Running title: Molecular dynamics of pathogen reproduction on plants

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Transcriptome and metabolite profiling the infection cycle of *Zymoseptoria tritici* on wheat (*Triticum aestivum*) reveals a biphasic interaction with plant immunity involving differential pathogen chromosomal contributions, and a variation on the hemibiotrophic lifestyle definition.

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Abstract

The “hemibiotrophic” fungus Zymoseptoria tritici causes Septoria tritici blotch disease of wheat (Triticum aestivum). Pathogen reproduction on wheat occurs without cell penetration, suggesting dynamic and intimate intercellular communication occurs between fungus and plant throughout the disease cycle. We used deep RNA sequencing and metabolomics to investigate the physiology of plant and pathogen throughout an asexual reproductive cycle of Z. tritici on wheat leaves. Over 3,000 pathogen genes, >7,000 wheat genes and >300 metabolites were differentially regulated. Intriguingly, individual fungal chromosomes contributed unequally to the overall gene expression changes. Early transcriptional down regulation of putative host defense genes was detected in inoculated leaves. There was little evidence for fungal nutrient acquisition from the plant throughout symptomless colonization by Z. tritici, which may instead be utilizing lipid and fatty acid stores for growth. However the fungus then subsequently manipulated specific plant carbohydrates, including fructan metabolites, during the switch to necrotrophic growth and reproduction. This switch coincided with increased expression of jasmonic acid (JA) biosynthesis genes and large scale activation of other plant “defense” responses. Fungal genes encoding putative secondary metabolite clusters and secreted effector proteins were identified with distinct infection phase-specific expression patterns, although functional analysis suggested many have overlapping / redundant functions in virulence. The pathogenic lifestyle of Z. tritici on wheat revealed through this study, involving initial defense suppression by a slow growing extracellular and nutritionally limited pathogen, followed by defense (hyper-) activation during reproduction, reveals a subtle modification on the conceptual definition of hemibiotrophic plant infection.
Introduction

Fungi are responsible for many of the most economically damaging diseases of plants which threaten future food security (Chakraborty and Newton, 2011; Fisher et al., 2012). The largest class of plant pathogenic fungi accounting for many of these diseases are the *Dothideomycetes*; members of which are predicted to utilize diverse and unique mechanisms for host infection leading to pathogen reproduction (Ohm et al., 2012).

For plant pathogens to reproduce effectively in host tissues each invading species must overcome several layers of plant immunity. After surpassing any physical barriers pathogens must then overcome the host plants molecular surveillance system which detects pathogen- or microbial associated molecular patterns (P/MAMPs), which trigger PAMP-triggered immunity (PTI). Successful pathogens suppress PTI by secreting protein effectors which interfere with perception or defense signaling. However plants can also detect the function of these effectors using resistance proteins which monitor the function of effector targeted proteins or recognize the effector directly. This gives rise to Effector-triggered immunity (ETI) which is multifaceted and strong, frequently involving localized plant cell death (Jones and Dangl, 2006). These inducible defenses are also associated with wide ranging transcriptional and hormonal reprogramming in the plant (Hammond-Kosack and Jones, 1996). This mechanism is particularly effective at preventing proliferation and reproduction of biotrophic pathogens (those which live and feed in association with living cells) in plant tissues.

Fungi from the genus *Mycosphaerella*, the largest genus of plant pathogenic fungi, belong to the *Dothideomycete* class. These fungi typically have long periods of symptomless intercellular (extracellular with respect to host cells) growth in infected plants (frequently described as “biotrophy”) followed by a rapid switch to “necrorophy” associated with host tissue death. Almost all *Mycosphaerella* fungi reproduce asexually (sporulate) in plant tissues undergoing this cell death, to initiate polycyclic diseases. Plant pathogenic *Mycosphaerella* species are thus often referred to as “hemibiotrophic” due to having long periods of initial colonization without stimulating host cell death where some form of biotrophic interaction is proposed (Luttrell, 1974). This initial asymptomatic phase is subsequently followed by host cell death associated with rapid fungal growth and subsequent reproduction (sporulation), a feature more commonly associated with successful colonization by necrotrophic pathogens.

Currently, one of the best studied *Mycosphaerella* fungi is *Zymoseptoria tritici* (also known as *Mycosphaerella graminicola* or *Septoria tritici*), which is exclusively pathogenic towards leaves of wheat (*Triticum aestivum*), causing Septoria tritici blotch (STB) disease. Typical of most *Mycosphaerella* plant pathogens, *Z. tritici* enters leaves via their natural openings, stomata, and completes its full asexual reproductive cycle without physically penetrating host cells (Kema et al., 1996; Pnini-Cohen et al., 2000; Deller et al., 2011; Dean et al., 2012). This strictly intercellular (or apoplastic) mode of colonization and reproduction differentiates *Z. tritici* from almost all other well studied plant pathogenic fungi with available genomic resources, including all species of *Magnaporthe*, *Fusarium*, *Ustilago*, *Colletotrichum*, *Blumeria* and *Puccinia*, all of which have at least one period of intracellular (invasive) growth within plant cells during infection (Brown and Hammond-Kosack, 2014). This lifestyle suggests that communication between the *Z. tritici* hyphae and the neighboring wheat cells must rely heavily on the exchange of secreted or cell surface localized molecules, but raises questions as to the exact nature of the dialogue throughout infection.

A single asexual reproductive cycle of *Z. tritici* on wheat leaves lasts at least two-three weeks from spores alighting on the leaf surface. During the early phase of colonization (lasting 7 days or more depending on the particular isolate-cultivar combination), the fungus grows extremely slowly in-between the mesophyll cell layer of the leaf. This growth is so slow that it has often proven difficult / impossible to detect significant increases in fungal biomass in infected tissue during this period (Kema et al., 1996; Pnini-Cohen et al., 2000; Keon et al., 2007). It has been recently demonstrated that
the function of a secreted LysM domain containing \textit{Z. tritici} effector protein, Mg3LysM, plays an important role in establishing this initial symptomless colonization through suppression of chitin (PAMP) recognition by the wheat chitin receptor proteins TaCEBiP and TaCERK1 (Marshall et al., 2011; Lee et al., 2014). However at some point after 7-10 days post infection, and for reasons that are currently unclear, wheat cells then suddenly begin to die. This response is strictly localized to the inoculated leaf area and shows many biochemical features of programmed cell death (PCD) (Keon et al., 2007; Rudd et al., 2008). The onset of host cell death coincides with a sharp increase in fungal growth rate and biomass in infected leaves. This may be supported by the increased apoplastic nutrient availability as a consequence of loss of host cell membrane integrity (Keon et al., 2007). \textit{Z. tritici} then ultimately enters its reproductive mode in the dying leaf tissue, forming masses of asexual spores, produced by pycnidia formed in sub-stomatal cavities. During high humidity and rainfall these spores are extruded through the stomatal aperture of dead leaf areas and are propagated via rain splash to establish further cycles of infection.

The fully sequenced 38Mb genome of the reference isolate of \textit{Z. tritici}, IPO323, comprises approximately 11,000 predicted genes spanning 21 chromosomes (Wittenberg et al., 2009; Goodwin et al., 2011). The eight smallest chromosomes harboring 646 genes have been shown to be conditionally dispensable for plant infection and are now referred to as either “dispensable” or “accessory” chromosomes. It is currently unknown what role these chromosomes have in the lifestyle of this fungus, although it has been suggested that they may function to facilitate more rapid evolutionary processes (Croll and McDonald, 2012; Croll et al., 2013). A notable feature of the overall gene composition of \textit{Z. tritici} is the unusually low numbers of genes encoding putative plant cell wall attacking proteins, perhaps as a consequence of its strictly extracellular mode of colonization. The \textit{Z. tritici} genome does however encode a large number of predicted secreted proteases and other classes of secreted proteins including many putative cysteine–rich effector candidates (Goodwin et al., 2011; do Amaral et al., 2012). In addition, despite the fungus triggering infection phase specific host cell death, no toxin (protein or metabolite) has thus far been described.

In contrast to \textit{Z. tritici}, the extremely large and repeat-rich genome sequence of hexaploid wheat is still being finalized (Eversole et al., 2014). However large numbers of EST’s, in addition to various individually sequenced chromosomes, are available to support molecular analyses (Wise et al., 2007; Eversole et al., 2014). In addition a limited number of studies have begun to address metabolomic responses of wheat towards pathogens, and / or pathogen effectors (Du Fall and Solomon, 2013). Many conserved plant responses to pathogens including the production of the phytohormones jasmonic and salicylic acid (JA and SA, respectively) can also be measured in wheat. Whilst various exceptions now exist, SA is often considered to trigger defenses effective against biotrophic pathogens involving localized hypersensitive cell death and the production of pathogenesis–related (PR) proteins, whilst JA triggers defenses more usually effective against necrotrophic pathogens (Hammond-Kosack and Parker, 2003; Glazebrook, 2005). Other conserved characteristic transcriptional defense responses of plants include activation of secondary metabolite pathways, such as the phenylpropanoid pathway, which can serve to strengthen plant cell walls via lignification thus preventing extensive cell wall hydrolysis induced by invading pathogens (Sticher et al., 1997). Monocotyledonous cereal plants also have various unique metabolite pathways which are transcriptionally activated during abiotic stresses. For wheat one particular example is the biosynthesis of fructans, which can serve as mobile carbon sources and have been implicated in plant tolerance of cold, drought and oxidative stresses (Hare et al., 1998; Gaudet et al., 1999; Valluru and Van den Ende, 2008; Van den Ende and Valluru, 2009; Keunen et al., 2013). However, to date no metabolomics analyses have been reported on the interaction of wheat with \textit{Z. tritici}.

RNA sequencing (RNAseq) technology has become more frequently used to profile fungal pathogen gene expression at particular phases of plant infection (de Jonge et al., 2012; Kleemann et al., 2012; O’Connell et al., 2012; Soanes et al., 2012; Cantu et al., 2013; Garnica et al., 2013; Hacquard et al., 2013; Meinhardt et al., 2014; Zhang et al., 2014). However
comparatively few studies (Kawahara et al., 2012; Yang et al., 2013; Yazawa et al., 2013) have simultaneously analyzed the responses of both pathogen and host, and very few have undertaken such an analysis throughout a complete pathogen reproductive cycle. One of the current most comprehensive studies of this type was performed, using a composite microarray, on the hemibiotrophic “fungal-like” filamentous oomycete Phytophthora capsici infecting tomato (a dicotyledonous plant), where many infection-phase specific transcriptional programs were identified for both pathogen and plant (Jupe et al., 2013). However, unlike the true fungus Z. tritici, P. capsici has a relatively short reproductive cycle on tomato and the initial “biotrophic” phase involves the generation of specific haustorial-like structures inside living plant cells. As representative members of two distinct taxonomic kingdoms it is probable that the suggested hemibiotrophic infection strategies differ for Z. tritici and P. capsici.

The unique temporal features of the Z. tritici - wheat interaction, and the available genomic and EST resources, make this an attractive model to investigate infection-phase specific transcriptional and physiological changes, where clearly defined periods can be relatively easily separated (Keon et al., 2007; Shetty et al., 2007; Kema et al., 2008). Three previous reports have used next generation RNAseq to study aspects of this interaction (Brunner et al., 2013; Yang et al., 2013; Kellner et al., 2014). Brunner et al. (2013), studied the expression of putative plant cell wall attacking enzymes (PCWDE) at three phases of infection by an isolate ST99CH3D7 of Z. tritici (not the publically available reference isolate IPO323) and demonstrated that the majority were strongly expressed during the necrotrophic phase. Yang et al. (2013) investigated both pathogen and host at two time points during the initial symptomless interaction (4- and 10-dpi) and at the onset of first symptoms (the transitional phase) which occurred at 13-dpi in these experiments. This study confirmed many previously published fungal transcriptional responses deriving from EST analysis, microarray and qRT-PCR based work (Keon et al., 2007; Kema et al., 2008; Rudd et al., 2010), and indicated that host defense responses may become activated at the disease transition. However, this analysis was somewhat limited by a number of features including (1) low overall depth of sequencing (< 13 million reads were generated per sample) leading to (2) very low coverage of fungal transcripts (<10% of the total predicted fungal genes had any RNAseq support), (3) the lack of analysis of fungus growing away from the plant (i.e. in culture) precluding the identification of fungal genes and processes up-regulated specifically during infection, (4) the absence of samples representing very early phases of the interaction, and (5) the lack of analysis of later phase of advanced necrotrophic colonization and asexual pathogen reproduction. Finally Kellner et al. (2014) described analysis of the Z. tritici transcriptome in axenic culture and also at a single time point of plant infection (during symptomless growth at 4-dpi) of wheat and a non-host plant. A notable observation from this work was that genes residing on the eight smallest accessory chromosomes were in general relatively low expressed (although differential expression was reported for some) compared with those on the larger “core” chromosomes 1-13. However, the sampling of only a single in planta time point made it impossible to determine whether this was a feature conserved throughout infection, and also prohibited the study of the dynamics of overall pathogen gene expression during the infection time course. There was no comparable analysis of the host transcriptome in this study.

The work presented here aimed to address the question of how the transcriptome and metabolome of Z. tritici and wheat leaves respond throughout clearly defined and distinct phases of infection, culminating in successful pathogen reproduction. The depth of RNAseq we achieved enabled us to report expression changes for >80% of all the current predicted fungal genes throughout infection. Analysis of two in vitro fungal culture conditions and corresponding mock inoculated wheat leaves formed effective baselines for simultaneous transcriptional and metabolite profiling the dynamics of the host-pathogen interaction. The large and comprehensive datasets we describe indicate that the early biotrophic phase of pathogen colonization does involve plant defense suppression but does not involve effective nutrient acquisition. Rapid fungal proliferation and asexual reproduction subsequently occurs in an environment with both transcriptional and hormonal features of an intense plant “defense” response. The data overall suggests a model
for initial pathogen subterfuge followed by extreme activation of plant immune responses occurring to facilitate reproduction in infected tissues. Moreover, different *Z. tritici* chromosomes, and chromosomal regions, appear to contribute differentially to this mechanism which offers an alternative and intriguing view on “hemibiotrophy” for plant pathogens that never physically penetrate host cells.

**Results and discussion**

**Identification and classification of differentially abundant *Z. tritici* and wheat leaf metabolites**

For our combined RNAseq and metabolite experimental pipeline (Supplementary Figure 1) we used two fungal culture media and five time points of plant infection. We chose the culture medium Czapek-Dox (CDB) as a defined medium which presents carbon as sucrose and nitrogen as nitrate only, whilst a second medium, PDB presents a rich and complex variety of carbon and nitrogen sources. Both media support fungal growth via a form of “yeast-like budding”, which occurs ~3-4 times faster in PDB than CDB (Keon et al., 2005). Five time points of leaf infection were selected to span key phases of the host – pathogen interaction (Figure 1). These included; 1 day (24h) post infection (1-dpi) where fungal spores had just germinated on the leaf surface; 4-dpi which represents early post-stomatal penetration and slow symptomless intercellular growth inside leaves; 9-dpi representing the “transition” phase associated with the onset of more rapid fungal intercellular growth and the first appearance of macroscopic disease symptoms on leaves; 14-dpi representing accelerating plant cell death with rapid fungal necrotrophic growth and the onset of sporulation. Finally we sampled tissue at 21-dpi when leaf material is fully defeated (having a dry necrotic appearance) and mature sporulation structures (pycnidia) are present containing abundant asexual spores.

A non-targeted metabolite profile analysis was performed on fungal growth in two culture media and mock and infected wheat leaves at every time point post inoculation, conducted utilizing a combination of three independent platforms: UHLC/MS/MS2 optimized for basic species, UHLC/MS/MS2 optimized for acidic species, and GC/MS. This led to the detection and quantification of 309 differentially abundant metabolites (Supplementary table 1). Thirty eight compounds were identified only as present in fungal cultures and were not detected at all in either mock treated or infected wheat leaves. Ten of these (over one quarter) were implicated in fatty acid and/or sterol metabolism (Supplementary table 2) highlighting that these are prevalent processes occurring in *Z. tritici* spores. We identified 56 metabolites which were found only in leaves (mock or inoculated) and not detected in cultured fungus (Supplementary table 2). These included many known plant-specific metabolites including sucrose, shikimate, salicylic acid and fructans, the latter of which function in abiotic stress responses and also as dynamic carbon stores in wheat (Hare et al., 1998; Gaudet et al., 1999; Valluru and Van den Ende, 2008; Van den Ende and Valluru, 2009). All remaining metabolites were detected in both fungal culture and plant (mock and inoculated) samples. However there were significant variations in the abundance of members of this group of metabolites between fungal culture and mock inoculated leaves. For example six metabolites were detected in at least 100-fold higher levels in fungal spores than untreated leaves (see mean abundance data present in Supplementary table 1). This group of metabolites included various sugar alcohols (arabitol, mannitol) and also stachydrine (proline betaine) and trehalose (Table 1).

**Accumulation of abundant fungal metabolites in infected leaves and RNAseq read mapping to the fungal genome chart the dynamics of disease progression**

RNAseq reads from each sample of fungal culture and the time course of leaf infection were then aligned to the fully sequenced *Z. tritici* genome template and the filtered (see methods) wheat NCBI Unigene build #60 set. A minimum of 80% of reads originating from fungal cultured material aligned to the fungal genome template (Table 2). Conversely very
small percentages of the total number of reads aligned to the fungal genome at both 1-dpi and 4-dpi (<4%), again highlighting the very low levels of quantifiable biomass during this period of leaf colonization. By 9-dpi, at the onset of first symptoms, an increased proportion of reads mapped to the fungal genome (<7%). Subsequently by 14-dpi the number of reads mapping increased significantly (<60%) and by 21-dpi the mapping obtained was comparable to the fungal cultured samples (~80%) indicating that the host RNA had been almost completely destroyed by this point (Keon et al., 2007). Particular subsets of metabolites showed a similar pattern of accumulation throughout infection, matching closely the overall RNAseq read mapping to the fungal genome. These metabolites included those which had been only detected in fungal cultures as well as those which we had determined to be >100 fold more abundant in fungal culture than in untreated leaves, including arabitol, mannitol, trehalose and stachydrine (Figure 2). These data suggest that quantities of these metabolites might accurately reflect the disease progression of *Z. tritici* infected leaves, and confirmed that they accumulate to high levels in the asexual spores of this species. These probably represent important metabolites in *Z. tritici*, as has been demonstrated for other fungal pathogens of wheat (Solomon et al., 2006; Lowe et al., 2009).

Specific whole *Z. tritici* chromosomes, and regions of individual chromosomes, contribute differentially to overall gene expression throughout the course of infection and asexual reproduction

In contrast to a previous study (Yang et al., 2013) the increased depth of our sequencing allowed us to detect significant expression of fungal genes in all samples and time points of infection, despite differing overall read mapping percentages. In summary, of the 11,017 predicted genes in the *Z. tritici* genome sequence, the following numbers were detected as expressed in each sample; CDB- 10,541; PDB- 10,437; Day 1- 9,740; Day4- 9,236; Day 9-10,041; Day14-10,464; Day21- 10,485 (Supplementary table 3). Therefore we were able to detect expression of at least 80% of all predicted fungal genes in all samples.

Prior to performing differential gene expression analyses between samples, we first calculated a mean value for relative expression (Fragments Per Kilobase Of Exon Per Million Fragments Mapped-FPKM) of all genes whose expression was detected and displayed them graphically across the 21 *Z. tritici* chromosomes using the OmniMapFree software (Antoniw et al., 2011). Figure 3A upper panel displays the physical distribution of all 11,017 currently predicted genes across the 21 chromosomes. We then sorted all genes on mean FPKM values (Supplementary table 3) and then subsequently displayed those with very low (mean FPKM <1.0) or no (FPKM=0) expression throughout the replicated experiments (Figure 3A lower panel). In total 260 currently predicted genes gave FPKM values of 0 in all samples, indicating that their expression level was below the limit of detection throughout this experiment. The majority of these genes lay in close physical proximity on chromosome 7, between nucleotides 1676643-2552085. PCR on genomic DNA spanning 3 independent intervals confirmed that the region was present in the experimental isolate (Figure 3A upper lower panel inset). This region currently contains 139 predicted genes and was also seen to have low overall expression at 4-dpi in an independent study (Kellner et al., 2014). The lack of expression support from this region across all replicated plant infections, suggests that high level expression of these genes is not essential for any phase of colonization or asexual reproduction by *Z. tritici*.

Genome-wide analysis also indicated that many genes present on the eight smallest “dispensable” or “accessory” chromosomes 14-21 also have low expression relative to those present on the first 13 “core” chromosomes. The calculated mean FPKM value / gene for each chromosome re-enforced this observation, highlighting that a gene present on any of the dispensable chromosomes was on average expressed 4 to 5-fold lower than an equivalent gene from the core chromosomes (Figure 3B). This observation was true for all samples at every time point (Supplementary table 3) thus confirming that the low expression previously reported for growth in one axenic culture and one time point of
infection (Kellner et al., 2014) is in fact seen throughout the entire asexual infection cycle (as sampled here) and in at least two additional culture media. Overall this suggests that expression of genes residing on the accessory chromosomes is significantly weaker at all phases of the pathogens asexual lifecycle in host tissue.

The most significant transcriptional reprogramming detected in Z. tritici occurs in response to the plant and involves genes primarily residing on core chromosomes

We next calculated the number of differentially expressed fungal genes between samples. The raw CuffDiff2 differential expression output was subjected to further filtering to increase the stringency of the analysis (see methods for details). We analyzed all differential gene expression changes relative to the levels found in the defined culture medium, CDB (Table 3). This demonstrated that the least number of differentially expressed genes (DEG) was between CDB and the other culture medium PDB (607). In contrast the highest number was between CDB and 9-dpi (1389). Of note were over 1,000 fungal genes differentially expressed within 1 day of alighting on the leaf surface; the majority being up-regulated. This indicated that major transcriptional re-programming takes place soon after contact with the host plant as has recently been suggested for the biotrophic fungal pathogen of corn Ustilago maydis (Lanver et al., 2014). This is reinforced by the fact we were able to identify >200 fungal genes which demonstrated a peak in expression levels as early as 1-dpi (Table 3).

In total 3046 genes exhibited significant differential expression across the experiment, representing 28% of the total genes predicted in the sequenced genome (Supplementary table 4). The majority of these genes located on the core chromosomes 1-13 with 2,955 genes showing differential expression (28% of all genes on chromosomes 1-13). In contrast only 79 differentially expressed genes were present on the accessory chromosomes 14-21, which represents 12% of the genes residing on these chromosomes. Taken together with the low overall relative expression of genes on chromosomes 14-21 (Figure 3), this would indicate that the fungus invests less transcribing sequences from the accessory chromosomes than from the core chromosomes at all phases of colonization and asexual reproduction.

Combined RNAseq and metabolite analysis of Z. tritici growth in axenic cultures and in planta suggests limited hexose and nitrate assimilation during early leaf infection.

In order to test correlations between changes in metabolite levels and changes in expression of genes, we performed an initial study focusing solely on in vitro growth by the fungus in CDB and PDB broths. To re-iterate, growth in both broths is morphologically indistinguishable however growth occurs approximately 4-5 times faster in PDB. CDB presents only sucrose as sole carbon source and nitrate as a sole nitrogen source. Materials were collected during log-phase growth from each broth.

The levels of almost all detected amino acids were significantly lower in cells grown in CDB than in PDB (Figure 4A) with the exception of glutamate and glutamine. This probably reflects slower fungal growth as a consequence of a single nitrogen source (NO$_3^-$). Coupled with this observation, two predicted plasma membrane transporters of the putative nitrate transporting nrt2-type (Galvan and Fernandez, 2001) were strongly transcriptionally up-regulated in CDB relative to PDB. This was paralleled by up-regulation of the Z. tritici nitrate reductase homologue (Figure 4B). Fungal growth on sucrose requires the activity of secreted invertase enzymes to generate extracellular glucose and fructose for uptake. One secreted invertase homologue was strongly up-regulated in CDB vs PDB as were three putative transmembrane hexose transporters (Figure 4B). The uptake of large amounts of hexoses derived from sucrose gave rise to large accumulations of both Gluc-6-P and its isomer Fruc-6-P in CDB grown cells (Figure 4B). In addition growth in CDB culture gave rise to increased levels of trehalose and mannitol in spores. A pathway to trehalose synthesis from Gluc-6-P was
also supported by increased UDP-glucose suggesting that this sugar may act as a storage compound for carbon in situations where nitrogen is limiting. The accumulation of high levels of Gluc-6-P and Fruc-6-P in CDB grown cells potentially arise due to a limitation on nitrogen to fuel downstream biosynthetic processes including amino acid biosynthesis. Perhaps most significantly, the putative transporters of nitrate and hexose which were highly expressed in CDB were strongly down-regulated during most phases of plant infection (Figure 4C). This probably indicates that more diverse or alternative nutrient sources are being utilized during infection, suggesting that simple hexose and nitrate uptake are not major extracellular nutrient sources, particularly not during the early symptomless phase of colonization (Figure 4C).

Transcriptome and metabolome analysis of symptomless colonization from 1-4 dpi

For Z. tritici

In order to begin to understand the dynamics of fungal transcriptional changes during infection we performed some global hierarchical clustering (K-means clustering) on expression. We separated all the genes with statistically significant differential expression into 12 clusters (clusters 1-12 displayed in Table 4). We then analyzed the composition of each cluster for the total numbers of genes, numbers of secreted proteins and numbers of different functional classes (represented by InterPro annotations- IPR).

K-means clusters 2, 5, 7, 10 and 11 (Table 4) all contained genes significantly up-regulated by 1-dpi relative to levels in CDB culture. The genes contained in each of these clusters are listed in Supplementary table 4. With respect to physiological processes up-regulated at 1-dpi the gene composition of clusters 5 and 7 were notable. The IPR annotations “Acyl-CoA dehydrogenase”; “AMP-dep synthetase / Ligase” and “Enoyl CoA hydratase” were numerically more prevalent among the total and peak up-regulated genes at 1-dpi. These functional groups are predominantly involved in the beta oxidation of fatty acids and lipids, which we also identified to be abundant in Z. tritici spores (Supplementary table 2). Two genes encoding proteins with IPR annotation “secretory lipase” were also represented in cluster 5 and 7 (Mycgr3G52069 and Mycgr3G43288). All genes in these clusters subsequently decrease in expression, particularly at 9- and 14-dpi. Inspection of the entire composition of genes in cluster 5 and 7 revealed that fatty acid generation from lipids, and their subsequent transport and beta oxidation, were prevalent processes occurring during early infection at 1- and 4-dpi (Supplementary table 4). Several predicted secreted cutinases also displayed strong up-regulation during this early period suggesting some degree of attach on host lipids. Moreover two key enzymes of the glyoxylate cycle, malate synthase (Mycgr3G70677) and isocitrate lyase (Mycgr3G83726) were present in expression cluster 5 with peak expression at 1- and 4-dpi, indicating the use of acetate, derived from fatty acid beta oxidation, is providing carbon skeletons for anabolic processes in Z. tritici during this early phase of infection.

An overall scheme, based on expression and K-cluster analysis, for the use of lipids as an initial energy source feeding the glyoxylate cycle and supporting the slow initial colonization of wheat leaves by Z. tritici is displayed in Figure 5. The importance of fatty acid beta-oxidation and the glyoxylate pathway has been functionally demonstrated via gene deletion analyses in a number of other plant and animal infecting fungi, including Leptosphaeria maculans (Idnurm and Howlett, 2002), Magnaporthe oryzae (Wang et al., 2003; Wang et al., 2007), Phaeosphaeria nodorum (Solomon et al., 2004) and Candida albicans (Lorenz and Fink, 2001). In fact it has been suggested that leaf surfaces represent a nutritionally poor environment for fungal pathogens (Tucker and Talbot, 2001), indicating that the use of stored energy sources is important to facilitate early differentiation which ultimately supports colonization. The fact that these transcriptional responses persist for at least 4 days post inoculation, and that during this period fungal growth is slow
and biomass low, suggests that \textit{Z. tritici} is not able to significantly re-program host physiology to gain additional nutrients during this period, suggesting that, in nutritional terms at least, it is a relatively poor biotroph.

We have previously characterized \textit{in silico}, the predicted protein secretome of \textit{Z. tritici} (do Amaral et al., 2012). From a total of 970 genes encoding predicted secreted proteins, 513 (>50\%) showed differential expression in at least one time point or condition within this experiment (Supplementary table 5). Three hundred and sixty six of these showed increased expression during at least one time point of leaf infection. The largest number of up-regulated genes of this type was detected at 9-dpi (235 genes) which fall within \textit{K}-clusters 10 and 11 (Table 4), with the least detected at 21-dpi (122). However, 155 genes encoding putative secreted proteins were up-regulated as early as 1-dpi (Supplementary table 5) again suggesting an early up-regulated protein secretion response on the host. The number of genes encoding predicted secreted proteins showing increased expression across multiple time-points of infection is shown in Figure 6. Only 39 of the 366 were up-regulated at every time point of infection (from 1-21-dpi Supplementary table 5) indicating that the majority are produced at distinct phases of the interaction. The largest overlap in identity of up-regulated genes occurred between 9- and 14-dpi (152 of the 366); followed by 4- and 9-dpi (140) then 1- and 4-dpi (124). Also of note are 49 genes up-regulated at 1-, 4- and 9-dpi suggesting an early and sustained up-regulation of gene expression throughout early infection for this group (Figure 6).

Recent genome and secretome comparative analysis involving >80 fungal species identified a family of secreted chloroperoxidases (possessing PFAM PF01328) which is expanded in number, in all analyzed \textit{Mycosphaerella} fungi (do Amaral et al., 2012). The predicted secretome of \textit{Z. tritici} contains 19 members of this family. These enzymes have been suggested to be involved in the synthesis of halogenated compounds including, for example, particular antibiotics (Hofrichter and Ullrich, 2006). In addition, chloroperoxidases can also potentially act upon harmful reactive oxygen species (\( \text{H}_2\text{O}_2 \)) which may be generated during plant resistance responses to pathogens (Wojtaszek, 1997; Apel and Hirt, 2004). Nine of the putative secreted fungal chloroperoxidases exhibited differential expression within this experiment. Eight of these fell into \textit{K}-cluster 7 (Table 4 and Supplementary Table 5) and displayed up-regulation by 1-dpi with peak up-regulation at 4-dpi, during the post-penetrative symptomless phase of leaf colonization. This indicates that this expanded family of genes may have functions important for the initial symptomless intercellular growth phase of \textit{Z. tritici}.

\textbf{For Wheat}

The most notable aspect of the wheat response to early intercellular colonization by \textit{Z. tritici} was down-regulation of specific gene classes characteristically associated with plant defense responses. At 1-dpi, whilst 101 unigenes out of 334 that were significantly down-regulated had no functional annotation, of the remaining 233 more than 60\% had predicted roles in plant defense responses (Table 5 and Supplementary table 6). It is likely that this early down-regulation of defense associated processes is a consequence of the initial fungal-plant recognition and communication. For example at 1-dpi down-regulation of a number of wheat genes encoding different pathogenesis-related (PR) protein classes including PR1, glucan endo-1,3-beta-glucosidases (PR2), basic chitinases (PR3), chitin-binding wheat win proteins (PR4) and thaumatin/osmotin-like proteins (PR5) was observed (Supplementary table 6).

In addition to the typical PR genes, we also observed down-regulation of other gene classes that have been linked with roles in activating plant defenses towards pathogens. This includes genes encoding WRKY transcription factors, subtilases (predicted plant equivalents of animal PCD-inducing caspases), and signaling receptor kinases of different classes such as leucine-rich repeat receptor-like kinases (RLKs), wall-associated Ser/Thr kinases (WAKs), cysteine-rich RLKs and lectin receptor kinases (LecRKs) (Supplementary table 6).
Oxylipins (e.g. 12-oxo-phytodienoic acid, OPDA and jasmonic acid, JA) are signaling molecules derived from α-linolenic acid that play multiple functions in plants including defense to pests and pathogens (Dong, 1998; Kunkel and Brooks, 2002; Bari and Jones, 2009). This includes C18 members e.g. 12-oxo-phytodienoic acid (OPDA) as well as the C12 molecules jasmonic acid (JA) and methyl jasmonate (MeJA). Peroxosomal OPDA reductase is a critical checkpoint in octadecanoid biosynthesis pathway as it controls metabolite flow from the C18- to the C12-group of compounds (Figure 7A). It is represented by a small gene family \((n = 3-10)\) in every plant species analyzed to date. Five wheat unigenes annotated as OPDA reductase were found to be significantly down-regulated at 1-dpi (Figure 7B and Supplementary table 6).

Lignin synthesis is an important plant defense mechanism against pathogens often induced at the site of pathogen attack. This response is most commonly effective during interactions with pathogens which seek to breach the plant cell wall. Cinnamyl alcohol dehydrogenase (CAD) is a key enzyme in lignin biosynthesis catalyzing the final step in the synthesis of monolignols (Figure 8A). CAD deficiency in grasses is known to result in alterations in lignin structure and its overall content (Saathoff et al., 2011; Fornale et al., 2012). Interestingly, three wheat unigenes Ta.24122, Ta.62207, and Ta.70656 annotated as CAD, were down-regulated at 1-dpi in addition to one unigene (Ta.9712) corresponding to caffeic acid 3-O-methyltransferase (COMT), another important lignin biosynthetic enzyme (Supplementary table 9, Figure 8B).

Fluctuations in the intracellular calcium ion concentration \([\text{Ca}^{2+}]_\text{i}\), represents a key second messenger pathway during plant immune response to pathogens (Nurnberger and Scheel, 2001; Rudd and Franklin-Tong, 2001), and calmodulin (CaM) and calcium-transporting ATPase proteins are important sensors and mediators of Ca2+-dependent signals. In total we also detected eleven unigenes belonging to this class which were significantly down-regulated at 1-dpi (Supplementary table 6).

Finally, at 1-dpi we also found coordinated down-regulation of genes encoding a multitude of proteins involved in potential detoxification of xenobiotics. This included several members of the glutathione transferase family (GST) \((n = 19)\), cytochrome P450’s (CYP450) \((n = 13)\), ABC transporter / multidrug resistance-associated protein family \((n = 9)\), and UDP glucosyl / glucuronol transferases (UGT) \((n = 5)\) (Supplementary table 6).

Notably all the genes discussed above were subsequently up-regulated at 9-dpi (or 9- and 14-dpi) when \(Z.\ tri tici\) transitions from asymptomatic to necrotic growth (described later). By contrast at 4-dpi only a very small number of wheat genes \((n = 87)\) showed significant change in expression compared to the corresponding mock-inoculated control plants (Table 5) suggesting that the plant cells have only limited “perception” of, and response to, the fungus. One possible explanation for this may be that by 4-dpi \(Z.\ tri tici\) has effectively deployed the range of effector proteins which were transcriptionally activated as early as 1-dpi (Supplementary table 5). It is conceivable that these may now be suppressing further recognition by the plant surveillance machinery and / or blocking defense signaling. In support of this the functionally characterized chitin-binding effector protein Mg3LysM (Marshall et al., 2011) was already significantly up-regulated at both 1- and 4-dpi (Supplementary tables 3 and 4).

**Overall summary for the symptomless phase**

Our experimental approach using fungal culture as an expression baseline identified over 1,000 fungal genes which were already differentially expressed within 24h of spores alighting on the plant surface, suggesting rapid adaptation to this environment. The most notable biological processes up-regulated at 1-dpi and also still at 4-dpi in \(Z.\ tri tici\) involves the metabolism of lipids and fatty acids, suggesting that this, as opposed to the uptake of external carbon sources, may support the early phase of colonization. For \(Z.\ tri tici\) to still require these pathways as long as 4 days post infection of the plant suggests that it remains in a nutrient limiting environment within the wheat leaf, which may be (at least in part)
responsible for the limited increase in biomass seen during symptomless colonization. Fungal secreted proteins are also prevalent amongst the up-regulated genes during this period including previously characterized LysM effectors which function to suppress PTI (Marshall et al., 2011; Lee et al., 2014). Consequently, at 1-dpi, the plant transcriptome analysis identified many defense-associated processes to be suppressed, possibly as a result of the functions of putative fungal secreted protein effectors. This suppression involved PR proteins, putative JA and Ca\(^{2+}\) signaling responses and cell wall strengthening. The enlarged gene family of secreted \textit{Z. tritici} chloroperoxidases was rapidly up-regulated by the fungus during this period, suggesting that it had also minimized the risk of exposure to oxidative stress in the plant environment. The overall scheme suggests a form of pathogen subterfuge with minimal utilization of host derived nutrients during this initial phase of colonization.

**Transcriptome and metabolome analysis of the transition to disease symptoms at 9-dpi**

*For \textit{Z. tritici}*

Nine days after inoculation the first symptoms of disease became visible (Figure 1), and the overall RNAseq read mapping to the fungal genomic template began to increase (Figure 2 and Table 2). The fungus was no longer up-regulating genes implicated in lipid metabolism and catabolism (Figure 5 and Supplementary Table 4), presumably as a consequence of increased availability of extracellular (plant-derived) nutrients.

The genome sequencing of \textit{Z. tritici} isolate IPO323, has previously predicted that it contains a large number of proteases (Goodwin et al., 2011), suggesting that proteolysis may be providing some essential nutrients at some point during infection. \(K\)-clusters 10 and 11 (Table 4) contain genes with peak expression at 9-dpi. The IPR classification “protease” is the most prevalent annotation in cluster 10, and third most prevalent in cluster 11, respectively. In total 60 genes with IPR annotation “protease” were present in the entire differentially expressed dataset (Supplementary table 4). Twenty nine of these fell into \(K\)-clusters 10 and 11. Notably, seventeen of these are predicted to be secreted (Supplementary table 5) suggesting that at 9-dpi, at the transition to disease symptoms, the fungus was producing its maximal secreted protease response. This may be in order to either attack host cells directly, or to begin hydrolysis of host proteins being released from dying cells.

Very high level of expression of many secreted proteins was a general and notable feature of the transition phase of infection. This was evident by simply sorting genes on transcript abundance from highest to lowest FPKM (Supplementary table 3), then counting the number of predicted secreted proteins in the top 50 most abundant transcripts. Figure 9 highlights that at 9-dpi almost 50% of all transcripts in this top abundance category encoded secreted proteins, of which 35% have no functional annotation. The two functionally characterized LysM domain effector proteins Mg3LysM and Mg1LysM lie in 12\(^{th}\) and 27\(^{th}\) place in the top 50 genes sorted on transcript abundance at 9-dpi (Supplementary table 3 and Supplementary table 7). Of the functionally unknown secreted proteins in this list are 13 genes encoding proteins with more than 5% cysteine, indicating they are cysteine-rich, a hallmark of apoplastic fungal effector proteins (de Wit et al., 2009). In total 68 genes encoding functionally un-annotated cysteine-rich effector proteins were differentially expressed within this study (Supplementary table 8 and Figure 9B), with the majority falling into \(K\)-clusters 10 and 11 shown in Table 4.

Five genes encoding candidate cysteine-rich effectors were selected for functional analysis through generating gene deletion/ disruption strains for each and testing for altered virulence on susceptible wheat leaves. All five genes were strongly up-regulated at 9-dpi and with high overall relative expression (highlighted by the light grey shading in Supplementary table 7). The genes selected all encoded mature proteins of between 47 and 127 amino acid residues containing between 6 and 10 cysteine residues. Supplementary Figure 2A displays the amino acid sequences of each
proteins along with the gene deletion strategy and demonstrates that many independent deletion strains were obtained for each gene targeted. However none of the mutant strains showed any detectable defect in virulence towards the susceptible wheat cultivar (Supplementary Figure 2B), indicating that a high level of genetic redundancy between these in planta expressed Z. tritici secreted protein effectors may exist. A similar problem in reverse genetics based functional studies of effectors has been encountered in other plant pathosystems, for example in Magnaporthe oryzae 78 different secreted fungal effector gene deletion strains were generated with only one (MC69) impacting upon virulence (Saitoh et al., 2012). That this level of redundancy may also occur in Z. tritici is perhaps emphasized by the fact that 115 genes encoding functionally unknown secreted proteins were shown to be up-regulated, often displaying peak up-regulation, at 9-dpi (Supplementary Table 5).

We then further analyzed the fungal genes present within K-clusters 1-12 of Table 4 to identify whether any were physically linked and thus potentially co-regulated gene clusters (or loci). We set a minimum cut-off of three adjacent genes falling into a single K-cluster as evidence for co-regulation at a locus. This identified 16 putative regions (Figure 10 and Table 6). In contrast to observations made for the plant pathogenic fungus Ustilago maydis (Kaemper et al., 2006), none of the putative physical clusters were predominantly composed of small secreted proteins. Instead many of the clustered genes had functions (IPR) related to metabolic processes, and in particular secondary metabolism. For example the largest putative co-expressed physical cluster of genes (Locus 11 in Table 6) was surrounding a polyketide synthase, PKS7 on chromosome 7, with a total of eight physically adjacent genes all falling into K-cluster 10 (Table 4 and Supplementary Table 3). This concerted up-regulation of all genes in the PKS7 cluster, and their peak expression at 9-dpi was confirmed by independent qRT-PCR analysis (Supplementary Figure 3). Interestingly the PKS7 cluster includes the ABC transporter ATR3, which has been previously shown to be dispensable for fungal virulence on wheat leaves (Stergiopoulos et al., 2003). Despite this, the PKS7 cluster represents a strong candidate region for the production of as yet unknown secondary metabolite generated during the period of disease symptom formation. The production of other currently unknown secondary metabolites at 9-dpi is also supported by the identification of two further, albeit smaller, physical clusters/loci of co-regulated genes. This included a three gene cluster containing a hybrid PKS-Non ribosomal peptide synthetase (HPS1) on chromosome 10 and a three gene cluster surrounding an isopentenyltransferase-like protein on chromosome 2 (physical cluster/loci 15 and 12 in Table 6 and Figure 10 respectively).

Overall these data suggest that Z. tritici is producing and perhaps secreting a diverse set of secondary metabolites at the onset of disease symptoms. We did not detect or resolve any candidate molecules in our metabolite analysis, possibly due to dilution, low quantities and/or unique structures. The role in infection of these putative metabolites is thus currently unknown, however functional studies on MgATR3 may indicate that, similarly to what we have shown for candidate protein effectors (Supplementary figure 2), they also have either redundant or perhaps cultivar-specific functions.

For Wheat

At 9-dpi the earlier suppression of plant defenses appears to be relieved and significant up-regulation of 2401 wheat transcripts was observed (Table 5 and Supplementary table 6). Over a third of these up-regulated genes currently have no functional annotation (Supplementary table 6). Similarly to the analysis of Z. tritici transcripts (Supplementary table 7) the relative abundance of wheat transcripts was sorted from highest to lowest for all samples and the 100 most highly expressed wheat genes were analyzed (Top 100). Four bona fide PR genes and one of the nepenthesin-like aspartic proteinase genes (genes known to play role in defense against various pathogens) were amongst the list of 100 most abundant wheat genes detected at 9-dpi (Supplementary table 8). Interestingly, a lipoxygenase-2 gene, a candidate for
the first component in the biosynthetic pathway of jasmonates (JA) (Figure 7), is also in the Top 100 list at 9-dpi and is ranked 67th (Supplementary table 8). So, it appears that at 9-dpi the infected plants start investing significant resources into the synthesis of defense-related compounds and proteins.

At 9-dpi we notably detected up-regulation of all three key enzymes of fructan, fructose-based polymer (fructan) biosynthesis, namely 1-SST (sucrose:sucrose 1-fructosyltransferase), 6-SFT (sucrose:fructan 6-fructosyltransferase), and 1-FFT (fructan:fructan 1-fructosyltransferase) (Supplementary table 6 and Figure 11). Cereal fructans are produced in the vacuoles and are then known to be transported to the apoplast. One of the important functions of fructans in the extracellular space is stabilization of cellular membranes during various abiotic stresses such as drought and cold (Hare et al., 1998; Gaudet et al., 1999; Valluru and Van den Ende, 2008; Van den Ende and Valluru, 2009; Keune et al., 2013). Fructans may also serve as principal short-term energy stores in plant leaves, bridging the temporal gaps between resource availability and demands. The transcriptional data suggests that fructan biosynthesis is stimulated during Z. tritici infection because the genes coding for 1-SST, 6-SFT and 1-FFT, were all found to be very highly up-regulated at 9-dpi (Supplementary table 6, Figure 11). In fact, these three biosynthetic genes reside in the top 5% of the most highly up-regulated genes detected at 9-dpi (Supplementary table 6). Metabolite analysis also began to detect statistically significant increases in fructan levels in infected leaves at 9-dpi (Supplementary table 1 and Figure 11).

Up-regulation of other groups of transcripts whose annotation suggests a role in response to pathogens and abiotic stresses was also observed (Supplementary table 6). These include transcripts encoding other PR proteins, cytoplasmic NB-LRR-like resistance proteins, receptor-like kinases (LRR-, lectin-, cysteine-rich-, leucine-rich and wall-associated kinases), cytoplasmic kinases (e.g. MPK3 which has previously been demonstrated to accumulate and be activated at the protein level in response to Z. tritici- (Rudd et al., 2008)) and phosphatases, potential cell death inducing subtilases, other proteases (AAA-type, serine-, cysteine-, aspartate- and metallo-proteases), plant ubiquitination machinery proteins (ubiquitin, E2 and E3 ubiquitin ligases and SCF/F-box proteins), ROS generating peroxidases, and WRKY type transcription factors. Genes encoding plastocyanin-like proteins, which are known to be associated with PCD, plant defense responses, and pathogen infection, were also found to be up-regulated at 9-dpi.

In addition, a large number of genes involved in biosynthesis of JA and ethylene; hormones known for their role in defense signaling in particular to necrotrophic pathogens; genes involved in biosynthesis of lignin and other phytoalexins, isoprenoid and flavonoid compounds, and numerous CYP450 genes potentially involved in synthesis of a variety of other secondary (defensive) metabolites, were significantly up-regulated during this infection time point (Supplementary table 6, Figure 7 and Figure 8). Quite noticeable also was up-regulation of many potential detoxification enzymes including ABC transporters and UDP-GTs, and enzymes involved in cell wall re-modelling such as callose biosynthesis genes, xyloglucan endotransglycosylases, and fasciclin-like arabinogalactan proteins (Supplementary table 6).

Interestingly, and in contrast to the earlier stage of the interaction (Table 5), only a relatively small number of transcripts were found to be down-regulated at this stage (n=181) and approximately 50% of these are unknowns (Supplementary table 6). The most noticeable was down-regulation of photosynthesis-related genes, genes involved in biosynthesis of tetrapyrrolics (active cores of chlorophyll), light signaling genes, protein biosynthesis genes, and genes encoding pentatricopeptide repeat (PPR) proteins. PPRs in the model plant Arabidopsis are known to play essential roles in biogenesis of mitochondria and chloroplasts (Lurin et al., 2004).

**Overall summary for the disease transition phase**
The onset of macroscopic cell death in the leaves had a profound effect upon the transcriptome of both the pathogen and the plant. The plant response comprised up-regulation of many "defense" pathways including defense-associated hormone biosynthetic processes as well as the characteristic agents of a plant counter attack, secreted PR-type proteins. The fungus was also putting maximum effort into secretion during this phase. The lack of altered infection phenotype for the five secreted candidate protein effectors, tested by generating single gene deletion strains, suggests that these may function in overlapping processes during the interaction. However protease secretion in particular might be compensating for the cessation of intracellular lipid degradation to provide energy to Z. tritici. Many candidate effector proteins had maximal expression at the transition time point as well as various putative physical and transcriptional co-regulated secondary metabolite biosynthetic clusters. We can envisage at least two potential roles for the large scale production of effectors and secondary metabolites at this stage; (1) they might be required to defend the fungus against the individual components of the plant counter-attack through inhibitory interactions or (2) they might be actively involved in stimulating the activation of plant defenses culminating in host cell death to facilitate necrotrophic colonization and reproduction (i.e. they may be putative toxins). Finally at this point infected wheat leaves have up-regulated the entire fructan biosynthetic pathway to either attempt to limit the degree of cell death or to remobilize carbohydrate stores away from the infection site. Concurrent to all the activated processes, the plant also now diverts energy away from any further photosynthesis in the infected tissue.

Transcriptome and metabolome analysis of rapid necrotrophic colonization and asexual reproduction by Z. tritici at 14-21dpi

For Z. tritici

At 14-dpi disease symptoms are clearly visible as the plants extensively undergo a form of widespread programmed cell death (PCD) with characteristics similar to hypersensitive cell death (Keon et al., 2007; Rudd et al., 2008; Dean et al., 2012). This process is complete at 21-dpi where the fungus is now asexually sporulating in defeated plant tissue (Figure 1).

Of the 115 predicted secreted protein effectors (functionally un-annotated proteins) which were strongly up-regulated at 9-dpi, 37 were no longer significantly up-regulated at 14-dpi. Of the remaining 78, a reduced, but still significant, level of up-regulation (vs CDB) was observed for 63 of them, with the remainder (15 genes) showing higher expression at 14-dpi than 9-dpi (Supplementary table 5). By 14-dpi only 13 genes encoding predicted secreted proteins were present in the top 50 abundant transcripts, with the remaining time points / materials having 9 or less (Supplementary table 3). By 21-dpi, 92 of the original 115 candidate effectors with peak expression at 9-dpi were no longer up-regulated relative to fungal culture, whilst 17 showed markedly decreased (but still significant) levels of up-regulation relative to fungal culture (Supplementary table 5). Only six genes encoding small secreted proteins show increased expression at 21-dpi, the majority building upon increased levels already detected at 14-dpi.

Overall this suggests a progressive "switching-off" of production of many previously up-regulated un-annotated candidate effector proteins in the lead-up to the complete defeat of plant tissue. This pattern is supported by the overall expression profiles of the 68 cysteine-rich effector candidates within this set (Figure 9B) many of which had peak expression at 9-dpi which was decreased by 14-dpi before being almost completely down to culture levels at 21-dpi. This trend is also mirrored by a comparable pattern of progressive down-regulation of secreted proteases suggesting a switch away from the digestion of host proteins for nutrition at 14- and 21 dpi (Figure 9C).

In contrast to the situation for proteases, the contents of K-clusters 1 and 2 (Table 4) highlight that a significant number of glycoside hydrolases, transporters and alcohol dehydrogenases have increased expression at 14-dpi, often with a
subsequent peak at 21-dpi. Combined with our previous in silico protein secretome analysis we detected 20 putative secreted plant cell wall attacking enzymes which were differentially expressed in our experiment (Table 7). Fourteen of these genes were present in K-clusters 1 and 2 (Table 4) suggesting that they are deployed late in the infection cycle, significantly associated with necrotrophic colonization. This agrees well with previous data describing expression of various PCWDE enzymes during wheat infection by a different Z. tritici isolate (Brunner et al., 2013). Overall, the genome sequence of Z. tritici contains relatively few genes encoding enzymes that may have the ability to attack plant cell walls to liberate simple sugars (Goodwin et al., 2011; do Amaral et al., 2012). Nevertheless the expression data here indicates a switch to putative complex carbohydrates as a more significant energy source very late in infection, potentially involving proteins whose substrate preferences are likely to include cellulose, hemicellulose and pectin, which are key components of plant cell walls (Table 7).

The most prominent functional category of genes with peak up-regulation at 21-dpi includes a variety of Zn^{2+} containing alcohol dehydrogenases. These functional classes are most prevalent in the genes listed in expression cluster 1 (Table 4). The function of many of these is currently unknown, however it is intriguing that the simultaneous analysis of metabolites in this tissue also identified very high levels of sugar alcohols including mannitol and arabitol at 21-dpi (Supplementary table 1 and Figure 2). Various other reductases (including aldo/keto) and dehydrogenases (glucose/ribitol) with undefined substrate specificities also show peak up-regulation at 21-dpi, suggesting an involvement in metabolism related to the accumulation or inter-conversion of abundant spore compounds. In support of this, the Z. tritici homologue of mannitol dehydrogenase (Mycgr3G104277), showed peak expression at 21-dpi, having already displayed significant up-regulation at 14-dpi (Supplementary table 4). Homologues of this gene, and mannitol production in general, have been shown to be involved in asexual sporulation of the related wheat infecting fungal pathogen Phaeosphaeria nodorum (Solomon et al., 2005; Solomon et al., 2006).

**For Wheat**

The most significant transcriptional re-programming in wheat detected in this study occurred in infected leaf tissue at 14-dpi, co-incident with large scale host cell death. As many as 6608 genes (i.e. 13.5% of the analyzed transcriptome) were found to be significantly up- or down-regulated at this stage of infection (Table 5).

Of 3056 up-regulated genes in total, 1055 were of unknown function (Supplementary table 6). Interestingly, the vast majority of these (94%) appear to be up-regulated >2 fold specifically at 14-dpi, implying that these are transcriptional programs associated with the fungus colonizing in its more rapid necrotrophic growth phase. Analysis of differentially expressed genes with functional annotation revealed that those up-regulated at 14-dpi can largely be grouped into the same overall functional categories as those up-regulated at 9-dpi with a few exceptions (Supplementary table 9). Thus, it appears that the main difference between lists of functionally annotated genes up-regulated at 14-dpi and 9-dpi is in numbers of genes residing in each functional category. The most noticeable difference were the larger numbers of genes belonging to the following functional categories: amino-acid, lipid, nucleotide and secondary (isoprenoids) metabolism, photosynthesis, and TCA cycle. Perhaps most significantly the average level of gene expression (fold change vs corresponding mock) at 14-dpi is noticeably higher (on average ~30 fold) than that at 9 dpi (~6 fold) suggesting that peak plant defense activation is occurring during this rapid fungal necrotrophic colonization phase. Conversely, transcripts encoding photosynthetic-related processes are strongly down-regulated at 14-dpi.

Comparative analysis of metabolite data at 14-dpi now highlights a very clear and large accumulation of wheat fructans as a consequence of continued transcriptional up-regulation of this biosynthetic pathway from 9- through to 14-dpi (Supplementary table 1, Supplementary table 6 and Figure 11). However, by 21-dpi when plant tissue appears necrotic,
the levels of fructans now detected in this tissue were diminished, suggesting that the fructan pool had either been metabolized by the fungus or mobilized away from the infection court. Similarly whilst transcriptional activation of plant secondary metabolism including lignification, was up-regulated at 9- and 14-dpi, key metabolites such as ferulate were detected maximally in the residual 21-dpi tissue (Supplementary table 1), suggesting that in contrast, the fungus had not effectively degraded / remobilized these metabolites, and that they are most probably embedded within the residual plant cell wall structures. Finally a sharp increase in the plant defense hormone salicylic acid (SA) was detected at 14-dpi which persisted to a lesser extent in the residual plant tissue at 21-dpi (Supplementary table 1 and Figure 12). This suggests that SA mediated defenses may be transiently triggered during the period of extensive host cell death involving the rapid necrotrophic colonization by Z. tritici.

**Overall summary of the necrotrophic phase and asexual reproduction**

The maximal plant defense response was detected at 14-dpi and this correlated inversely with a decreased overall expression of putative fungal effectors, which were ultimately switched off entirely by sporulating Z. tritici at 21-dpi. During this period the fungus appeared less reliant on secreted proteases and began to invest more effort in producing its putative cell wall attacking capability, despite this genome feature being unusually low in numbers for a necrotrophic pathogen (Goodwin et al., 2011; do Amaral et al., 2012). This suggests some degree of effort to utilize carbohydrates from plant cell walls late in infection. Despite this, the plant continues to attempt to re-enforce cell walls via lignification and continues to maintain up-regulation of JA pathway and potentially also SA mediated defenses (perhaps transiently). Wheat fructan metabolites are now clearly detected at high levels at 14-dpi suggesting use by the plant to either minimize cell death or to remobilize intracellular carbohydrates. This transcriptional and metabolite analysis highlights a clear overlap with abiotic stress responses in wheat (Valluru and Van den Ende, 2008). By 21-dpi, upon complete defeat of plant tissues, major fungal metabolites accumulate, which can be associated with asexual spore formation, and certain classes of enzymes which may be involved in generating and / or interconverting spore storage compounds show peak up-regulation. Overall these data suggest that at 14-dpi the fungus thrives in plant tissue which is in fact undergoing a massive defense response involving localized cell death. The fact that the pathogen reproduces in this environment suggests that a form of hijack of plant cell death and defense activation are a feature of successful Z. tritici colonization perhaps facilitating reproduction during this period.

**Conclusions**

This study describes an in depth analysis of the interaction between Z. tritici and wheat leaves, via combined genome-wide RNAseq and global LC-MS- and GC-MS-based metabolite profiling, spanning a full asexual disease cycle of the pathogen on its host as well as two in vitro growth conditions. This analysis allowed several novel key findings on the Z. tritici-wheat interaction to be made: (1) relatively minor contributions of genes on dispensable/accessory chromosomes to overall gene expression at any infection phase; (2) rapid and large scale in planta up-regulation of fungal genes encoding candidate effector proteins; (3) different infection phase-specific fungal nutritional programs; (4) novel co-expressed secondary metabolite clusters, and (5) early repression followed by specific activation of plant “defense” responses involving biosynthesis of fructans, SA, JA, and cell wall re-enforcement at the switch to necrotrophy and pathogen reproduction.

The collective temporal regulation of all these processes indicate that, whilst nutritionally, Z. tritici appears to be a relatively poor biotroph, it uses an effective biphasic interaction with plant immunity involving an initial subterfuge strategy which is followed by an apparent hijack through sudden acute activation. How this is achieved for a strictly extracellular colonizing (apoplastic) filamentous pathogen remains unclear, but may involve any number of the strongly
up-regulated secretion responses (proteins and/or metabolites) detected in this study which are now open to functional analyses. This strategy may serve as an intriguing model for many other plant-infecting fungi which are currently perceived to have hemibiotrophic or necrotrophic lifestyles.

Materials and Methods

Biological materials / samples
The fungal isolate IPO323 and wheat cultivar Riband were used in all experiments. All materials were freeze-dried prior to RNA or metabolite isolation. Fungal cultures were propagated in shake flasks (220 rpm) at 18°C for 3 Days (for Potato Dextrose broth) or 5 Days (for Czapek-Dox broth) and then harvested via filtration. These incubation periods were determined to be within the logarithmic growth phase Z. tritici. Plant inoculation experiments were done as previously described (Keon et al., 2007) using a spore density of 1 x 10^6 spores / ml in 0.01% Tween 20 in sterile water. Mock inoculations of plants were made using just the Tween 20 water solution.

Each biological replicate plant sample for either RNA or metabolite isolation was made up of 5 leaves collected from 5 independent plants randomly distributed in a single walk-in temperature and humidity controlled glasshouse. Leaves were immediately frozen in liquid nitrogen, freeze-dried, then ground to fine powder in liquid nitrogen.

Metabolomics

Metabolite analysis was performed at Metabolon Inc., Research Triangle Park, North Carolina, USA. The global unbiased metabolic profiling platform consists of a combination of three independent platforms: UHLC/MS/MS2 optimized for basic species, UHLC/MS/MS2 optimized for acidic species, and GC/MS. These platforms have been described in detail in previous publications (Evans et al., 2009; Ohta et al., 2009). Essentially, 20 mg of each sample was thawed on ice and extracted using an automated MicroLab STAR system (Hamilton Company) in 400 mL of methanol containing recovery standards. UPLC/MS was performed using a Waters Acquity UHPLC (Waters Corporation) coupled to an LTQ mass spectrometer (Thermo Fisher Scientific Inc.) equipped with an electrospray ionization source. The extracted samples were then split into three equal aliquots for analysis by the three methods. For the two LC methods, chromatographic separation followed by full scan mass spectra was carried out to record retention time, molecular weight (m/z) and MS/MS^2 of all detectable ions presented in the samples (Evans et al., 2009). For GC/MS, bis-trimethyl-silyl-trifluoroacetamide derivatized samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole MS operated at unit mass resolving power (Ohta et al., 2009). Compounds were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (m/z), preferred adducts, and in-source fragments, as well as their associated MS/MS2 spectra (Dehaven et al. 2010; 2012). This library allowed the rapid identification of metabolites in the experimental samples with high confidence. Comparison of experimental samples to process blanks (water only) and solvent blanks allowed the removal of signal derived from artefacts.

Data Imputation and Statistical Analysis: The missing value for a given metabolite was imputed with the observed minimum detected value, based on the assumption that they were below the limits of detection / sensitivity of the instrument. Statistical analysis of the data was performed using JMP (SAS, http://www.jmp.com) and “R” (http://cran.r-project.org). Welch’s two-sample t-tests were performed on the log-transformed data to compare the treatment and control groups.
at each time point. Multiple comparisons were accounted for with the false discovery (FDR) rate method, and each FDR was estimated using q-values. For the convenience of data visualization, the raw area counts for each biochemical were re-scaled by dividing the value for a specific biochemical in each sample by the median value observed for that specific biochemical (median scaled).

RNA sequencing

Total RNA was isolated from freeze-dried tissues using the Trizol procedure incorporating a final LiCl₂ precipitation. RNA was further purified using the Qiagen Plant RNA isolation kit columns. The mRNA was isolated from four micrograms of total RNA, then subjected to fragmentation followed by cDNA synthesis. The ends of the cDNA fragments were repaired and A-tails were added. Sequencing adapters containing individual index sequences were ligated to the A-tailed fragments. The adapter ligated cDNA library was amplified using twelve cycles of PCR. All reactions were done using NEB’s NEBNext modules. The libraries were run on an Agilent Technologies Bioanalyzer to determine the size and concentration, and then normalized to 10 nM for sequencing. Sequencing was done using an Illumina HiSeq 2000 sequencer to generate 100 base single end reads. Five libraries were run per lane, with an average yield 45,000,000 reads per sample (range 30,000,000-67,000,000 reads per sample).

In order to remove bias from targets such as ribosomal sequences (22 *Mycosphaerella* rRNA and 99 wheat unigenes), these were removed from the analysis before proceeding with data normalization to get the read count (expression values). All short reads (single-end) were mapped with TopHat (v2.0.6) against *Z. tritici* genome (-G *Mycosphaerella graminicola.MG2.16.gtf*) (Trapnell et al., 2012). Cufflinks (v2.1.1) was used to calculate FPKM values for reference annotations (-G *Mycosphaerella graminicola.MG2.16.gtf*) but excluding genes annotated as rRNA (-M rRNA.genes.gtf). Differential expression analysis was done with Cuffdiff (cuffdiff -u -M rRNA.genes.gtf -b *Mycosphaerella graminicola.MG2.16.dna.toplevel.fa*). We extracted all significant genes (p<0.05) between CDB and all other samples (PDB, 1-dpi, 4-dpi, 9-dpi, 14-dpi and 21-dpi). Analysis was performed on both the 3 biological replicates experiment and a 2 biological replicates experiment. This was due to biological replicate 3 disease tracking slightly faster than 1 and 2 and also because biological replicate 3 was sequenced on a different platform (generating only 75-bp reads) 2 years previously. Only genes which were consistently differentially expressed in both analyses were considered further. The total output of the differentially expressed genes from *Z. tritici* were further filtered to remove any predicted differentially expressed gene where very low expression levels overall were detected. This involved removal of genes where FPKM values never exceeded <5.0 in all samples. This minimum cut off was selected following a dispersal analysis on the range of mean FPKM values for fungal growth in both media and at every time point of plant infection (Supplementary Figure 4A and B). A further cut-off was then applied at a 2-fold change in expression. These steps were taken to increase the stringency of identifying robust differential expression events and led to 3034 *Z. tritici* candidate genes. This list of fungal differential expressed genes was then subjected to K-means clustering using the csCluster method in cummeRbund (v.2.0.0). We tested different *K*-values ranging from 1 to 16. We selected *K*=12 as this produced a set of distinct profiles with about 60% of the total original dissimilarity.

Transcriptomes of *T. aestivum* leaves infected with *Z. tritici* (1-dpi, 4-dpi, 9-dpi and 14-dpi) along with the mock (buffer)-inoculated *T. aestivum* leaves of identical age (i.e. corresponding to 1-dpi, 4-dpi, 9-dpi and 14-dpi) were also studied. We pre-processed all reads by removing reads that mapped to wheat rRNA sequences or *Z. tritici* transcripts (bowtie2 --very-fast --ungz filtered.fastq.gz). The remaining reads were mapped with bowtie2 to a wheat transcriptome reference sequence to generate raw counts. This wheat transcriptome reference was constructed using wheat Unigene Build #60 (http://www.ncbi.nlm.nih.gov/UniGene/) by filtering out all the contaminating non-plant sequences i.e. those with >80% coverage of unigene and >90% similarity to genome of the known wheat pathogens e.g. *Zymoseptoria tritici*, *Fusarium*
graminearum, Puccinia spp. and Blumeria spp. The final filtered wheat unigene data set comprised 48,878 sequences. We then functionally annotated these unigene sequences using the Mercator pipeline (Lohse et al., 2014), which uses the MapMan ‘BIN’ ontology (Thimm et al., 2004), tailored for functional annotation of plant ‘omics’ data.

The resulting raw counts data was subjected to in house statistical analysis for identification of differentially expressed genes (DEGs) as follows. Eight treatments were used forming a two by four factorial set. The two factors were Treatment (mock-inoculated control or infected wheat leaves) and Time (1-dpi, 4-dpi, 9-dpi and 14-dpi). Three separate replicated biological experiments, forming three statistical blocks, were done. In the first two blocks, both control and treated samples were observed, but in the third block only data from genes of the control samples were observed. Hence, there were three replicates of control, but only two replicates of infected samples at the four time points, making 20 RNAseq samples altogether. Regression analysis was used on a per gene basis to assess the significance of differences between the Treatments, Times and interaction between these two factors. A generalized linear model to the count data for each gene was generated, assuming a Poisson distribution with a log link and accounting for the difference in sequencing depth by way of an estimated offset variable in the model:

\[
\text{Log}(\text{count}_{ijk}) = \log(d_{ijk}) + \text{Constant} + \text{Block}_i + \text{Treatment}_j + \text{Time}_k + (\text{Treatment}.\text{Time})_{jk}
\]

where, count\(_{ijk}\) is the count for Block \(i\), \(i = 1, 2, 3\); Treatment \(j\); \(j = 1\) (mock) or \(2\) (infected) and Time \(k\), \(k = 1\) (1-dpi), \(2\) (4-dpi), \(3\) (9-dpi) or \(4\) (14-dpi); and where \(d_{ijk}\) is the offset variable with values estimated as the proportion of total count per sample using a set of genes proposed not to be differentially expressed and therefore contributing only to assessment of sequencing depth, based on a Poisson goodness of fit statistic. The analysis took account of variance due to the three experiments as statistical blocks, with two degrees of freedom (df), before testing the effects of Treatment (with 1 df), Time (with 3 df) and the interaction (with 3 df). Therefore, with 20 observations, F-tests (rather than Chi-squared) for the effects were based on 10 (residual) df, these F-tests being appropriate to account for any overdispersion in the data, above that expected for a Poisson distribution.

Following the analysis, the \(p\)-values from the F-test results for the differences between Treatments, Time and the interaction were adjusted using the Benjamini and Hochberg (1995) method (B-H) to account for false discovery at the 5% level of significance (Benjamini and Hochberg, 1995) given 48,878 genes. Predicted means back on the raw Illumina read counts scale for these genes were then also output, and compared using the approximate least significant difference (LSD) value at the 5% level of significance. This allowed 12,901 differentially expressed wheat genes with a significant interaction between Treatment and Time \((p<0.05, \text{F-test} \text{ and } \rho_{\text{adj-B-H}}<0.05)\) to be output. The LSD values were approximated as the model used was non-linear. In particular, these values were likely to have been poorly estimated for those unigenes represented by very low Illumina read counts. Therefore, these very lowly expressed genes (with RPM \(<2\)) were removed from further analyses. A further cut-off was then applied at a 2-fold change in expression. This resulted in identification of 7,441 statistically and biologically most important wheat DEGs.

**Real-Time qRT-PCR**

In order to confirm selected transcriptional changes in planta, quantitative real time PCR (qPCR) was conducted on cDNA synthesized from RNA from illumina-analyzed replicates combined. A 200 ng aliquot of total RNA was used for each sample and cDNA was synthesized using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit according to the manufacturer’s instructions. In order to check for genomic contamination, a standard PCR assay was conducted using intron-spanning primers. Only cDNA samples for which a single band of the correct size was observed were used. The cDNA samples were diluted 1:3 and subjected to qPCR using SYBR Green JumpStart Taq ReadyMix (Sigma) in

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accordance with the manufacturer’s instructions. Thermocycler settings were as follows: 2 mins 95 °C, followed by 50 cycles of 15 s 95 °C, 30 s 60°C and 45 s 72’. Melt curves were run for all primer pairs (Supplementary Table 10) in order to check for dimerization. Data were analyzed using the Applied Biosystems 7500 software version 2.0.1 and the Z. tritici β-tubulin gene was used as a reference.

**Fungal transformation**

Agrobacterium-mediated fungal transformation was performed as previously (Motteram et al., 2009) using the Ku70 modified form of isolate IPO323 (Bowler et al., 2010). Constructs designed to either delete or disrupt candidate secreted protein effector genes were generated in vector pCHYG (Motteram et al., 2009). Several independent transformants with each gene disrupted were validated by PCR on genomic DNA. All primer sequences used for the design of KO constructs and targeting validation of transformants are shown in Supplementary Table 10.

**Cryo-Scanning Electron Microscopy (CSEM)**

Sections of inoculated wheat leaves (c. 5 mm x 5 mm) were excised using a sterile blade and attached to cryo stubs using a smear of OCT compound (Agar Scientific). Slotted stubs were used to enable the freeze fracturing of leaves. The mounted samples were plunged into pre-frozen liquid nitrogen then transferred under vacuum to the cooled (-180 °C) cryo-chamber.

Fractured surfaces were prepared and imaged using the JSM LVSEM 6360 scanning electron microscope (Jeol UK). Leaves were fractured using the cold blade mounted in the Alto 2100 (Gatan, UK) cryo-chamber, followed by sublimation to remove any ice on the surfaces. This was performed by raising the temperature of the stage to -95°C for 1 minute. The temperature was allowed to recover to -150°C before coating the samples with gold for 1 min (~ 10 nm thickness). The samples were transferred to the scanning electron microscope with the Gatan stage temperature maintained at -150°C for examination and imaging in high vacuum mode.

Imaging of leaf surfaces inoculated with the fungal pathogen was done under cryo-mode in a JSM FEGSEM 6700 scanning electron microscope (Jeol, UK). Sample preparation in the attached Alto 2500 (Gatan, UK) cryo-chamber was performed in a similar way to remove any contaminating ice and the samples were coated with Au Pd (~5 nm thickness).

**Supplementary files**

The following additional data are available with the online version of this paper.

**Supplementary Figure 1.** Schematic overview of the experimental workflow (.ppt)

**Supplementary Figure 2.** (A) The amino acid sequences and gene deletion strategy for five candidate Z. tritici protein effectors. PCR validation of subsequent transformed Z. tritici strains subsequently tested for altered plant infection (B) representative plant infection assays monitored at 21-dpi (.ppt)

**Supplementary Figure 3.** Quantitative PCR expression validation across wheat leaf infection of all eight genes present within the co-expressed PKS7 gene cluster from Z. tritici (.ppt)

**Supplementary Figure 4.** (A) Boxplots display the range of FPKM values of all Z. tritici genes for all conditions and time points. The data confirmed that there was no inflation (or deflation) of values at Day 1 or Day 4 as a consequence of less
sequencing depth. (B) Density plot displaying the number of genes at each mean FPKM value from each condition and time point. The solid bold vertical line highlights the mean FPKM minimum cut-off of 5.0 used in the subsequent analysis of differential gene expression (.ppt)

Supplementary Table 1. Excel spreadsheet containing all details on the 305 differentially abundant metabolites identified during this study. (.xls)

Supplementary Table 2. Lists of metabolites detected only in either Z. tritici or in wheat leaves throughout the course of profiling experiments (.xls)

Supplementary Table 3. Excel spreadsheet displaying the Mean FPKM value for all Z. tritici genes in all samples. The spreadsheet contains gene codes retrievable from the JGI genome website (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html) and from Ensembl (http://fungi.ensembl.org/Zymoseptoria_tritici/Info/Index) together with a prediction of encoded protein secretion and available functional annotation. (.xls)

Supplementary Table 4. Excel spreadsheet containing 3046 Z. tritici genes with differential expression detected during this study. The spreadsheet contains gene codes retrievable from the JGI genome website (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html) and from Ensembl (http://fungi.ensembl.org/Zymoseptoria_tritici/Info/Index) together with a prediction of encoded protein secretion and available functional annotation. Expression clusters (k-clusters) to which each gene belongs are also indicated. Expression values are presented as Log2 fold changes. (.xls)

Supplementary Table 5. All differentially expressed Z. tritici genes encoding predicted secreted proteins. The spreadsheet contains gene codes retrievable from the JGI genome website (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html) and from Ensembl (http://fungi.ensembl.org/Zymoseptoria_tritici/Info/Index) together with a prediction of encoded protein secretion and available functional annotation. Expression values are presented as Log2 fold changes. (.xls)

Supplementary Table 6. All differentially expressed wheat (T. aestivum) unigenes detected during this study. Fold changes vs corresponding mock inoculated leaves are shown together with available functional annotation. Sequences are retrievable using gene codes from http://www.plexdb.org/index.php (.xls)

Supplementary Table 7. The “Top 50” Z. tritici genes ranked on highest relative expression at 9-dpi. Gene codes are retrievable from Ensembl (http://fungi.ensembl.org/Zymoseptoria_tritici/Info/Index). Light grey shading indicated gene was subjected to functional characterization by gene deletion (see Suppl Figure 2). Dark grey shading indicates LysM effectors. (.doc)

Supplementary Table 8. Excel spreadsheet containing The “Top 100” wheat (T. aestivum) unigenes ranked on highest relative expression with available functional annotation. Sequences are retrievable using gene codes from http://www.plexdb.org/index.php (.xls)

Supplementary Table 9. Excel spreadsheet containing comparison of wheat (T. aestivum) functional categories up-regulated relative to mock inoculated leaves at 9 and 14-dpi. Sequences are retrievable using gene codes from http://www.plexdb.org/index.php (.xls)

Supplementary Table 10. All primer sequences used in this study. (.xls)
Acknowledgements

The authors would like to thank members of the Rothamsted Research Horticultural support team. The work was supported by the Biotechnology and Biological Sciences Research Council of the UK (BBSRC) through the Institute Strategic Program grant “20:20 Wheat®” (BB/J/00426X/1) awarded to Rothamsted Research (JJR, KK, KH-P, AA, JD, AL, MS, SP, JH and KEH-K) and performed in collaboration with Syngenta Biosciences (LA, AB, AF, RAD and MC). The study was also supported by a Syngenta sponsored Industrial Case (BBSRC) PhD Studentship (MD).

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Figure Legends

Figure 1. The time course of infection of *Z. tritici* on wheat. Leaf materials were collected and subsequently analyzed by both RNA-seq, GC-MS and LC-MS

Figure 2. Abundant fungal metabolite accumulation and RNAseq read mapping to the fungal genome chart the progress of disease. Red lines represent *Z. tritici* infected leaves. Blue lines represent mock treated leaves. ** = significant increase at Padj<0.05

Figure 3. The small “accessory” chromosomes and a region on chromosome 7 have low transcriptional activity throughout infection. A- Upper panel displays the position of all current predicted *Z. tritici* genes across the 21 chromosomes of isolate IPO323. A- Lower panel shows all genes with very low or no (FPKM<1.0) detected expression throughout infection. Inset displays PCR on genomic DNA confirming that the low/ non transcribed region on chromosome 7 was present in the experimental isolate IPO323. B- Displays the mean value of expression per gene from each chromosome averaged from the entire dataset.

Figure 4. Metabolite and transcriptome analysis of fungal culture illuminates metabolism during early plant infection. A- Fungal growth in CDB reduces levels of amino acids. Blue font signifies decreased levels in CDB, yellow indicates increased levels (Padj<0.05). B- Growth in CDB increases levels of Hexose 6-Ps, trehalose and mannitol, accompanied by up-regulation of genes encoding hexose and nitrate transporters. C- Low level expression of genes involved in nitrate and hexose uptake and assimilation during phases of plant infection. Numbers represent the mean FPKM values at each time point of infection.

Figure 5. Early leaf infection (1-4-dpi) induces expression of *Z. tritici* genes involved in the beta-oxidation of lipids and fatty acids and up-regulation of the glyoxylate pathway. Red coloring indicates up-regulation; no shading represents no significant change relative to expression levels in CDB culture (Padj<0.05)

Figure 6. Expression profiles of 366 *Z. tritic* genes encoding putative secreted proteins up-regulated during leaf infection.

Figure 7. Fungal-induced changes in expression of the wheat jasmonate biosynthesis pathway. A- The schematic pathway of jasmonic acid (JA) biosynthesis in plants. B- The relative expression changes of JA pathway components in wheat leaves from 1-14 dpi with *Z. tritici*. Green shading indicates significant down-regulation (Padj<0.05) whilst red shading indicates up-regulation (Padj<0.05)

Figure 8. Fungal-induced changes in expression of the wheat lignin biosynthesis pathway. A- The schematic pathways leading to lignin formation in plants. Metabolite analysis of *Z. tritici* infected wheat leaves identified increased levels of phenylalanine and ferulate during the late stages of infection. B- The relative expression changes of lignin biosynthetic pathway components in wheat leaves from 1-14-dpi with *Z. tritici*. Green shading indicated significant down-regulation (Padj<0.05) whilst red shading indicates up-regulation (Padj<0.05)

Figure 9- Expression profiles of *Z. tritici* secreted protein sub-classes throughout plant infection. A- Percentage of secreted and un-annotated proteins present in the top 50 most abundant transcripts at each time point. B- Expression profile of 68 un-annotated small cysteine-rich secreted proteins. C- Expression profile of 31 differentially expressed secreted proteases.
Figure 10. The physical position of putative co-expressed *Z. tritici* gene clusters. A- All currently predicted *Z. tritici* genes. B- The position of 16 putative co-expressed physical gene clusters shown in Table 6.

Figure 11. Fructan biosynthesis pathway and fructan metabolite accumulation are triggered in wheat leaves during the onset of disease symptoms induced by *Z. tritici*. A- Quantification of fructan metabolites during the course of infection (** = significant at Padj<0.05). B- Transcriptional up-regulation of the fructan biosynthetic pathway. Green shading indicated significant down-regulation (Padj<0.05) whilst red shading indicates up-regulation (Padj<0.05).

Figure 12. Salicylic acid (SA) accumulation coincides with the necrotrophic phase of fungal colonization. Red line = fungal inoculated leaves; Blue = mock inoculated leaves. ** = significant at Padj<0.05.
Tables

<table>
<thead>
<tr>
<th>Biochemical</th>
<th>Platform</th>
<th>Mean values mock leaves</th>
<th>Mean values fungal culture</th>
<th>Ratio fungal culture/ mock leaves</th>
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Table 1. Metabolites detected in fungal culture at levels >100 fold mock inoculated wheat leaves.
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<th># reads</th>
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<th>% reads mapped to Z. tritici genome</th>
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<td>CDB-2</td>
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Table 2. RNAseq read counts and % mapping statistics to the *Z. tritici* genome.
Table 3. The number of *Z. tritici* differentially expressed genes relative to levels in CDB culture. The number of genes showing their peak up-regulation / time point is also indicated.

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<td>1389</td>
<td>1114</td>
<td>1331</td>
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<td>627</td>
<td>771</td>
<td>819</td>
<td>640</td>
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<td>224</td>
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<td>Chloroperoxidase (4.4) Cytochrome P450 (4.4) Glucose/riboitol dehydrogenase (3.3) Major facilitator superfamily (3.3) Zn-finger (3.0) remainder all appear once in the list</td>
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**Table 4.** Analysis of expression clusters of *Z. tritici* genes (K-clusters) throughout the course of infection.
<table>
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<tr>
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<th>1-dpi</th>
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<th>9-dpi</th>
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<td>up-regulated</td>
<td>352</td>
<td>36</td>
<td>2401</td>
<td>3056</td>
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<td>down-regulated</td>
<td>334</td>
<td>51</td>
<td>181</td>
<td>3552</td>
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<tr>
<td>Total</td>
<td>686</td>
<td>87</td>
<td>2582</td>
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Table 5. The numbers of differentially expressed wheat genes in *Z. tritici* infected leaves vs mock inoculated leaves at 1-14-dpi. 21-dpi was not analyzed due to the lack of wheat RNA in the heavily diseased necrotic leaves.
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<th>Expression Pattern</th>
<th>Ensembl Id</th>
<th>Chromosome Location</th>
<th>IPR Description</th>
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Table 6. Physical clusters of co-expressed *Z. tritici* genes
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Table 7. Expression profiles of differentially expressed *Z. tritici* secreted plant cell wall attacking enzymes
Figure 1. The time course of infection of *Z. tritici* on wheat. Leaf materials were collected and subsequently analysed by both RNAseq, GC-MS and LC-MS
Figure 2. Abundant fungal metabolite accumulation and RNAseq read mapping to the fungal genome chart the progress of disease. Red lines represent *Z. tritici* infected leaves. Blue lines represent mock treated leaves. ** = significant increase at Padj<0.05.
Figure 3. The small “accessory” chromosomes and a region on chromosome 7 have low transcriptional activity throughout infection. A- Upper panels displays the position of all current predicted *Z. tritici* genes across the 21 chromosomes of isolate IPO323. A- Lower panel shows all genes with very low or no (FPKM<1.0) detected expression throughout infection. Inset displays PCR on genomic DNA confirming that the low/ non transcribed region on Chr 7 was present in the experimental isolate IPO323. B- Displays the mean value of expression per gene from each chromosome averaged from the entire dataset.
Figure 4. Metabolite and transcriptome analysis of fungal culture illuminates metabolism during early plant infection. A- Fungal growth in CDB reduces levels of amino acids. Blue font signifies decreased levels in CDB, yellow indicates increased levels (Padj<0.05). B- Growth in CDB increases levels of Hexose 6-Ps, trehalose and mannitol, accompanied by up-regulation of genes encoding hexose and nitrate transporters. C- Low level expression of genes involved in nitrate and hexose uptake and assimilation during phases of plant infection. Numbers represent the mean FPKM values at each time point of infection.
Figure 5. Early leaf infection (1-4dpi) induces expression of Z. tritici genes involved in the beta-oxidation of lipids and fatty acids and up-regulation of the glyoxylate pathway. Red colouring indicates up-regulation; no shading represents no significant change relative to expression levels in CDB culture (P_{adj}<0.05)
Figure 6. Expression profiles of 366 *Z. tritici* genes encoding putative secreted proteins up-regulated during leaf infection.
Figure 7. A- The schematic pathway of Jasmonic acid (JA) biosynthesis in plants. B- The relative expression changes of JA pathway components in wheat leaves from 1-14 dpi with Z. tritici. Green shading indicated significant down-regulation (Padj<0.05) whilst red shading indicates up-regulation (Padj<0.05).
Figure 8. A- the schematic pathways leading to lignin formation in plants. Metabolite analysis of Z. tritici infected wheat leaves identified increased levels of phenylalanine and ferulate during the late stages of infection. B- The relative expression changes of lignin biosynthetic pathway components in wheat leaves from 1-14 dpi with Z. tritici. Green shading indicated significant down-regulation (Padj<0.05) whilst red shading indicates up-regulation (Padj<0.05).
Figure 9- Expression profiles of *Z. tritici* secreted protein sub-classes throughout plant infection. A- % of secreted and unannotated proteins present in the top 50 most abundant transcripts at each time point. B- Expression profile of 68 unannotated small cysteine-rich secreted proteins. C- Expression profile of 31 differentially expressed secreted proteases.
Figure 10. The physical position of putative co-expressed *Z. tritici* gene clusters. A- All currently predicted *Z. tritici* genes. B- The position of 16 putative co-expressed physical gene clusters shown in Table 6.
Figure 11. Fructan biosynthesis pathway and fructan metabolite accumulation are triggered in wheat leaves during the onset of disease symptoms induced by Z. tritici. A- Quantification of fructan metabolites during the course of infection (** = significant at Padj<0.05). B- Transcriptional up-regulation of the fructan biosynthetic pathway. Green shading indicated significant down-regulation (Padj<0.05) whilst red shading indicates up-regulation (Padj<0.05).
Figure 12. Salicylic acid (SA) accumulation occurs coincident with the necrotrophic phase of fungal colonisation. Red line = fungal inoculated leaves; Blue= mock inoculated leaves. **= significant at Padj<0.05