Comparative Transcriptomics Reveals How Wheat Responds to Infection by *Zymoseptoria tritici*

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The fungus *Zymoseptoria tritici* causes septoria tritici blotch (STB) on wheat, an important disease globally and the most damaging wheat disease in Europe. Despite the global significance of STB, the molecular basis of wheat defense against *Z. tritici* is poorly understood. Here, we use a comparative transcriptomic study to investigate how wheat responds to infection by four distinct strains of *Z. tritici*. We examined the response of wheat across the entire infection cycle, identifying both shared responses to the four strains and strain-specific responses. We found that the early asymptomatic phase is characterized by strong upregulation of genes encoding receptor-like kinases and pathogenesis-related proteins, indicating the onset of a defense response. We also identified genes that were differentially expressed among the four fungal strains, including genes related to defense. Genes involved in senescence were induced during both the asymptomatic phase and at late stages of infection, suggesting manipulation of senescence processes by both the plant and the pathogen. Our findings illustrate the need, when identifying important genes affecting disease resistance in plants, to include multiple pathogen strains.

The ascomycete fungus *Zymoseptoria tritici* (formerly known as *Mycosphaerella graminicola*) causes septoria tritici blotch (STB), the most damaging disease on wheat in Europe (Hardwick et al. 2001; O'Driscoll et al. 2014), leading to expenditure of \$1.2 billion each year for fungicides to control the disease (Torriani et al. 2015). As a result of frequent recombination, high gene flow, and large sizes, *Z. tritici* populations are typically very diverse and evolve rapidly in response to deployment of resistant wheat cultivars and fungicides (McDonald and Linde 2002; Wittenberg et al. 2009; Zhan and McDonald 2004; Zhan et al. 2003), making *Z. tritici* an appropriate model to analyze the responses of hosts to different naturally occurring strains of the same pathogen. *Z. tritici* has a complex infection cycle. After penetrating leaves through stomata, the fungus begins a long period of asymptomatic growth traditionally considered to represent a biotrophic phase, though it was recently proposed that it

may, instead, be simply a long latent period (Sánchez-Vallet et al. 2015). During this latent phase that lasts for 9 to 14 days, *Z. tritici* is thought to evade host recognition, aided by LysM effectors that prevent chitin recognition by the plant (Marshall et al. 2011). Following the latent period, *Z. tritici* switches rapidly to a necrotrophic phase of infection that leads to a rapid increase of fungal biomass inside the plant (Rudd et al. 2015).

Most plants are thought to use a two-branched immune system to defend against pathogen attacks. Pathogen-associated molecular patterns (PAMPs) are recognized by transmembrane pattern recognition receptors (PRRs), resulting in PAMP-triggered immunity (PTI) (Nicaise et al. 2009). In return, pathogens can deploy effectors to interfere with PTI. These effectors can be recognized by specific resistance (R) proteins inside the cell, resulting in effector-triggered immunity (ETI) (Rajamuthiah and Mylonakis 2014). ETI can enhance defense against pathogens by triggering a hypersensitive cell death response (HR) (Greenberg and Yao 2004; Coll et al. 2011; Tao et al. 2003; Thilmony et al. 2006). Some R proteins can also recognize the apoplastic effectors produced by pathogens (Win et al. 2012). In contrast with the traditional ETI, these immune responses happen at the cell surface, are relatively slow, and are not associated with a rapid, orchestrated HR (Thiruganasambandam et al. 2011; Valent and Khang 2010). To distinguish it from PTI and ETI, this resistance is referred to as effector-triggered defense (ETD) (Stotz et al. 2014; Win et al. 2012). Unlike PTI and ETI, ETD reduces the growth of the pathogen but does not stop it completely.

To detect the presence of pathogens, plants use a particular group of receptor-like kinases (RLKs) that consist of a serine/threonine kinase domain with a specific extracellular domain and a group of special R proteins as the major PRRs (Afzal et al. 2008; Dardick et al. 2012; Goff and Ramonell 2007; Sanabria et al. 2010; Shiu and Bleecker 2001; Win et al. 2012). In plants, most RLKs have a conserved arginine aspartate domain, called an RD domain, that plays a role in autophosphorylation and activation. These RD-RLKs affect development in various ways. In a second group of RLKs, the non-RD RLKs, the arginine in the RD domain is substituted by another amino acid (Krupa et al. 2004). These non-RD RLKs can act as PRRs to recognize pathogens (Dardick et al. 2012; Johnson et al. 1996). RLKs can be divided into several subclasses based on their specific protein domain compositions, e.g., S domain RLKs (Nasrallah et al. 1988), leucine-rich repeat (LRR) RLKs (Takemoto et al. 2000), lectin-like RLKs (Lannoo and Van Damme 2014; Shiu and Bleecker 2003) and wall-associated kinase (WAK)-type RLKs (Decreux and Messiaen 2005; Wagner and Kohorn 2001). In addition to PAMP recognition, RLKs also participate in the recognition of pathogen avirulence proteins (Lee et al. 2006). For example, the wheat *Stb6* gene that recognizes the AvrStb6 protein of *Z. tritici* (Zhong et al. 2017) was recently shown to encode a WAK-type

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RLK (Saintenac et al. in press). Through their multiple critical functions, the expression of RLK-related genes can affect the immune responses of plants.

Pathogenesis and plant development can affect each other (Grant and Jones 2009; Häffner et al. 2015). Plant senescence, the process of aging in plants that leads to nutrient remobilization and degradation and death of tissue, is often activated in response to pathogens. Plants utilize a senescence-like process to resist biotrophic pathogens and prevent early senescence as a defense against necrotrophic pathogens. Conversely, biotrophic pathogens delay the senescence process to keep the host cells alive (Häffner et al. 2015; Mengiste 2012) while necrotrophic pathogens induce the senescence process to accelerate destruction of the host cells (Häffner et al. 2015; Mengiste 2012). The role of senescence in hemibiotrophic pathogens like *Z. tritici* that exhibit both biotrophic and necrotrophic characteristics is poorly understood.

Taking advantage of RNA-Seq, several studies focused on the transcription profiles associated with plants colonized by fungi (Kawahara et al. 2012; Palma-Guerrero et al. 2016, 2017; Yazawa et al. 2013). One of the most comprehensive studies of pathogen-plant interactions investigated the transcriptome and metabolome of *Z. tritici* in wheat leaves during the infection cycle (Rudd et al. 2015). The authors analyzed the expression of specific catalogs of defense-related genes in wheat infected by a single *Z. tritici* strain. However, how wheat responds to different *Z. tritici* strains remains largely unknown.

To investigate the dynamics of the wheat transcriptome in response to infection by different *Z. tritici* strains, we infected bread wheat (*Triticum aestivum* L.) with four different *Z. tritici* strains in a common garden design (de Villemereuil et al. 2016), i.e., in a uniform biotic and abiotic environment shared across all host and pathogen strains, and performed a comparative transcriptomic study that covered the entire infection cycle. To our knowledge, this is the first experiment to explore the response of wheat to different strains of a fungal pathogen. We found that the earliest stage of infection was dominated by the upregulation of defense-related genes, including genes encoding RLK and pathogenesis-related (PR) proteins. Large numbers of genes that were differentially expressed (DEGs) among fungal strains were detected during each stage of the infection cycle. The most significant differentially regulated genes that were shared among all four strain infections provided insight into the basal defense response against *Z. tritici*. Many genes associated with senescence were found to be upregulated, both at the early asymptomatic stage of infection and during the latest stage of infection. We propose that *Z. tritici* triggers senescence in wheat as an important component of its invasion strategy.

RESULTS

Analysis of the wheat transcriptome of infected wheat.

We compared the transcriptome of wheat leaves from 'Drifter' (*Triticum aestivum* L. cv. Drifter) infected with four different strains of *Z. tritici* (3D7, 3D1, 1A5, and 1E4). These four fungal strains are known to differ in their levels of virulence and in their rates of disease progression (Stewart et al. 2016; Palma-Guerrero et al. 2017; Zhan et al. 2002, 2005). Samples were analyzed at four distinct timepoints, covering asymptomatic, necrotrophic, and saprotrophic phases of the *Z. tritici* life-cycle. These same samples, at the same timepoints, were used previously to analyze the fungal transcriptomes (Palma-Guerrero et al. 2017).

Using a customized bioinformatics analysis (Supplementary Fig. S1), we generated over 20 million raw reads for all the samples (Table 1). The percentage of reads mapped to the wheat genome decreased during the disease cycle at different rates for

different pathogen strains (Fig. 1A), in a pattern consistent with the increase in fungal reads reported previously for these same samples (Palma-Guerrero et al. 2017). The decrease was slow during the latent period of infection, when plants showed few symptoms, but, starting at 14 days postinfection (dpi), the alignment rates dropped dramatically in the 3D7, 1A5, and 1E4 infections. This decline occurred later in the infections with 3D1, which exhibits a delayed disease progression (Palma-Guerrero et al. 2017). At 28 dpi, fewer wheat reads could be detected in the infected plants because the infected leaves at this timepoint were mostly dead. As reported earlier, most of the reads at 28 dpi were of pathogen origin (Palma-Guerrero et al. 2017).

The sharp decrease in wheat reads at 14 dpi is consistent with the rapid increase in fungal biomass following the transition to the necrotrophic phase of infection. Symptoms expressed in the leaves were consistent with the measured changes in mapped reads (Palma-Guerrero et al. 2017). Infections with strain 3D7 showed the fastest decline in read mapping ratio, while the 3D1 infections showed the highest percentage of mapped wheat reads. Strains 1A5 and 1E4 did not have clear differences in mapped reads between 12 and 14 dpi.

We used an early version of the wheat transcriptome comprising 99,385 genes (International Wheat Genome Sequencing Consortium (IWGSC) 2014) for all analyses. The cluster analysis (Fig. 1B) of all transcriptome samples showed good correlations among the biological replicates. The 28-dpi transcriptomes differed strongly from other timepoints. Thus, we separated the samples into two super clusters: the asymptomatic phase and the acute disease phase. At 14 dpi, only genes from 3D1 infections did not cluster into the acute disease group, which is consistent with the slower disease progression and resulting slower decline of mapped wheat reads in 3D1 infections.

Strain-specific gene expression in the wheat response to *Z. tritici* infection.

To study the plant response to the different strains, we investigated the DEGs between infections at each timepoint. At 7 and 28 dpi, there were relatively few DEGs between the different strain infections (Fig. 2A). In contrast, at 12 and 14 dpi, the transcriptome profiles of wheat infected with the different strains differed strongly. The 3D1 strain with the slowest disease progression induced the greatest differences in wheat gene expression. Overall, the infections induced the greatest differences in expression profiles at 14 dpi, most likely reflecting the asynchronicity of disease development among pathogen strains. Wheat plants infected with the 1A5 and 1E4 strains had very few DEGs at 7 and 14 dpi, but, at 12 dpi, there were almost 3,500 DEGs between these two strain infections (Fig. 2A). Both the dynamics of alignment rates against the wheat genome and the significant differences in transcriptomes likely reflect differences in levels of pathogen virulence and the speed of disease progression among the four infections.

We analyzed the expression profiles of several defense-related genes that are known to play important roles in plant immunity (Adhikari et al. 2007; Bolton et al. 2008; Coram et al. 2008; Hammond-Kosack and Rudd 2008; McGrann et al. 2009; Orton et al. 2016; Ray et al. 2003; Rostoks et al. 2003; Shetty et al. 2009). Comparisons were based on the maximum expression of each gene across the disease cycle to reduce the influence of asynchronicity of disease development among fungal strains (Fig. 2B). Clear strain-specific expression was found for some of these genes. For example, *PR-1* was highly expressed in 3D7, 1A5, and 1E4 infections (with average maximum counts per million reads [CPM] values of 171, 286, and 135, respectively) but was less expressed in 3D1 infections (with an average maximum CPM value of 58). Given that the 3D1 infections achieved maximum expression of *PR-1* at 12 dpi, followed by a

rapid drop in expression at 14 dpi, we can discount the possibility that this lower expression is due to the asynchronicity of infection. The *PR-3* gene showed higher maximum expression in 1A5 infections (average maximum CPM value of 200) compared with the other three strain infections (41 CPM in 3D7, 8 CPM in 3D1, and 38 CPM in 1E4). In this case, the maximum expression in 1A5 infections was observed at 14 dpi, as observed also for 1E4 infections, whereas 3D7 and 3D1 infections showed the maximum expression at 12 dpi. These strain-specific differences in gene expression were found not only for PR genes but also in other gene categories, including a cysteine protease and the *MLO* gene that were much less expressed in 3D1 infections and much more expressed in 3D7 infections (Supplementary Table S1).

The biological function of most *MLO* genes is not known in detail (Kusch and Panstruga 2017), but they are associated with disease susceptibility. A xylanase inhibitor protein (XIP-1) showed higher expression values in 3D1 infections (average maximum CPM value of 96) than in the other three strain infections (19 CPM in 3D7, 26 CPM in 1A5, and 24 in 1E4). This higher expression value for 3D1 infections was only observed at 28 dpi, whereas, at earlier timepoints, the expression of this gene was lower in 3D1 infections than for the other three strain infections. XIPs are thought to provide an important plant defense against pathogenic fungi (Juge 2006). The strain-specific expression of these defense-related genes indicates that different defense responses are induced by different strain infections.

Table 1. Summary of the transcriptomic dataset

Strain	Infection stage ^a	Library ID	Replicate	Total clean paired reads	Uniquely mapped paired reads ^b	Alignment rate (%) ^c
3D7	7	3D7-2-7d	1	64,436,956	40,318,100	62.57
	7	3D7-8-7d	2	79,117,442	48,194,686	60.92
	7	3D7-10-7d	3	79,731,275	50,462,620	63.29
	12	3D7-2-12d	1	41,178,383	23,840,446	57.90
	12	3D7-3-12d	2	92,887,591	53,146,652	57.22
	12	3D7-6-12d	3	30,837,244	18,173,978	58.94
	14	3D7-4-14d	1	52,530,897	13,024,118	24.79
	14	3D7-5-14d	2	31,946,821	14,030,480	43.92
	14	3D7-10-14d	3	44,781,425	4,034,961	9.01
	28	3D7-1-28d	1	42,108,770	711,902	1.69
	28	3D7-5-28d	2	31,124,289	599,632	1.93
	28	3D7-10-28d	3	26,186,577	493,082	1.88
3D1	7	3D1-1-7d	1	53,927,155	33,696,664	62.49
	7	3D1-2-7d	2	54,371,387	33,940,169	62.42
	7	3D1-3-7d	3	72,634,605	45,081,800	62.07
	12	3D1-2-12d	1	37,859,198	22,290,066	58.88
	12	3D1-5-12d	2	29,322,054	17,680,121	60.3
	12	3D1-8-12d	3	38,183,710	23,760,239	62.23
	14	3D1-7-14d	1	38,057,118	22,307,621	58.62
	14	3D1-9-14d	2	45,694,973	26,006,331	56.91
	14	3D1-10-14d	3	53,612,066	32,417,156	60.47
	28	3D1-1-28d	1	27,115,882	879,474	3.24
	28	3D1-4-28d	2	23,741,543	736,157	3.1
	28	3D1-4-28d	3	28,746,538	1,049,064	3.65
1A5	7	1A5-1-7d	1	72,853,335	44,533,689	61.13
	7	1A5-7-7d	2	69,670,022	43,195,098	62
	7	1A5-10-7d	3	85,838,058	52,598,094	61.28
	12	1A5-3-12d	1	31,154,153	17,779,660	57.07
	12	1A5-5-12d	2	30,887,965	18,836,586	60.98
	12	1A5-7-12d	3	43,295,985	25,916,167	59.86
	14	1A5-8-14d	1	30,718,325	16,582,799	53.98
	14	1A5-9-14d	2	29,767,185	10,668,015	35.84
	14	1A5-10-14d	3	35,614,454	16,552,916	46.48
	28	1A5-3-28d	1	26,334,680	1,249,259	4.74
	28	1A5-6-28d	2	42,378,178	886,761	2.09
	28	1A5-7-28d	3	30,641,850	700,266	2.29
1E4	7	1E4-4-7d	1	85,872,533	51,901,691	60.44
	7	1E4-5-7d	2	72,247,223	43,197,267	59.79
	7	1E4-6-7d	3	108,111,684	54,656,373	50.56
	12	1E4-3-12d	1	43,473,395	23,984,384	55.17
	12	1E4-6-12d	2	34,526,903	20,580,614	59.61
	12	1E4-7-12d	3	29,724,987	17,322,914	58.28
	14	1E4-5-14d	1	26,086,427	13,008,131	49.87
	14	1E4-8-14d	2	29,653,868	14,013,955	47.26
	14	1E4-10-14d	3	29,254,210	12,163,119	41.58
	28	1E4-3-28d	1	34,784,411	540,972	1.56
	28	1E4-4-28d	2	46,193,137	772,324	1.67
	28	1E4-6-28d	3	46,477,794	615,169	1.32
Mocks	3	C1-3 dpi	1	46,663,407	29,134,401	62.44
	3	C2-3 dpi	2	53,385,742	32,773,739	61.39
	3	C3-3 dpi	3	34,647,240	21,802,134	62.93
	24	C2-24 dpi	1	37,170,117	23,448,782	63.09
	24	C3-24 dpi	2	38,362,408	23,116,911	60.26
	24	C5-24 dpi	3	36,899,443	23,550,917	63.82

^a Shown in days postinoculation.

^b The number of reads mapped to the wheat genome.

^c The percentage of reads mapped to the wheat genome with respect to the total number of reads sequenced (wheat + fungus).

Timecourse transcriptome analysis of *Z. tritici* infections.

Because we anticipated that normal leaf development would influence the wheat transcriptome, we chose mock infections at 3 and 24 dpi as external controls. The DEGs between younger (3-dpi) and older (24-dpi) wheat leaves were considered as development-related genes. Thus, these 19,082 DEGs were used as a standard “baseline” to exclude the genes that were not triggered by the pathogen but were differentially expressed as part of normal plant development. Thus, for the timecourse analysis, we focused only on those genes that are equally expressed between younger and older leaves in the control plants, to identify the genes involved in the response to the pathogen.

We divided the timecourse of the disease into three phases (Fig. 3A). In phase 1 (7 to 12 dpi), 1E4 infections showed the most DEGs among the four strain infections. The fact that 1E4 infections had the lowest rate of mapped RNA-Seq reads (Fig. 1A) suggests that wheat responds earlier to the 1E4 strain, even though symptom development for this strain was intermediate among the four strains (Palma-Guerrero et al. 2017). The number of DEGs in 1E4 infections decreased strongly in phase 2 (12 to 14 dpi). The 3D7 and 1A5 infections, which did not show as many DEGs as in 1E4 infections in phase 1, showed the highest differences in their transcriptomes in phase 2, with about 15,000 DEGs in 3D7 infections and 10,000 DEGs in 1A5

infections. In phase 3 (14 to 28 dpi), the number of DEGs in 3D7 and 1A5 infections were similar to 1E4 infections. For the *Z. tritici* strain with the slowest disease progression, 3D1, there were fewer DEGs in phases 1 and 2, but, in phase 3, there were about 18,000 DEGs, indicating that wheat responds to the 3D1 strain much later compared with the other strains. We conclude that the different number of DEGs in the different strain infections largely reflects differences in virulence and rates of disease progression among the pathogen strains. The large proportion of downregulated genes in phase 3 is consistent with the fact that the infected leaves were mostly dead at 28 dpi.

Both RD and non-RD RLKs play important roles in the wheat response to pathogen infection.

We hypothesized that the genes that show the largest changes in expression levels between different phases of the infection cycle play the greatest role in the host response to the fungal infection. We focused on the top 200 Gene Ontology (GO)-annotated DEGs for all conditions in the timecourse analysis and performed a GO enrichment analysis (Fig. 3B; Supplementary Fig. S2). Interestingly, the most upregulated genes in phase 1 and the most downregulated genes in phase 3 were enriched in serine/threonine kinases. We grouped these genes for all four strain infections in phase 1 (upregulated) and phase 3 (downregulated) separately

Fig. 1. Wheat transcriptome analysis. **A**, Mapping of transcriptome data to the wheat genome. The x axis shows the timepoints (days postinfection [dpi]) at which samples were taken, while the y axis indicates the percentage of Illumina reads that could be mapped to the wheat genome at the different timepoints. Different colors indicate different strains of *Zymoseptoria tritici* that were used to infect wheat. Bars show standard errors. **B**, Hierarchical clusters of wheat transcriptomes in the infected samples. Samples in the green rectangle were considered in the asymptomatic phase, while samples in the orange rectangle were considered in the acute phase.

(Supplementary Table S2). These genes encode both RD and non-RD RLKs, which indicate the essential functions of both RLKs in the defense response of wheat (Fig. 3C). Because non-RD RLKs were already shown to act as PRRs to recognize pathogens, we postulate that the strong upregulation of non-RD RLKs indicates that the plant recognized the pathogen and initiated a defense response in phase 1. Besides non-RD RLKs, we found that several types of RD-RLKs are also induced following infection.

To gain a better understanding of the kinds of RLKs involved in wheat defense responses, we generated phylogenetic trees for the RLK genes identified in phases 1 and 3 (Fig. 3D). We found several different types of RLKs that were upregulated in phase 1 and downregulated in phase 3, consistent with the previously described functions of RLKs in plant defense (Decreux and Messiaen 2005; Lannoo and Van Damme 2014; Nasrallah et al. 1988; Shiu and Bleecker 2003; Takemoto et al. 2000; Wagner and Kohorn 2001). These RLKs included LRR domains-RLKs, wall-associated RLKs, lectin L-type RLKs, and S-receptor-like serine/threonine protein kinases. Interestingly, the RD and non-RD

RLKs were scattered across the phylogenetic trees. In each RLK clade, we found both RD and non-RD RLKs. The RD RLK gene *Traes_5BL_2AFC8B15* is particularly interesting because its homolog was predicted to have an antifungal function in ginkgo, which suggests it may play an important role in the wheat-*Z. tritici* interaction. This gene was sharply upregulated during phase 1 in all strain infections and sharply downregulated during phase 3 in the 3D7 and 1A5 infections. In the more virulent 3D7 and 1A5 infections, *Traes_5BL_2AFC8B15* was expressed at a higher level, suggesting that it may play an important role during the asymptomatic and transition phases (Supplementary Fig. S3).

The expression of GTPase genes also changed during the different stages of infection, being downregulated in phase 2 and upregulated in phase 3. There were 27, 18, 27, and 28 genes encoding GTPases upregulated in phase 3 in the 3D7, 3D1, 1A5, and 1E4 infections, respectively. Thirteen of these GTPases were shared by all strain infections. In phase 2, there were only five to 15 GTPase genes downregulated in 3D7, 1A5, and 1E4 infections but none were downregulated in 3D1 infections. Two

Fig. 2. Strain-specific defense responses in wheat infected by *Zymoseptoria tritici*. **A**, Differentially expressed genes between pairs of strain infections at each timepoint. Blue shows the number of downregulated genes, while red shows the number of upregulated genes. **B**, Expression of known defense-related genes in each strain infection based on maximum expression of each gene across the disease cycle. The expression values are shown as $\log_2$ -transformed normalized counts per million reads.

Fig. 3. Timecourse transcriptome analysis of wheat. **A**, Numbers of differentially expressed wheat genes (DEGs) resulting from infection by four pathogen strains across the disease cycle. Blue indicates downregulated DEGs and red indicates upregulated DEGs in every phase. **B**, Significantly enriched Gene Ontology (GO) terms of the top 200 upregulated annotated genes in phase 1. The x axis indicates the $\log_{10}$ -transformed values of adjusted P values, while the y axis indicates the GO terms. Different colors represent different strain infections. The GO terms of protein serine/threonine kinase activity are highlighted with pink rectangles. **C**, Multiple alignment of receptor like kinase (RLK) genes among the top 200 upregulated genes for any strain in phase 1. The arginine aspartate (RD) domain is marked with a pink rectangle. All the non-RD RLKs are in blue. RD-minus RLKs are in green. **D**, Phylogenetic tree of RLKs in the top 200 upregulated genes for any strain in phase 1. The different colors indicate the predicted domains of proteins encoded by these genes, based on the blast analysis. The blue stars indicate the non-RD RLKs.

GTPase genes in 3D7 infections and three GTPase genes in 1E4 infections were downregulated in phase 2 and upregulated in phase 3. GTPases were shown to be involved in plant immunity by providing a switch at the binding site of G proteins, which is likely to trigger signal transduction and induction of downstream immune responses (Kawano et al. 2014; Zhang et al. 2012). A special type of GTPase was shown to affect leaf senescence (Kwon et al. 2009). Here, we find that GTPase genes were differentially expressed during the transition period and during saprotrophic growth of the pathogen, indicating an important role for GTPases during the acute disease stage.

Wheat recognizes *Z. tritici* during the latent period and activates its defense responses.

To identify genes that are conserved in wheat's response to *Z. tritici*, we studied the top DEGs shared by all strain infections in each phase. We defined the shared top DEGs at each phase as follows: i) genes were in the top 200 DEGs in at least two strain infections, ii) genes were also in the top 250 DEGs in all four strain infections. Following this strategy, we compiled a list of the shared top DEGs for all phases of the disease cycle. To infer possible functions of these genes, we identified their closest homologs in *Arabidopsis* and rice.

In phases 1 and 3, a large fraction (101 and 121 genes with GO annotations) of the top DEGs were shared by all four strain infections (Table 2). Most of the shared upregulated top DEGs at phase 1 were predicted to encode RLKs (Fig. 4A), which were significantly enriched in the GO analysis (Supplementary Table S4). Of these, 13 RLKs have RD domains and three have non-RD domains. Six additional genes encoded resistance gene analogs. There were two HR-related genes. One was an HR-like lesion-induced gene (Traes_4AL_708E7D22B) and the other was a gene induced during the HR (Traes_1BL_6D953AB6D). These findings strongly suggest that, from 7 to 12 dpi, the plants recognized all four pathogen strains and triggered both PTI and ETI (or ETD), causing an HR-like response to resist the infection. An HR-like cell death was described earlier during the compatible interaction with *Z. tritici*, and it was suggested that this allows the pathogen to transition to the necrotrophic stage of growth (Keon et al. 2007).

To further investigate the defense response during early stages of the infections, we compared the genes that showed the greatest differential expression between the 3D1 infection and the other three infections at 7 dpi. Because of the delayed disease progression in 3D1, these DEGs may be conserved in all strain infections and, thus, represent the early defense response of wheat.

At 7 dpi, there were 199 DEGs between the 3D1 infection and the other three infections, of which 98 were upregulated and 101 were downregulated in 3D1 infections. The upregulated genes at 7 dpi included four genes related to cysteine synthase (Fig. 4B). Cysteine is the metabolic precursor of essential antioxidants and many defense compounds, which makes cysteine a major contributor to the plant defense system (Alvarez et al. 2012; Romero et al. 2014). Other defense-related genes were also upregulated in the early stage of infection, including 24 RLKs (seven of which

are non-RD RLKs), two resistance genes, and three PR genes (Fig. 4C). The upregulation of defense-related genes indicates that wheat recognizes *Z. tritici* and activates its defenses by 7 dpi, during the latent period when no symptoms of infection are yet visible.

Genes associated with senescence exhibit complex regulation during a *Z. tritici* infection.

Many genes related to senescence are significantly upregulated during phase 1 (Fig. 4A). These genes encode cytochrome P450s, ethylene responses, glutathione *S*-transferase, F-box protein domains, MYC-type helix-loop-helix domains, and WRKY transcription factors. These upregulated genes suggest that wheat undergoes a senescence-like process during the early stage of the infection cycle. On the other hand, five senescence-associated genes (SAG) were downregulated at 7 dpi, suggesting that processes that delay senescence also are activated early in the infection cycle (Fig. 4C).

The expression of RLK genes and disease resistance genes dropped dramatically during phase 3 (Fig. 4A). By 28 dpi, the leaves were mainly completely infected and strongly necrotic. Three genes involved in wound responses were significantly downregulated in phase 3, suggesting that the plant is losing its ability to repair itself. The largest groups of shared upregulated genes during phase 3 were predicted to encode heat shock proteins (15 genes), ubiquitin expression proteins (four genes), tubulin (six genes), and actin (six genes) (Fig. 4A). These genes all play essential roles in plant senescence (Häffner et al. 2015; Miller et al. 1999; Sherman 2010), indicating that *Z. tritici* specifically triggers senescence late in the infection cycle to promote the death of the host cells.

We identified 142 wheat genes that have SAG homologs in *Arabidopsis*. These can be separated into two distinct clusters, namely SAGs that are upregulated in phase 1 and SAGs that are upregulated late in the infection cycle (Fig. 5). Corresponding to the findings above, this two-wave regulation of SAGs suggests that senescence plays important roles during both early and late stages of the infection cycle.

DISCUSSION

When confronted by genetically diverse pathogen populations, identical plants may display different responses, resulting from the unique cocktail of effectors produced by each pathogen strain. We performed a comparative transcriptomics analysis for a wheat cultivar infected by four genetically distinct strains of *Zymoseptoria tritici* to differentiate conserved host responses and strain-specific host responses to STB infection. By analyzing the dynamics of gene expression across the entire infection cycle for four pathogen strains, we deciphered the complex interplay between host and pathogen responses. One major finding was strong evidence for recognition of the pathogen and activation of host defenses during the extended asymptomatic phase of infection. We also found different transcriptional responses of wheat to the different pathogen strains.

Defense-related genes are upregulated during the asymptomatic latent period.

A wheat plant infected by *Z. tritici* experiences a prolonged latent period lasting 9 to 14 days, during which the plant remains asymptomatic while the pathogen grows its hyphae throughout the apoplast. Earlier studies suggested that *Z. tritici* escaped recognition by the plant during this latent period (Goodwin et al. 2011). Our data indicate that wheat had already recognized the fungus and initiated a defense response by 7 dpi, well within the typical latent period. Several genes involved in pathogen response, including genes encoding RLKs, PR proteins, and cysteine biosynthesis (Alvarez et al. 2012;

Table 2. Number of shared top differentially expressed genes in each phase of infection

Phases	With GO ^a	Without GO
Phase 1 upregulated	73	64
Phase 1 downregulated	28	33
Phase 2 upregulated	2	4
Phase 2 downregulated	2	4
Phase 3 upregulated	97	53
Phase 3 downregulated	24	24

^a GO = Gene Ontology.

Romero et al. 2014), were upregulated by 7 dpi, leading us to conclude that wheat defense responses are already active at 7 dpi. The decreased expression of photosynthesis-related genes suggests that the pathogen was already affecting expression of plant genes by 7 dpi. Genes involved in methylation also changed their expression by 7 dpi, suggesting that epigenetic changes may also take place during the asymptomatic phase of infection (Fig. 4C).

Previous studies showed that several types of defense-related genes change their expression during the transition from asymptomatic infection to necrotrophy (Rudd et al. 2015), a period we called phase 1. We found that a large fraction of the top 0.2% of significantly upregulated genes in phase 1 encoded various forms of both RD and non-RD RLKs, including LRR RLKs, nucleotide-binding-ARC domains, S-receptor RLKs, lectin-L RLKs, and wall-associated RLKs. We interpret this to mean that wheat cells near the spreading *Z. tritici* hyphae begin to recognize the pathogen, probably mediated through non-RD RLKs, and initiate a defense response in which RD RLKs likely play downstream roles.

We conclude that, although visible symptoms of infection are absent, there is already a plethora of interactions underway between wheat and *Z. tritici* during the asymptomatic phase of infection. This differs considerably from what has been

reported for endophytic fungi, which also grow asymptotically within their hosts. In a recent study of the transcriptome response of *Lolium arundinaceum* to the fungal endophyte *Epichloë coenophiala*, it was observed that the plant shows remarkably few changes at the transcriptome level in the presence of the fungus (Dinkins et al. 2017). The *Lolium* study also found that genes encoding WRKY transcription factors associated with pathogen defense responses were highly represented among the DEGs when the endophyte was present, suggesting that WRKYs may play a role in symbiotic stability. We also found genes encoding WRKY transcription factors upregulated at 7 dpi (Fig. 4A), and we hypothesize that they may play a similar role during the latent phase of *Z. tritici*.

After the pathogen switches from asymptomatic infection to necrotrophic infection, the plant loses its ability to defend itself against the pathogen. In the acute stage of disease development, most RLKs and resistance gene analogs were significantly downregulated, suggesting a collapse of the plant defense system.

Differential response of wheat to different fungal strains.

Previous infection experiments showed clear phenotypic differences across the infection cycle for the four strains used in

Fig. 4. Defense response in the early and late phase of the infections. **A**, Predicted functions of shared top differentially expressed genes (DEGs). The y axis indicates the counts of genes predicted to encode each protein specifically shown on the x axis. Only annotations with gene counts >1 were kept. The red arrows represent the genes encoding proteins involved in plant senescence. **B**, Significant Gene Ontology enrichments of DEGs in 3D1 infections compared with the other three strain infections at 7 days postinfection (dpi). **C**, Defense response at 7 dpi. The y axis indicates the counts of genes predicted to encode each protein specifically shown on the x axis. Only annotations with gene counts >1 were kept. The red arrows represent the genes encoding proteins involved in plant senescence.

our study (Palma-Guerrero et al. 2017). This was reflected in the number of RNA-Seq reads that could be mapped to the wheat genome; we could consistently map higher numbers of wheat reads for the slower growing strains, and we found that the DEGs at different timepoints differed strongly between strains. We also detected DEGs between strain infections at every timepoint, with the 3D1 strain being slowest to induce a defense response. The highest degree of asynchronicity in disease response was at 14 dpi, when the pathogens were in the process of switching from asymptomatic growth to necrotrophic growth. Thus, the phenotypic differences in disease progression between the *Z. tritici* strains were clearly reflected in the wheat gene expression data. Apart from the asynchronicity among strains, we also found that the maximum expression of some defense-related genes, including *PR-1* and *PR-3*, a cysteine-protease, and *MLO* differed among the four strain infections, suggesting that different responses are induced by different strains of the same pathogen. Interestingly, XIP-1 showed higher expression for the least virulent strain, 3D1. Endoxylanase inhibitors like XIP-I and TAXI are specific inhibitors of bacterial and fungal endoxylanases and are thought to be important contributors to plant defenses against pathogenic fungi (Juge 2006). However, given that the higher expression for this gene in the 3D1 strain infection was only observed at 28 dpi, a timepoint when the plant cells were mostly

dead, this higher expression was probably not contributing to wheat defense against the pathogen.

Senescence-related processes play an important role across the entire infection cycle.

The relationship between senescence and resistance in necrotrophic and biotrophic interactions was described earlier (Häffner et al. 2015). In general, host plants use senescence to fend off biotrophic pathogens, while necrotrophic pathogens deliberately induce senescence to kill their host plants. Interestingly, our study revealed an even more complex pattern, possibly because *Z. tritici* acts like a hemibiotrophic pathogen.

Based on our transcriptome analyses, we postulate that there are two waves of senescence-related processes occurring during an infection cycle (Figs. 4A and C and 5). The senescence processes occurring during the asymptomatic “biotrophic” phase may be triggered by pathogen recognition and used by the plant as an early defense strategy. These early response SAGs were upregulated in infected plants at 7 dpi and were then downregulated at later stages of infection. As a key marker for senescence, the downregulation of photosynthesis genes in phase 1 implies that senescence-related processes occur during the asymptomatic phase of disease development. We interpret this senescence-like process as part of the plant strategy to resist biotrophic pathogens. A good example of this is shown by

Fig. 5. Expression of senescence-associated genes (SAGs). The SAGs were clustered and filtered into two groups (early or late response). Genes with log₂-transformed counts per million reads under -4 were considered as not expressed and are marked with blue. Yellow to red indicate gene expression values from low to high.

the wheat gene *Lr34*, which provides durable resistance to fungal pathogens by stimulating senescence-like processes in wheat (Krattinger et al. 2009). Interestingly, we also found that five SAGs were downregulated after pathogen recognition at 7 dpi. We hypothesize that this downregulation was induced by the pathogen to slow down the senescence process and keep plant cells alive. We propose that these conflicting patterns in the regulation of SAGs reflect antagonism between plant and pathogen at the beginning of the infection cycle, with both plant and pathogen regulating senescence processes for their own interests.

Late in the infection cycle, at 28 dpi, *Z. tritici* is in the saprotrophic phase, converting wheat biomass to fungal biomass while building many fruiting bodies, and disease symptoms are acute. Consistent with the observed symptoms, we found that most genes involved in senescence were significantly upregulated during phase 3 (Fig. 4A). Heat-shock proteins are mainly involved in the stress response, which would include the senescence process (Al-Whaibi 2011; Sherman 2010). Upregulation of actin, tubulin, and ubiquitin genes also reflects the senescence process at this timepoint (Belknap and Garbarino 1996; Breeze et al. 2011a). The upregulation of actin and tubulin during plant senescence was observed previously, and it was suggested that plant autophagy requires activity of microtubules and microfilaments, explaining the increased expression of actin and tubulin late in senescence when the autophagic degradation of cellular compounds is underway (Breeze et al. 2011b). Two waves of H₂O₂ production by the plant were reported during a compatible interaction in susceptible cultivars challenged by *Z. tritici*, with a smaller one early in the infection cycle and a stronger one during the onset of necrosis. The latter wave could be associated with the HR response (Shetty et al. 2003). This regulation of HR responses fits with the senescence processes reported here. Taken together, these data indicate that senescence plays an important role in the wheat response to *Z. tritici* infection.

Conclusion.

These experiments provided important insight into how wheat responds to an infection by *Z. tritici*. Some defense responses were conserved across all four pathogen strains, while other responses were highly strain-specific. Future studies should include additional wheat cultivars and *Z. tritici* strains to identify “master genes” that are central to the defense response of wheat against this pathogen.

MATERIALS AND METHODS

mRNA sequencing data and alignment to wheat genome.

The experimental design included leaves from wheat cultivar Drifter infected with four different strains of *Z. tritici*, namely, 3D7, 3D1, 1A5 and 1E4, under common garden conditions, in the same greenhouse chamber under uniform conditions, as previously described in a previous paper that described the fungal transcriptome for the same samples (Palma-Guerrero et al. 2017). RNA was extracted at 7, 12, 14, and 28 dpi, covering the asymptomatic (i.e., biotrophic), necrotrophic, and saprotrophic phases of the *Z. tritici* infection cycle. To serve as controls, RNA was extracted from uninfected plants (mock infections) growing in the common garden at 3 and 24 dpi. All the infected and mock samples were collected at the same time of day to avoid photo-period effects associated with different times during a day. We generated three biological replicates for each treatment.

Methods used for mRNA sequencing were described in an earlier publication oriented around comparisons of the fungal transcriptomes in the four strains (Palma-Guerrero et al. 2017). The same datasets (National Center for Biotechnology Information (NCBI) SRA SRP077418) were used in this study. To align the reads to the wheat genome, we first used Trimmomatic

v0.33 (Bolger et al. 2014) to trim the sequencing adapters and remove low-quality reads, using the following command: ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 LEADING:10 TRAILING:10 SLIDINGWINDOW:4:15 MINLEN:50. The trimmed reads were aligned against a wheat pseudo-genome sequence, which was produced by concatenating predicted coding sequences (CDS). The wheat CDS were obtained from the Wheat Portal's Seq Repository (International Wheat Genome Sequencing Consortium (IWGSC) 2014). Individual CDS were concatenated into pseudo-chromosomes in which individual CDS were separated from each other by a 150-bp random sequence. We used STAR (Dobin et al. 2013) to perform the alignment using the command: `–outFilterMultimapNmax 5 –outFilterMatchNminOverLread 0.2 –outFilterScoreMinOverLread 0.2 –outFilterMismatchNmax 5 –outFilterMatchNmin 50 –sjdbOverhang 125`.

Transcriptome analyses.

HTSeq v0.6.0 (Anders et al. 2015) was used to obtain counts for every gene with a unique alignment. The parameter “–s reverse” was adjusted to use strand information. Hierarchical clusters were produced with the “hclust” function in R, using the log-transformed gene counts generated in the R package DESeq2 v1.11.1 (Love et al. 2014). We applied the TMM (trimmed mean of M values) method (Robinson and Oshlack 2010) implemented in Bioconductor EdgeR v3.12.0 (Robinson et al. 2010) to normalize gene counts. The TMM-normalized CPM values were log₂-transformed and were used to generate heatmaps with the R package pheatmap (Kolde 2015). ggplot2 (Wickham 2009) was used to generate the other plots.

Analysis of DEGs.

Genes without at least 0.5 CPM in at least three samples were removed before conducting pairwise analyses of differential gene expression (Anders et al. 2013). We used the exactTest function in the edgeR v3.12.0 package (Robinson and Smyth 2008) to identify DEGs under two conditions. The Benjamin-Hochberg false discovery rate correction was used to adjust *P* values based on the exact Fisher test.

Functional annotations and phylogenetic trees.

TopGO v2.22.0 (Rahnenfuhrer 2010) was used to perform a GO enrichment analysis based on GO annotations retrieved from Ensemble. To complement the functional annotations of wheat genes, we manually annotated some of the genes among the top DEGs in one or more of the analyses, based on the rice and *Arabidopsis* gene annotations. The wheat genes with senescence-associated roles were also manually annotated, based on *Arabidopsis* gene annotations. GO term sizes were set to contain at least five annotated genes. The BLAST command line application was installed from NCBI (Altschul et al. 1990). The predicted protein sequences of rice (version 6) were obtained from the PlantGDB database, while *Arabidopsis* protein sequences (version 10) were obtained from The Arabidopsis Information Resource database. Clustal X (Larkin et al. 2007) was used to perform multiple alignments of sequences for specific proteins. These aligned sequences were used to produce phylogenetic trees with a maximum likelihood approach implemented in the software MrBayes (Huelsenbeck and Ronquist 2001; Larget and Simon 1999). We used all the protein models available in MrBayes and a reversible jump Monte Carlo Markov chain (Larget and Simon 1999). The heterogeneity of substitution rates among different sites was modeled using a gamma distribution.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The Arabidopsis Information Resource: www.arabidopsis.org
 PlantGDB database: plantgdb.org/OsGDB
 Wheat Portal Seq Repository database:
wheat-urgi.versailles.inra.fr/Seq-Repository