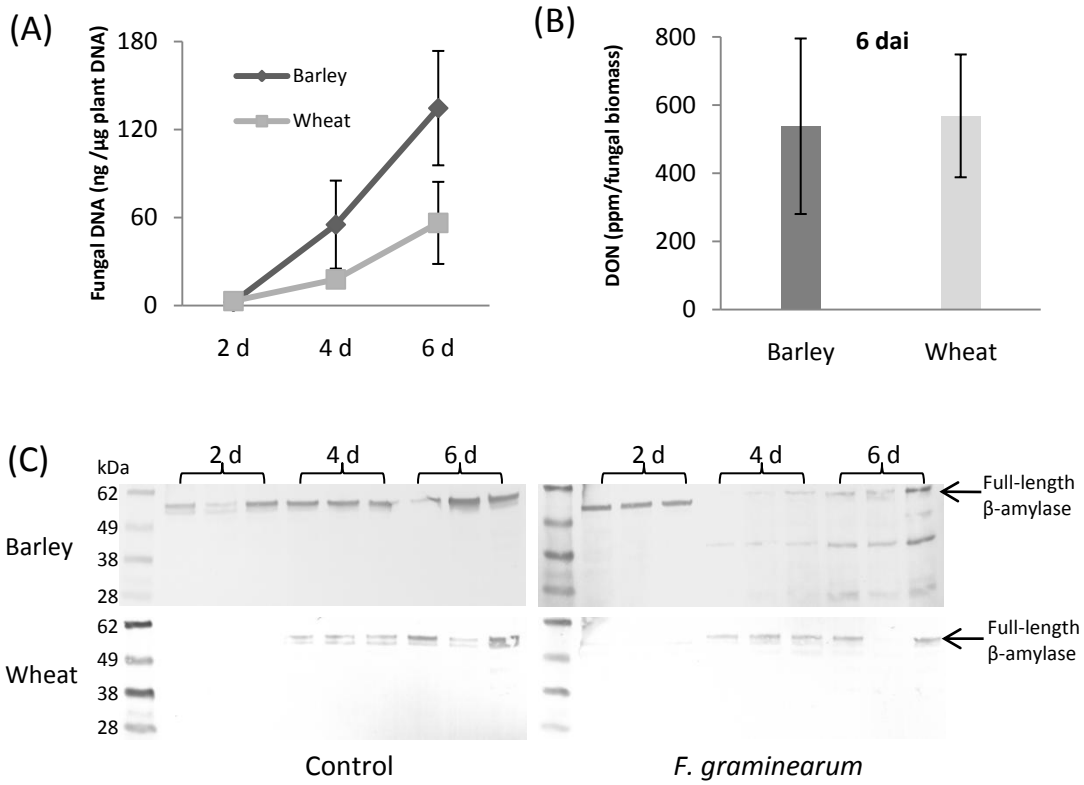
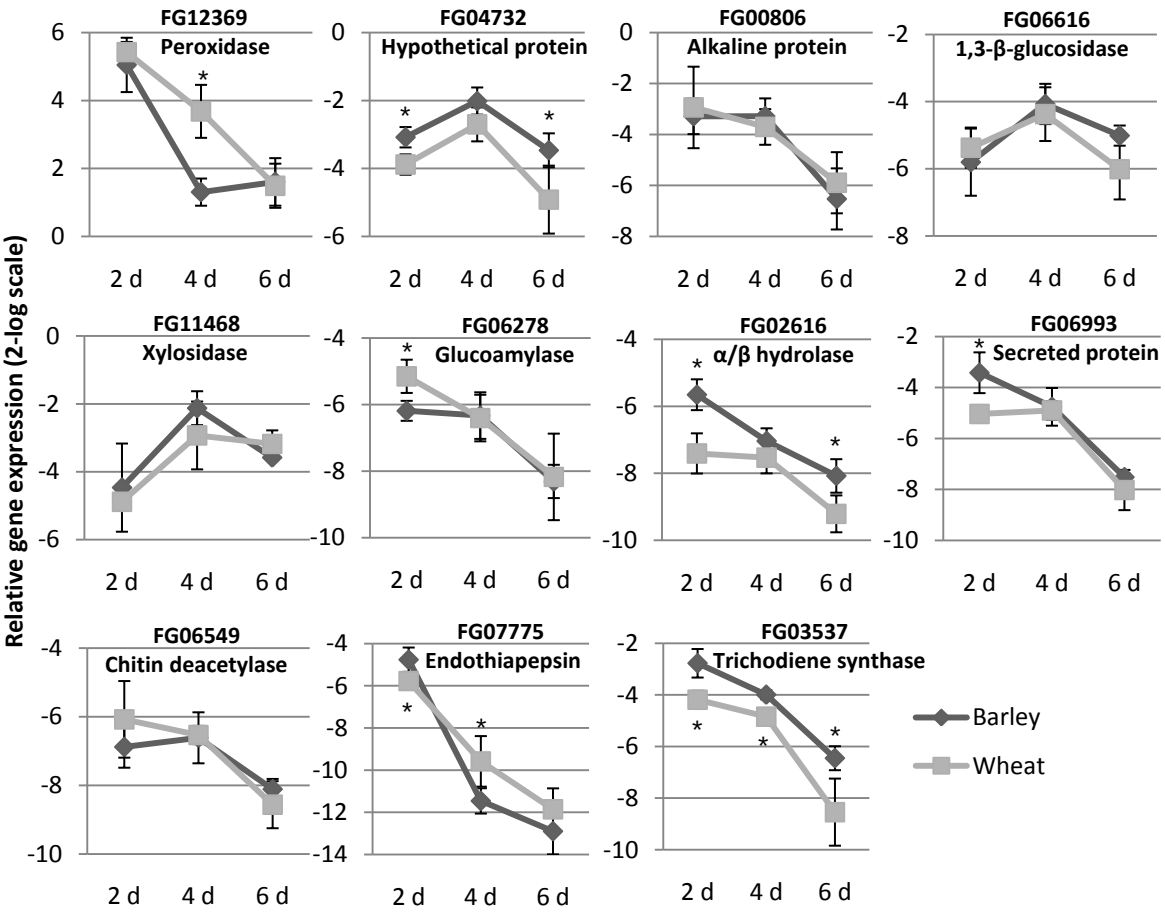

(A)
Fungal DNA (ng /µg plant DNA)
180
120
60
0
Barley
Wheat
2 d
4 d
6 d
(B)
6 dai
DON (ppm/fungal biomass)
800
600
400
200
0
Barley
Wheat
(C)
kDa
2 d
4 d
6 d
62
49
38
28
Barley
Wheat
Full-length
β-amylase
Control
F. graminearum

Fig. 3



	Fold change						
	FG12369	**FG04732**	**FG06278**	**FG02616**	**FG06993**	**FG07775**	**FG03537**
2 d	–	-1.8	2.1	-3.3	-3.4	-2.1	-2.7
4 d	5.5	–	–	–	–	4.2	-1.8
6 d	–	-2.4	–	-2.2	–	–	-3.4

Fig. 4

6 dai

Barley

Wheat

Control *F. graminearum*

Fig. S1

Table S2 Primer sequences for qRT-PCR analysis of fungal genes.

Gene	Accession no[a]	Primer	Sequences (5'→3')
GAPDH	FGSG_06257	F	CGTCTCCGTTGTTGACTTGA
		R	GTAGGCCAGAACACCCTTGA
Peroxidase	FGSG_12369	F	CTTATTGTTCTCGGCGGTGT
		R	TCCTGAGTAGCATCGACACG
Hypothetical protein	FGSG_04732	F	TTATTCGAAAGGTGGCCAAG
		R	GTCGCTGAACACTTCAAGCA
Alkaline protease	FGSG_00806	F	TTCAACAACGCTGTCGAGTC
		R	GAGCAGAGGCAGGAGAAGTG
Glucan 1,3-beta-glucosidase GLUC78	FGSG_06616	F	GTATCAGCACCAAGGCCAGT
		R	AAAAGTTGTTGCGGTTGTCC
Xylosidase	FGSG_11468	F	ATTCTGGAGCCAGTTGATGG
		R	CCTGGCGAAGATAGTCCTTG
Glucoamylase	FGSG_06278	F	CAATATCCCCGCTCTTGGTA
		R	AGAGAGATGGTTGCCTTCCA
α/β hydrolase	FGSG_02616	F	GTCACTTTGGAGGCGACACT
		R	CAAATTGACCGCTTCCTCAT
Secreted protein	FGSG_06993	F	ACTGCACCTACGCCAAGAGT
		R	GCATAGAGCCGAGAGCAGTC
Chitin deacetylase	FGSG_06549	F	TCAGACTGTACCACCCACCA
		R	GGCAAGTGTAGCCAGTGTCA
Endothiapepsin	FGSG_07775	F	GCACAGACACTCAACCTGGA
		R	CTTGGCAGGGTTGTACTCGT
Trichodiene synthase	FGSG_03537	F	CCTGCCATGAGATCACTCTG
		R	CATCACCTGAGGGTCCTTGT

[a] Accession numbers in *Fusarium graminearum* gene index from Broad Institute

Chapter 5

Expression, purification and characterization of a barley PR17a protein and a *Fusarium graminearum* hypothetical protein

1. Introduction

A large array of defense response can be activated in plant attacked by the pathogens including cell wall fortification, production of antimicrobial secondary metabolites such as phytoalexins, accumulation of pathogenesis-related (PR) proteins and programmed cell death (McDowell and Dangl, 2000). Among those, PR proteins are generally believed to have an important role in the resistance to pathogen. PR proteins can exert the anti-fungal activity through inhibition of hypal growth and spore germination, spore lysis, reduction in viability of germinated spore, permeabilization of fungal membranes or triggering plant defense (Campos *et al*., 2008). Based on structural and functional properties, up to 17 families of PR proteins have been identified so far with functions involved in hydrolysis of fungal cell walls (PR2, β-1,3-glucanase; PR3, chitinase), plant cell wall rigidification (PR9, peroxidase) and signal transduction (PR15, oxalate oxidase) (Christensen *et al*., 2002). Most of them are the extracellular proteins (Christensen *et al*., 2002).

The first step to discover the role of the protein in defense is the phenomena of accumulation upon infection. During barley-*Fusarium graminearum* interaction, several PR transcripts or proteins including PR3, PR4, PR5, PR9 and PR15 were found to be upregulated (Yang *et al*., 2010). Interestingly, two spots identified as secreted hypothetical protein (accession no: gi2266664) increased in intensity in that study (Yang *et al*., 2010). This gene transcript was shown to be induced in barley in response to *Blumeria graminis* and the protein product belongs to PR17 family defined as PR17a (Christensen *et al*., 2002). Some proteins such as NtPRp27 from tobacco and WCI-5 from wheat belonging to PR17 family were found to respond to viral or fungal infection, respectively (Christensen *et al*., 2002). However, the function of the PR17a in plant defense to pathogen is still unknown.

Currently two fungi *Bipolaris sorokiniana* and *Septoria tritici* have received much attention since they are the main pathogens resulting in the most serious foliar disease and significant yield losses in cereal worldwide. *B. sorokiniana* can cause foliar spot blotch, root rot, and black point on cereal grains, as well as head blight and

seedling blight of wheat and barley (Kumar *et al*., 2002). As a hemibiotrophic pathogen, *B. sorokiniana* exerts a biotrophic and a subsequent necrotrophic growth phase. *B. sorokiniana* development intracellularly and intercellularly includes cuticle and cell wall penetration followed by the development of hyphae within the invaded and living epidermal host cell (biotrophic phase) and hyphae invasion into the mesophyll layer, accompanied by epidermal and mesophyll cell death (necrotrophic phase) (Kumar *et al*., 2002). Toxins are produced to kill the tissue and aid the infection (Kumar *et al*., 2002). The disease symptoms occur in few days. *Septoria tritici*, a hemibiotrophic fungus, mainly causes speckled leaf blotch of wheat (Shetty *et al*., 2003). *Septoria tritici* invades the host through stomata and grows only intercellularly. Usually no disease symptom can be observed for a long time (10-15 days) and suddenly the plant tissue dies (Shetty *et al*., 2003). Thus, in the present study of the recombinant PR17a protein was made to elucidate its molecular function in cereal resistance to these two pathogens.

Additionally, the study on two *Fusarium graminearum* mutants which have been knocked out transcription factors *YAP* and *AP*, respectively, showed more aggressiveness than wildtype *in planta*. A hypothetical protein (accession no: FG11033) was strongly upregulated in both mutants (Jensen, unpublished data, see Appendix II). In order to gain some insights of the function of this protein and its relation to the transcription factors and pathogenesis, we expressed and functional characterized the protein in the effect on appearance of wheat leaves.

2. Materials and Methods

2.1 Cloning of two genes

Two purchased plasmids pCR2.1 containing cDNAs encoding proteins after removal of signal peptides with restriction enzyme sites *Nde*I (5’ site) and *Bam*HI (3’ site) were digested and cloned into pET-15b expression vector (Novagen). The resulting constructs were introduced into *Escherichia coli* strain DH5α® cells and then into *Escherichia coli* expression strain Rossetta® cells.

2.2 Purification and properties of recombinant proteins

Rossetta® cells were grown in LB medium supplemented with 100 µg/mL ampicillin and 5 µg/mL chloramphenicol at 37 °C until the absorbance at 600 nm reached to 0.6. Expression of His_6-tagged barley protein and fungal protein was induced with 100 µM isopropyl-β-D-thiogalactopyranoside (IPTG) at 20 °C overnight and at 37 °C for 3 h, respectively. Protein was extracted from cell pellets by mixing with Bugbuster reagent and Benzonase (Novagen) for barley protein and by mixing with buffer containing 8 M urea and 0.5 M

Tris-HCl, pH 8.3 for fungal protein prior to purification by a 5 mL His-Trap HP column (GE Healthcare) according to manufacturer's protocols. After the eluted fractions for two proteins were dialysed with buffer containing 100 mM NaCl, 50mM Tris-HCl, pH 8 and 1mM EDTA twice at 4°C overnight and concentrated to 5 mL by ultrafiltration through Centriprep® centrifugal filter (mass cut off: 3 kDa), they were purified on Superdex® 75 column (GE Healthcare) according to manufacturer's instruction. The proteins were analysed by SDS-PAGE followed by staining with Coomassie blue or Sypro Ruby solution. Protein concentration (unit: M) was determined by the following formula: $(OD_{280}-OD_{320})$/extinction coefficient. Mass spectrometric analysis of intact proteins was performed as described (Shahpiri *et al*., 2008). IEF for recombinant proteins was performed using Novex IFE gels (pH3–10) according to manufacturer's instruction. Protein sequences were further subjected to BLAST search in NCBI.

2.3 Free thiol assay

Thiol quantification was determined as described (Ellman, 1959) with some modifications. Fifty µL of samples were mixed with 50 µL of reagent containing 6 M guanidium hydrochloride, 100 mM Tris-HCl, pH 8 and 400 µM DTNB and incubated for 10 min at room temperature in dark. If no free thiol was detected from native protein, fifty µL of samples were mixed with 50 µL of buffer containing 8M urea, 100 mM Tris-HCl, pH 8 and 20 mM DTT and incubated for 30 min at room temperature. Afterwards the sample was applied to NAP-5 column (GE Healthcare) which was equilibrated with 10 mL of buffer containing 4 M urea, 50 mM Tris-HCl, pH 8 and 0.2 mM EDTA followed by washing with 400 µL and elution with 500 µL of equilibration buffer. N-acetyl cysteine was used as standard. The concentration of the free thiol was determined by measuring absorbance at 412 nM.

2.4 Inoculum preparation and plant growth

The spore suspension of *Septoria tritici* isolate IPO323 (10^6 spores/mL) and *Bipolaris sorokiniana* isolate CP1623 (4×10^3 spores/mL) were prepared as described (Jørgensen *et al*., 1996; Shetty *et al*., 2003). Wheat cv. Sevin and barley cv. Pallas near-isogenic line P0-1 were cultivated as described (Shetty *et al*., 2003).

2.5 Infiltration of protein and plant inoculation

When plants were 14-day-old, leaves were infiltrated with 300 µL of protein solution containing 2, 4 or 7 µg barley protein by using a plasitic syringe until complete saturation. Water was used as control. Inoculum of

Bipolaris sorokiniana was immediately sprayed onto the fixed wheat and barley leaves after infiltration whereas *Septoria tritici* inoculum was sprayed only onto wheat leaves. Leaves were harvested at 1, 3 and 6 days after inoculation (dai) for *Bipolaris sorokiniana* inoculation and 6 and 15 dai for *Septoria tritici* inoculation. At least three leaves were prepared for each treatment at each time point. Additionally, two wheat or barley leaves were infiltrated with 2, 4, 7 µg barley protein or water as control without inoculation and harvested after 3, 6 and 15 days. For fungal protein, one 14-day-old wheat leaf was infiltrated with 300 µL protein solution containing 0.2, 1, 2, 4, 8 µg protein or water as control. Samples were harvested after 8 days.

3. Results and discussions

3.1 Barley recombinant protein

3.1.1 Protein purification

Upon the SDS-PAGE gel of the purified barley protein, two bands were visualized with molecular weight around 25 and 51 kDa when stained with Sypro Ruby (Fig. 1A), which might be monomer and dimer, respectively. The results were in agreement with the theoretical mass.

3.1.2 Experimental molecular weight and pI

Two groups of peaks were observed in mass spectrum of barley protein (Fig. 2A). The mass of the first peak indicating a singly charged ion in the first group was around 25122 Da which was expected for barley protein with removal of methionine (Kim *et al.*, 2001). A singly charged ion corresponding to an additional 176 Da in mass, 25298 Da, was observed for the major peak. This additional mass might be resulted from spontaneous α-*N*-6-phosphogluconoylation in *E.coli* (Geoghegan *et al.*, 1999). The mass range of the second group of peaks with poor resolution was from around 50244 to 50672 Da (Fig. 2A), which could be the protein dimers. IEF analysis of barley protein revealed two major bands at pI 5.1 and 5.4 (the theoretical pI) as well as several weak bands (Fig. 3), suggesting the possible degradation of protein or post-translational modification at His-tagged fragment or internal protein sequence.

3.1.3 Amino acid sequence analysis

By BLAST search in NCBI with protein sequence, recombinant barley protein has 58% and 67% homology to barley PR17c and PR17d proteins, respectively (Fig. 4A). Comparing the previous published sequences of so-called PR17b which were also upregulated in barley in response to the fungus *Blumeria graminis* up to 96 hours after inoculation (hai) (Christensen *et al*., 2002), the proteins share 60% identify (Fig. 4A). It has been reported that five regions (Fig. 4A) are highly conserved in the family. The first and second regions are significantly similar to zinc metalloproteinases covering the zinc-binding motif, the active site and the peptide-binding groove. The third and fifth regions contain protein kinase C phosphorylation site and the tyrosine residue possibly to be a proton donor, respectively. Additionally, the free thiol experiment showed that the only cysteine in the sequence was not modified.

3.1.4 Effect of barley protein on fungal pathogen infection of plants

Disease symptoms caused by *Bipolaris sorokiniana* developed more rapidly in wheat than barley at early stage of infection (Fig. 5A, Fig. 5B). Less disease symptom in barley and wheat leaves during 1 to 6 dai with infiltration of barley protein with amount up to 7 µg was observed than control (Fig. 5A, Fig. 5B). Growth of *Septoria tritici* was slower than *Bipolaris sorokiniana in planta* (Fig. 5). However, wheat leaves with infiltration of barley protein showed different responses to *Septoria tritici* infection at 15 dai that more disease severity was observed in most of leaves (Fig. 5C). The reason for the different effects on growth of two pathogens *in planta* is still unknown. Further, to examine the effect of protein on plant growth, the pathogen was not introduced to plants after infiltration of protein. No significant influence by barley protein on appearance of plants was observed (Fig. 5D).

3.2 Fungal recombinant protein

3.2.1 Recombinant protein purification

There was one visible band for purified fungal protein on SDS-PAGE gel stained with Coomassie blue (Fig. 1B), which had molecular weight around 12 kDa in agreement with theoretical mass.

3.2.2 Experimental molecular weight and pI

A group of peaks was revealed in the spectrum of fungal protein (Fig. 2B). The mass of the first singly charged ion was the theoretical mass with removal of methionine. Two peaks with addition of mass 178 and 258 Da to

first peak mass, respectively, were observed. It was reported that extra 178 or 258 Da mass in His-tagged fusion protein occurs resulted from spontaneous α-*N*-6-phosphogluconoylation in *E.coli* (Geoghegan *et al*., 1999). One band at pI 7.45 was shown on IEF gel for fungal protein which was lower than the expected pI 8 (Fig. 3).

3.2.3 Amino acid sequence analysis

Blast searches revealed no obvious identity to the recombinant protein (Fig. 4B). Two cysteines in the protein were modified. However, after reduction with DTT, the free thiol was still not detectable. The reason was unknown.

3.2.4 Effect of fungal protein on wheat leaves

There was no visible difference on the appearance of leaves between control and the ones infiltrated with fungal protein with variable amounts (Fig. 6).

4. Conclusion

PR17a gene has been cloned and expressed *in E.coli* since its transcript was accumulated strongly in barley in response to *Blumeria graminis*. Despite of its similarity to aminopeptidase N from eukaryotes and thermolysin from bacteria in the sequence analysis, the recombinant protein did not show any protease activity in the previous study (Christensen *et al*., 2002). In order to illustrate the function of this protein during plant-pathogen interaction, we expressed and characterized the PR17a protein in the present study. The results revealed that the recombinant protein might inhibit fungal pathogen *Bipolaris sorokiniana* infection of barley and wheat leaves and promote *Septoria tritici* infection of wheat leaves, which may relative to the different pathogenicity mechanisms of two pathogens. The mechanism by which PR17a functions to control and restrict invasion of *Bipolaris sorokiniana* and structure-based function characterization will be focused on in the future research. Different proteases assays from the ones in the previous study will be tested on this protein as well.

The recombinant *Fusarium graminearum* protein (FG11033) was successfully expressed in *E.coli* and purified for the first time. No visible effect of this protein on appearance of wheat leaves was observed. Future work will focus on metabolic changes triggered by this protein in plant leaves if any.

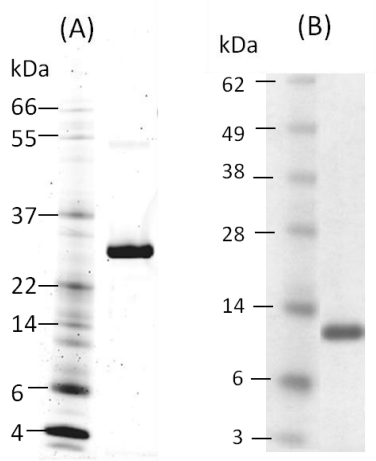


Fig. 1. Purity of recombinant barley protein (A) and fungal protein (B) analysed by SDS-PAGE stained by Sypro Ruby and Coomassie blue staining, respectively. Molecular size markers are indicated.

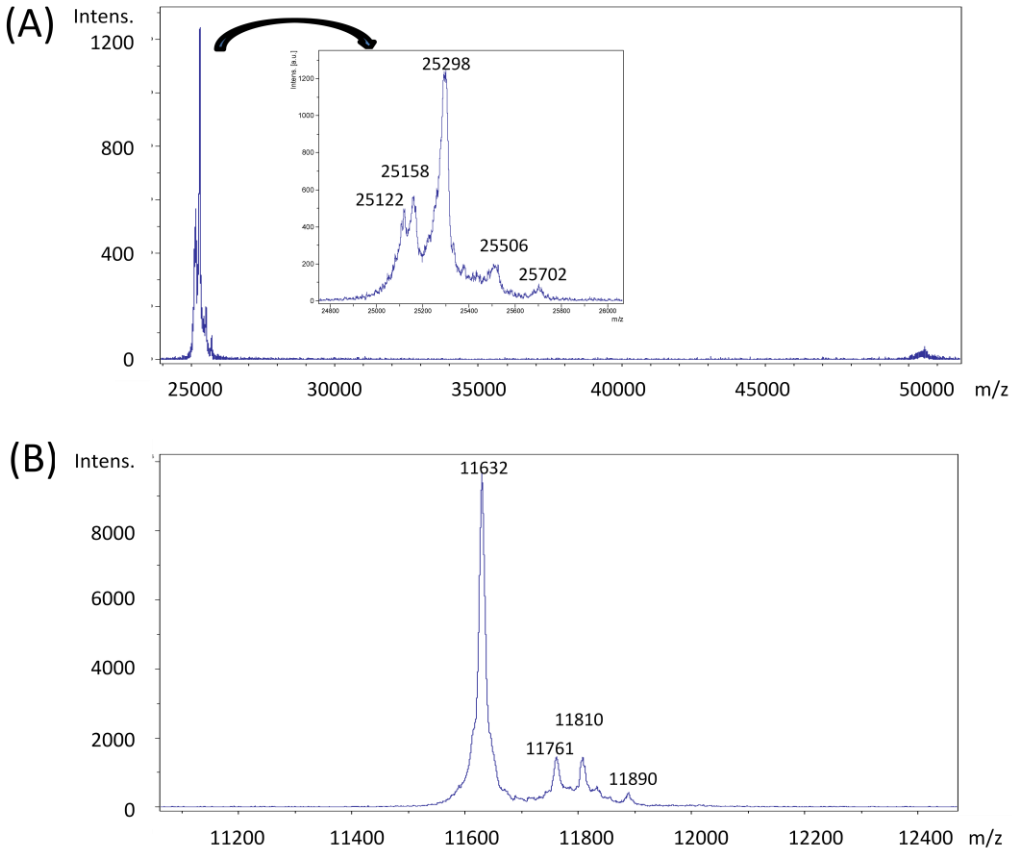


Fig. 2. MS analysis of purified recombinant barley protein (A) and fungal protein (B). The mass (Da) of each peak is indicated.

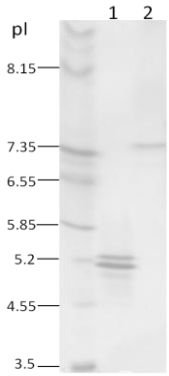


Fig. 3. Coomassie blue-stained IEF electrophoresis analysis of recombinant barley protein (lane 1) and fungal protein (lane 2). pI markers are indicated.

(A)

```
PR17b                          MKPQVATVAF---FLLVTMAATARAVTFDASNTASGTAGGQRFDNAVGLAYSKQVLSDAS 57
gi|157093712|PR17c             MKLQVATVAS---FLLVALAATAQAVTFDASNKASGTSGGRRFEQAVGLPYSKKVLSEAS 57
gi|157093716|PR17d             MKIAIAAAAAAPLLLLLALAGTARAVTFDATNTVPDSAGGQRFNQDVGVDYAKQVLSDAS 60
Recombinant protein(PR17a)     MGSSHHHHHH----SSGLVPRGSHMVTFDVTNEASSTAGGQRFDREYGAAYAKQVLSDAS 56
                                                  ****.:* ...::**:**:.  *  *:*:***:**

PR17b                          TFIWNTFNQRAAADRKPVD--AVTLVVED-IGGVAFASGNGIHLSAKYVGGYSGD-VKKE 113
gi|157093712|PR17c             AFIWKTFNQRAVGDRKPVN--AVTLVVED-ISGVAFTSANGIHLSAQYVASISGD-VKKE 113
gi|157093716|PR17d             SFIWTTFNQPNPGDRRDYD--SVTLAVVDNIEPVAQTVGNAIQLRAQYVAGFDGD-VKQE 117
Recombinant protein(PR17a)     SFTWGIFNQPDPSDRRPADGDTVTLAVRD-TNGIASTSGSTIELSARSVGGITGDNLKEQ 115
                               :* *  ***   .**:  :  :***.* *    :* : .. *.* *: *..  ** :*::

                                        Region 1                    Region 2
PR17b                          VTGVLYHEATHVWQWNGR-GTANGGLIEGIADYVRLKAGLAPGHWRPQGSGDRWDQGYDI 172
gi|157093712|PR17c             VTGVLYHEATHVWQWNGQ-GKANGGLIEGIADYVRLKAGFAPGHWVKPGQGDRWDQGYDV 172
gi|157093716|PR17d             VKGVLYHEATHVWQWIDH-YGEKPGLFEGIADYVRLKADLAPGHWVKDGGGDRWDQGYDV 176
Recombinant protein(PR17a)     VDGVLYHEVVHVWQWGLQDYHEHHGIFEGIADYVRLKAGYVAANWVKEGGGSRWDEGYDV 175
                               * ******..*****  :    : *::***********. ...:*   * *.***:***:

                                Region 3        Region 4                    Region 5
PR17b                          TARFLDYCDSLMPGFVAQLNAKMKSGYSDDFFAQILGKNVQQLWKDYKAKFGG--- 225
gi|157093712|PR17c             TARFLDYCDSLKPGFVAQLNAKMKSGYTDDFFAQILGKNVQQLWRDYKSKFGA--- 225
gi|157093716|PR17d             TARFLDYCDSLKPGFVAEMNGKLKDGYSDDYFVQILGKSVDELWSDYKAKYPQPQS 232
Recombinant protein(PR17a)     TARFLDYCDSRKPGFVAEMNGKLKDGYNDDYFVQILGTSADQLWNDYKAKYSQG-- 229
                               **********  *****::*.*:*.**.**:*.****...::** ***:*:
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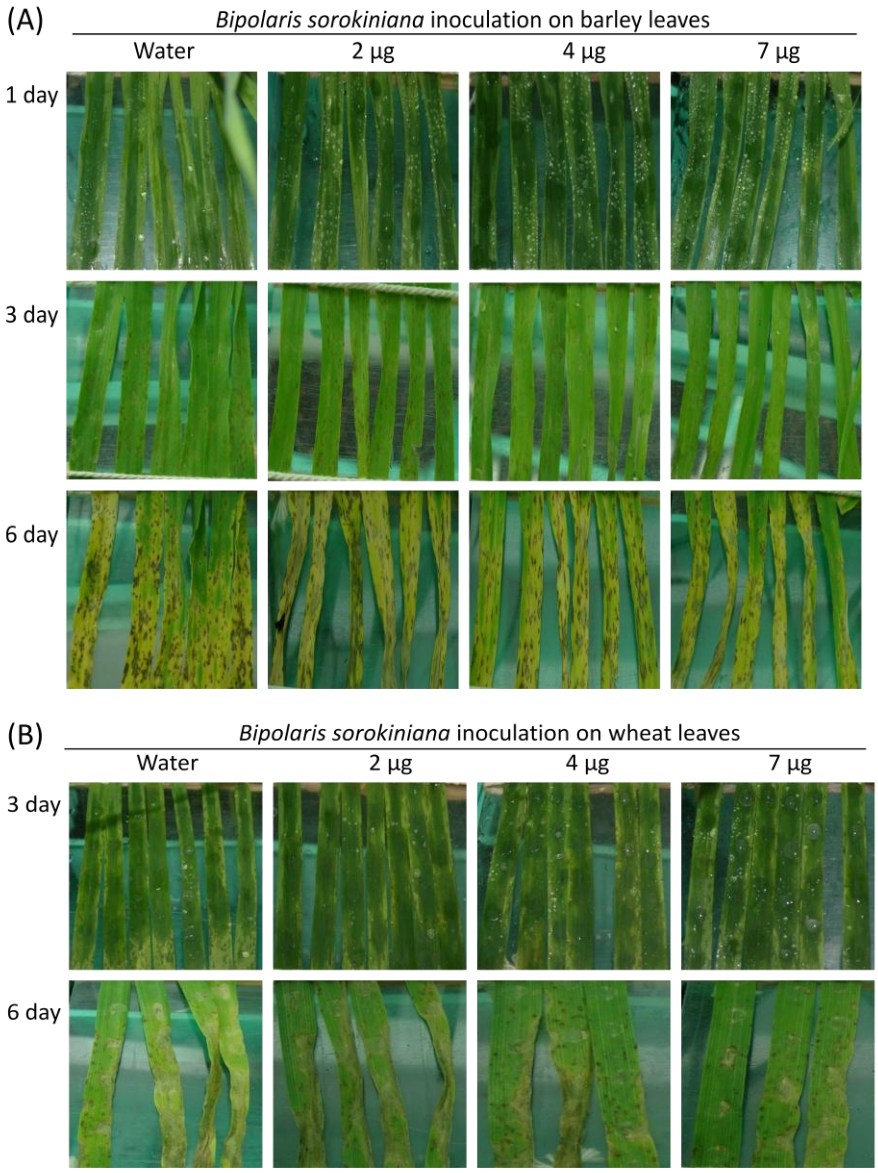
(B) Recombinant fungal protein

MGSSHHHHHHSSGLVPRGSHMASIKVNFYSDTGCRNFIGSRFIDYNANQGGTYHTGGPAG 60

SRGGLYVDSNNSGLSYRGFSNHADGSSPFTGNVRDGQCIGTLDGLYAVFTV 111

Fig. 4. Sequence analysis of barley protein (A) and fungal protein (B). Predicted signal peptides are highlighted in blue. Additional his-tags are highlighted in purple. Cysteine is highlighted in red. * indicates identical residues. ':' and '.' indicates 'strong' and 'weaker' conservation, respectively, as defined in the ClustalX documentation. '-' indicates sequence gaps. Highly conserved regions are boxed.


(A)
Bipolaris sorokiniana inoculation on barley leaves
Water
2 µg
4 µg
7 µg
1 day
3 day
6 day
(B)
Bipolaris sorokiniana inoculation on wheat leaves
Water
2 µg
4 µg
7 µg
3 day
6 day

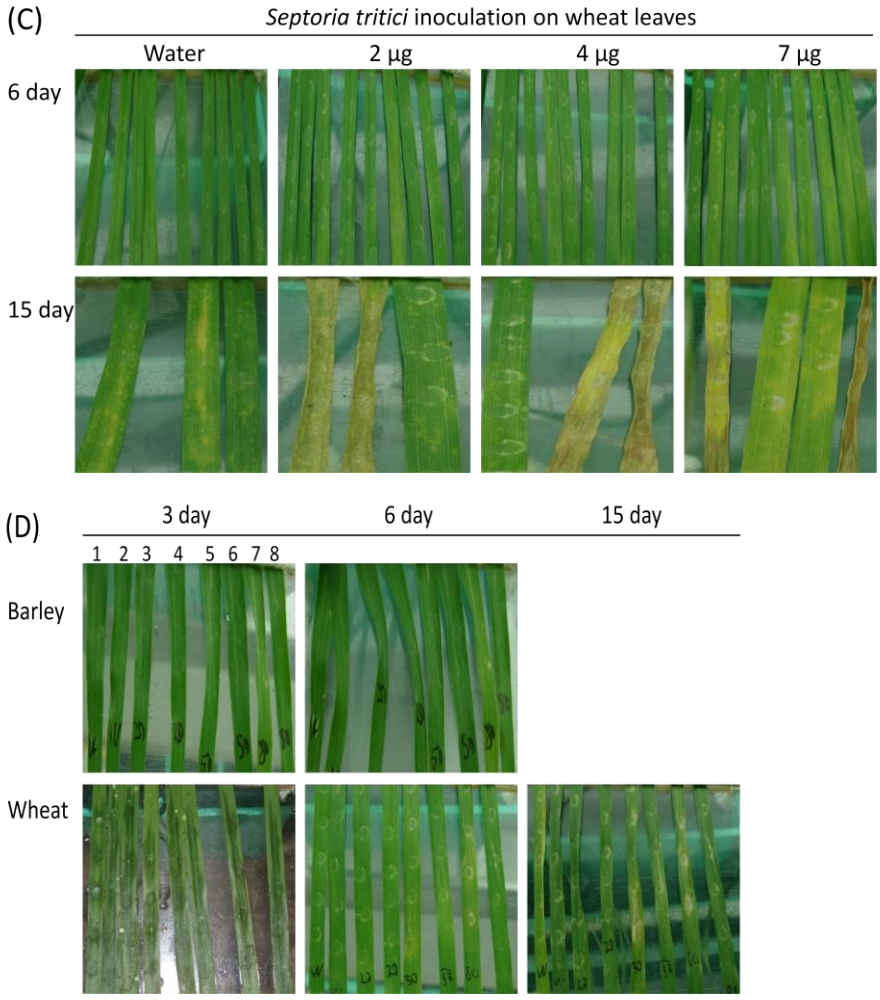


Fig. 5. Effect of the recombinant barley protein (2, 4 and 7µg) on the growth of *Bipolaris sorokiniana* on barley leaves (A) at 1, 3 and 6 dai and wheat leaves (B) at 3 and 6 dai and *Septoria tritici* on wheat leaves (C) at 6 and 15 dai. Water is used as control. Barley and wheat leaves after infiltration of protein or water were not inoculated and harvested after 3, 6 and 15 days (D). Leaf 1 to 8: water, water, 2, 2, 4, 4, 7, 7 µg of protein.

Fig. 6. Effect of the recombinant fungal protein on the appearance of wheat leaves. Leaf 1 to 6: water, 0.2, 1, 2, 4, 8 µg of protein.

Acknowledgement

We thank Aida Curovic (Department of Systems Biology, Technical University of Denmark) for technical assistance in cloning, expression and purification of proteins and Irene Skovby Rasmussen (University of Copenhagen, Denmark) for helping doing *in planta* experiments.

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Chapter 6

Gene expression of *Fusarium graminearum* during infection in wheat and barley spikelets

In collaboration with Prof. Corby Kistler, Cereal Disease Laboratory, Minnesota, US and Senior Researcher Laurent Gautier, Center for Biological Sequence Analysis (CBS), Technical University of Denmark

1. Introduction

Secreted enzymes have an important role in pathogenicity for *Fusarium graminearum* which is a causal fungal pathogen causing destructive disease namely Fusarium head blight in wheat and barley. In Chapter 4, fungal secreted proteins were identified in the culture medium containing barley or wheat grain flour. Ten genes encoding proteins expressed *in vitro* in addition to *Tri5* were examined by qRT-PCR for transcription analysis in *F. graminearum*-infected spikelets of barley and wheat from 2 to 6 dai.

Another approach to study gene expression patterns *in planta* is DNA microarray. In 2003 *F. graminearum* genome sequence was publicly released by the Broad Institute, which provides good opportunity for designing Affymetrix GeneChip. Differential transcript accumulation has been detected using GeneChip which represented putative genes (~14000) when fungi were grown in culture under three nutritional regimes and in infected barley spikelets (Güldener *et al.*, 2006). Recently, a more comprehensive GeneChip covering approximate 18000 genes has been developed and released by the group of Prof. Corby Kistler. They have applied the new DNA chip to *Fusarium graminearum*-infected wheat to further detect the fungal pathogenicity genes. The experiments including fungal spores preparation, plant inoculation, RNA extraction, labeling and hybridization were conducted as described (Güldener *et al.*, 2006, personal communication). Wheat was point-inoculated and harvested at 24, 48, 72, 96, 144 and 192 hours after inoculation (hai) whereas barley was spray-inoculated and harvested at the same time point without 192 dai. RNA from water treated plants from the last time point was used as control. Three biological replicates were prepared for each time point (personal communication with Prof. Corby Kistler).

The aim of this study is to compare microarray results with qRT-PCR results of these 11 genes firstly and to profile the major *F. graminearum* global gene expression patterns in the infected barley and wheat secondly. Microarray data of *F. graminearum* gene expression during infection in wheat and barley spikelets was given by Prof. Corby Kistler. Senior Researcher Laurent Gautier from CBS, Department of Systems Biology, DTU helped visualizing and analyzing the data.

2. Results and discussion

The expression patterns for the 11 genes from 24 h to 96 h were similar in barley and wheat (Fig. 6.1). However, from 96 h to 144 h most genes were decreased expressed in wheat but stable or increased expressed in barley (Fig. 6.1). The results can indicate that fungus establishes colonization more rapidly in wheat than barley which has been demonstrated by the globe comparison of fungal transcriptome data in the infected wheat and barley (personal communication with Corby Kistler). The explanation may be the different inoculation approaches in barley and wheat that point-inoculation is more efficient than spray inoculation for infection. Alternatively, the reason can be partially that the experiments for two plants were conducted by different persons at different time. Thus, the same inoculation method and growth condition for two plants are essential for more accurate comparison. Furthermore, the expression patterns analysed by microarray and qRT-PCR were different (see Chapter 4). It may due to that microarray analysis reflects the accumulation of absolute transcript abundance during infection process rather than gene regulation in qRT-PCR analysis.

The major gene expression patterns included 360 and 348 fungal genes in *F. graminearum*-infected barley (Fig. 6.2, Supplementary Table 6.1 in CD-ROM) and wheat (Fig. 6.3, Supplementary Table 6.1 in CD-ROM), respectively. The patterns in barley and wheat (Fig. 6.2, Fig. 6.3) showed that 72 h seemed to be an important timepoint for interaction since most genes were increased expressed up to 72 h whereas variable expression profiles could be obtained after 72 h. This timepoint is when *F. graminearum* switches from a biotrophic to a necrotrophic phase (Kang and Buchenauer, 1999). In barley, the genes with decreased or stable expression from 72 h to 144 h were mainly annotated as carbohydrate-degraded enzymes, trichothecene biosynthesis enzymes, cytochrome P450, heat shock proteins and elongation factors (Cluster 1–5, 11, 12, 16–18, 20, 21, 25). The major genes with increased expression from 72 h encoded enzymes including proteases, chitin binding proteins, housekeeping enzymes and antioxidant enzymes such as thioredoxins and catalases (Cluster 6–10, 13-15, 19, 22–24). In wheat, the genes with decreased or stable expression from 72 h were mainly annotated as carbohydrate-degraded enzymes, trichothecene biosynthesis enzymes and housekeeping enzymes such as glyceraldehyde 3-phosphate dehydrogenase (Cluster 2, 6, 11–15, 18, 20–23, 25). The genes with increased expression after 72 h mainly functioned as oxidoreductases and antioxidant enzymes such as thioredoxins (Cluster 1, 3–5, 7–10, 16, 17, 19, 24).

3. Reference

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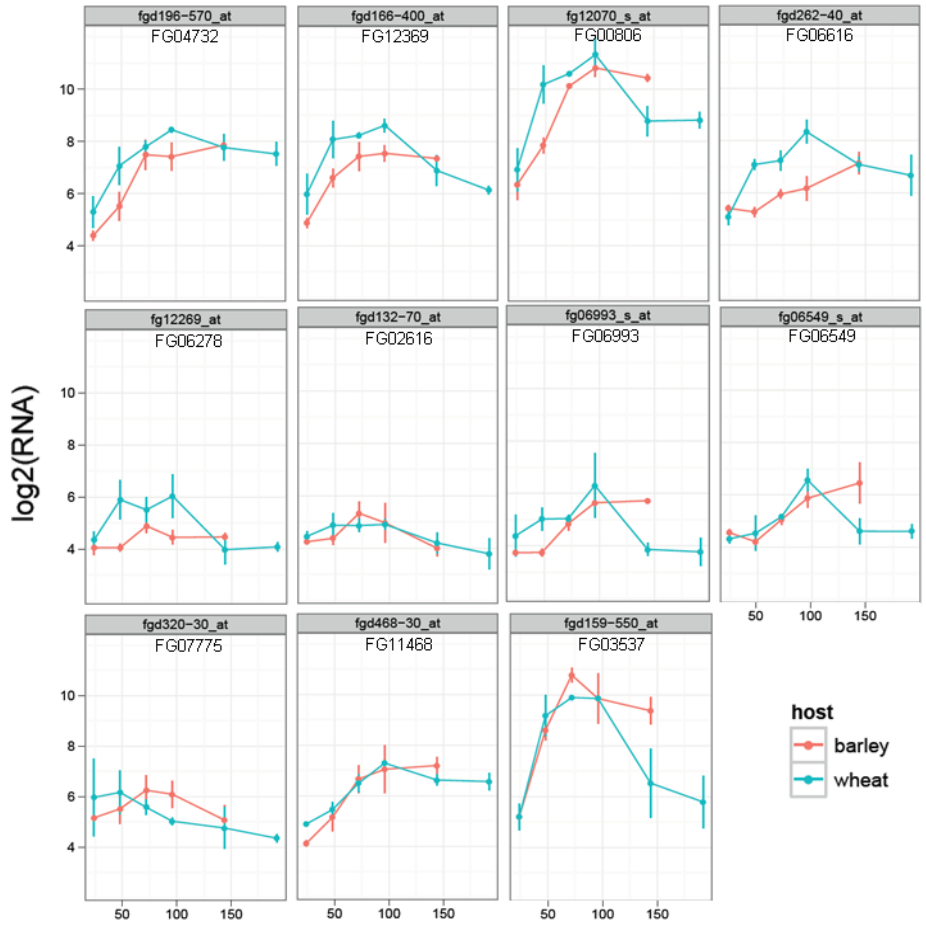

Fig. 6.1. Expression profiles of eleven fungal genes in *F. graminearum*-infected barley and wheat spikelets at 24, 48, 72, 96, 144 and 192 hai. No 192 hai is shown for barley. Each node corresponds to one time point. Probe ID (_at) and accession number (FG) for gene are indicated.

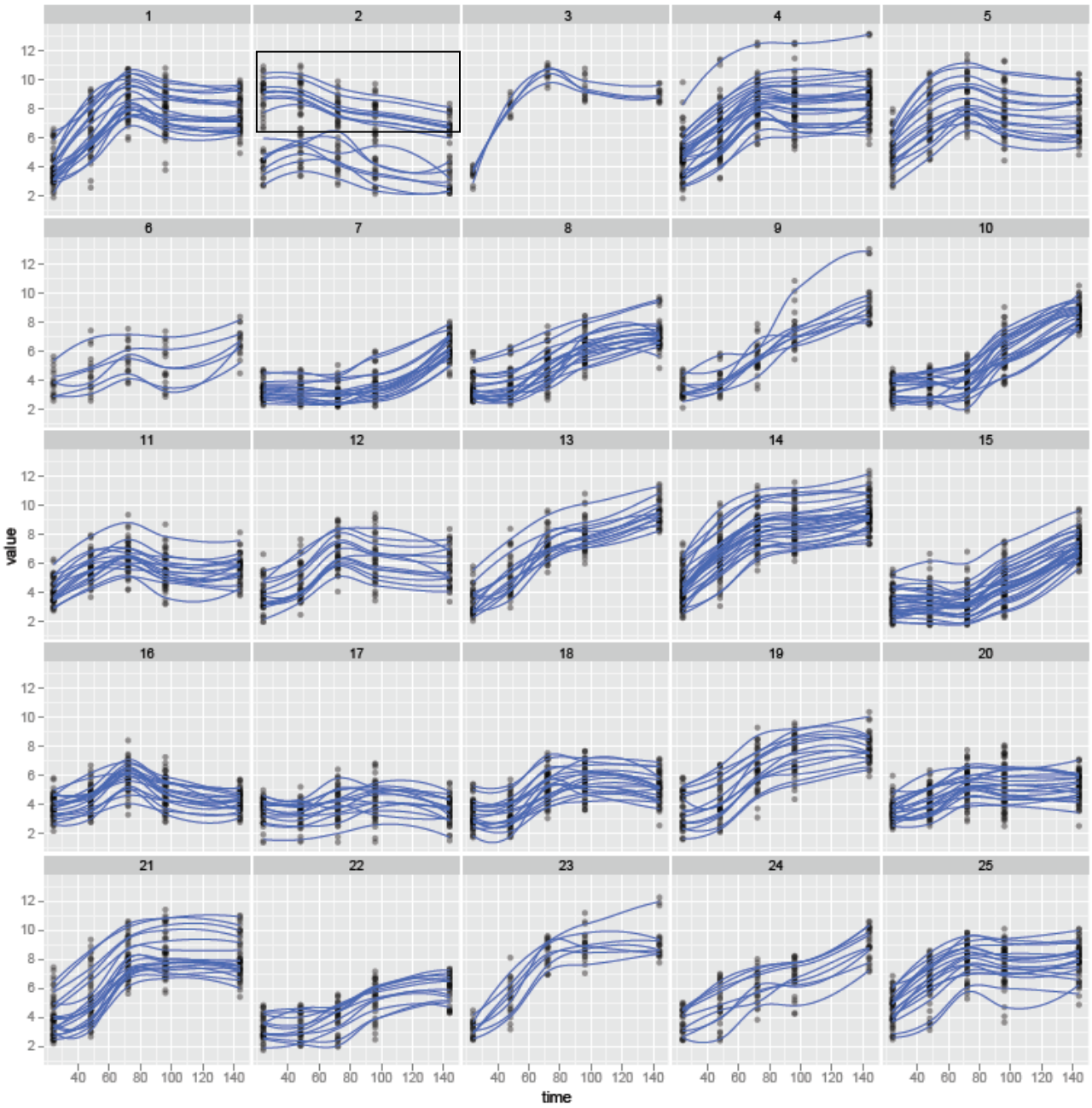

Fig. 6.2. Twenty-five patterns of gene expression in *F. graminearum*-infected barley spikelets at 24, 48, 72, 96 and 144 hai. Cluster numbers are indicated. Each trace corresponds to one gene and each node corresponds to one time point. The genes in the box in the Cluster 2 belong to *E.coli* or rice genes which are designed to be on gene chip.

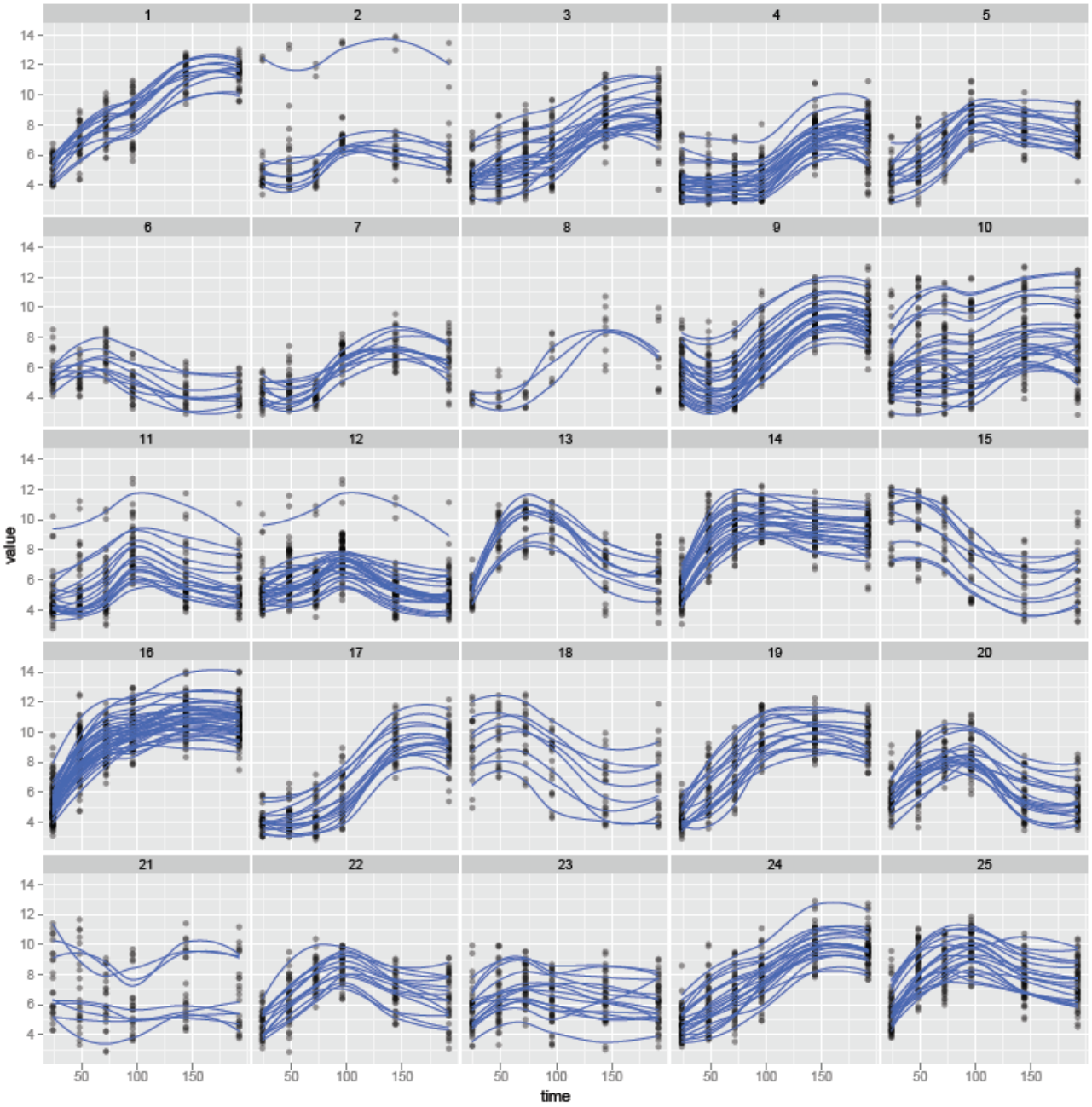

Fig. 6.3. Twenty-five patterns of gene expression in *F. graminearum*-infected wheat spikelets at 24, 48, 72, 96, 144 and 192 hai. Cluster numbers are indicated. Each trace corresponds to one gene and each node corresponds to one time point.

Concluding remarks and perspectives

FHB mainly caused by *Fusarium graminearum* in cereals leads to huge loss of grain yield and quality with mycotoxin contamination worldwide. The problems have increased in Denmark during the recent years, especially the toxins. Fungicides and crop management practices are ineffective in reducing infection. Fungicides may even lead to accumulation of mycotoxins by stressing the pathogens. Highly resistant cereal cultivars are not available yet. Therefore, it is highly required to understand the underlying interaction between cereal plants and *Fusarium graminearum* at molecular level to control *Fusarium graminearum* infection and minimizing toxin levels.

In order to solve the problem and achieve the goals, the present Ph.D. project was carried out with gel-based proteomic approaches from both plant and pathogen sides. We have investigated the severity of FHB in the susceptible barley using two different amounts of nitrogen fertilizer in the first study. The results showed that there was a good correlation among fungal biomass, mycotoxins and levels of proteome degradation caused by fungus suggesting FHB was more severe in barley grown with low N than with high N. However, the reason why lower level of N fertilizer causes higher level of infection is still not clear, which will be the focus in the future research. Proteomic analysis of barley infected by *Fusarium graminearum* from 1 to 3 dai showed the upregulation of pathogenesis-related proteins, increased energy metabolism and changes of the secondary metabolism and protein synthesis in the infected barley. Barley β-amylases were shown to be markers for infection at proteome level. The identified proteins can be the targets to further study the functional role in plant defense. Therefore, a PR17 protein which was upregulated in barley in response to *F. graminearum* was chosen for expression. It was shown that there was an effect of this protein on growth of *Bipolaris sorokiniana* and *Septoria tritici in planta*. By profiling the *in vitro* secreted proteome of *F. graminearum* on the 2-D gels in the presence of substrates of barley or wheat grain, it was possible to identify 69 unique fungal proteins in either medium mainly including enzymes involved in degradation of cell walls, starch and protein. The expression of some genes encoding *in vitro* protein was examined in the infected wheat and barley. This result was also compared to microarray data of *F. graminearum* during infection of wheat and barley. Furthermore, a fungal hypothetical protein which was strongly upregulated in *YAP*- or *AP*-knockout *F. graminearum* mutant compared to wildtype (collaboration with Jens D. Jensen) were expressed and initial bioassays were tested.

In conclusion, gel-based proteomics has been shown to be a useful tool for understanding cellular mechanisms, plant pathology and plant-microbe interactions in the present studies. However, there are certain limitations to this method. For instance, only most abundant proteins are detected on 2-D gels whereas the key proteins during interaction expressed in low abundance can not be identified. This issue may be partially

addressed by using high throughput LC-MS/MS coupled to quantification techniques such as ICAT and iTRAQ. Another major problem in proteomic analysis is the fact that identification is usually achieved when the genome sequence or a large amount of sequence data is available in public databases. Although identification can also be performed by *de novo* sequencing, it requires more sophisticated instruments and bioinformatics tools. These technical limitations in proteomics studies need to be overcome in order to enlarge the knowledge on cellular mechanisms during plant-pathogen interaction. Currently high throughput proteome techniques are continuing to develop for identification of novel targets for increased resistant plants.

Another important aspect in classical proteome analysis is the follow-up study of the identified proteins, which will be validation of the expression of the genes, the elucidation of the functional activities of proteins in pathogenicity and disease resistance and how they interact with other proteins. Several techniques such as qRT-PCR, microarray, protein crystallization and structure determination, yeast two hybrids, protein chips and gene knockout or overexpression experiments can be applied and significantly complementary to proteomics-based research.

Here we have mainly focused on the proteomics in understanding of barley-*F. graminearum* interaction. However, in order to fully comprehend all the diverse and complicated cellular activities that are involved in interaction, it is important to integrate all the information generated from studies of genomics, transcriptomics, metabolomics, proteomics, traditional plant pathology and genetic engineering. This will ultimately contribute to the development of novel disease resistant cultivars of cereals.

Appendix I

Implications of high-temperature events and water deficits on protein profiles in wheat (*Triticum aestivum* L. cv. Vinjett) grain

Implications of high-temperature events and water deficits on protein profiles in wheat (*Triticum aestivum* L. cv. Vinjett) grain

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Abbreviations: **dpa**, days post anthesis; **PR**, pathogenesis-related; **ROS**, reactive oxygen species

Keywords: Environmental stress / Grain protein fractions / Proteomics / Wheat

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Abstract

Increased climatic variability is resulting in an increase of both the frequency and the magnitude of extreme climate events. Therefore, cereals may be exposed to more than one stress event in the growing season, which may ultimately affect crop yield and quality. Here, effects are reported of interaction of water deficits and/or a high temperature event (32 ^{o}C) during vegetative growth (terminal spikelet) with either of these stress events applied during generative growth (anthesis) in wheat. Influence of combinations of stress on protein fractions (albumins, globulins, gliadins and glutenins) in grains and stress-induced changes on the albumin and gliadin proteomes were investigated by two-dimensional gel electrophoresis and mass spectrometry. The synthesis of individual protein fractions was shown to be affected by both the type and time of the applied stresses. Identified drought or high-temperature-responsive proteins included proteins involved in primary metabolism, storage and stress response such as late embryogenesis abundant proteins, peroxiredoxins and α-amylase/trypsin inhibitors. Several proteins *e.g.* heat shock protein and 14-3-3 protein changed in abundance only under multiple high temperatures.

1 Introduction

Climate change is likely to become an even more acute problem than previously anticipated [1]. Accelerated climatic variability is resulting in an increase of both the frequency and the magnitude of extreme events [2, 3]. More frequent extreme climatic episodes result in cereals being exposed to several stress events during the growing season, ultimately affecting cereal production [4–6]. The most sensitive growth stages in cereals are stem elongation, booting and anthesis [7]. Extreme heat episodes after anthesis increase development of the crops, shorten the grain filling period [8] resulting in reduction of kernel weight and of grain quality parameters (starch and protein) [9]. Moderately high temperatures after cold winter periods increase cereal yields [10]. An extreme heat event at the double-ridge stage did not affect the response of wheat to heat stress at anthesis [2]. Drought has also become a limiting factor for cereal production worldwide resulting in significant grain yield losses [4, 11, 12] and increased heavy rainfalls lead to waterlogging and nutrient leaching resulting in reductions of cereal growth and quality [5, 13].

Wheat grain quality receives increasing attention because of its economic and nutritional importance [14]. Protein fractions including albumins, globulins, gliadins and glutenins are the most important components of wheat grains for end-use quality [15]. In order to maintain grain yield and quality under increased climatic variability it is important to understand both the effect of single environmental constraints and of the combination of extreme climate events on grain protein composition. It has been shown that increases in temperature induce synthesis of gliadins at the expense of glutenins in wheat [16]. Furthermore, high temperature applied from anthesis to maturity decreased the contents of the albumin and globulin fractions, while drought applied after anthesis decreased the albumin and globulin fraction, but had no significant effect on gliadins and glutenins [17]. Several studies have been performed to investigate the response of wheat grain proteins to either long-term drought during vegetative growth or long-term high temperature applied during grain filling, showing up-regulation of pathogenesis-related (PR) proteins, allergens, protease inhibitors, heat shock proteins, and proteins involved in oxidative stress and starch biosynthesis [14, 18, 19]. However, little is known about the effect of the combination of short-term (days) water deficits and high temperature during grain development on wheat grain proteins. Although grain protein content and composition - the key parameters of grain quality - are primarily genetically controlled, environmental factors can influence the synthesis of protein throughout grain growth [20]. The interactions of multiple episodes as well as multiple forms of stress so far have not been investigated extensively.

The aim of the present study was to investigate implications of the interactions of short-term drought and high temperature events applied at two different growth stages of wheat (terminal spikelet and anthesis). Changes in grain proteins were monitored by 2-DE MALDI-MS based proteomics. This approach has been shown to be very useful in characterizing changes in protein profiles of plants under stress episodes [19]. The results are discussed in relation to the general use of proteomics to identify traits relevant for stress tolerant cereals and identification of wheat cultivars with drought and high-temperature tolerance.

2 Materials and methods

2.1 Plant growth

A pot experiment was conducted at the 'semifield' station of Research Center Flakkebjerg, Department of Genetics and Biotechnology, Faculty of Agricultural Sciences, Aarhus University, Denmark in the growing season of 2007. Pots with both depth and diameter of 25 cm were filled with 4.2 kg of a 1:2:1 (v/v/v) mixture of peat substrate, loamy soil and sand. A dose of 5.25 g K_2SO_4, 3.5 g $(NH_4)_2SO_4$, 4.67 g NH_4NO_3, 1.9 g $CaSO_4$, 1.9 g $MgSO_4$, 0.4 g $MnSO_4$, 0.4 g $CuSO_4$ and 11.67 g $CaCO_3$ per pot was mixed in the soil. Spring wheat (*Triticum aestivum* L. cv. Vinjett) was sown (15 seeds per pot) and thinned to 5 seedlings per pot at the three-leaf stage. The main shoot of each plant was labeled. From the third-leaf stage, three to five seedlings were taken for observation of spike initiation by dissection at an interval of about two to three days, until the terminal spikelet stage was identified.

An automatic irrigation system was used, and the total (not for individual pots) applied water was recorded. Water deficits and high temperature stresses were implemented at terminal spikelet (the first stage, denoted A) and anthesis (the second stage, denoted B) as specified below. At the first stage, three treatments were implemented in three growth chambers, namely temperate conditions as control (cA), water deficits (wdA) and high temperature (htA). At the second stage, plants from each of the above treatments were further exposed to three treatments in the three chambers, as control (cB), water deficit (wdB) and high temperature (htB). In total 9 combinations of stress types were implemented: cA-cB, cA-wdB, cA-htB, wdA-cB, wdA-htB, htA-cB, htA-wdB, htA-htB and wdA-wdB. The experiment was a completely random block design with three biological replicates.

In the high temperature treatment at both stages the temperature in the growth chambers was set to increase from 24 ^{o}C at night (8 h) to a maximum of 32 °C for 2 h during the day (16 h). The temperature in the control and water deficits treatments was set to increase from 12°C to 20 ^{o}C at the first stage and from 16 °C to 24 ^{o}C at the second stage. The heat treatment lasted for 10 d. Water supply was withheld in the pots in the semifield for 7 d (at the 3-leaf stage) before the first stage and for 6 d (just after the spike emerged) before the second stage. Thereafter the pots were moved into the growth chambers in order to apply the different treatments.

2.2 Protein extraction and quantification

Grain fresh weight and raw protein% (derived from total N * 5.7) were measured and shown in supplementary Figs. s1 and s2. Kernels from 5 individual ears of the main tillers in 5 individual pots were harvested at 19, 26, 32 and 52 days (at maturity) post anthesis (dpa). For each treatment, biological triplicates of wheat kernels were freeze dried and ground to a fine powder in liquid nitrogen. Based on the solubility in a series of solvents, the classified grain protein fractions of albumins, globulins, gliadins and glutenins [15] were extracted as described in [21] with minor modifications. Briefly, albumins were

extracted from 100 mg flour that was mixed continuously with 1 mL of buffer containing 25 mM sodium phosphate (pH 7.5) for 60 min at 4 °C. The flour was re-extracted with 1 mL of a solution containing 0.1 M NaCl and 20 mM DTT by incubation in an ultrasonic bath (2210 BRANSON) for 60 min at 4 °C to obtain the globulins. The gliadins were extracted from the resulting pellet using 1 mL of a solution containing 50% (v/v) 1-propanol, 1% (v/v) acetic acid, 2% (w/v) DTT by incubating the samples in the ultrasonic bath for 60 min at 4 °C. Then the glutenins were extracted from the resulting pellet with 1 mL of 1 mM HCl by mixing for 60 min followed by ultrasonication for 1 min at 4 °C. After each extraction, the suspension was collected by centrifugation at 20800 x *g* for 10 min. The residue was washed and re-extracted with the same buffer twice before the extraction of the following fraction. Protein concentration of albumins, globulins and gliadins from supernatants was determined using the Amido black method [22] with bovine serum albumin as standard. As this method is not ideal for determination of the concentration of acid-soluble proteins, the concentration of glutenins was determined using the Bradford assay [23]. Statistical analysis of differences in contents of protein fractions between stress and control treatments was performed by Student´s *t*-test ($p < 0.05$).

2.3 Two-dimensional gel electrophoresis

2-DE was run according to [24] with minor modifications. Albumins (150 µg depending on the sample in a volume of 200–300 µL) were precipitated by 4 volumes of acetone at -20 °C overnight. The albumin pellets or gliadins (100 µg corresponding to a volume of 25-30 µL depending on the sample) were added 350 µL of rehydration buffer (8 M urea (GE Healthcare), 2% (w/v) CHAPS (Sigma), 0.5% (v/v) IPG buffer pH 3–10 (GE Healthcare), 0.3% (w/v) DTT (Sigma) and a trace of orange G). The solution was thoroughly vortexed and centrifuged (20800 x *g* for 10 min). The supernatant was applied to an IPG strip pH 3–10 (18 cm; GE Healthcare) and IEF was run (Ettan IPGphor; GE Healthcare) as described [25]. After IEF, the strips were equilibrated first with a solution containing 6 M urea (GE Healthcare), 30% (v/v) glycerol (GE Healthcare), 50 mM Tris (Sigma) pH 8.8, 0.01% (w/v) Bromophenol Blue (Sigma), 2% (w/v) SDS (BDH/Merck) with 1% (w/v) DTT (Sigma) for 15 min and then with a similar solution without DTT, but containing 2.5% (w/v) iodoacetamide (Sigma) for 15 min. The strips were placed on top of 12.5% acrylamide gels (3% C/0.375% bisacrylamide) and the second dimension was run (Ettan DALTsix electrophoresis unit; GE Healthcare) according to the manufacturer's protocol. The gels were stained in Blue Silver stain [26]. One gel was run for each biological replicate.

2.4 Image analysis

The gels were scanned using a ScanMaker 9800XL, Microtek at 300 dpi resolution in both color and grayscale (16 bits). All grayscale gel images were imported into the Progenesis SameSpots software (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK). One gel image from the cA-cB control treatment was chosen as a reference image and all gel images were warped and matched to the reference image. Three gel

images representing three biological replicates for each treatment were grouped to obtain the average volume of each spot. Images from each stress treatment were compared to images from cA-cB. A threshold of ANOVA (p) < 0.05 and at least 1.5-fold change in average spot volume between a stress treatments and the corresponding control treatment was used to select the protein spots for further MS analysis.

2.5 Enzymatic digestion and mass spectrometry

In-gel digestion was performed as described [25], albumins and gliadins being digested by trypsin (Promega) and chymotrypsin (sequencing grade, Roche) [27], respectively. The peptide mixtures were prepared for MS analysis on a washed MALDI 600 µL AnchorChip target plate (Bruker-Daltonics, Bremen, Germany) as described [25]. A tryptic digest of β-lactoglobulin was used for external calibration. Tryptic and chymotryptic peptides were analysed in Ultraflex II MALDI-TOF-TOF mass spectrometer (Bruker-Daltonics, Bremen, Germany) in positive reflector mode for peptide mass mapping or peptide fragment ion mapping. Internal calibration was carried out using trypsin autolysis products (*m/z* 842.51 and *m/z* 2211.10). An in-house Mascot server (http://www.matrixscience.com) was used for database searches in the NCBInr (National Center for Biotechnology Information) and the TaGI wheat gene index Release 11.0 (http://compbio.dfci.harvard.edu/tgi). The following parameters were used for searching: allowed global modification, carbamidomethyl cysteine; variable modification, oxidation of methionine; missed cleavages, 1; peptide tolerance, 80 ppm and MS/MS tolerance ±0.5 Da. The positive identification had to meet the following criteria: a significant MASCOT score and at least four matched peptides in MS analysis or two matched peptides in MS/MS analysis.

3 Results and discussion

Overall, no significant differences in final grain weights have been found (Supplementary data, Fig. s1). When heat was applied in the vegetative stages (htA), the grain weight at 12 dpa was significantly higher than in the other treatments (Fig. s1 C), probably indicating enhanced growth during these treatments. No significant differences in the total protein content of these grains have been found, although the protein content was more variable when heat was applied during the vegetative stage (Supplementary data, Fig. S2).

3.1 The effect of stress on accumulation of protein fractions

The albumin content in all treatments decreased significantly (56–80%) during grain filling (Fig. 1A, 1E). At 19 dpa, all stress treatments applied had decreased the albumin fractions significantly, where the lowest contents were found in the htA-htB treatment. By contrast, at 32 dpa the albumin content in all of the stress treatments were higher than for the control and at maturity, only the albumin contents in cA-wdB and wdA-cB treatments were significantly higher than in the control. High-temperature stress resulted in higher albumin contents at maturity when applied at anthesis than at terminal spikelet. By contrast, no significant difference in albumin contents was observed when drought was applied at both growth stages.

The relatively low globulin content increased differently (22–135%) under treatments during grain filling. Compared to the earlier growth stages, greater differences in globulin contents between stress and control were observed at maturity (Fig. 1B, 1F). The stress treatments except htA-cB (Fig. 1F) significantly increased the contents of globulins at maturity. A larger increase in globulins at maturity was observed when drought was applied at anthesis or terminal spikelet than high temperature (Fig. 1B, 1F).

Gliadins showed with the exception of the htA-cB treatment increased (39–166%) contents during grain filling, but stress treatment did not significantly affect the rate of accumulation of gliadins (Fig. 1C, 1G). At maturity only htA-cB treatment decreased gliadin contents, similar to globulins. The highest contents of gliadins were observed at 19 dpa in htA-htB and at maturity in cA-wdB, cA-htB and in wdA-cB.

The glutenins accumulated during grain filling increased (85–159%) in response to different stress treatments (Fig. 1D, 1H). The htA-htB treatment resulted at maturity in higher contents of glutenins as well as gliadins, which is contrary to findings by others that in wheat high temperature can induce synthesis of gliadins at the expense of glutenins [16]. As with the globulins, stress caused greater differences in glutenin contents at maturity than at 19 dpa. In contrast to the albumin and gliadin fractions, multiple high temperature events (htA-htB, Fig. 1D) or water deficits (wdA-wdB, Fig. 1H) applied at both terminal spikelet and anthesis resulted in significantly higher contents of glutenins at maturity than in the single stress treatments.

At maturity the highest contents of albumins, globulins and gliadins were obtained under the single drought stress applied at either stage whereas glutenins increased under the htA-htB treatment, indicating differences in the response of grain protein fractions to drought and heat stress. High temperature was capable of causing substantial changes in the accumulation level of gluten proteins during grain filling, in agreement with previous findings [9]. In addition, higher contents of albumins, globulins, gliadins and glutenins were observed when high temperature was applied at anthesis than terminal spikelet. This is in contrast to the finding of no significant differences in protein fractions under drought stress applied at either stage, which indicates that grain protein contents were greatly affected by both the type and the exposure time of the stress applied. During the period from terminal spikelet to anthesis, high temperature stress is reported to have a damaging effect on the viability of pollen, resulting in failure of fertilization [20]. As a consequence, the poor development of fertilized ovaries into caryopses may affect protein synthesis during grain filling. Water deficit was found to inhibit photosynthesis at both stages, which can influence the formation of generative organs and consequently grain protein synthesis [20, 28]. It has previously been shown that both grain yield and grain number were influenced more by stress at anthesis than at heading [9]. Furthermore, it was observed that the combination of drought and heat stress applied at two stages did not have a greater effect on the albumin, globulin and gliadin contents during grain filling, compared with either of the stresses applied individually. It has been reported that different stresses may require antagonistic responses and that a combination of drought and heat stress may alter plant metabolism resulting in a new pattern of defense response compared to single stress [20, 29]. Thus, the combination of different stresses might not cause more changes to grain protein content than a single stress event.

3.2 Drought or high-temperature-responsive albumins

Albumins and gliadins from mature grains (52 dpa) in the cA-wdB, cA-htB, htA-htB and cA-cB (control) treatments were chosen for detailed 2D-based proteome analysis, because these stress scenarios resulted in significant increases of albumins, globulins, gliadins and glutenins at maturity.

The representative 2D protein patterns of albumins and gliadins for all the treatments (Fig. 2 and 3), allowed detection of approximately 250 and 100 spots, respectively. ANOVA was applied to evaluate spots differing significantly in abundance between stress treatments and control and to confirm the reproducibility of the data. Based on the selection criteria (at least 1.5-fold change, see section 2.4), in the cA-wdB, cA-htB and htA-htB treatments, 16/5, 10/11 and 31/8 spots exhibited significant changes (between 1.5 and 2.4-fold) in intensity in the albumin/gliadin fractions due to stress, respectively. All differentially displayed spots were identified by MS except spot a3 of decreased intensity in cA-wdB (Fig. 2) and spots e14, e18 and e26 in htA-htB (Fig. 2 and Table 1). The difference of pI and MW between experimental and theoretical data may have resulted from experimental error, post-translational modification or protein turnover occurring in the cell. According to the function the proteins identified from different stress treatments were mainly involved in stress response, carbohydrate metabolism and storage in addition to protein synthesis.

3.2.1 Stress-related albumins

The first major group of the responsive albumins had functions related to stress and defense. A late embryogenesis abundant protein which is associated with desiccation stress was up-regulated in response to drought stress (1.8 fold, spot a9, Fig. 2), possibly due to its role in protection of proteins and cell membranes from disruption or damage in the near-dry state [30]. A number of proteins related to oxidative stress showed a different expression behavior in response to temperature or drought stress. For instance, 1-Cys peroxiredoxin was down-regulated under drought treatment (2.4 fold, spot a4, Fig. 2), whereas up-regulation of 1-Cys peroxiredoxin (1.9 fold, spot b1, Fig. 2) and a 27 kDa hypothetical protein belonging to the thiol reductase superfamily (1.5 fold, spot b7, Fig. 2) and down-regulation of a hypothetical protein belonging to the thioredoxin superfamily (1.5 fold, spot b5, Fig. 2) were observed in plants under single high temperature stress but not with multiple high temperature stress. During exposure to abiotic stress, ROS are often produced in plants and can cause oxidative damage to proteins, DNA and lipids in the cells [19, 31]. The quantitative alteration of the above ROS-scavenging enzymes suggests their different roles in oxidative stress. Similar observations were made in wheat leaves after a 7-day exposure to drought [31]. Lipoxygenase which is considered to be partly responsible for the formation of lipid peroxidation products as well as ROS was down-regulated with high temperature probably as a consequence of the inhibition of ROS production [32]. Additionally, lipoxygenase is known to be involved in jasmonic acid biosynthesis, which often accumulates in plants in response to biotic and abiotic stress [33].

Several spots changing in abundance were identified as defense-related proteins, including α-amylase inhibitors, α-amylase/trypsin inhibitors CM1, CM3, CM17, serpin and cystatin. Similar results were also observed in seeds of durum wheat subjected to heat for five days after anthesis [34]. The α-amylase/trypsin protease inhibitors have been shown to guard against digestive enzymes of insects and fungi and are often accumulated in response to biotic stress [19, 34]. Interestingly, α-amylase inhibitor in one spot (1.5 fold, spot a8, Fig. 2) was up-regulated and down-regulated in another spot (1.5 fold, spot a11, Fig. 2) under drought. This may be due to PTMs of the protein such as dephosphorylation resulting in pI shift from acid to base. It is well known that the phosphorylation of proteins can change in response to stress [31]. Furthermore, the up-regulation of three α-amylase inhibitors and the down-regulation of α-amylase inhibitor CM1 and cystatin under htA-htB may reflect their different roles in response to high-temperature treatments.

3.2.2 Albumins involved in primary metabolism

The second group of identified albumins is involved in primary metabolism. In response to drought, 3-phosphoglycerate kinase involved in the glycolysis was up-regulated (1.6 fold, spot a16, Fig. 2) and glucose/ribitol dehydrogenase, which is related to glucose degradation and has a role in desiccation and salinity tolerance in barley seeds [35, 36] (2.1 fold, spot a12, Fig. 2) was down-regulated, suggesting changes in carbohydrate metabolism. In addition, enzymes such as triosephosphate isomerase (1.5 fold, spot b10, Fig. 2) and succinyl-CoA ligase (1.9 fold, spot b2, Fig. 2) participating in glycolysis and in citric acid cycle, respectively, were down-regulated by a single high temperature event, whereas fructose-6-phosphate-2-kinase (2.0 fold, spot e8, Fig.2) enolase 2 (1.9 fold, spot e10, Fig. 2) and β-amylase (1.6 fold, spot e7, Fig. 2) increased and aldose reductase decreased (1.9 fold, spot e16, Fig. 2) in abundance with multiple high temperature events. It has been reported by others that several glycolytic enzymes including triosephosphate isomerase were increased in the grain in response to high temperature initiated at 10 dpa [19]. This is in contrast to the present results and be explained by the duration of stress exposure or be cultivar-specific. Furthermore, changes in the abundance of 40S ribosomal protein S8 and glutamine synthetase in the single high temperature treatment and of ribosomal protein S2, methionine synthase and glutamate-cysteine ligase in the multiple high temperature treatment may reflect alterations in protein and amino acid biosynthesis.

3.2.3 Albumins with other functions

The identified calmodulin TaCaM2-2 decreased in response to drought (1.7 fold, spot a5, Fig. 2). Many extracellular signals such as biotic and abiotic stress can elicit changes in cellular Ca^{2+} concentration in plants [37]. Calmodulin is a Ca^{2+} binding protein involved in calcium signaling under stress conditions [37]. Several proteins changed in abundance only under multiple high temperatures including up-regulated cinnamoyl-CoA reductase, translationally-controlled tumor protein, cell division control protein and heat

shock cognate 70 (1.6 fold, spot e28; 1.5 fold, spot e24; 1.9 fold, spot e3; 1.9 fold, spot e19, respectively), and the down-regulated 14-3-3 protein (1.9 fold, spot e22, Fig. 2). Cinnamoyl-CoA reductase is involved in lignin biosynthesis. Lignin is an important factor in plant defense response because pathogens are not able to degrade this mechanical barrier [38]. It has recently been reported that the expression of genes encoding translationally-controlled tumor protein which is related to diverse cellular processes such as apoptosis, microtubule organization and ion homeostasis in eukaryotes was enhanced by stress including high temperature and salt in cabbage [39]. Up-regulation of cell division control protein suggests enhanced cell division as respond to high temperature stress. Heat shock proteins, known as molecular chaperones assisting in the correct folding of polypeptides, have been shown to have a protective role during abiotic and biotic stress [40]. 14-3-3 proteins function as major regulators of primary metabolism and cellular signal transduction in plants. They were shown to regulate target proteins that are involved in response to stress [41]. Several 14-3-3 genes were differentially regulated in rice in response to biotic and abiotic stress [41]. Transcripts encoding proteins belonging to the 14-3-3 family accumulated in barley after biotic stress [34]. Hurkman *et al*. [19] and Laino *et al*. [34] have observed the decreased abundance of the spots belonging to the 14-3-3 family in wheat after heat stress. The down-regulation of 14-3-3 protein in the present study may indicate that 14-3-3-interactor negatively regulates factors that mediate stress response.

3.3 Drought or high-temperature-responsive storage proteins

Expression of several storage proteins were modulated under stress as well. Some storage proteins including α-gliadin, γ-gliadin, low molecular weight glutenin and globulins decreased and one globulin and one low molecular weight glutenin increased in abundance in response to water deficits. Several α-gliadins, γ-gliadins and low molecular weight glutenins increased and α/β-gliadin, ω-gliadin and globulins decreased in abundance in response to high temperature. Our results indicate that stress could increase the abundance of some gliadins at expense of other gliadins which have highly variable sequences [27]. However, the reason why different components of gliadins and globulins vary in response to abiotic stress is still unknown.

3.4 Comparison of plant responses under three different stress events

No common spot from the albumin and gliadin fractions was found to respond to both drought and high temperature stress. Furthermore, 1-Cys peroxiredoxin was regulated in opposite ways at single drought and high temperature events. The results indicate different responses of wheat grain protein to drought and to high temperature events. Drought can cause stomatal closure, decrease of photosynthetic activity, increased oxidative stress, alteration of cell wall elasticity and generation of toxic metabolites causing plant death [31]. Here, proteins involved in stress/defense, signaling pathways, redox regulation and energy metabolism were found. High temperature stress often results in increases of stomatal conductance,

respiration, leaf transpiration and oxidative stress [42], shortens the duration of grain filling in cereals and enhances gluten protein accumulation and starch synthesis [19]. Plants respond to heat by signaling via abscisic acid, ethylene and salicylic acid, scavenging of ROS via production of antioxidants, and transcriptional activation of stress-related proteins [43]. Here, a number of proteins including heat shock proteins, carbohydrate metabolism and storage proteins were found to be responsive to high temperature.

Albumin proteins involved in primary metabolism did not change in abundance between the cA-htB and htA-htB treatments. However, a 27 kDa hypothetical protein belonging to thiol reductase superfamily was regulated in opposite ways between these two treatments. Several proteins with other functions than involvement in stress and primary metabolism were found to be regulated only in response to two high temperature events.

4 Concluding remarks

The combination of drought and heat stress applied at two stages of grain filling did not have a significant effect on albumin contents, compared with either of the stresses applied individually. Globulins and gliadins showed with exception htA-cB increased protein fraction contents from 19 dpa. The glutenins accumulated during grain filling in response to different treatments. Some stress treatments can significantly modify contents of individual proteins in grain. Changes of grain protein contents that strongly depended on the type of stress applied. It was possible to identify several proteins responsive to drought and high temperature episodes. Few common proteins were observed responding to single and multiple high temperature events. The identified proteins play key roles in anti-desiccation, antioxidation, defense, carbohydrate metabolism and storage. These protein markers identified in this study might be relevant for plant breeding. Thus the present findings indicating effects of more than one stress event and type on the grain protein composition, will contribute to the identification of cultivars with increased tolerance to increasing climate variability.

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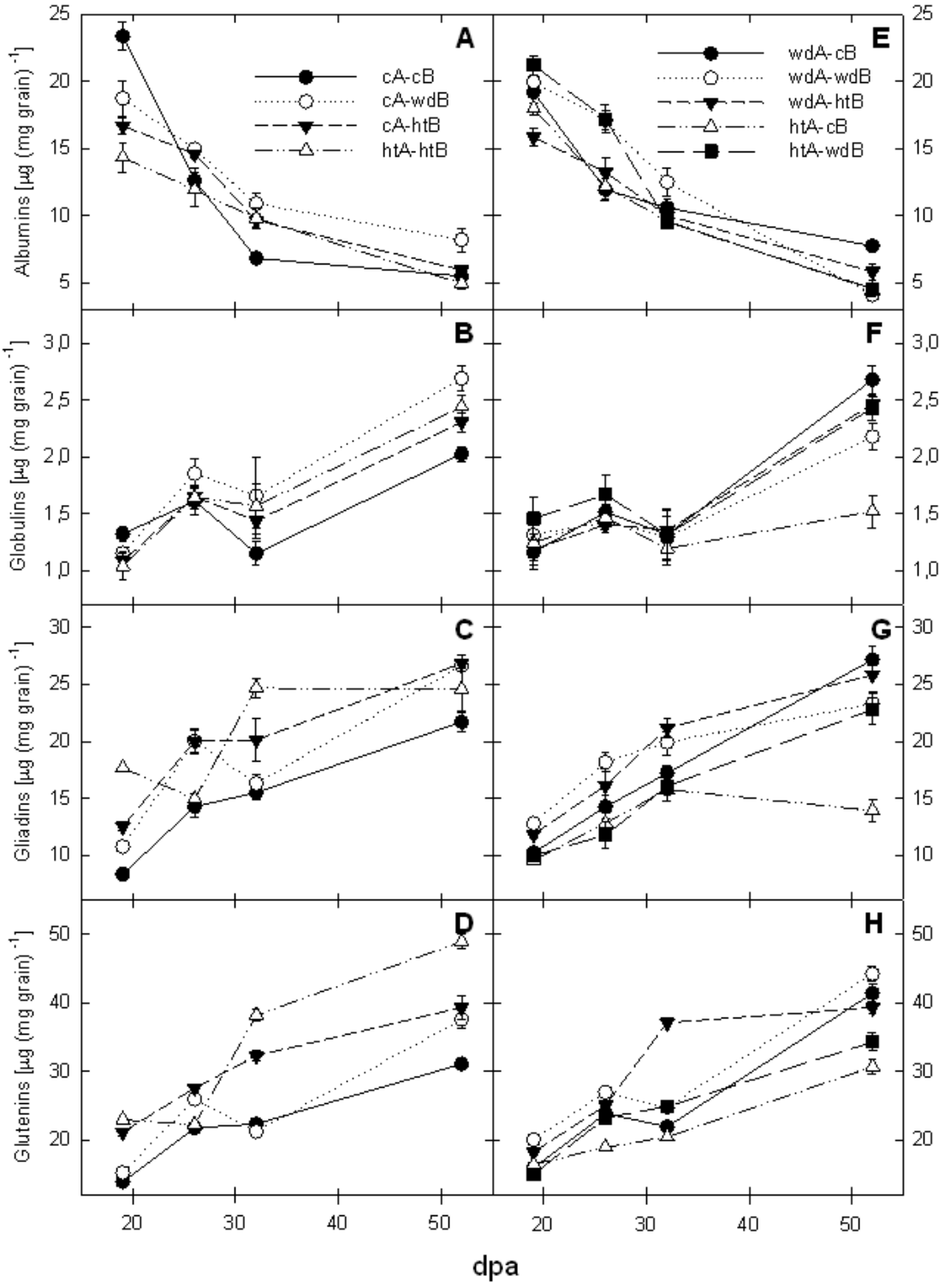
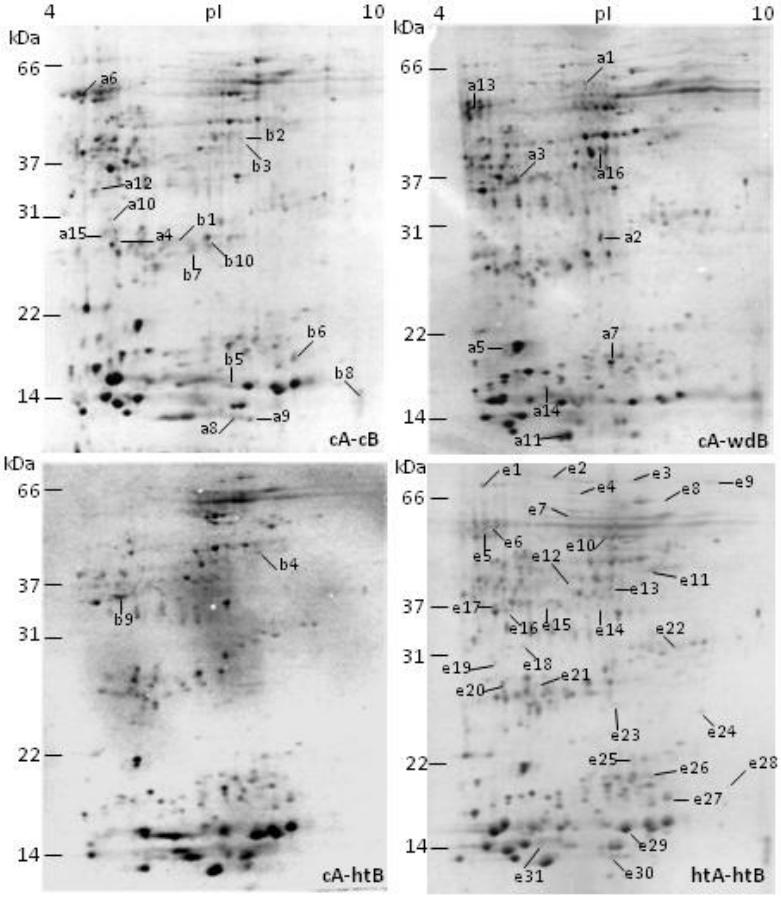
Figure and table legends

Figure 1. The accumulation of albumins (A+E), globulins (B+F), gliadins (C+G) and glutenins (D+H) in wheat grain under high temperature and/or water deficit stress treatments applied at terminal spikelet and/or anthesis. The treatments are denoted as outlined in Materials and Methods. The cA-cB, cA-wdB, cA-htB and htA-htB treatments selected for proteomic analysis are shown in left column, other treatments are shown in right column.

Figure 2. Albumin profiles of mature wheat grain. The representative 2D-gels (pI range 4–10) are shown for control (cA-cB) and samples under water deficit (cA-wdB), single high temperature (cA-htB) and multiple high temperatures (htA-htB) treatments. Molecular size markers are indicated. Protein spots changing in intensity in response to stress are numbered.

Figure 3. Gliadin profiles of mature wheat grain. The representative 2D-gels (pI range 4–10) are shown for control (cA-cB) and samples under water deficit (cA-wdB), single high temperature (cA-htB) and multiple high temperatures (htA-htB) treatments. Molecular size markers are indicated. Protein spots changing in intensity in response to stress are numbered.


A
B
C
D
E
F
G
H
cA-cB
cA-wdB
cA-htB
htA-htB
wdA-cB
wdA-wdB
wdA-htB
htA-cB
htA-wdB
Albumins [µg (mg grain) -1]
Globulins [µg (mg grain) -1]
Gliadins [µg (mg grain) -1]
Glutenins [µg (mg grain) -1]
dpa


4
pI
10
kDa
66
37
31
22
14
a6
b2
b3
a12
a10
a15
a4
b1
b7
b10
b6
b5
b8
a8
a9
cA-cB
a1
a13
a3
a16
a2
a5
a7
a14
a11
cA-wdB
b4
b9
cA-htB
e1
e2
e4
e3
e8
e9
e7
e6
e5
e10
e12
e11
e13
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e28
e27
e29
e31
e30
htA-htB

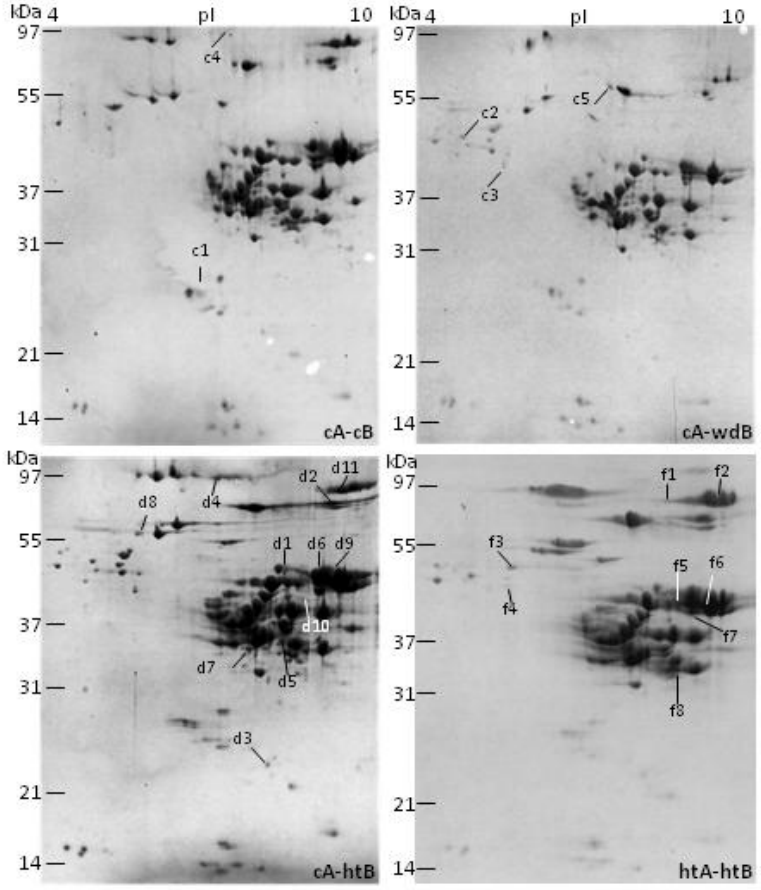
kDa
4
pI
10
97
55
37
31
21
14
c4
c1
cA-cB
c5
c2
c3
cA-wdB
d2
d11
d8
d4
d1
d6
d9
d10
d7
d5
d3
cA-htB
f1
f2
f3
f5
f6
f4
f7
f8
htA-htB

Table 1. Identification of wheat grain proteins in response to high temperature (ht) and water deficit (wd)

Spot no[a]	Spot relative intensity[b]	MS score[c]	Number of matched peptides	Sequence coverage (%)	Theor. MW(kDa)/ pI	Exp. MW(kD)/ pI	Accession number[d]	Organism[e]	Protein and conserved domain	MS/MS (sequence of matched peptides)[f]
cA-wdB treatment										
a8	1.5	78	7	34	16.6/7.45	13.0/7.40	TC264942	*Triticum aestivum*	Monomeric α-amylase inhibitor	
a9	1.8	86	7	66	10.0/5.50	12.8/7.67	gi|1169516	*Triticum aestivum*	Late embryogenesis abundant protein H2	
a16	1.6	82	8	31	31.3/5.05	45.7/7.77	gi|28172913	*Hordeum vulgare*	Cytosolic 3-phosphoglycerate kinase	
a1	1.9	72	6	14	72.6/5.63	62.5/7.63	gi|218683839	*Isomeris arborea*	Phytochrome B	
a2	1.6	102	12	42	26.4/5.58	30.1/7.70	TC235043	*Oryza sativa*	Embryo-specific protein	
a7	1.6	75	7	35	66.3/7.78	17.0/7.70	TC234137	*Triticum aestivum*	Embryo globulin	
a4	-2.4	89	8	44	24.0/6.31	28.6/5.78	TC265183	*Triticum aestivum*	1-Cys peroxiredoxin	
a11	-1.5	75	5	30	13.6/5.37	12.9/6.53	gi|134034615	*Triticum aestivum*	Monomeric α-amylase inhibitor	
a14	-1.7	89	7	57	18.9/7.44	15.3/6.75	gi|123957	*Triticum aestivum*	α-amylase/trypsin inhibitor CM3	
a12	-2.1	77	6	20	31.9/6.54	37.1/5.53	gi|7431022	*Hordeum vulgare*	Glucose and ribitol dehydrogenase	
a10	-2.1	133	10	52	25.5/6.66	31.3/5.70	gi|16903082	*Triticum aestivum*	Small Ras-related GTP-binding protein	
a5	-1.7	76	5	22	16.9/4.10	19.3/5.90	gi|1754999	*Triticum aestivum*	Calmodulin TaCaM2-2	
a6	-2.1	137	16	28	49.9/6.16	54.8/5.27	TC246874	*Zea mays*	Globulin-like protein	
a13	-1.6	93	15	36	49.9/6.16	56.1/5.98	TC246759	*Zea mays*	Globulin-like protein	
a15	-1.5	108	10	22	49.9/6.16	29.4/5.60	TC246874	*Zea mays*	Globulin-like protein	
c3	1.5	72	5	40	34.6/8.71	43.6/5.39	BQ243369	*Triticum aestivum*	Low molecular weight glutenin	
c2	-1.5	72	5	38	32.8/8.50	47.5/5.04	BQ242949	*Triticum aestivum*	α-gliadin	
c4	-1.5	76	4	37	34.6/8.71	68.6/7.37	BQ243369	*Triticum aestivum*	Low molecular weight glutenin	
c5	-1.5				32.3/7.16	61.3/7.20	TC250312	*Triticum aestivum*	γ-gliadin	RWDIHVAHGW, ATNPRDYAGKW
c1	-1.5				32.3/7.16	26.3/6.50	BJ233925	*Triticum aestivum*	γ-gliadin	TRGMTPMTQVCIVARGY, TRGMTPMTQVCIVARGYSCM GSTVVVEMVE
cA-htB treatment										
b1	1.9	72	7	31	24.1/6.31	28.4/6.60	gi|1710077	*Hordeum vulgare*	1-Cys peroxiredoxin PER1	
b7	1.5	102	8	31	23.9/6.06	27.1/6.85	TC250400	*Triticum aestivum*	27K protein, pfam03227, γ-interferon inducible lysosomal thiol reductase	
b4	1.5	76	9	22	43.5/5.11	46.1/8.03	gi|224589268	*Triticum aestivum*	Serpin 2	
b6	1.8	76	5	35	16.5/5.07	17.2/8.30	gi|21711	*Triticum aestivum*	α-amylase/trypsin inhibitor CM17	

b3	-1.8	75	8	17	50.5/5.68	45.3/7.57	gi|1495812	*Solanum tuberosum*	Lipoxygenase	
b5	-1.5	73	6	51	14.8/5.68	14.9/7.37	gi|242032659	*Sorghum bicolor*	Hypothetical protein, cd02947, thioredoxin family	
b2	-1.9	72	7	22	45.6/6.08	46.1/7.57	gi|226500228	*Zea mays*	Succinyl-CoA ligase β-chain	
b10	-1.5	109	11	39	26.8/5.38	29.0/7.07	TC246911	*Triticum aestivum*	Triosephosphate isomerase	
b9	-1.5	81	9	18	39.4/5.38	40.5/5.93	gi|1419094	*Nicotiana tabacum*	Glutamine synthetase	
b8	-1.8	72	5	20	24.8/10.41	14.1/9.24	TC264449	*Oryza sativa*	40S ribosomal protein S8	
d1	1.5				32.8/8.50	47.5/8.25	BQ244309	*Triticum aestivum*	α-gliadin	PIEPGIFPTAFATISIRPGL, GFCPASTIAPVRGNRDPRVPD VPCNVQCL
d6	1.5	77	4	33	32.8/8.50	46.2/8.83	BQ242040	*Triticum aestivum*	α-gliadin	
d7	1.7	79	5	31	34.6/6.62	34.4/7.61	BQ167777	*Triticum aestivum*	α/β-gliadin A-V	
d9	1.6	72	4	23	32.8/8.50	45.6/9.01	BE424082	*Triticum aestivum*	α-gliadin	
d10	1.7	74	4	25	34.6/6.62	42.5/8.48	BQ245636	*Triticum aestivum*	α/β-gliadin A-V	
d11	1.6	72	4	23	32.8/8.50	71.9/9.18	BE424082	*Triticum aestivum*	α-gliadin	
d2	1.8				32.3/7.16	61.3/9.01	TC250312	*Triticum aestivum*	γ-gliadin	RWDIHVAHGW, ATNPRDYAGKW
d3	1.6				34.6/8.71	22.2/7.84	BQ243369	*Triticum aestivum*	Low molecular weight glutenin	KKIGSPL, GVHSILGGGTPTGGAMCFPTP TAVGGGNW
d4	1.9				34.6/8.71	67.6/7.08	BQ243369	*Triticum aestivum*	Low molecular weight glutenin	KKIGSPL, GVHSILGGGTPTGGAMCFPTP TAVGGGNW
d5	1.9				34.6/8.71	35.8/8.07	BQ243369	*Triticum aestivum*	Low molecular weight glutenin	WAVVQNPP, GVHSILGGGTPTGGAMCFPTP TAVGGGNW
d8	-2.0	75	5	17	34.6/6.62	54.0/5.77	TC249891	*Triticum aestivum*	α/β-gliadin	
htA-htB treatment										
e27	1.6	76	6	35	16.5/5.07	17.1/8.13	gi|21711	*Triticum aestivum*	α-amylase inhibitor CM17	
e30	1.6	115	7	65	13.7/6.18	14.5/7.38	gi|134034577	*Triticum aestivum*	Monomeric α-amylase inhibitor	
e29	1.5	101	8	72	13.7/5.23	14.0/7.58	gi|56480630	*Triticum aestivum*	0.19 dimeric α-amylase inhibitor	
e8	2.0	74	6	14	84.8/6.05	70.3/8.17	TC249217	*Oryza sativa*	Fructose-6-phosphate-2-kinase	
e10	1.9	72	9	31	48.4/5.70	50.9/7.34	gi|162460735	*Zea mays*	Enolase 2	
e7	1.6	94	11	19	59.6/5.66	60.5/6.89	TC249933	*Hordeum vulgare*	β-amylase	
e11	2.0	86	10	28	40.9/9.35	42.0/8.24	gi|81176532	*Triticum aestivum*	Ribosomal protein S2	
e28	1.6	77	6	46	18.7/8.62	18.3/8.92	gi|15822545	*Triticum aestivum*	Cinnamoyl-CoA reductase	
e24	1.5	79	5	33	18.9/4.55	28.7/9.61	gi|75246527	*Triticum aestivum*	Translationally-controlled tumor protein	
e3	1.9	81	10	13	89.7/5.12	88.7/7.73	TC233240	*Oryza sativa*	Cell division control protein 48 homolog E	

e19	+	1.9	96	11	37	71.6/5.30	32.8/5.86	TC264180	*Oryza sativa*	Heat shock cognate 70 kDa protein
e23	+	1.5	72	4	20	18.7/6.04	29.6/7.49	TC237069	*Triticum aestivum*	NADH dehydrogenase subunit J
e13	+	1.6	72	7	22	41.2/6.60	38.5/7.40	gi|242046234	*Sorghum bicolor*	Hypothetical protein, pfam03214, reversibly glycosylated polypeptide
e12	+	2.0	87	6	23	37.4/5.85	38.7/6.85	gi|218197438	*Oryza sativa*	Hypothetical protein
e25	+	2.2	72	5	51	15.2/11.62	21.9/7.69	CA739630	*Triticum aestivum*	Predicted protein
e9	+	1.7	78	11	12	134.3/9.05	87.5/8.75	gi|224099935	*Populus trichocarpa*	Predicted protein
e20	-	2.2	112	7	35	23.3/6.06	31.4/5.97	gi|30793446	*Triticum aestivum*	27K protein, pfam03227, γ-interferon inducible lysosomal thiol reductase
e31	-	1.9	82	5	55	13.7/6.73	13.7/6.45	gi|253783731	*Triticum aestivum*	α-amylase inhibitor CM1
e21	-	2.0	117	8	34	26.6/6.37	31.1/6.40	gi|90959771	*Triticum aestivum*	Multidomain cystatin
e16	-	1.9	113	12	34	35.8/6.51	36.1/6.00	TC264995	*Hordeum vulgare*	Aldose reductase
e4	-	1.5	72	5	11	84.8/5.68	77.2/7.03	gi|50897038	*Hordeum vulgare*	Methionine synthase
e5	-	1.6	94	15	32	59.5/6.27	54.1/5.71	gi|3913791	*Solanum lycopersicum*	Glutamate--cysteine ligase, chloroplastic
e22	-	1.9	95	8	40	29.4/4.83	33.8/8.13	gi|40781605	*Triticum aestivum*	14-3-3 protein
e1	-	1.6	72	8	16	71.2/6.25	82.7/5.71	TC252572	*Oryza sativa*	Ste20-related protein
e17	-	1.9	75	9	20	66.7/7.78	36.6/5.86	gi|215398470	*Triticum aestivum*	Globulin 3
e15	-	1.6	92	8	19	66.6/7.78	36.6/6.56	gi|215398470	*Triticum aestivum*	Globulin 3
e6	-	1.9	75	9	15	49.9/6.16	55.3/5.81	TC246703	*Zea mays*	Globulin-like protein
e2	-	1.7	73	6	15	57.8/8.19	91.3/6.61	gi|225450579	*Vitis vinifera*	Hypothetical protein, cl03252, PPR repeat
f1	+	1.6	75	4	23	32.8/8.50	71.9/8.46	BE424082	*Triticum aestivum*	α-gliadin
f2	+	1.8	81	4	23	32.8/8.50	71.9/9.05	BE424082	*Triticum aestivum*	α-gliadin
f5	+	1.5				32.3/7.16	39.2/8.20	TC250312	*Triticum aestivum*	γ-gliadin	VPPECSIIRAPF, VPPECSIIRAPFASIVAGIGGQ
f6	+	1.5	73	4	33	32.8/8.50	39.2/8.67	BQ242040	*Triticum aestivum*	α-gliadin
f7	+	1.9				35.0/8.60	38.4/8.52	BQ241281	*Triticum aestivum*	Low-molecular-weight glutenin subunit group 4 type II	HNSNHVHSNNNHHYRSNNNHHF, HCNKHYRTTNNNNPSNNNHNHF
f8	+	1.6	72	4	8	32.3/7.16	32.3/8.30	TC249991	*Triticum aestivum*	γ-gliadin
f3	-	2.0				34.6/6.62	47.9/5.44	BQ244076	*Triticum aestivum*	α/β-gliadin	SSRSAKPSPMLSML, SVLTEKKITL
f4	-	1.6				44.6/6.12	44.0/5.33	CA726637	*Triticum aestivum*	ω-gliadin	AMFIAMARRTNMMK, IAMARRTNMMKVL

[a] Spot numbers a, b and e refer to Fig. 2 (albumin fractions), spot numbers c, d and f refer to Fig. 3 (gliadin fractions).
[b] +: spots increasing in intensity under stress treatments; -: spots decreasing in intensity under stress treatments.

[c] Significant MS score is above 71 for NCBI and HvGI.
[d] gi: accession number in NCBI; TC, BQ, BE, BJ: accession number in the TaGI wheat gene index Release 11.0.
[e] When the identification was based on EST sequence, the organism with the most homologous sequence is given. The theoretical pI and MW are calculated from the homologous sequence of that organism.
[f] Significant MS/MS score is above 26 for TaGI.

Appendix II

Secretome analysis of *YAP*- or *AP*-knockout mutant of *Fusarium graminearum*

Collaboration with Ph.D. student Jens Due Jensen, Department of Plant Biology and Biotechnology, University of Copenhagen

YAP and *AP* are known as transcription factors which regulate genes encoding antioxidants such as catalases and superoxide dismutases and are required for the response to oxidative stress (personal communication with Jens Due Jensen). Two *Fusarium graminearum* mutants have been knocked out *YAP* and *AP* genes, respectively, constructed by Jens Due Jensen, to investigate the role of antioxidants in pathogenesis. In *in planta* experiments, these two mutants are more aggressive than wildtype strain during infection of wheat. In order to gain some insights into the molecular mechanism of infection in the mutants, we conducted *in vitro* secretome analysis with the substrate of wheat grain flour due to difficulty of obtaining fungal proteins *in planta* and the important role of secreted proteins in pathogenicity. The methods of isolation of fungal secreted proteins, 2-DE, image analysis and protein identification were performed as described in Chapter 4. In total, 38 and 34 spots changed in intensity in *AP* and *YAP* mutants compared to the wildtype, of which 35 and 31 spots were identified, respectively. There was one spot identified as wheat abundant protein serpin in either mutant. The identified fungal proteins were mainly involved in the degradation of plant substrate cell wall, starch and protein. The 2-D patterns of mutants as well as wildtype and protein identification were shown in Fig. S1 and Table S1, respectively.

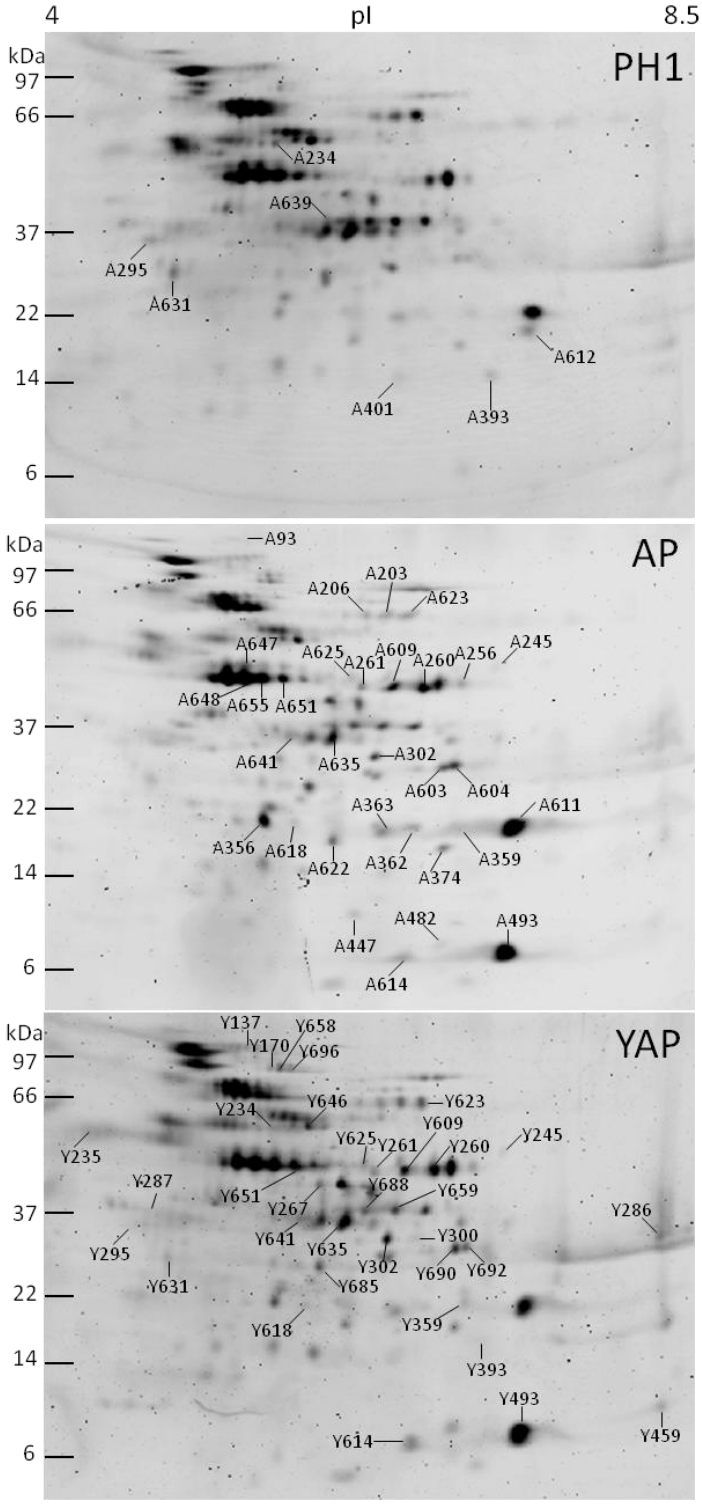

Fig. S1. 2-DE Sypro-Ruby stained gels from the secretome of wildtype (PH1), *AP*-knockout and *YAP*-knockout *F. graminearum* grown in medium containing wheat grain flour. Molecular size markers and pI ranges are indicated. Protein spots changing in intensity between the *AP* or *YAP* mutant and wildtype are numbered as Axx and Yxx, respectively.

Table S1. Identification list of *F. graminearum* proteins varying between mutants and wildtype

Spot no	Spot relative intensity[a]	MS score	Number of peptides matched	Sequence coverage (%)	Theor./Exp. pI	Theor./Exp. MW (kDa)	Accession number[b]	Protein and conserved domain	Signal peptide[c]
***AP* mutant**									
A256	1.8	55	4	10	6.40/6.86	43.72/45.07	FGSG01596	Hypothetical protein, pfam00150, cellulase superfamily	Yes
A260	2.6	58	3	8	6.38/6.59	45.90/44.69	FGSG01596	Hypothetical protein, pfam00150, cellulase superfamily	Yes
A356	2.9	58	5	7	9.01/5.52	37.65/23.00	FGSG02658	Endoglucanase-5	Yes
A295	-2.1	55	5	17	4.70/4.58	40.51/36.26	FGSG05292	GPI-anchored cell wall β-1,3-endoglucanase EglC	Yes
A93	1.7	63	7	9	4.72/5.25	94.93/142.12	FGSG11097	Hypothetical protein, cl02568, WSC superfamily involved in carbohydrate binding, cl09101, sugar utilizing enzymes	Yes
A651[d]	2.1	57	3	6	5.37/5.65	48.84/46.38	FGSG03842	α-amylase 1	Yes
A604[d]	2.5	91	7	29	5.62/6.81	33.63/32.57	FGSG01818	Leucine aminopeptidase	Yes
A603[d]	2.7	57	5	24	5.62/6.72	33.63/32.57	FGSG01818	Leucine aminopeptidase	Yes
A261[d]	2.5	56	4	11	5.51/6.19	66.83/44.69	FGSG03467	Extracellular elastinolytic metalloproteinase precursor	Yes
A647	1.9	58	5	15	5.39/5.43	49.76/48.54	FGSG04527	Carboxypeptidase S1	Yes
A655[d]	1.8	96	7	21	5.39/5.52	49.76/46.38	FGSG04527	Carboxypeptidase S1	Yes
A648	1.6	100	8	26	5.39/5.41	49.76/46.38	FGSG04527	Carboxypeptidase S1	Yes
A234	-3.1	81	5	8	5.81/5.52	50.54/54.80	FGSG04936	Aminopeptidase Y	Yes
A639	-1.5	64	5	22	5.82/5.85	36.60/38.33	FGSG05738	Hypothetical protein, cd01310, TatD_DNAse, cl00281,metallo-dependent-hydrolases superfamily	No
A362	2.1	71	6	22	5.58/6.50	28.59/21.85	FGSG04826	L-xylulose reductase	No
A623[d]	-2	61	5	13	6.68/6.52	48.03/65.26	FGSG06549	Chitin deacetylase	Yes
A203	-1.8	78	6	16	6.68/6.36	48.03/65.26	FGSG06549	Chitin deacetylase	Yes
A206	-1.5	57	4	12	6.68/6.23	48.03/65.26	FGSG06549	Chitin deacetylase	Yes
A374[d]	2.1	172	7	39	6.31/6.72	19.61/18.46	FGSG00777	Cyclophilin, mitochondrial	No
A611[d]	2.2	194	15	77	6.92/7.17	18.61/23.00	FGSG04074	Hypothetical protein, cell wall protein	Yes
A363[d]	1.7	62	5	21	6.92/6.30	18.61/21.85	FGSG04074	Hypothetical protein, cell wall protein	Yes
A359[d]	1.8	113	8	44	6.92/6.77	18.61/23.57	FGSG04074	Hypothetical protein, cell wall protein	Yes
A612[d]	1.7	62	7	26	6.92/7.17	18.61/20.70	FGSG04074	Hypothetical protein, cell wall protein	Yes
A631	-2.1	56	4	17	5.08/4.80	33.22/29.83	FGSG07603	Hypothetical protein, cl09931, NADB_Rossmann superfamily	No
A622[d]	1.8	112	6	52	5.71/5.96	21.80/19.57	FGSG01403	Hypothetical protein, cl00438, FMN_red superfamily	No
A393	-1.6	58	4	32	6.23/6.90	16.84/16.30	FGSG09570	Predicted protein	Yes
A641	-1.8	55	5	14	5.37/5.70	48.21/36.26	FGSG08833	Hypothetical protein	No
A493[d]	12	415	4	58	6.94/7.12	9.46/6.37	FGSG11033	Predicted protein	Yes
A614[d]	3.8	254	4	58	6.94/6.41	9.46/5.96	FGSG11033	Predicted protein	Yes
A302	3.8	68	5	18	5.86/6.28	35.21/33.63	FGSG11204	Hypothetical protein	Yes
A609	4.3	59	6	12	6.36/6.39	49.24/44.69	FGSG01637	Hypothetical protein	Yes
A245	2.2	64	5	13	7.82/7.06	40.30/48.09	FGSG02721	Hypothetical protein , pfam10282, DUF2394 unknown	Yes

								function	
A482	1.5	85	5	76	6.10/6.68	10.22/6.83	FGSG04000	Hypothetical protein	No
A401	1.8	94	6	45	6.11/6.32	13.43/14.26	FGSG04741	Hypothetical protein	Yes
A635	-1.5	100	10	24	6.64/5.96	50.57/36.26	TC253068	Serpin	No
***YAP* mutant**									
Y658	2	58	7	13	5.11/5.59	84.17/83.26	FGSG06616	Glucan 1,3-β-glucosidase GLUC78	Yes
Y696	2.2	55	7	13	5.11/5.67	84.17/83.26	FGSG06616	Glucan 1,3-β-glucosidase GLUC78	Yes
Y170	1.7	55	7	13	5.11/5.46	84.17/83.26	FGSG06616	Glucan 1,3-β-glucosidase GLUC78	Yes
Y295	-1.6	56	5	17	4.70/4.58	40.51/34.42	FGSG05292	GPI-anchored cell wall β-1,3-endoglucanase EglC	Yes
Y267	2.2	57	4	12	5.84/5.82	47.50/41.45	FGSG08265	β-1,6-glucanase precursor	Yes
Y260[d]	2	58	3	8	6.40/6.59	43.72/44.35	FGSG01596	Hypothetical protein, pfam00150, cellulase superfamily	Yes
Y300	1.6	62	5	15	6.22/6.47	45.48/32.48	FGSG00047	Hypothetical protein,cl09107, esterase_lipase superfamily	No
Y646	-1.8	75	5	9	5.43/5.76	56.08/54.61	FGSG11386	Hypothetical protein, cl09107, esterase-lipase superfamily	Yes
Y286[e]	2				6.80/8.15	40.85/35.38	FGSG00806	Alkaline protease	Yes
Y261	2.3	56	4	11	5.51/6.20	66.83/44.35	FGSG03467	Extracellular elastinolytic metalloproteinase precursor	Yes
Y692	1.7	63	4	18	5.62/6.80	33.63/31.51	FGSG01818	Leucine aminopeptidase	Yes
Y690	2.1	58	5	24	5.62/6.72	33.63/31.51	FGSG01818	Leucine aminopeptidase	Yes
Y234	-1.9	81	5	8	5.81/5.50	50.54/52.66	FGSG04936	Aminopeptidase Y	Yes
Y651[d]	2.5	128	9	27	5.39/5.67	49.76/44.98	FGSG04527	Carboxypeptidase S1	Yes
Y137	1.6	68	8	16	5.19/5.29	92.40/99.56	FGSG06572	Hypothetical protein, pfam00082, peptidase_S8, Subtilase family, pfam06280, DUF1034	Yes
Y359	1.6	93	7	43	6.92/6.80	18.61/22.46	FGSG04074	Hypothetical protein,cell wall protein	Yes
Y685	-1.9	74	5	22	5.58/5.80	28.59/28.05	FGSG04826	L-xylulose reductase	No
Y623	-1.6	68	5	15	6.68/6.51	48.03/64.43	FGSG06549	Chitin deacetylase	Yes
Y659	-1.8	60	5	19	5.87/6.34	32.32/37.33	FGSG06257	Glyceraldehyde-3-phosphate dehydrogenase	No
Y688	-1.9	73	7	31	5.87/6.13	32.32/37.33	FGSG06257	Glyceraldehyde-3-phosphate dehydrogenase	No
Y631	-1.9	56	4	17	5.08/4.79	33.22/29.05	FGSG07603	Hypothetical protein, cl09931, NADB_Rossmann superfamily	No
Y235	1.6	56	4	14	4.83/4.33	38.37/51.75	FGSG08037	Hypothetical protein, cl01383, intradiol_dioxygenases superfamily	Yes
Y245	2	62	5	13	7.82/7.06	40.30/47.71	FGSG02721	Hypothetical protein , pfam10282, DUF2394 unknown function	Yes
Y641	-1.7	55	5	14	5.37/5.67	48.21/34.90	FGSG08833	Hypothetical protein	No
Y493[d]	15	310	4	58	6.94/7.14	9.46/6.33	FGSG11033	Predicted protein	Yes
Y614[d]	5.9	260	3	49	6.94/6.43	9.46/5.66	FGSG11033	Predicted protein	Yes
Y459	1.5	58	3	49	6.94/8.10	9.46/9.14	FGSG11033	Predicted protein	Yes
Y302	4	63	5	18	5.86/6.26	35.21/32.48	FGSG11204	Hypothetical protein	Yes
Y393	-1.7	58	4	32	6.23/6.89	16.84/15.54	FGSG09570	Predicted protein	Yes
Y609	3.6	59	6	12	6.36/6.38	49.24/44.35	FGSG01637	Hypothetical protein	Yes
Y635[d]	-1.5	85	13	27	6.64/6.01	50.57/34.90	TC253068	Serpin	No

[a] +: increasing in intensity in the mutants; -: decreasing in intensity in the mutants
[b] FGSG: accession number in *Fusarium graminearum* gene index from Broad Institute; TC: accession number in the TaGI wheat gene index Release 11.0
[c] If the signal peptide is contained, the theoretical pI and MW are calculated after removal of signal peptide
[d] At least one peptide additionally confirmed by MS/MS analysis
[e] Identified by MS/MS analysis