Proteome catalogue of *Zymoseptoria tritici* proteins captured during pathogenesis in wheat

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Abstract

Zymoseptoria tritici is an economically important pathogen of wheat. However, the molecular basis of pathogenicity on wheat is still poorly understood. Here, we present a global survey of the proteins secreted by this fungus in the apoplast of resistant (cv. Shafir) and susceptible (cv. Obelisk) wheat cultivars after inoculation with reference *Z. tritici* strain IPO323. The fungal proteins present in apoplastic fluids were analyzed by gel electrophoresis and by data-independent acquisition liquid chromatography/mass spectrometry (LC/MS^E) combined with data-dependent acquisition LC-MS/MS. Subsequent mapping mass spectrometry-derived peptide sequence data against the genome sequence of strain IPO323 identified 665 peptides in the MS^E and 93 in the LC-MS/MS mode that matched to 85 proteins. The identified fungal proteins, including cell-wall degrading enzymes and proteases, might function in pathogenicity, but the functions of many remain unknown. Most fungal proteins accumulated in cv. Obelisk at the onset of necrotrophy. This inventory provides an excellent basis for future detailed studies on the role of these genes and their encoded proteins during pathogenesis in wheat.

Keywords: *Zymoseptoria tritici*, apoplastic fluid, pathogenesis, proteomics, liquid chromatography/mass spectrometry, fungal proteins

1. Introduction

Proteomics has become a powerful tool for providing knowledge on plant-pathogen interactions by disclosing important information about pathogenicity and virulence factors, thus opening up new possibilities for crop disease diagnosis and crop protection (Rampitsch and Bykova, 2012). It is also frequently used to catalogue proteins that reside in the plant apoplast (Delaunoy et al., 2014). In fact, recent studies have suggested that the apoplast is one of the first compartments for interaction between proteins of fungal and host origin during pathogenesis (Doehlemann and Hemetsberger, 2013). It is therefore an important resource for the discovery of key determinants in virulence and avirulence (Joosten and de Wit, 1999; Rep, 2005; Thomma et al., 2005; Kamoun, 2006; Houterman et al., 2007; Bolton et al., 2008; Stergiopoulos and de Wit, 2009; de Jonge et al., 2010). For example, in the biotrophic tomato pathogen *Cladosporium fulvum*, the analyses of apoplastic fluids (AFs) identified several

effector proteins that play crucial roles in pathogenicity (Joosten and de Wit, 1988; Van den Ackerveken et al., 1992; Laugé et al., 2000; Bolton et al., 2008).

Zymoseptoria tritici (Desm.) Quaedvlieg & Crous (Quaedvlieg et al., 2011) is the causal agent of septoria tritici blotch (STB), which is currently the major wheat disease in Europe and the Mediterranean Basin (this issue). *Z. tritici* has a hemibiotrophic lifestyle with a relatively long latent period during which fungal development is slow and strictly apoplastic and a necrotrophic phase that is characterized by massive host killing and fungal proliferation culminating in the production of pycnidia, producing massive amounts of asexual pycnidiospores (Kema et al., 1996; Duncan and Howard, 2000). Detailed histological studies showed that the apoplast is a major unexplored reservoir for interaction in the *Z. tritici*-wheat pathosystem (Kema et al., 1996; Rudd and Hammond-Kosack, 2008).

The transition between the biotrophic and necrotrophic phases is reminiscent of the hypersensitive response (HR) and is accompanied by the differential regulation of wheat mitogen-activated protein kinase cascades (Keon et al., 2007; Rudd and Hammond-Kosack., 2008). As a result, the contents of killed plant cells leak into the apoplast, boosting rapid fungal proliferation in collapsing tissue that starts around 10-14 days post-inoculation (dpi), which is associated with appearance of macroscopically visible chlorotic and eventually coalescing necrotic blotches in the foliage. During the onset of lesion formation, Keon et al. (2007) identified the accumulation of glucose and fructose and amino acids in wheat AFs, whereas fungal proliferation also culminates in the massive formation of pycnidia that occupy the necrotic lesions until full symptom development at 21 dpi (Kema et al., 1996; Duncan and Howard, 2000). During these later phases of infection, cell wall-degrading enzymes (CWDEs) are increasingly expressed (Kema et al., 2008; Siah et al., 2010).

In the incompatible interaction, macroscopic symptoms vary greatly from none detectable to leaf chlorosis, but without significant fungal proliferation (Kema et al., 1996; Ware, 2006; Rudd and Hammond-Kosack., 2008). Despite the absence of HR (Kema et al., 1996; Ware, 2006; Rudd and Hammond-Kosack., 2008), which is a hallmark of resistance in biotrophic host-pathogen interactions (Greenberg, 1997; Heath, 2000), H₂O₂ accumulation - often used as a marker of HR- was reported, which negatively affects *Z. tritici* development at later stages of infection, but apparently does not hamper early development (Shetty et al., 2003; Shetty et al., 2007).

Here, we present a global *in planta* proteome survey of *Z. tritici* in both compatible and incompatible interactions, resulting in a foliar apoplastic catalogue of total and secreted proteins. For this purpose, the fungal proteins present in AFs were analyzed by gel

electrophoresis and by data-independent acquisition liquid chromatography/mass spectrometry (LC/MS^E) combined with data-dependent acquisition LC-MS/MS approaches thereby providing an excellent basis for future detailed studies on the role of the identified *Z. tritici* genes and their encoded proteins inducing susceptibility or resistance in wheat.

2. Material and Methods

2.1 Fungal strain, plant infections and isolation of apoplastic fluids

The sequenced Dutch *Z. tritici* reference strain IPO323 was used throughout this study to inoculate seedlings of wheat cvs. Obelisk (susceptible) and Shafir (resistant) (Kema et al., 1996; Kema et al., 2000; Brading et al., 2002; Goodwin et al., 2011). Experimental procedures for plant and fungal materials and pre/post-inoculation conditions were as described previously (Kema et al., 1996; Ware, 2006). The inoculated primary leaves of both cultivars were collected at 0, 7, 14 and 21 days post-inoculation (dpi). AFs of 160 plants (16 pots with 10 plants/pot) were harvested at these time points for each cultivar, according to de Wit and Spikman (1982), with slight modifications, yielding eight samples for SDS-PAGE and LC-MS proteome analysis. The samples were subsequently filtered through a 0.22 µm filter (Millipore. Corp., Bedford, MA), frozen, lyophilized and stored at -20°C until further use.

2.2 Protein extractions for SDS-PAGE and LC-MS analysis

Protein extractions started with resuspending AFs in 0.2 ml of 8 M urea, 2 M thiourea, 5 mM diothreitol (DTT) (Sigma-Aldrich Chemie, Steinheim, Germany) followed by incubation for 1 hr at 30°C in a shaker (GFL, Burgwedel, Germany), alkylation of proteins with 15 mM iodoacetamide (IAA) (GE Healthcare, UK) for 30 min. at room temperature (in the dark) and centrifugation (15 min, 15,000 g). A fraction of the supernatant (50 µl, corresponding to AF of 40 plants), was used for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. For this purpose proteins were precipitated by adding nine volumes of cold acetone, after which the air-dried pellet was heated for 5 min at 95°C in SDS sample buffer. SDS-PAGE was performed on 15% Midget gels (Pharmacia, Co. Sweden, Laemmli 1970) and gels were stained with Coomassie Brilliant Blue R-250 (CBB). Subsequently, the gel was sliced, and gel slices containing (stained) proteins of interest were

excised from the lanes for in-gel tryptic digestion and peptide sequencing. Each gel slice was first destained in 40% acetonitrile and 200 mM ammonium bicarbonate for two times for 30 min and then air-dried by vacuum centrifugation (Savant Speedvac, USA) after which trypsin (10 ng/μl) (Sequence grade modified, Promega, WI, USA) was added, followed by overnight incubation at 37°C. Tryptic peptides were extracted with 50% acetonitrile containing 0.1% trifluoroacetic acid (TFA) (Fluka, Buchs, Switzerland) and air-dried by vacuum centrifugation (Shevchenko et al., 1996).

For comparative LC-MS analysis protein all AF samples were digested in solution by trypsin. The remainder of the supernatant (150 μl; see above) was diluted with three volumes of 0.1 M ammonium bicarbonate and proteolytic digestion was initiated by adding 2 μl of trypsin (0.2 μg/μl). Each sample was subsequently incubated overnight at 37°C, after which the tryptic digestion was terminated by adding TFA to a final concentration of 0.5% (v/v). After centrifugation at 15,000 g for 10 min, the supernatant was loaded on a SupelClean™ LC-18 1 ml SPE column (Supelco, Bellefonte, USA) equilibrated with 0.1% TFA and peptides were eluted with 84% acetonitrile (ACN) (HPLC Supra-gradient, Biosolve, Valkenswaard, The Netherlands), containing 0.1% formic acid (FA) (Merck, Darmstadt, Germany) and dissolved in 40 μl 0.1% FA prior to LC-MS analysis.

2.3 Comparative LC-MS/MS and LC-MS^E

To identify fungal proteins, we used two proteomic techniques; the label-free data-independent acquisition (DIA) LC/MS^E combined with data-dependent acquisition (DDA) LC-MS/MS approaches that enable detailed identification and quantification of complex protein samples (America et al., 2006; America and Cordewener, 2008; Cappadona et al., 2010). LC-MS/MS preferentially identifies the most abundant components in a peptide mixture, as co-eluting low-abundant peptides will most likely not be chosen for MS/MS fragmentation. In contrast, LC-MS^E has no precursor selection before fragmentation and is therefore less biased for identification of predominant peptides resulting in a wider coverage of proteins present in the AFs. For peptide separation a nanoAcquity UPLC system (Waters Corporation, Manchester, UK) was used with a BEH C18 column (75 μm x 25 cm with 1.7 μm particles, Waters, UK) and a 65 min linear gradient from 3 to 40% ACN (in 0.1 % FA) at a flow rate of 200 nl/min. The eluting peptides were on-line injected into a Synapt Q-TOF MS instrument (Waters Corporation, Manchester, UK). MS analyses were performed in positive

mode using Electrospray ionization (ESI) with a NanoLockSpray source. As lock mass [Glu¹]fibrinopeptide B (1 pmol/μl, Sigma) was delivered from a syringe pump (Harvard Apparatus, location, USA) to the reference sprayer of the NanoLockSpray source at a flow rate of 0.2 μl/min and the lock mass channel was sampled every 30s. Accurate LC-MS data were collected with the Synapt Q-TOF MS instrument operating in either the MS/MS or MS^E mode (DDA and DIA) using low (6 eV) and elevated (ramp from 15 to 35 eV) energy spectra every 0.6 s over a 140-1900 m/z range, respectively. LC-MS/MS was performed by peptide fragmentation on the three most intense multiple charged ions that were detected in the MS survey scan within 0.6 s over a 300-1400 m/z range and a dynamic exclusion window of 60 s with an automatically adjusted collision energy based on the observed precursor m/z and charge state.

2.4 Data base construction and data analysis

LC-MS/MS and LC-MS^E data were processed using ProteinLynx Global Server software (PLGS version 2.4, Waters Corporation, Manchester, UK) and the resulting list of masses containing all the fragment information was searched for matching proteins using a merged non-redundant database including all gene models of *Z. tritici* IPO323 (Goodwin et al., 2011) and from COGEME (<http://cogeme.ex.ac.uk/sequence.html>). Sequences from wheat [TaGI v11 071508] were obtained from the Gene Index Databases, Dana-Farber Cancer Institute, Boston, MA 02115 USA (URL: <http://compbio.dfci.harvard.edu/tgi>) (Perteau et al., 2003; Lee et al., 2005) and sequences of common contaminants, such as trypsin and keratin, were also included. These databases were merged into a single database using an in-house developed FastaFileMerger tool (FFM) that only maintained the longest protein sequence with 100% homology from repeated sequences. This eventually resulted in a non-redundant database for *Z. tritici* and wheat comprising 238,013 sequences.

For LC-MS^E, the search was performed using the following parameters: a minimum of five fragments ions per peptide and a minimum of nine fragments ions per protein, a minimum of one peptide match per protein and a maximum of one missed trypsin cleavage. Furthermore, we used (i) carbamidomethylation as fixed modification, (ii) oxidation as variable modifications, and (iii) a false positive threshold of four percent. For LC-MS/MS analysis the peptide mass tolerance was set to 50 ppm and a fragment mass tolerance of 0.1 Da. Carbamidomethylation was used as fixed modification, and deamidation and oxidation as variable modifications. The AutoMod search was applied as secondary search to the database

search results. This analysis tool increases protein coverage by taking into account missed trypsin cleavages and non-specific cleavages, post-translational modifications, and amino acid substitutions. In case of in-gel digested samples, the database search was performed with MASCOT on the FFM merged database, but with precursor and fragment mass tolerance at 0.8 Da and carbamidomethylation as fixed modification and deamidation, oxidation and dioxidation as variable modifications. Finally, the LC-MS/MS and LC-MS^E outputs were further merged and wheat proteins that were identified only once across all time points were not considered for further analysis. Since we used all gene models of *Z. tritici*, additional filtering steps were performed for proteins with alternative models (based on additional peptides obtained but not covered by the present gene models of the *Z. tritici* data base at the United States Department of Energy-Joint Genome Institute, DOE-JGI) and eventually only best (revised) models were used. Furthermore, the identification of *Z. tritici* proteins was certified if a protein was assigned on at least two peptides with a LC-MS/MS score ≥ 50 and/or MS^E > 5 . However, for proteins with only one assigned peptide, particularly for the small secreted proteins (SSPs), these were kept only when the MS^E score was > 6 and/or for LC-MS/MS when the score was ≥ 50 . Single peptides that overlapped with other proteins with the same identified peptide were excluded (for the single hit protein) from further analysis.

2.5 Protein quantification using Progenesis

For quantitative analyses, the LC-MS^E data were processed in Progenesis LC-MS V2.6 (Nonlinear Dynamics, Newcastle, UK), which imported high resolution raw profile data (with lock-spray mass-calibration) into custom mzNLD format and extracted peptide intensity features from MS^E data. The LC-MS^E data of cvs. Shafir and Obelisk from all duplicated time points were used for quantification. The LC-MS^E chromatograms of all acquired datasets were aligned against a chosen reference run (cv. Obelisk at 14 dpi). Retention time alignment by warping allowed the creation of a single aggregate peak map containing all peak data from all samples of which only 2+, 3+ and 4+ charged peptides were included in the comparison. After peak detection on the aggregate map the intensities were determined for each peak per individual run. The peak intensity data were normalized using the average of peak intensity ratios for each run versus the reference, after removing the outliers (majority of differential peaks). The generation of so-called feature tables allowed then quantitative comparisons of peak intensities between samples. Peptide identification results from ProteinLynx database searches of LC-MS^E data were imported into Progenesis for linking identified peptides to their

corresponding peak features. In Progenesis subsequent filtering steps were carried out, *i.e.* number of peptide hits ≥ 2 over all runs and a ProteinLynx Global SERVER (PLGS) peptide score > 5 . Consequently, a relative expression profile was generated per peak each sample (cultivars) at each time point. The expression profiles, when at least two or more peptides were assigned to a protein of interest, were checked and peptides were filtered when the alignment was not clear. Multivariate principal component analysis (PCA) was performed with GeneMaths (Applied Maths, Belgium) on the peak area data (log transformed and pareto scaled) to examine the overall differences between the samples.

2.6 Characterization of proteins and the identification of conserved domains

Functional annotation was performed using the Blast2GO v2 program (Götz et al., 2008), which enables the annotation of each protein with a GO number that categorizes them to molecular function, biological process or cellular component. Sequences were directly annotated using BLASTp against the NCBI non-redundant protein sequence database using a cut-off E-value of $1e^{-6}$ and functional domains were searched with InterPro Scan, SignalP 3.0 (Bendtsen et. al., 2004a), PSORT II, and TMHMM-based algorithms and resulted in level 2 and level 3 analyses, illustrating general functional categories.

The MultiLoc program was used for subcellular localization prediction (Torsten et al., 2009). We used the obtained sequence information to search for conserved domains using the InterPro Scan program (<http://www.ebi.ac.uk/Tools/InterProScan/>) and online software (Marchler-Bauer et al., 2009) (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) to determine the classifications and possible functions of identified hypothetical proteins. In addition to classically secreted proteins predicted with SignalP 3.0, the SecretomeP1.0 Server (<http://www.cbs.dtu.dk/services/>) (Bendtsen et. al., 2004b) predicted non-classically secreted proteins and we used a score above 0.6 as a high probability threshold for such alternative secretion mechanisms. Eventually, proteins were narrowed down to secreted proteins by meeting the following requirements: (i) extracellular prediction by PSORT II, (ii) containing a signal peptide and signal peptide cleavage site according to SignalP 3.0 with a minimal D-Score of 0.5 and (iii) absence of internal transmembrane helix (TM = 0).

2.7 RT-PCR of candidates

The expression of 16 proteins, comprising hypothetical proteins and cell-wall degrading enzymes for which we had quantitative proteomic data, was analyzed by Real-time qPCR. The susceptible cv. Obelisk was inoculated with *Z. tritici* strain IPO323 in three biological replicates and infected leaves were collected at 2, 4, 8, 12, 16 and 20 days post-inoculation, flash-frozen in liquid nitrogen and kept at -80°C until use. Total RNA was isolated from ~0,7 ml ground leaf tissue with one ml of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in 2 ml tubes according to the manufacturer's instruction. To remove contaminated DNA, total RNA was treated with RNase-free DNase I (Promega, Madison, USA). First-strand cDNA was generated using Superscript III Reverse Transcriptase (Invitrogen, San Diego, CA, USA) and further diluted (5x) and finally used for SYBR® Green qPCR (Applied Biosystems, Foster City, CA). For each reaction a 1 µl aliquot of cDNA was used in a 25 µl PCR volume with primers at a final concentration of 0.30 µM at an annealing temperature of 60°C using an ABI 7500 Real-Time PCR System (Applied Biosystems). The expression was normalized with the constitutively expressed *Z. tritici beta-tubulin* gene (See Table S1 for a list of the used primers).

3. Results and discussion

3.1 Outline of methodology

The apoplastic space is a rich plant-pathogen interface containing a plethora of proteins for mutual molecular communication (Delaunoy et al., 2014). We isolated AFs from the resistant (Shafir) and susceptible (Obelisk) wheat cvs. at several time points after inoculation with *Z. tritici* strain IPO323 to identify proteins that are crucial during pathogenesis in wheat. The total AF protein load was initially analyzed by SDS-PAGE and subsequently unravelled by label-free LC-MS^E and data dependent acquisition LC-MS/MS techniques. Obtained protein sequences were eventually mapped to the *Z. tritici* IPO323 reference genome for gene identification and localization.

3.2 Differential symptom development induced by *Zymoseptoria tritici* strain IPO323 on susceptible and resistant wheat cultivars

We followed the symptom development of *Z. tritici* strain IPO323 on cvs. Obelisk and Shafir until 21 dpi (Fig. 1A). Until seven dpi, resistant and susceptible plants did not show

any visible disease symptoms. In the resistant cultivar, patchy chlorosis developed at 14 dpi that remained constant until 21 dpi without any formation of pycnidia. During compatibility, the first macroscopically visible symptoms appeared at 10 dpi as greyish sunken necrotic lesions that coalesced into larger ones that eventually covered the entire leaf and gradually showed increasing numbers of pycnidia at 14 dpi until symptom development was completed at 21 dpi. The AF volumes from the inoculated susceptible and resistant cultivars (0.5-0.6 ml/gr fresh weight) were similar until 7 dpi. From 14 dpi onwards, AF volumes of the susceptible cultivar increased to 2.3 ml/gr fresh weight vs 0.8 ml/gr fresh weight for the resistant cultivar, probably reflecting increased leakage of soluble wheat proteins during the onset of the necrotic phase.

3.3 Analysis of the apoplastic proteome

3.3.1 SDS-PAGE

SDS-PAGE was used to investigate qualitative differences in the apoplastic protein profiles of susceptible (S) and resistant (R) cultivars (Fig. 1B). At 0 dpi, the apoplastic protein profiles of both cultivars looked quite similar, although some proteins (bands around 25 and 90 kDa) were more abundant in cv. Shafir compared to cv. Obelisk. Since the samples were pools of 160 plants, it was not obvious from these data whether all resistant plants showed (already before infection) an increased level of certain proteins compared to susceptible plants. Fig. 1B illustrates a clear reduction of the apoplastic protein content after 7 dpi in susceptible plants, but not in resistant plants, whose AF protein profile dramatically changed during this first phase of pathogenesis. A number of gel slices was subsequently analysed by mass spectrometry for protein identification, which yielded 15 different proteins (Fig. 1C). Most of these originated from wheat and primarily comprised PR proteins with reported antifungal activity such as β -1,3-glucanases (PR-2), chitinases (PR-3) and peroxidases (PR-9) that were previously described during *Z. tritici* pathogenesis (Muthukrishnan et al., 2001; Shetty et al., 2009).

SDS-PAGE also identified three *Z. tritici* proteins (Fig. 1C). Two proteins contained a secretion signal with strong homology to α -L-arabinofuranosidase and a six-hairpin glycosidase from *Pyrenophora tritici-repentis*. The third protein showed strong homology with a methionine synthase of *C. fulvum*, which is particularly required for methionine synthesis during infection (Solomon et al., 2000).

3.3.2 Quantitative analysis of LC-MS^E data by Progenesis

As expected, the AF samples harvested at 0 dpi deviated strongly from all other samples. Consequently, the software was not able to align the 0 dpi samples properly to the reference sample, and therefore these samples were not considered for further analysis. The single aggregate map containing all MS peak data from the 7, 14 and 21 dpi comprised a total of 6,921 features. The PCA revealed a clear separation over the vertical axis (PCA2) of the protein samples collected from the AFs of the resistant and susceptible cultivars (Fig. 2A). Replication accuracy is demonstrated by the close proximity of the two 21 dpi replicates. Furthermore, on the x-axis (PCA1) the 21 dpi samples were distinct from the 7 dpi and 14 dpi samples; the latter two were much more proximate to each other, indicating that major changes occur during the later phases of pathogenesis (Fig. 2A, B).

3.3.3 Peptide identification with LC-MS/MS and LC-MS^E

LC-MS/MS analyses of all eight sample runs resulted in 544 unique peptide sequences that matched with 439 wheat peptides and 93 *Z. tritici* peptides (Table S2). A total of 4,735 peptide sequences were identified by LC-MS^E from which the majority (4,009) matched with wheat proteins, while 665 peptides matched with *Z. tritici* proteins (Table S2). The percentage of unique peptide sequences identified from the LC-MS^E analysis was much higher (~89%) compared with LC-MS/MS analysis, but we also noticed that a substantial part of the peptide sequences resulting from the LC-MS^E analysis originated from in source fragmentation of the precursor (tryptic) peptide. Irrespective of the technology used, the number of peptides matching with wheat proteins was approximately six times higher than those matching with *Z. tritici* proteins. Subsequent analyses focused on these fungal proteins.

3.3.4 Fungal proteins

In total, 85 fungal proteins were identified during sampled stages of infection, particularly in the cv. Obelisk during the later phases of pathogenesis. The identified proteins were grouped in different biological processes and functional categories (Table S3, Fig. 3). Sixty-two proteins were functionally annotated of which 22 were predicted to encode metabolic enzymes. A detailed GO description of all identified and annotated *Z. tritici* proteins is provided in the Supplementary Information (Table S3).

3.3.5 Fungal secreted proteins

SignalP predicted that 47% (40/85) of the *Z. tritici* proteins were secreted (Table 1). This estimate only includes proteins with N-terminal signal sequences that are targeted or predicted as constituents of the classical secretory pathway via these signal sequences. Additionally, 15 proteins were predicted to be secreted via non-classical secretory pathways (Table S3; see Cleves, 1997 and Nickel, 2005). Although the presence of these fungal proteins lack canonical signal peptides in the fungal secretome, their roles were demonstrated or hypothesized during fungal pathogenesis. For example, metalloproteases are implicated in key pathogenic processes in many fungi (Monod et al., 2002) and superoxide dismutase, an antioxidant enzyme involved in the scavenging system of ROS (Fridovich, 1995) plays a role in virulence of *Candida albicans* (Hwang et al., 2002). One protein with no GO term was also identified in the resistant cultivar during the early stage of infection (7 dpi). In addition, some fungal proteins appeared to be of cytoplasmic origin, such as the 40s ribosomal protein S15, 3-isopropylmalate dehydratase and aldehyde dehydrogenase and likely result from either cell leakage during sample preparation or from senescing mycelium or mesophyll cell collapse during the later stages of pathogenesis.

However, only proteins secreted via the classical secretory pathway were included for further analysis. The majority of these proteins is associated with pathogenesis and comprised many CWDEs that were identified during the late stages of infection, including α -L-arabinofuranosidases, β -glucosidases, β -xylosidases, endoglucanases, endo- β -xylanases and pectinesterases, suggesting active cell wall hydrolysis. CWDEs are of special interest because they are not only implicated in cell wall hydrolysis, thereby releasing nutrients for the colonizing pathogen (De Lorenzo et al., 1997), but also contribute to virulence (Bateman and Basham, 1976; Kapat et al., 1998; Kang and Buchenauer, 2000; Wanjiru et al., 2002). In addition, it has been reported that some fragments of decomposing cell walls, such as β -1,3-glucans and oligolacturonides elicit defence responses in plants (Esquerré-Tugayé et al., 2000; De Lorenzo et al., 1997). There is increasing evidence that CWDEs are involved in the pathogenesis of *Z. tritici* in wheat (Cohen and Eyal, 1993; Kema et al., 1996; Duncan and Howard, 2000; Shetty et al., 2003; Douaiher et al. 2007; Shetty et al., 2007; Kema et al., 2008; Siah et al., 2010; Brunner et al., 2013), and particularly likely during the switch from biotrophy to necrotrophy, which might be associated with the production of these enzymes that could release toxic cell wall fragments triggering cell-death (Goodwin et al., 2011). Two

out of the five cutinase genes in the genome of *Z. tritici* have been identified in this proteome study. Cutinases have been reported to play roles in cuticular penetration (Li et al., 2003), spore attachment (Deising et al., 1992; Pascholati et al., 1993), generation of cutin monomers as signaling molecules (Woloshuk and Kolattukudy, 1986; Podila et al., 1988; Francis et al., 1996) and even in protection of the host plant (Wang et al., 2000). More recently, Cut2, one of the cutinases of *Magnaporthe grisea*, was reported to be involved in surface sensing, thereby mediating appressorium differentiation and formation of the penetration peg (Skamnioti and Gurr, 2007, 2008). In *Z. tritici* that enters its host via stomata without appressorium formation (Kema et al., 1996) cutinases most likely have another function, such as lipase activity (Rudd et al., 2015).

Besides CWDEs, our proteome analysis confirms the secretion of a large number of proteases into the apoplast, particularly in the susceptible cv. Obelisk. Several different classes of proteases were identified including aspartic proteases, aminopeptidases and carboxypeptidases. Secreted proteases are potential virulence factors in fungi (Monod et al., 2002) and can be involved in degradation of cell wall-embedded proteins, but can also play regulatory roles in defence as has been shown for cysteine proteinase avrRpt2, which triggers RPS2-mediated defence that also requires RIN4 (Axtell and Staskawicz, 2003). In addition, proteases might inactivate PR proteins, although many PR proteins are known to be fairly resistant to proteases (Edreva, 2005). Goodwin et al. (2011) compared gene families and PFAM domains of *Z. tritici* with several other fungi and reported that peptidases and α -amylases were significantly expanded, suggesting alternative modes of nutrition that may include hydrolysis of proteins and starch, respectively, during the biotrophic phase of infection. In addition, the presence of proteins potentially involved in transport and metabolism of amino acids, lipids and coenzymes at 7 dpi might give more insight in the mechanism of nutrient retrieval from the environment during the biotrophic phase. Interestingly, Rudd et al. (2015) observed an up-regulation of biological processes that mainly involve the metabolism of lipids and fatty acids, during the early phases of colonization.

Furthermore, we identified proteins that are associated with the protection against anti-fungal oxidative stress responses of plants. The generation of reactive oxygen species (ROS) by plants is an early response to invading pathogens. ROS are not only toxic on their own at fairly high concentrations, but they can also serve as signal molecules at lower concentrations triggering different plant defence responses, including the production of PR proteins and phytoalexins (Lamb and Dixon, 1997; Mellersh et al., 2002; Torres and Dangl, 2005; Torres et al., 2006). As a consequence, pathogens use antioxidant defence systems or detoxify ROS

(Skamnioti et al., 2007). Catalases and peroxidases are among these oxidative stress-related proteins that protect against oxidative stress and are therefore essential for fungal survival during host invasion, but even more so during the necrotrophic phase when massive amounts of ROS, including H₂O₂, are present (Skamnioti et al., 2007). It has been suggested that *Z. tritici* is able to colonize susceptible wheat plants by avoiding recognition, *i.e.* suppressing or scavenging H₂O₂ (Shetty et al., 2003; Shetty et al., 2007). Indeed, the antioxidant catalases and peroxidases might degrade H₂O₂ accumulation during various phases of infection. We identified two of the three *Z. tritici* catalase/peroxidase genes during infection of the susceptible plant of which one appears to be secreted. Indeed, similar to other pathosystems (Garre et al., 1998; Schouten et al., 2002; Robbertse et al., 2003), we have shown that only a knock-out mutant of the non-secreted catalase/peroxidase was impaired in pathogenicity (MirzadiGohari and Kema, unpublished).

A large number of proteins with unknown function is frequently identified in proteomic studies. Here, we identified 12 secreted cysteine-rich proteins (3-10 cysteine residues) of which nine were small secreted proteins (SSPs) (less than 300 amino acids, MirzadiGohari et al., 2015). Several SSPs have shown to exhibit effector functions in various pathosystems (Rep, 2005; Houterman et al., 2007; Bolton et al., 2008). One of the identified SSPs at 21 dpi showed homology with the PhiA protein from *A. nidulans*, which was also identified in AFs of *C. fulvum*-infected tomato (Bolton et al., 2008), where it is involved in phialide and conidium formation, a function that is likely very basic in filamentous ascomycetes (Melin et al., 2003). In addition, it also mapped to the quantitative trait locus for pycnidium development in cv. Obelisk on chromosome 5 (Ware, 2006; Mizadi Gohari et al., 2015). We therefore hypothesize that it may also function during pycnidium formation and conidiogenesis in *Z. tritici*. The majority of the SSPs did not have any functional domain, were partially unique to *Z. tritici* and appeared to reside mainly in repetitive DNA stretches located in Transposable Element-rich regions, a phenomenon also observed for *Leptosphaeria maculans* SSPs (Rouxel et al., 2011). Recent analyses of five SSPs however, showed the complexity of functional analyses, as several top-candidates were dispensable for pathogenesis, thereby underscoring potential redundancies and questioning current effector candidate finding strategies (MirzadiGohari et al., 2015; Rudd et al., 2015).

We anticipate identifying more proteins in the future by applying more sensitive proteome analyses, particularly during the biotrophic phase where fungal proliferation is slow and biomass is low. Additionally, we will focus on proteins playing a crucial role in the lifestyle switch from biotrophy to necrotrophy where fungal proliferation is enormous (Kema

et al., 1996; Ware, 2006; Rudd and Hammond-Kosack., 2008). Obviously, absence of protein sequences in the database will preclude their identification, but lowly abundant fungal proteins could also be easily obscured by highly abundant plant proteins, such as photosynthetic enzymes and structural proteins. The use of a 6 frame translation of the *Z. tritici* genome sequence would help in part to solve this problem, as it identifies non annotated genes and/or corrects erroneous gene models.

After examining changes in the individual functional groups, we re-evaluated the LC-MS^E data by analyzing the overall response pattern of several *Z. tritici* proteins for which we had quantitative data (Fig. S1) and 16 selected proteins were additionally validated by Real-time qPCR in order to determine their expression profile at 2, 4, 8, 12, 14 and 20 dpi of the susceptible cv. Obelisk. Both proteomic and real-time qPCR data provided evidence for increased expression during infection of several proteins (Fig. 4) except for a few proteins that were either lowly expressed or varied across samples. The majority of the studied genes was strongly expressed during plant infection, mostly peaking during the necrotrophic phase corresponding to fungal proliferation and pycnidia development, thereby corroborating the proteomic results. These included xylanases, cutinase and hypothetical proteins that showed a consistent accumulation from 7 dpi to 21 dpi in the susceptible cv. Obelisk while others were highly expressed during the biotrophic phase of colonization, suggesting that these proteins may function as effectors during host colonisation.

4. Conclusions

In this study, we present a first catalogue of *Z. tritici* proteins secreted in the wheat apoplast during pathogenesis in wheat that complements recent genome analyses (Goodwin et al., 2011). The identified plethora of proteins provides a rich resource for detailed analysis of their role(s) during *Z. tritici* pathogenesis. Clearly, the apoplastic host-pathogen interface reveals mainly stress-related proteins, proteases and CWDEs, which are an important resource for effector discovery and validation. Subsequent experiments will uncover their exact function during pathogenesis.

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Supplementary material

Supplementary Table 1. QPCR primers used in this study

Supplementary Table 2. Total number of unique peptide sequences identified with LC-MS/MS and LC-MS^E in the apoplastic fluids obtained from the resistant (Shafir) and susceptible (Obelisk) wheat cvs. at four time points after inoculation with *Zymoseptoria tritici* strain IPO323 (0, 7, 14 and 21 days post-inoculation).

Supplementary Table 3. Summary of the *Zymoseptoria tritici* proteins that were identified in the apoplastic fluids isolated from the resistant (Shafir) and susceptible (Obelisk) wheat cvs. at four time points after inoculation with *Z. tritici* strain IPO323 (0, 7, 14 and 21 dpi).

Supplementary Figure 1. Quantitative analysis of differentially expressed proteins from *Zymoseptoria tritici* identified in apoplastic fluids isolated at 7, 14 and 21* (*duplicates) days post-inoculation with *Z. tritici* strain IPO323 from the resistant (Shafir) and susceptible (Obelisk) wheat cvs. The Y-axis corresponds to standardized normalized value; red lines correspond to matching peptides assigned to a particular protein or a category of proteins displayed from top to bottom; **(A)** Peptides originating from hypothetical secreted proteins corresponding to estExt_fgenes1_kg.C_chr_30051; estExt_fgenes1_pg.C_chr_130030 and fgenes1_pg.C_chr_11000104; **(B)** Peptides originating from two xylanases corresponding to gene models estExt_fgenes1_kg.C_chr_60237 and estExt_fgenes1_kg.C_chr_70317; **(C)** Peptides originating from catalase peroxidase corresponding to gene model estExt_fgenes1_pg.C_chr_11443; **(D)** Peptides originating from cutinase corresponding to gene model estExt_fgenes1_kg.C_chr_20529.

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Fig. 1. A. Symptom development of septoria tritici blotch on resistant (Shafir) and susceptible (Obelisk) wheat cultivars after inoculation with *Zymoseptoria tritici* strain IPO323. Note that leaf tissue of cv. Obelisk has become necrotic at 14 and 21 days post-inoculation which is associated with fungal proliferation and the development of increasing numbers of pycnidia (black structures on the leaves). Leaves of resistant cv. Shafir show only some slight chlorosis. **B.** SDS-PAGE gel stained with Coomassie blue showing protein profiles present in apoplastic fluids that were isolated from primary leaves of the susceptible (S; Obelisk) and resistant (R; Shafir) wheat cultivars at 0, 7, 14 and 21 days post-inoculation with *Z. tritici* strain IPO323. Molecular masses of marker proteins are indicated on the left (in kDa). **C.** Proteins identified by mass spectrometry in gel slices I-V excised from the SDS-PAGE containing proteins present in apoplastic fluids.

Fig. 2. Principal Component Analysis (PCA) based on LC-MS peak areas from all features detected in the LC-MS^E analysis from three time point samples (7, 14 and 21 days post-inoculation with *Zymoseptoria tritici* strain IPO323) from the susceptible (S; Obelisk) and resistant (R; Shafir) wheat cvs. (time point 0 dpi is not included and the samples at 21 dpi were duplicated, indicated by the asterisk). **A.** Score plot. **B.** Loading plot, just visualizing the

identified peptide features. Red spots are *Z. tritici* proteins, green spots are wheat proteins and spot size is related to the peptide identification score. Clearly, most fungal derived peptides occur in the lower left corner, related to gene expression in the susceptible cv. Obelisk at later time points. Many wheat peptides are located in the upper part of the plot, indicating that the protein composition of the AF undergoes major changes also in the resistant cv. Shafir at later time point.

Fig. 3. A. Number of *Zymoseptoria tritici* proteins identified with LC-MS/MS and LC-MS^E, present in the apoplastic fluids that were obtained from the primary leaves of the resistant (Shafir) and susceptible (Obelisk) wheat cultivars, respectively, at 0, 7, 14 and 21 days post-inoculation with *Z. tritici* strain IPO323. **B.** Gene ontology analysis based on biological processes. **C.** GO based on molecular function, of 85 *Z. tritici* proteins that were identified in apoplastic fluids. The pie charts show the percentage of identified proteins in the various functional categories (filtered by #Seq=1.0 and level 2) and do not take un-annotated proteins into account. The different categories (level 2, indicated in bold) are shown in more detail (level 3) below.

Fig.4. Real-Time qPCR analysis on 16 selected genes during fungal infection of susceptible wheat leaves (S; Obelisk) that were inoculated with *Zymoseptoria tritici* strain IPO323 and sampled during the course of infection (2, 4, 8, 12, 16 and 20 days post-inoculation). Bars and numbers indicate the relative expression levels together with the variation. The *Z. tritici* *beta-tubulin* gene was used for expression normalisation.

Table 1

Summary of the secreted *Zymoseptoria tritici* proteins that were identified in the apoplastic fluids isolated from the resistant (Shafir) and susceptible (Obelisk) wheat cvs. at four time points after inoculation with *Z. tritici* strain IPO323 (0, 7, 14 and 21 dpi)

JGI Header ^{a)}	Pep. No ^{b)}	Cov. % ^{c)}	Pep. Score ^{d)}		Mol. Func. ^{e)}	Description	Cv. & time points ^{f)}	Total aa ^{g)}
			Min Score	Max Score				
estExt_fgenes1_kg.C_chr_20529	21	74	5,4	85,5	hydrolase activity	Cutinase	S, 14 & 21dpi	224
e_gw1.6.1246.1	14	63	5,1	79,5	hydrolase activity	Cutinase	S, 21dpi	232
estExt_fgenes1_kg.C_chr_70027	10	23	7,5	80	hydrolase activity	Gpi-anchored cell wall protein; β -endoglucanase	S, 21dpi	480
estExt_fgenes1_kg.C_chr_100051	3	23	5,2	6,0	hydrolase activity	Glycoside hydrolase, family 12; endoglucanase a precursor	S, 21dpi	246
estExt_fgenes1_kg.C_chr_60237	25	73	5,1	94,3	hydrolase activity	Glycoside hydrolase, family 11; endo- β -xylanase	S, 21dpi	225
estExt_fgenes1_kg.C_chr_70317	28	61	5,2	78,1	hydrolase activity	Glycoside hydrolase, family 10; endo- β -xylanase	S, 14 & 21dpi	347
estExt_fgenes1_pg.C_chr_40433	13	28	6,3	56,6	hydrolase activity	Glycoside hydrolase, family 3; β -xylosidase	S, 21dpi	769
estExt_fgenes1_pg.C_chr_40440	2	3	5,0	5,1	hydrolase activity	Glycoside hydrolase, family 3; β -glucosidase	S, 21dpi	979
estExt_fgenes1_pg.C_chr_50362	16	40	5,9	79,3	hydrolase activity	Glycoside hydrolase, family 7 ; cellobiohydrolase d	S, 21dpi	444
estExt_fgenes1_pm.C_chr_30480	7	22	5,2	7,0	hydrolase activity	Glycoside hydrolase, family 62; α -L-	S, 14dpi	331

						arabinofuranosidase		
estExt_fgenes1_pg.C_chr_100384	7	18	6,2	70,9	hydrolase activity	Glycoside hydrolase, family 62; α -l-arabinofuranosidase	S, 21dpi	690
estExt_Genewise1.C_chr_21868	22	55	5,5	81,3	hydrolase activity	Glycoside hydrolase, family 62; α -n-arabinofuranosidase	S, 14 & 21dpi	321
estExt_fgenes1_pg.C_chr_130228	12	29	5,1	84,6	hydrolase activity	Glycoside hydrolase, family 31	S, 14 & 21dpi	991
estExt_fgenes1_kg.C_chr_80005	5	13	5,4	6,7	hydrolase activity	Glycoside hydrolase, family 43; secreted arabinase	S, 21dpi	319
estExt_Genewise1.C_chr_12866	12	52	5,0	6,8	hydrolase activity	Pectinesterase	S, 14 & 21dpi	330
fgenes1_pg.C_chr_1001469	34	31	5,0	62,6	hydrolase activity	Glycoside hydrolase, family 3	S, 14 & 21dpi R, 21dpi	1055
fgenes1_pg.C_chr_3000807	1	7	52,9	52,9	hydrolase activity	Glycoside hydrolase family, β -mannanase	S, 14dpi	363
estExt_fgenes1_kg.C_chr_110051	5	42	5,2	60,8	hydrolase activity	Lipases ; acetyl xylan esterase	S, 14 & 21dpi	301
estExt_fgenes1_pg.C_chr_70016	3	10	5,5	5,9	Peptidase activity	Peptidase A1, pepsin, aspartyl protease	S, 14dpi	441
fgenes1_pg.C_chr_4000424	2	10	5,0	5,2	Peptidase activity	Peptidase A1, pepsin, Aspartic Protease	S, 21dpi	460
estExt_fgenes1_kg.C_chr_70011	5	24	5,2	62,6	Peptidase activity	Peptidase A4, Asparatic protease, scytalidopepsin B	S, 14 & 21dpi	269
estExt_fgenes1_pg.C_chr_10314	5	19	5,3	35,5	Peptidase activity	Peptidase; aminopeptidase	S, 21dpi	552
estExt_fgenes1_pg.C_chr_50636	19	52	5,1	74,0	Peptidase activity	Peptidase S8 and S53, subtilisin, kexin, sedolisin	S, 21dpi	400
estExt_fgenes1_pg.C_chr_60048	9	27	5,1	6,2	Peptidase activity	Peptidase M14, carboxypeptidase A	S, 21dpi	426

estExt_fgenes1_pg.C_chr_110083	21	27	7,6	52,9	oxidoreductase activity	FMN-dependent dehydrogenase	S, 14 & 21dpi	504
estExt_Genewise1.C_chr_90838	1	3	55,5	55,5	oxidoreductase activity	Glucose-methanol-choline oxidoreductase	S, 21dpi	555
estExt_fgenes1_pg.C_chr_11443	39	39	7,3	71,9	peroxidase activity	Catalase peroxidase hpi	S, 14 & 21dpi	796
estExt_fgenes1_pg.C_chr_20902 [∞]	32	49	5,8	74,5	catalytic activity	No significant hits Six-hairpin glycosidase (<i>Pyrenophora tritici-repentis</i> Pt-1C-BFP) XP_001940715.1 e=0.0	S, 21dpi	667
fgenes1_pm.C_chr_4000019 ^Θ	1	7	71,4	71,4	---NA---*	Hypothetical protein FG11205.1 (<i>Gibberella zeae</i> PH-1) 2e ⁻³⁵	S, 21dpi	149
estExt_fgenes1_kg.C_chr_110087 [#]	1	10	7,6	78,1	---NA---	Unnamed protein product (<i>Podospora anserina</i> S mat+) 3e ⁻¹¹	S, 14 & 21dpi	156
estExt_fgenes1_kg.C_chr_120103 ^{**}	1	6	73,5	73,5	---NA---	Conserved hypothetical protein (<i>Verticillium albo-atrum</i> VaMs.102) 4e ⁻⁴⁵	S, 21dpi	238
estExt_fgenes1_pg.C_chr_130030 ^{**}	16	31	7,0	80,2469	---NA---	Conserved hypothetical protein (<i>Pyrenophora tritici-repentis</i> Pt-1C-BFP) 2e ⁻¹⁵³ XP_001930959.1	S, 14 & 21dpi	483
fgenes1_pg.C_chr_11000104 ^{**}	9	28	5,6	6,2	---NA---	Conserved hypothetical protein (<i>Pyrenophora tritici-repentis</i> Pt-1C-BFP) 1e ⁻¹⁷⁴	S, 21dpi	497

estExt_fgenes1_kg.C_chr_50387 ^ϕ	2	28	5,7	8,1	---NA---	Hypothetical protein FG08238.1 (<i>Gibberella</i> <i>zeae</i> PH-1) 2e ⁻²⁵	S, 21dpi	149
estExt_fgenes1_kg.C_chr_70220 [⊕]	1	7	49,3	72	---NA---	Hypothetical protein UREG_02418 (<i>Uncinocarpus reesii</i> 1704) 7e ⁻¹¹	S, 21dpi	188
						PhiA protein (<i>Emericella</i> <i>nidulans</i>) 3e ⁻¹⁰		
estExt_fgenes1_kg.C_chr_90057	14	25	5,1	82,7	---NA---	Low homology to IgE- binding protein (<i>Penicillium marneffe</i>) 3e ⁻¹⁵	S, 21dpi	198
estExt_fgenes1_pg.C_chr_40369 [#]	5	39	5,1	79,7	---NA---	No significant hits	S, 14 & 21dpi	154
estExt_fgenes1_kg.C_chr_30051 [⊗]	15	25	5,3	85,6	---NA---	Hypothetical protein SNOG_07926 (<i>Phaeosphaeria nodorum</i> SN15)	S, 14 & 21dpi	519
						stress response protein Rds1(<i>Pyrenophora tritici-</i> <i>repentis</i> Pt-1C-BFP)		
estExt_fgenes1_kg.C_chr_30295 ^ϕ	2	15	5,5	6,2	---NA---	Hypothetical protein MGCH7_ch7g1093 (<i>Magnaporthe oryzae</i> 70e ⁻¹⁵)	S, 21dpi	177
estExt_fgenes1_kg.C_chr_60114 [#]	1	7	87,3	87,3	---NA---	No significant hits	S, 14dpi	157

^{a)} JGI headers from <http://genomeportal.jgi-psf.org/Mycgr3/Mycgr3.home.html> (search across all models)

^{b)} Maximum number of identified peptides, including generated in source fragments

^{c)} Coverage percentage of amino acid sequence with the matched peptides

^{d)} Peptide score (min and max) based on both DDA and MS^E. The score range for significant matches is for MS^E ≥ 5 and for DDA ≥ 50

^{e)} GO term provided for molecular function from Blast2Go functional annotation tool

^{f)} S: Susceptible cultivar (Obelisk), R: Resistant cultivar (Shafir)

^{g)} Amino acid length

*--NA--: no GO term identified

Unique Small Secreted Protein (SSP)

** Conserved SSP

φ Lowly conserved SSP

∞ IPR000421: Possible cell adhesion protein

⊖ IPR010829 Cerato-platanin

⊕ Essential for phialid development in *Aspergillus*

⊘ Homology to stress response protein rds1

NB. Proteins identified with only one peptide have lower confidence level than those identified with more than one peptide

Figure 1

Figure 2

Figure 3

Figure 4