ORIGINAL RESEARCH

Examining the Transcriptional Response in Wheat *Fhb1* Near-Isogenic Lines to *Fusarium graminearum* Infection and Deoxynivalenol Treatment

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Abstract

Fusarium head blight (FHB) is a disease caused predominantly by the fungal pathogen *Fusarium graminearum* that affects wheat and other small-grain cereals and can lead to severe yield loss and reduction in grain quality. Trichothecene mycotoxins, such as deoxynivalenol (DON), accumulate during infection and increase pathogen virulence and decrease grain quality. The *Fhb1* locus on wheat chromosome 3BS confers Type II resistance to FHB and resistance to the spread of infection on the spike and has been associated with resistance to DON accumulation. To gain a better genetic understanding of the functional role of *Fhb1* and resistance or susceptibility to FHB, we examined DON and ergosterol accumulation, FHB resistance, and the whole-genome transcriptomic response using RNA-seq in a near-isogenic line (NIL) pair carrying the resistant and susceptible alleles for *Fhb1* during *F. graminearum* infection and DON treatment. Our results provide a gene expression atlas for the resistant and susceptible wheat–*F. graminearum* interaction. The DON concentration and transcriptomic results show that the rachis is a key location for conferring Type II resistance. In addition, the wheat transcriptome analysis revealed a set of *Fhb1*-responsive genes that may play a role in resistance and a set of DON-responsive genes that may play a role in trichothecene resistance. Transcriptomic results from the pathogen show that the *F. graminearum* genome responds differently to the host level of resistance. The results of this study extend our understanding of host and pathogen responses in the wheat–*F. graminearum* interaction.

FUSARIUM HEAD BLIGHT, which is caused by the fungal pathogen *Fusarium graminearum*, affects small grains around the world. This disease results in bleaching and necrotic lesions on the spikelets and trichothecene mycotoxin accumulation, which decreases grain yield and quality in wheat (McMullen et al., 1997; Goswami and Kistler, 2004). Deoxynivalenol, the most common trichothecene that accumulates in *F. graminearum*-infected cereal grain, is deleterious to the health of humans and animals. Thus, the European Union and the Food and Drug Administration in the US have enacted polices to limit the amount of DON in food (Desjardins 2006; The European Commission, 2006). Accordingly, breeders have sought, with limited success, to develop FHB-resistant varieties.

Fusarium head blight infection occurs between flowering and the soft dough stages in wheat when *F.*

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graminearum spores infect the host through the stomata, wounds, between the lemma and palea, or through areas with thinner cell wall surfaces such as the base of glumes (Leonard and Bushnell, 2003). Symptoms of infection can be seen 3 d after inoculation and include necrosis, water-soaked lesions, and bleached tissue (Kang and Buchenauer, 2000; Trail, 2009; Leonard and Bushnell, 2003). Deoxynivalenol, which is a virulence factor, suppresses cell wall thickening at the rachis nodes and inhibits protein synthesis (Desjardins and Hohn, 1997; Ilgen et al., 2009; Jansen et al., 2005). Loss-of-function mutations in the *TRI5* gene (the first step in the trichothecene biosynthesis pathway) in *F. graminearum* result in the lack of DON production and reduced virulence (Proctor et al., 1995). *TRI5* mutants are restricted at the thickened cell walls of the rachis node, leading to reduced spread of infection, demonstrating the importance of trichothecenes during infection (Jansen et al., 2005). Using a *F. graminearum* strain with a green fluorescent protein (GFP) fused to a *TRI5* promoter resulted in high levels of *TRI5* induction at the rachis node and lower levels in the rachis, indicating the rachis node is important in wheat defense (Ilgen et al., 2009).

Many quantitative trait loci (QTL) have been identified for FHB resistance in wheat (Liu et al., 2009). The major QTL on chromosome 3BS (*Fhb1*) is derived from the Chinese cultivar Sumai 3 and explains up to 60% of the phenotypic variation in FHB resistance. *Fhb1* contributes to Type II resistance (resistance to the spread of infection) and is a source of resistance for many wheat breeding programs (Anderson et al., 2001; Bai et al., 1999; Buerstmayr et al., 2002; Kolb et al., 2001; Liu et al., 2006; Pumphrey et al., 2007; Waldron et al., 1999; Zhou et al., 2002). The *Fhb1* locus has been fine-mapped to a 1.2-cM region between the markers *STS3B-189* and *STS3B-206* (Liu et al., 2006). *Fhb1* is associated with DON resistance and the conversion of DON to DON-3-*O*-glucoside, indicating that *Fhb1* may encode a uridine diphosphate (UDP)-glucosyltransferase or regulate a similar enzyme (Lemmens et al., 2005). Notably, UDP-glucosyltransferase in *Arabidopsis thaliana* (L.) Heynh., barley (*Hordeum vulgare* L.), *Brachypodium distachyon* (L.) P.Beauv., rice (*Oryza sativa* L.), and sorghum [*Sorghum bicolor* (L.) Moench] have been isolated that exhibit resistance to DON (Poppenberger et al., 2003; Schweiger et al., 2010; 2013a; Shin et al., 2012). Although *Fhb1* has received extensive study and the current data point to a trichothecene detoxification mechanism, to date, the gene that underlies *Fhb1* is unknown.

The host response in wheat to *F. graminearum* infection has been an active area of research over more than a decade. Defense, stress response, and pathogenesis-related genes are often reported to be upregulated during *F. graminearum* infection of wheat spikes (Bernardo et al., 2007; Golkari et al., 2007; Gottwald et al., 2012; Kong et al., 2005; 2007; Pritsch et al., 2000; 2001; Yu and Muehlbauer, 2001; Zhou et al., 2005; Foroud et al., 2012). In addition, genes involved in ethylene and jasmonate

signaling have been identified and, in some cases, associated with the resistant response (Li and Yen, 2008). These studies have provided an overview of the host response to *F. graminearum* infection but do not compare related genotypes (e.g., NILs) carrying a resistant or susceptible allele for a resistant locus. Several recent studies have compared the host response in NIL pairs carrying resistant or susceptible alleles for major FHB-resistant QTL (Ding et al., 2011; Gunnaiah et al., 2012; Jia et al., 2009; Kugler et al., 2013; Schweiger et al., 2013b; Steiner et al., 2009; Xiao et al., 2013). These studies have identified numerous genes that are differentially expressed between the resistant and susceptible genotype but a comprehensive understanding of host resistance has been elusive.

Gene expression patterns in *F. graminearum* during infection have also been studied in barley and wheat using an *F. graminearum* Affymetrix GeneChip (Güldener et al., 2006). During infection in barley, 7132 probe sets were detected at one or more time points (24, 48, 72, 96, and 144 h after inoculation) after infection. Two classes of gene transcripts that were identified that are likely to be involved in infection encoded cell wall-degrading enzymes and trichothecene biosynthetic enzymes (Güldener et al., 2006). Similar to the case in barley, during infection on wheat, *F. graminearum* genes that encode proteins for plant cell wall degradation or modification and trichothecene biosynthesis were expressed (Lysøe et al., 2011; Zhang et al., 2012). These studies have identified the *F. graminearum* genes that are expressed during infection of barley and wheat; however, the gene expression patterns on resistant and susceptible host genotypes are unknown.

The overall aim of this study was to examine gene expression patterns in wheat and *F. graminearum* in an *Fhb1* NIL pair carrying either the resistant or susceptible allele. The three specific objectives were to: (i) compile a gene expression atlas of the wheat–*F. graminearum* interaction, (ii) identify *Fhb1*-responsive genes during *F. graminearum* infection in the spikelets and rachis, and in the spikelets after DON treatment, and (iii) identify *F. graminearum* genes that are differentially expressed during *F. graminearum* infection of the *Fhb1* NIL pair.

Materials and Methods

Plant and Fungal Materials

The wheat NIL pair 260-1-1-2 (*Fhb1*+) and 260-1-1-4 (*Fhb1*–) was used for this study. *Fhb1*+ carries the resistant allele and *Fhb1*– carries the susceptible allele for *Fhb1*. The development of the NIL pair was described previously in Pumphrey et al. (2007). Briefly, the FHB-resistant line Sumai 3 and the moderately FHB-susceptible line 'Stoa' were crossed and a recombinant inbred line population was derived. An FHB-resistant recombinant inbred line (RIL63) from this population was crossed with an FHB-susceptible line, 'MN97448'. An F7-derived line that was heterozygous for the *Fhb1* region was selfed and progeny that were homozygous for

Fhb1+ and *Fhb1–* were derived and referred to as a *Fhb1* NIL pair. The *F. graminearum* isolate Butte 86ADA-11 (supplied by R. Dill-Macky, University of Minnesota) that produces DON and 15-acetylated DON was used for *F. graminearum* inoculations (Evans et al., 2000).

Growth Conditions

Seed for the NIL pair was planted at five seeds per square pot (16.24 cm along each side) in Sunshine MVP potting mix (SunGro Horticulture, Agawam, MA) and grown in a growth chamber under 16 h of light at 20°C and 8 h of darkness at 18°C. Light intensity in the growth chamber at pot level was 170 ± 20 μE m^{-2}s^{-1}. The plants were fertilized with Osmocote Plus 15–9-12 (5 mL) (Scotts Company, Marysville, OH) 1 wk after planting and watered daily.

Experimental Design

To examine the transcriptomes of wheat and *F. graminearum* during infection and the wheat transcriptome after DON treatment, RNA-seq experiments were conducted on spikelet and rachis tissue after inoculation with *F. graminearum* and on spikelet tissue after DON and water treatment. For the spikelet and rachis tissue from *F. graminearum*-inoculated plants, four central spikelets on 10 spikes per genotype were inoculated at anthesis with 10 μL *F. graminearum* inoculum (100,000 macroconidia per mL in water) or 10 μL sterile water and sampled at 96 h after inoculation (hai). For the spikelet samples, the rachis was not removed. For the rachis samples, inoculated spikelets were removed immediately before sampling and the exposed rachis was sampled at 48 and 96 hai (Supplemental Fig. 1). For the DON- and water-inoculated plants, four central spikelets on 10 spikes per genotype per treatment were inoculated at anthesis with 2 μg DON 10μL^{-1} or 10 μL sterile water and the spikelets and the associated rachis were sampled at 12 hai. For all inoculations, spikes were covered with a small clear plastic bag until sampling. Three biological replications with a completely random design were conducted for each experiment.

Deoxynivalenol concentration was determined on the *F. graminearum*- and water-inoculated spikelet samples at 96 hai, *F. graminearum*-inoculated rachis samples at 48 and 96 hai and DON- and water-inoculated spikelet samples at 12 hai. Ergosterol concentration was determined on the *F. graminearum* and water-inoculated spikelet samples at 96 hai and *F. graminearum*-inoculated rachis samples at 48 and 96 hai. These experiments and the samples from each experiment are summarized in Supplemental Table 1.

To assess the disease phenotypes of the NIL pair, three replications of four central spikelets on five spikes per genotype per time point were inoculated at anthesis with 10 μL *F. graminearum* inoculum (100,000 macroconidia per mL in water). Spikes were covered with a small clear plastic bag for 48 h. Deoxynivalenol and ergosterol concentration was determined for the spikelet and associated rachis tissue from *F. graminearum*-inoculated

Table 1. Differentially expressed genes (DEGs) of wheat between the *Fhb1* near-isogenic line pair carrying either the resistant or susceptible allele.

	Sample		Number of DEGs			
Tissue	Inoculum	Timepoint	Up *Fhb1+*[†]	Only *Fhb1+*[‡]	Up *Fhb1–*[§]	Only *Fhb1–*[¶]
Spikelet	*F. graminearum*	96 hai	1097	442	1660	644
Rachis	*F. graminearum*	96 hai	158	83	472	57
Spikelet	DON[††]	12 hai	136	37	299	63
Spikelet	Water	12 hai	14	4	48	35

[†] Differentially expressed genes that were upregulated in the resistant genotype.

[‡] Differentially expressed genes that showed expression only in the resistant genotype.

[§] Differentially expressed genes that were upregulated in the susceptible genotype.

[¶] Differentially expressed genesthat showed expression only in the susceptible genotype.

[††] hai, h after inoculation; DON, deoxynivalenol

spikelets sampled at 1, 4, 7, 12, and 21 d after inoculation (dai). To assess disease severity, infected spikelets were counted at 7, 14, and 21 dai. Disease severity was reported by dividing the number of infected spikelets by the total number of spikelets (% infected spikelets).

Deoxynivalenol and Ergosterol Analysis

All DON and ergosterol analyses were performed by Yanhong Dong (University of Minnesota). Deoxynivalenol and ergosterol concentration were measured by gas chromatography–mass spectrometry (Dong et al., 2006; Mirocha et al., 1998). Deoxynivalenol and ergosterol concentrations were analyzed by multiple comparisons of means by Tukey's Honest Significant Difference method using the R statistics package (R Development Core Team, 2010).

RNA-seq

RNA was extracted for sequencing using the RNeasy Plant Mini Kit (QIAGEN, Valencia, CA) from each replication of the experiments described above. RNA from the three replications of the *F. graminearum*-inoculated spikelet samples at 96 hai were sequenced separately. RNA from the three replications of the *F. graminearum*-inoculated rachis samples at 96 hai and the DON- and water-inoculated spikelet samples at 12 hai were pooled for sequencing. RNA samples were submitted to the University of Minnesota Genomics Center for quality control, library creation, and sequencing. Sequencing was performed using the Illumina HiSeq 2000 (Illumina, Inc., San Diego, CA) to produce 100-bp paired-end reads with a ~200-bp insertion size. The average Q-score for all pass-filtered reads was above Q30 for all samples.

Per sample, RNA-seq reads were mapped on the chromosome-sorted whole-genome shotgun assembly (IWGSC, 2014) and the underlying gene annotation (version 1.0) was used to assign reads to the respective gene models (ftp://ftpmips.helmholtz-muenchen.de/plants/wheat/IWGSC/genePrediction_v1.0/survey_sequence_gene_models_MIPS_feb2013.zip, accessed 11 Nov. 2015). Where available, the corresponding mapped genes for

the version 2.1 gene models were also included. For mapping, the following programs and versions were used: Bowtie (version 2.0.6; Langmead et al., 2009), SAMtools (version 0.1.18; Li et al., 2009), and TopHat (version 2.0.7; Trapnell et al., 2009). Based on the summarized read counts per gene, differentially expressed genes (DEGs) of selected comparisons (i.e., resistant vs. susceptible genotypes) were calculated with Cuffdiff (version 2.1.1; Trapnell et al., 2010). A q-value (false discovery rate (FDR)-adjusted p-value) of <0.05 and a twofold change in FPKM value were used to classify DEGs. Supplemental Fig. 2 shows a schematic overview of the analysis pipeline.

RNA-seq reads for *F. graminearum*-inoculated spikelet and rachis samples were mapped using Bowtie (version 0.12.8) with default parameters against the *F. graminearum* coding sequences (CDS), taken from the file FGDB_v32.orf, found at ftp://ftpmips.gsf.de/fungi/Fusarium/FGDB/v32/ (accessed 19 Nov. 2015), a collection of 13,826 gene models. For mapping, Bowtie (version 0.12.8) was used with default parameters. Customized Python scripts were used to count the number of *F. graminearum* CDS and reads matching *F. graminearum* gene models. A q-value (FDR-adjusted p-value) of <0.05, a twofold change in FPKM value, and an FPKM value of at least 10 in one of the samples were used to classify DEGs.

Gene Ontology Enrichment Analysis

Gene Ontology annotations for wheat were taken from the wheat genome sequence version 1.0 (IWGSC, 2014). To test for enrichment, we applied hypergeometric tests using the GOstats package (Falcon and Gentleman, 2007), considering the Gene Ontology graph structure (conditional = TRUE) and keeping over-represented terms below an FDR-adjusted p-value of 0.05. Only terms linked to molecular functions and biological processes were considered. For *F. graminearum*, gene annotation and assignment to the Munich Information Center for Protein Sequences (MIPS) Functional Catalogue were as previously described (Güldener et al., 2006). Gene enrichments were categorized using the hypergeometric distribution as implemented at http://mips.helmholtz-muenchen.de/funcatDB/search_main_frame.html (accessed 19 Nov. 2015).

Results

Graphical Genotype of a *Fhb1* NIL Pair

A NIL pair carrying either the *Fhb1*-resistant or susceptible locus was previously developed through self-pollinating an F7-derived line that was heterozygous for the *Fhb1* locus and selecting homozygous resistant and susceptible progeny (Pumphrey et al., 2007). To assess the extent of the near-isogenicity of these lines and the introgressed *Fhb1* region, the NIL pair carrying either the *Fhb1*-resistant or susceptible allele was genotyped with simple sequence repeat (SSR) and single-nucleotide polymorphism (SNP) markers. The NIL pair was genotyped using four SSR markers in the 3BS *Fhb1*

region (*gwm533*, *gwm493*, *barc133*, and *barc87*) and 20 additional randomly selected SSR markers, one for each chromosome. The NILs were polymorphic for the *Fhb1* markers and monomorphic for the markers on the other 20 chromosomes. The lines were also genotyped using a Wheat iSelect SNP assay (Cavanagh et al., 2013; Fig. 1). Of the 6293 SNPs, 5934 were placed on the wheat map and only 74 were polymorphic between the NILs, indicating that the lines were 98.8% identical. Fourteen (18.9%) of the polymorphic SNPs mapped to chromosome 3BS spanning the *Fhb1* region and encompassing approximately 31.5 cM. Small blocks of polymorphic SNPs were also seen on chromosomes 1A, 1B, and 7D; an additional polymorphic SNP was found on both 3A and 3D. The polymorphic regions on chromosomes 1A and 1B spanned approximately 9 and 18 cM, respectively, and did not correspond to the QTL for FHB severity that have been mapped to chromosomes 1A and 1B (Buerstmayr et al., 2009). Additional major QTL regions that had been previously identified on chromosomes 5A and 6BS (*Fhb2*) (Buerstmayr et al., 1999; Cuthbert et al., 2007) were not polymorphic between the NILs.

Phenotypic Characterization of the *Fhb1* NIL Pair

To examine the phenotypic differences between the wheat *Fhb1* NIL pair carrying either the resistant (*Fhb1*+) or susceptible (*Fhb1*−) allele at the *Fhb1* locus, disease severity was measured on point-inoculated spikes at 7, 12, and 21 dai (Fig. 2A). An increase in infected spikelets was observed over time from 7 to 21 dai, with a greater severity seen in the susceptible genotype. At 21 dai, the percentage of infected spikelets was significantly different between the resistant (41.2% ± 11.2%) and susceptible (80.2% ± 8.3%) genotypes ($P \leq 0.05$). These results confirm previous studies showing that the *Fhb1*-resistant allele contributes to Type II resistance (Jia et al., 2009; Liu et al., 2006).

To further characterize the wheat *Fhb1* NIL pair, we analyzed DON and ergosterol concentration on spikelet and rachis tissue from *F. graminearum*-inoculated spikelets at 1, 4, 7, 12, and 21 dai (Fig. 2B,C). In both genotypes, DON was not detectable at 1 dai. Deoxynivalenol and ergosterol concentration increased in the inoculated spikelets and rachis from 1 to 21 dai in both genotypes (Fig. 2B,C). Deoxynivalenol concentration in both the resistant and susceptible genotypes was significantly greater in the spikelets than in the rachis at 7 dai ($p \leq 0.01$ and $p \leq 0.001$). Deoxynivalenol was also significantly greater in the spikelet than in the rachis at 12 dai in the susceptible genotype ($p \leq 0.001$). Similarly, ergosterol concentration was significantly greater in the spikelets than in the rachis at 7 dai for both genotypes ($p \leq 0.001$ for resistant and susceptible genotypes), and ergosterol was significantly greater in the spikelets than in the rachis of the susceptible genotype at 12 dai ($p \leq 0.001$). Comparing the resistant and susceptible genotypes, DON concentration was significantly greater in the susceptible genotype at 21 dai in both the spikelets ($p \leq 0.05$) and the rachis ($p \leq 0.01$). Additionally, ergosterol
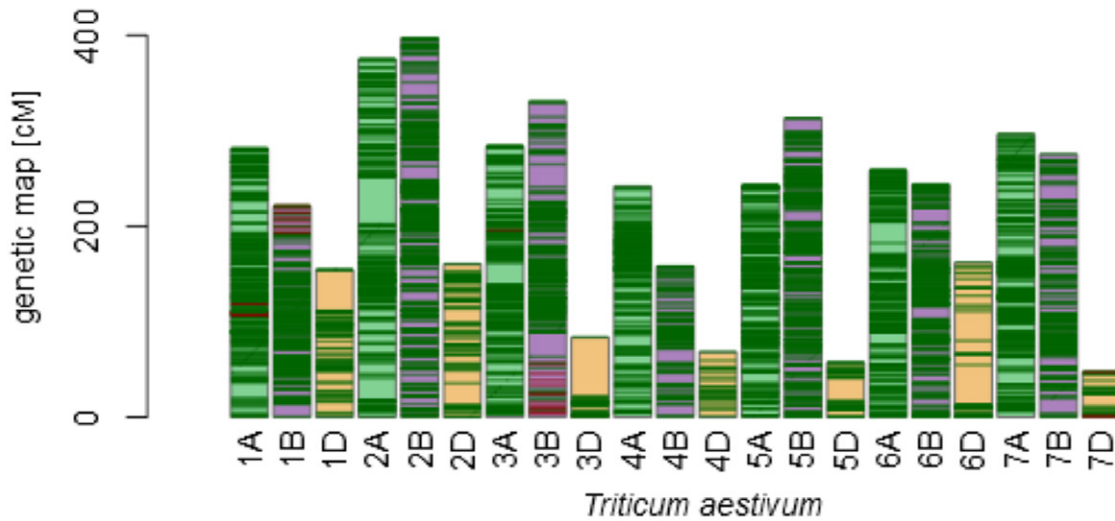
Fig. 1. Genetic characterization of the *Fhb1* near-isogenic line (NIL) pair in wheat. Graphical genotype for the NIL pair *Fhb1+* (*Fusarium* head blight-resistant) and *Fhb1–* (susceptible). Regions depicted in red contain single-nucleotide polymorphisms that are polymorphic between the NILs. Regions shown in dark green are not polymorphic; regions in light green, purple, and yellow represent the A, B and D genomes, respectively.
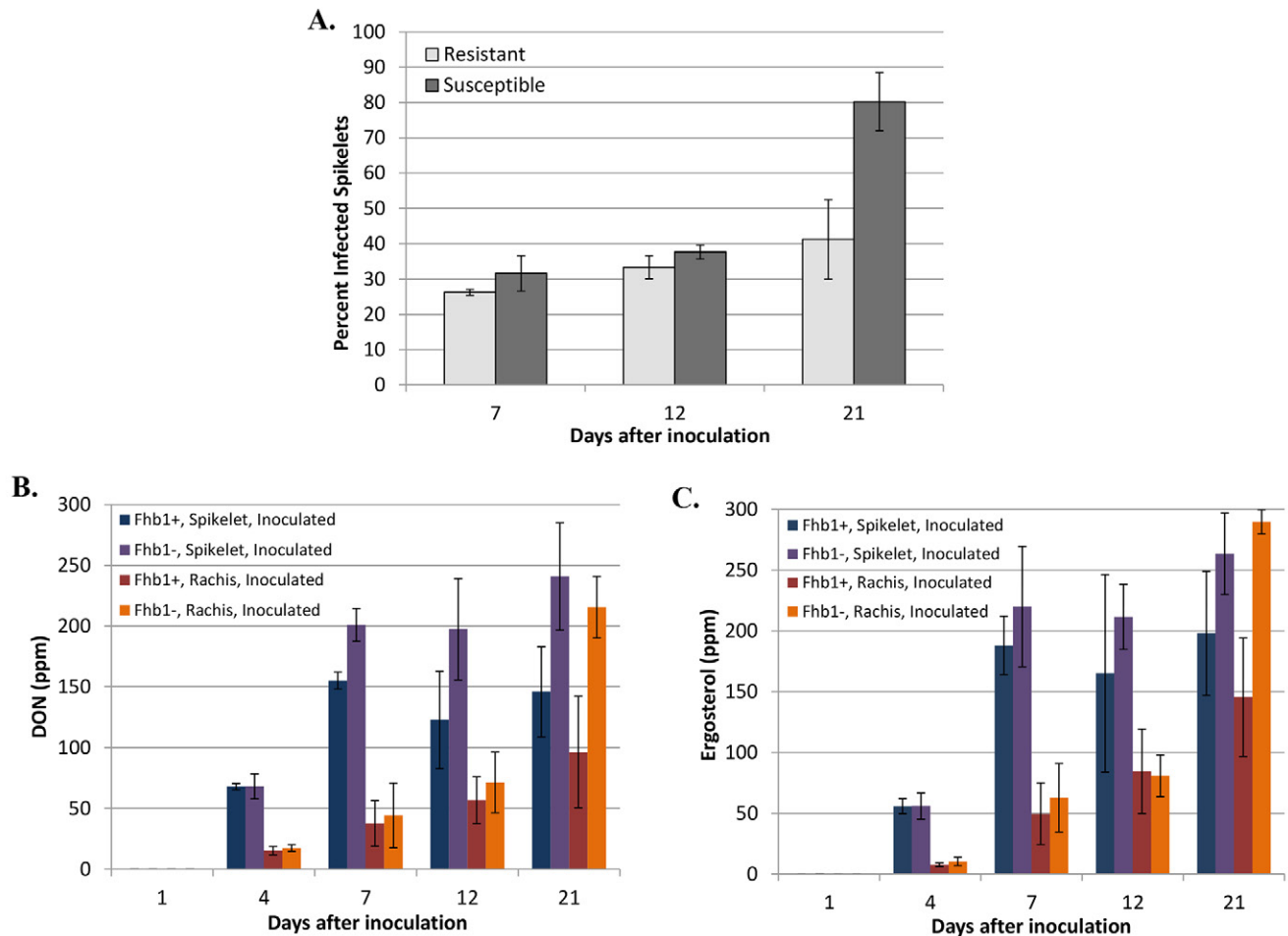

Fig. 2. Disease severity, deoxynivalenol (DON), and ergosterol levels in the *Fusarium graminearum*-infected wheat spikes for the *Fhb1* NIL pair carrying either the resistant or susceptible allele. A. Percentage of infected spikelets on *Fhb1+* (resistant) and *Fhb1–* (susceptible) at 7, 12, and 21 d after inoculation (dai). B. Deoxynivalenol concentration (ppm) of inoculated *Fhb1+* and *Fhb1–* spikelets and associated rachis at 1, 4, 7, 12, and 21 dai. C. Ergosterol concentration (ppm) of inoculated *Fhb1+* and *Fhb1–* spikelets and associated rachis at 1, 4, 7, 12, and 21 dai.

was significantly greater in the susceptible rachis than in the resistant rachis at 21 dai ($p \leq 0.001$). The increased levels of DON and ergosterol in the rachis in *Fhb1*– compared to *Fhb1*+ at 21 dai indicates that the rachis is a key tissue for conferring *Fhb1*-derived resistance and is consistent with the disease severity results (Fig. 2A).

Wheat Response to *F. graminearum* Infection

To compare the host response in plants carrying the resistant allele to those with the susceptible allele for *Fhb1*, we conducted RNA-seq on the *Fhb1* NIL pair inoculated with *F. graminearum*, DON, or water. Four sets of tissue samples were collected from both genotypes: spikelet samples from *F. graminearum*-inoculated spikelets at 96 hai (FgS96), rachis samples from *F. graminearum*-inoculated spikelets at 96 hai (FgR96), and spikelets sampled from DON (D12) and water-inoculated (W12) at 12 hai. To verify that these plants were inoculated with *F. graminearum* and DON and to determine if the samples were collected at a biologically appropriate time, we conducted DON and ergosterol analysis on the sampled tissue. Our results showed that DON and ergosterol were present in all of the *F. graminearum*-inoculated samples, DON was present in the DON-inoculated sample, and neither DON nor ergosterol was detected in the water-inoculated samples (Supplemental Table 2).

We paired-end sequenced RNA from each sample using the Illumina HiSeq 2000 (Illumina). We generated 314.1 million, 111.8 million, 83.6 million, and 45.3 million 100-bp reads from the FgS96, FgR96, D12, and W12 samples from the resistant genotype per tissue sample, respectively, and 301.9 million, 92.6 million, 130.6 million, and 50.4 million reads from the FgS96, FgR96, D12, and W12 samples for the susceptible genotype, respectively. The data have been deposited into the Sequence Read Archive database under the accession SRP052836. The RNA-seq reads were mapped to the chromosome arm sorted WGS contigs (IWGSC, 2014), FPKM values obtained for each transcript, and differential expression analysis was performed between the resistant and susceptible line for each sample. Genes were classified as differentially expressed with a *q*-value of <0.05 and at least a twofold change in expression. Differentially expressed genes for all experiments are summarized in Table 1.

Genomic Location of DEGs

The genomic distribution of the DEGs was examined for each of the three wheat genomes: A (Supplemental Fig. 3), B (Fig. 3), and D (Supplemental Fig. 4). Figure 3 shows a large number of genes differentially expressed in the *Fhb1* location on the short arm of chromosome 3B for the FgR96 and D12 samples. Differentially expressed genes from the FgS96 sample were found throughout the genome, including the *Fhb1* region. Differentially expressed genes that were upregulated in both the resistant and susceptible genotypes were found in the regions exhibiting polymorphisms between the two genotypes identified in the SNP analysis, including chromosome 1B (Fig. 3; Supplemental Fig. 3; Supplemental Fig. 4).

Differentially Expressed Genes Found in All Samples

To identify gene expression similarities in the various treatments, we examined DEGs that were significantly upregulated in the resistant or susceptible genotypes in each of the four samples (Supplemental Table 3–Table 6). A Venn diagram of the DEGs upregulated in the resistant genotype from all of the samples (Fig. 4A) showed four genes that were differentially expressed. These genes encoded a DnaJ homolog subfamily C member 25 homolog, a 2-oxoglutarate and Fe(II)-dependent oxygenase superfamily protein, a ubiquitin-conjugating enzyme 22, and a protein with unknown function (Table 2). Two of the four genes map to chromosome 3B and the other two genes map to chromosomes 1BL and 7AS. Differentially expressed genes that were upregulated in the susceptible genotype from all of the samples identified three genes (Fig. 4B) that encoded a dephospho-Coenzyme A kinase, WD-repeat-containing protein-like protein (WD indicates the increased frequency of tryptophan and aspartic acid pairs in an amino acid), and a protein with unknown function (Table 2). Two of these three genes show no expression in the resistant genotype. Two of the three genes map to chromosome 3DS and the other gene maps to 7AS.

Differentially Expressed Genes Identified in F. graminearum-*Inoculated or DON-Treated Plants*

To identify genes that only responded to DON or infection, we compared the DEGs from DON- and *F. graminearum*-inoculated plants. By comparing DEGs in the FgS96, FgR96, and D12 samples, we found 12 DEGs that were upregulated in the resistant genotype (Fig. 4C). These 12 genes include, in addition to the four DEGs found higher in the resistant genotype in all samples, a tetratricopeptide repeat protein, a WD-repeat protein, and receptor protein kinases (Table 3). Nine of the 12 genes were found on chromosome 3B and the other three genes on 1BL, 7AS, and 7DL. Additionally, there were three genes in this group that did not show expression in the susceptible genotype in all three samples and two genes that showed no expression in the susceptible genotype in two of the three samples. We also found 13 DEGs that were upregulated in the susceptible genotype in the FgS96, FgR96, and D12 samples (Table 3; Fig. 4D). In addition to the DEGs found to be upregulated in the susceptible genotype of all samples, this group of genes contained three *O*-methyltransferases and a UDP-*D*-glucose epimerase. The 13 genes are found on chromosomes 1BL (five), 3B (two), 3DS (three), 6BS (one), 7AS (one), and 7DS (one). Four of the 13 genes showed no expression in the resistant genotype in all of the samples.

Fig. 3. Distribution of differentially expressed genes (DEGs) on the wheat subgenome B. Tracks (a) to (d) summarize DEGs with higher expression in the resistant genotype: Track (a) illustrates DEGs for *Fusarium graminearum*-infected spikelets at 96 h after inoculation (hai), (b) *F. graminearum*-infected rachis at 96 hai, (c) deoxynivalenol (DON)-infected spikelets at 12 hai, and (d) water-inoculated spikelets at 12 hai. Tracks (e) to (h) summarize DEGs with higher expression in the susceptible genotype: Track (e) illustrates DEGs for *F. graminearum*-infected spikelets at 96 hai, (f) *F. graminearum*-infected rachis at 96 hai, (g) DON-infected spikelet at 12 hai, and (h) water-inoculated spikelets at 12 hai. The heat map in the outer track illustrates the genetically anchored genes obtained from IWGSC (2014) with a maximum of 608 genetically anchored genes per bin. The red rectangle indicates the putative location of the *Fhb1* gene. Tracks (a) to (h) represent the relative amount of genes compared to the overall amount of genes in one bin (range: 0–100%).

Differentially Expressed Genes Identified in the Spikelet and Rachis in F. graminearum-Inoculated Plants

The rachis is thought to be a major determinant of FHB resistance. Therefore, we compared the DEGs found in the FgS96 and FgR96 samples (Supplemental Table 3, Supplemental Table 4). We expected to see a subset of genes in common because of the sampling of the spikelets included the associated rachis in the sampled tissue. We found 23 DEGs that were upregulated in the resistant genotype in common between the FgS96 and FgR96 samples, which included the DEGs found in common

in the previous analyses. It is possible that the number of overlapping DEGs was lower than expected because the spikelet tissue comprised a larger amount of the total sample than the rachis tissue in the spikelet samples. The DEGs that were upregulated in the resistant genotype that were common to both the FgS96 and FgR96 samples included an acetylglucosaminyltransferase and MYB transcription factors. Genes often found associated with *F. graminearum* infection, such as cytochrome P450 family proteins and glutathione transferases, were found to be upregulated in the susceptible genotype in the FgS96 and FgR96 samples. Two cytochrome P450
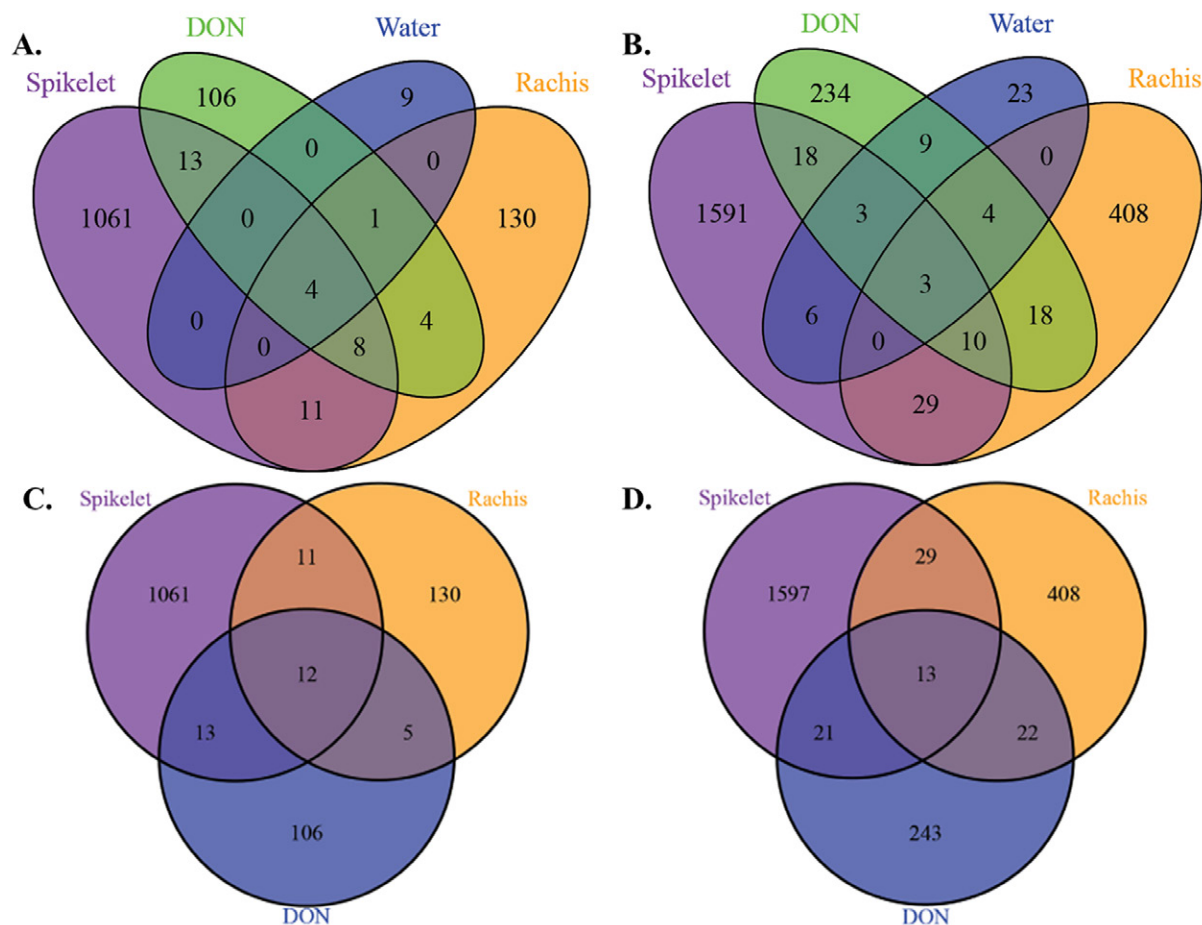
Fig. 4. Expression of the *Fhb1* near-isogenic line (NIL) pair during *Fusarium graminearum* infection. A. Venn diagram of differentially expressed genes (DEGs) that were upregulated in the *Fhb1+* (resistant) compared to *Fhb1−* (susceptible) for the *F. graminearum*-inoculated wheat spikelet and rachis samples at 96 h after inoculation (hai) and the deoxynivalenol (DON)- and water-inoculated spikelet samples at 12 hai. B. Venn diagram of DEGs that were upregulated in *Fhb1−* compared to *Fhb1+* for the *F. graminearum*-inoculated spikelet and rachis samples at 96 hai and the DON- and water-inoculated spikelet samples at 12 hai. C. Venn diagram of DEGs that were upregulated in *Fhb1+* compared to *Fhb1−* for the *F. graminearum*-inoculated spikelet and rachis samples at 96 hai and DON-inoculated spikelet samples at 12 hai. D. Venn diagram of DEGs that were upregulated in *Fhb1−* compared to *Fhb1+* for the *F. graminearum*-inoculated spikelet and rachis samples at 96 hai and DON-inoculated spikelet samples at 12 hai.

Table 2. Differentially expressed genes of wheat that were common to all comparisons.[†‡]

IWGSC gene version 1.0	IWGSC gene version 2.1	CHRM[§]	Spikelet *Fhb1+*	Spikelet *Fhb1−*	Rachis *Fhb1+*	Rachis *Fhb1−*	DON *Fhb1+*	DON *Fhb1−*	Water *Fhb1+*	Water *Fhb1−*	Annotation
						FPKM					
Ta1blLoc006658	Traes_1BL_F247C7924	1BL	61.52	12.97	41.69	11.62	45.34	3.23	100.25	11.36	Unknown protein
Ta3bLoc011967		3B	47.62	3.13	17.12	0.85	2.88	0.36	219.74	5.13	DnaJ homolog subfamily C member 25 homolog
Ta7asLoc012784	Traes_7AS_A6B40EA20	7AS	86.1	2.28	39.43	0	38.42	0	143.22	0	2-oxoglutarate and Fe(II)-dependent oxygenase superfamily protein
Ta3bLoc032788	Traes_3B_E77959B79	3B	14.65	2.78	9.05	1.79	18.05	4.76	16.67	4.82	Ubiquitin-conjugating enzyme 22
Ta3dsLoc008571	Traes_3DS_1DE20CFF3	3DS	0	2.27	0	4.88	0	12.39	0	26.99	Dephospho-CoA kinase, putative, expressed
Ta7asLoc002401	Traes_7AS_23DFA827E	7AS	1.5	15.13	1.07	9.1	1.93	18.6	1.68	20.08	WD repeat-containing protein-like protein
Ta3dsLoc008200	Traes_3DS_3DAAA7A72	3DS	0	3.3	0	2.84	0	28.39	0	19.7	Unknown protein

[†] Samples represented in the table are *Fusarium graminearum*-inoculated wheat spikelets sampled at 96 h after inoculation (hai), *F. graminearum*-inoculated rachis sampled at 96 hai, deoxynivalenol-inoculated spikelets sampled at 12 hai, and water-inoculated spikelets sampled at 12 hai for the *Fusarium* head blight-resistant (*Fhb1+*) and susceptible (*Fhb1−*) genotypes for each treatment.

[‡] Genes were classified as differentially expressed with a *q*-value less than 0.05 and a twofold change in expression.

[§] CHRM, chromosome arm; FPKM, fragments per kilobase of transcript per million mapped reads; CoA, Coenzyme A; WD, term indicating increased frequency of tryptophan and aspartic acid pairs in an amino acid.

Table 3. Differentially expressed genes of wheat that were common to all samples except the water-inoculated samples.†‡

IWGSC gene version 1.0	IWGSC gene version 2.1	CHRM§	Spikelet Fhb1+	Spikelet Fhb1−	Rachis Fhb1+	Rachis Fhb1−	DON Fhb1+	DON Fhb1−	Annotation
					FPKM				
Genes upregulated in the resistant genotype									
Ta1blLoc006658	Traes_1BL_F247C7924	1BL	61.52	12.97	41.69	11.62	45.34	3.23	Unknown protein
Ta3blLoc003627	Traes_3B_3375519C8	3B	8.94	0.73	5.13	0.71	4.88	0.89	WD-repeat protein, putative
Ta3blLoc011967		3B	47.62	3.13	17.12	0.85	2.88	0.36	DnaJ homolog subfamily C member 25 homolog
Ta3blLoc028859	Traes_3B_A7DD93C38	3B	23.3	0	2.05	0	4.34	0	Receptor-like protein kinase
Ta3blLoc032788	Traes_3B_E77959B79	3B	14.65	2.78	9.05	1.79	18.05	4.76	Ubiquitin-conjugating enzyme 22
Ta3blLoc039717		3B	8.23	2.75	11.48	1.47	7.66	1.2	Metal tolerance protein C3
Ta3blLoc042226	Traes_3B_8D85D1E9F	3B	14.85	0.26	4.92	0	2.08	0	Tetratricopeptide repeat protein, tpr, putative
Ta3blLoc047728	Traes_3B_04B913527	3B	20.6	0.35	13.09	0.4	2.28	0.4	Subtilase
Ta3blLoc047729	Traes_3B_A1EB9A90A	3B	9.24	0	3.68	0	3.17	0	Chaperone protein DnaJ, putative
Ta3blLoc048370		3B	29.33	0.05	14.13	0	11.36	0	Receptor-like protein kinase
Ta7asLoc012784	Traes_7AS_A6B40EA20	7AS	86.1	2.28	39.43	0	38.42	0	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
Ta7dlLoc025634	Traes_7DL_6B4FA59C6	7DL	9.06	0	7.71	0	2.45	0	MYB transcription factor
Genes upregulated in the susceptible genotype									
Ta1blLoc001983	Ta1blMIPSv2Loc100107	1BL	2.6	175.6	0	128.38	0	212.23	*O*-methyltransferase ZRP4
Ta1blLoc010801	Traes_1BL_4F99047F1	1BL	55.17	156.94	19.89	156.18	35.12	90.34	UDP-*D*-glucose epimerase 1
Ta1blLoc018184	Traes_1BL_5E6CEFBC5	1BL	0	3182.1	21.37	447.42	1.23	7.28	ATP-dependent zinc metalloprotease FTSH 5, mitochondrial
Ta1blLoc018757	Traes_1BL_D60867F6B	1BL	0	18.14	0	13.98	0	6.74	*O*-methyltransferase
Ta1blLoc020932	Traes_1BL_3699A930D, Ta1blMIPSv2Loc108787	1BL	0.24	26.28	0	15.28	0	12.1	*O*-methyltransferase
Ta3blLoc016873	Traes_3B_B74F61671	3B	0.72	40.13	0.68	25.18	0.18	12.82	Protein C20orf11, putative
Ta3blLoc043552	Traes_3B_05EEE7D3F1	3B	1.07	79.55	0.73	127.75	0	76.05	Sarcoplasmic reticulum histidine-rich calcium-binding protein, putative, expressed
Ta3dsLoc008200	Traes_3DS_3DAAA7A72	3DS	0	3.3	0	2.84	0	28.39	Unknown protein
Ta3dsLoc008571	Traes_3DS_1DE20CFF3	3DS	0	2.27	0	4.88	0	12.39	Dephospho-CoA kinase, putative, expressed
Ta3dsLoc008798	Traes_3DS_4D6C03FCF	3DS	0	6.53	0	14.03	0	39.35	60S ribosomal protein l9
Ta6bsLoc019663		6BS	3.99	17.24	2.07	9.57	0.42	7.22	Unknown protein
Ta7asLoc002401	Traes_7AS_23DFA827E	7AS	1.5	15.13	1.07	9.1	1.93	18.6	WD repeat-containing protein-like protein
Ta7dsLoc011242	Traes_7DS_A7468F6DA	7DS	2.56	56.78	2.36	82.64	0.54	13.95	Heat-shock protein, putative

† Samples represented in the table are *Fusarium graminearum*-inoculated spikelets sampled at 96 h after inoculation (hai), *F. graminearum*-inoculated rachis sampled at 96 hai, and deoxynivalenol (DON)-inoculated spikelets sampled at 12 hai for the *Fusarium* head blight-resistant (*Fhb1+*) and susceptible (*Fhb1−*) genotypes for each treatment.

‡ Genes were classified as differentially expressed with a *q*-value less than 0.05 and a twofold change in expression.

§ CHRM, chromosome; WD, term indicating increased frequency of tryptophan and aspartic acid pairs in an amino acid; CoA, Coenzyme A; UDP, uridine diphosphate; ATP, adenosine triphosphate; FPKM, fragments per kilobase of transcript per million mapped reads.

family proteins and a glutathione transferase were found to be upregulated in the susceptible genotype of both the FgS96 and FgR96 samples. Genes with the same annotations were upregulated in the resistant genotype of the FgS96 and FgR96 samples but the specific genes were found only in either the FgS96 or the FgR96 samples. The gene designated *WFhb1_c1* has been annotated as a pectin methyl esterase inhibitor and has been proposed to be a candidate of *Fhb1* (Zhuang et al., 2013). Genes annotated as having pectin esterase activity were found only in the DEGs from the FgS96 sample; however, they were upregulated in the susceptible genotype and did not have any sequence similarity to *WFhb1_c1*.

Wheat Response to DON

To gain an understanding of the wheat response to DON, we compared the D12 and W12 samples from the resistant and susceptible genotypes. We found 1228 DEGs in the resistant genotype between the D12 and W12 samples (Supplemental Table 7) and 1012 DEGs in the susceptible genotype between the D12 and W12 samples (Supplemental Table 8). There were 979 genes upregulated in the D12 sample of the resistant genotype and 754 genes upregulated in the D12 sample of the susceptible genotype. Of these genes, 447 were upregulated in the D12 samples of both genotypes and 281 of the 447 genes were expressed only in the D12 samples. The 281 genes that were induced by DON inoculation in both the resistant and susceptible genotypes included detoxification and

transport genes such as glutathione-*S*-transferases, UDP-glycosyltransferases, adenosine triphosphate-binding cassette (ABC) transporters, and cytochrome P450 genes.

Gardiner et al. (2010) found 255 barley transcripts with increased accumulation and three with decreased accumulation after DON inoculation. When the 1228 DEGs between D12 and W12 samples from the resistant genotype and the 1012 DEGs between the D12 and W12 samples from the susceptible genotype were compared against the transcripts identified in Gardiner et al. (2010), there were bireciprocal matches to 14 out of the 255 barley transcripts (www.vmatch.de, accessed 12 Nov. 2015; parameters: seed length,12; exdrop, 2; L, 200; identity, 87). Additionally, Gardiner and associates (2010) found 40 transcripts that also responded to trichothecene-producing *F. graminearum* in barley from Boddu et al. (2007). One of these transcripts, which showed a trichothecene-specific response, had sequence similarity to a DEG in our study. This transcript was annotated by Gardiner et al. (2010) as a serine/threonine phosphatase. The lack of a substantial overlap between wheat and barley treated with DON indicated that wheat and barley responded fundamentally differently to DON.

Fhb1-*specific Response to DON*

To identify the *Fhb1*-specific response to DON, we compared the DEGs that were upregulated in *Fhb1+* or *Fhb1−* in the D12 samples (Supplemental Table 5). In total, we found 136 and 299 genes that were upregulated in the resistant and susceptible genotypes, respectively. We found 102 DEGs that were upregulated in *Fhb1+* and 226 DEGs that were upregulated in *Fhb1−* of the D12 samples and were not found in any other sample, indicating that most of these genes responded specifically to DON application (Supplemental Table 9). Of the 102 DEGs that were upregulated in *Fhb1+*, 22 showed no expression in the susceptible genotype and 32 of the 226 DEGs showed no expression in the resistant genotype. These DEGs were distributed across all of the chromosomes, with 36 located on chromosome 3B. Some of the DEGs may be involved in DON detoxification, including multiple genes encoding cytochrome P450s, glutathione *S*-transferases, glycosyltransferases, UDP-glucosyltransferase, an ABC transporter, and an *O*-methyltransferase.

Gene Ontology Enrichment Analysis

To gain a genome-wide understanding of the gene expression differences between the resistant and susceptible genotypes, we calculated Gene Ontology enrichments based on the DEGs. Gene Ontology terms were considered to be over-represented when the FDR-adjusted *p*-value was below 0.05. Differentially expressed genes that were upregulated in the resistant genotype in the FgS96 sample had 45 terms that were classified as significant and included terms for stress response, peptidase activity, and monooxygenase activity. Differentially expressed genes that were upregulated in the resistant genotype derived from FgR96 sample at 96 hai had 34 terms classified as
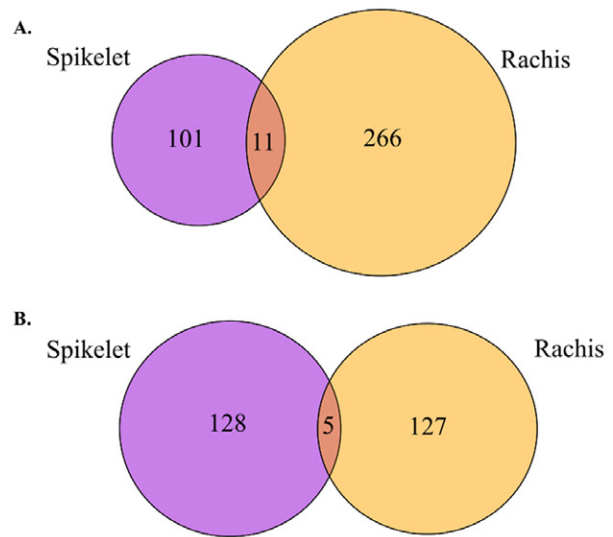

Fig. 5. *Fusarium graminearum* gene expression during infection of the *Fhb1* near-isogenic line (NIL) pair. A. Two-way Venn diagram of *F. graminearum* genes that were differentially expressed between *Fhb1+* (*Fusarium* head blight-resistant) and *Fhb1−* (susceptible) wheat genotypes in *F. graminearum*-inoculated spikelets sampled at 96 h after inoculation (hai) and rachis sampled at 96 hai that were upregulated in *Fhb1+*. B. Two-way Venn diagram of *F. graminearum* genes differentially expressed between *Fhb1+* and *Fhb1−* in *F. graminearum*-inoculated spikelets sampled at 96 hai and rachis sampled at 96 hai that were upregulated in *Fhb1−*.

significant and were highly enriched for terms including transport, methyltransferase activity, and monooxygenase activity. Differentially expressed genes with higher expression in the resistant genotype for the D12 sample had 40 Gene Ontology terms classified as significant and were enriched for defense response genes.

Pathogen Response during Infection

To examine the *F. graminearum* transcriptome during infection, Illumina RNA-seq reads from the FgS96 and FgR96 samples were mapped to the *F. graminearum* CDS and the number of DEGs between the resistant and susceptible genotypes was determined. Genes were considered to be differentially expressed with a *q*-value ≤ 0.05, at least a twofold change in expression values, and at least one sample expression value ≥10 FPKM. We found 245 and 409 DEGs in the FgS96 and FgR96 samples, respectively. In the FgS96 samples, 112 DEGs were upregulated in the resistant genotype and 133 DEGs were upregulated in the susceptible genotype (Supplemental Table 10). In the FgR96 samples, 277 and 132 DEGs were upregulated in the resistant and susceptible genotypes, respectively (Supplemental Table 11). Of the DEGs that were upregulated in the resistant genotype, 11 were found in both sample comparisons (Fig. 5A). Of the DEGs that were upregulated in the susceptible genotype, five were found in both sample comparisons (Fig. 5B). Higher expression levels were seen in the same genotype for the FgS96 and FgR96 samples in 16 of the 24 common DEGs (Table 4). The DEGs differing in their direction of differential

Table 4. *Fusarium graminearum* genes that were differentially expressed in both *F. graminearum*-inoculated wheat spikelet and rachis samples.[†‡]

Gene code	Spikelet *Fhb1*+	Spikelet *Fhb1*−	Rachis *Fhb1*+	Rachis *Fhb1*−	Gene	Annotation
			FPKM[§]			
FGSG_01770	9.57	19.58	19.15	6.29		Conserved hypothetical protein
FGSG_03213	2.64	17.8	1.74	24.63		Related to gibberellin 20-oxidase
FGSG_04435	7.26	16.15	11.48	4.41		Conserved hypothetical protein
FGSG_07636	4.75	11.31	18.68	8.29		Related to dimethylaniline monooxygenase
FGSG_03303	25.45	9.73	36.67	17.74		Related to alcohol dehydrogenase homolog Bli-4
FGSG_10706	17.76	7.95	49.93	11.71		Probable ATP-binding multidrug cassette transport protein
FGSG_11395	15.33	6.58	56.8	18.27	NPS14	Related to AM-toxin synthetase
FGSG_00799	19.4	415.69	530.69	113.45		Conserved hypothetical protein
FGSG_04581	372.14	185.59	606.72	219		Conserved hypothetical protein
FGSG_07386	46.68	21.17	55.85	27.07		Conserved hypothetical protein
FGSG_07597	27.5	11.54	177.25	64.34		Conserved hypothetical protein
FGSG_07836	12.59	50.94	3.75	13.11		Related to dopamine-responsive protein
FGSG_09088	73.21	23.73	209.17	87.89		Related to peroxisomal short-chain alcohol dehydrogenase
FGSG_09977	81.27	36.22	200.7	52.44		Conserved hypothetical protein
FGSG_10593	11.38	27.87	7.4	21.65		Conserved hypothetical protein
FGSG_10754	517.29	1,435.32	40.01	1457.3		Conserved hypothetical protein
FGSG_05820	9.11	35.59	29.23	64.65		Conserved hypothetical protein
FGSG_07986	0.00	78.82	107.15	0.00		Hypothetical protein
FGSG_06582	23.81	0.54	16.04	0.00		Hypothetical protein
FGSG_12896	27.77	528.36	271.79	25.3		Hypothetical protein
FGSG_11026	10.19	0.00	16.50	7.29	NPS1	Nonribosomal peptide synthetase
FGSG_08056	30.86	15.25	0.00	10.70		Hypothetical protein
FGSG_02288	0.00	310,247.00	10.57	1.58		Related to ECM32- DNA dependent ATPase/DNA helicase B
FGSG_13878	1,181,920.00	0.00	82.62	28.28		Hypothetical protein

[†] Samples represented in the table are *F. graminearum*-inoculated spikelets sampled at 96 h after inoculation (hai) and *F. graminearum*-inoculated rachis sampled at 96 hai for the *Fusarium* head blight-resistant (*Fhb1*+) and susceptible (*Fhb1*−) genotypes for each treatment.

[‡] Genes were classified as differentially expressed with a *q*-value less than 0.05 and at least a twofold change in expression.

[§] FPKM, fragments per kilobase of transcript per million mapped reads; ATP, adenosine triphosphate.

expression showed higher expression in the susceptible line in the FgS96 sample and higher expression in the resistant line in the FgR96 sample. Nonribosomal peptide synthetases NPS1 and NPS14 were expressed at higher levels in the resistant genotype in both the FgS96 and FgR96 samples. NPS1 potentially encodes a SidC ferrichrome siderophore (Bushley et al., 2008) that is involved in high-affinity iron binding. Although the function of NPS14 is currently unknown (Hansen et al., 2012), it is related to the AM-toxin synthetase gene, which is a major virulence factor in *Alternaria alternata,* which causes *Alternaria* blotch on apple (*Malus domestica* Borkh.)(Johnson et al., 2000).

Of the 245 DEGs in the FgS96 samples, 57 showed no expression in the resistant genotype and 41 showed no expression in the susceptible genotype. Of the 409 DEGs in the FgR96 samples, nine showed no expression in the resistant genotype and seven showed no expression in the susceptible genotype. Trichothecene biosynthesis genes were found to be differentially expressed in both tissue samples. TRI9 and NPS9, another nonribosomal peptide synthase related to AM-toxin synthase, were upregulated in the resistant genotype in the FgS96 sample. In the FgR96 sample, a large set of genes related

to sesquiterpene biosynthesis were upregulated in the resistant genotype including the trichothecene biosynthetic genes *TRI1, TRI3, TRI4, TRI5, TRI8, TRI11, TRI12, TRI14*, and the terpene cyclase CLM1, encoding longiborneol synthase (McCormick et al., 2010).

Functional Catalogue Enrichment Analysis

To determine whether particular categories of *Fusarium* DEGs were over-represented in any of the treatments, we determined their enrichment compared to their representation in the genome as a whole. In the disease-susceptible wheat genotype, few gene categories were significantly over-represented, whether in the spikelet or in the rachis. One exception was the category for "unclassified proteins" with unknown function in the susceptible rachis (MIPS 99; $P = 6.82 \times 10^{-4}$). Paradoxically, a category that was highly over-represented in both resistant spikelet and resistant rachis tissue was "disease, virulence, and defense" (MIPS 32.05; $P = 0.0033$ and 6.18×10^{-5}, respectively). Secondary metabolism (MIPS 01.20; $P = 1.98 \times 10^{-4}$) and ABC transporters (20.03.25; $P = 8.51 \times 10^{-5}$) were categories that were also significantly enriched among DEGs in resistant rachis tissue.

Discussion

In this study, we examined the disease severity, DON accumulation, and gene expression in a wheat NIL pair carrying either the resistant or susceptible allele for the *Fhb1* QTL. We used RNA-seq to examine gene expression to expand on and validate the results of the Jia et al. (2009) study, which used the same NIL pair but was limited by the wheat GeneChip platform to identify differential transcript accumulation. Our results provide a gene atlas of expression in a wheat NIL pair carrying either *Fhb1+* or *Fhb1–* during *F. graminearum* infection and DON application. We also identified wheat genes that were differentially expressed in the *Fhb1* NIL pair during *F. graminearum* infection in the spikelets and rachis and after DON treatment; we then used this information to identify *Fhb1–* and DON-responsive genes.

Fhb1 Exhibits Type II Resistance Mediated at the Rachis Node

Plants carrying the *Fhb1+* allele exhibited a high level of Type II resistance, defined by the observation that disease symptoms were restricted to the initial site of infection. Several lines of evidence pointed to the rachis node as being an important site of Type II resistance. Savard et al. (2000) found that the rachis had a higher concentration of DON than the spikelets when measured from 4 to 25 d after *F. graminearum* inoculation. *F. graminearum* mutants in the *TRI5* gene (the first gene in the trichothecene biosynthetic pathway) were restricted in their ability to pass through the cell walls of the rachis node to enter the rachis and spread to neighboring spikelets (Jansen et al., 2005; Maier et al., 2006). *F. graminearum* strains carrying the GFP reporter gene driven by the *Tri5* promoter showed increased GFP expression at the rachis node in Type II resistant genotypes (Ilgen et al., 2009), indicating that the rachis node induces *Tri5* gene expression and trichothecene biosynthesis. We observed higher concentrations of DON in the rachis in the susceptible genotype compared to the Type II resistant genotype at 21 dai (Fig. 2B). Consistent with the accumulation of DON in the rachis, we identified the expression of eight *F. graminearum* trichothecene biosynthetic genes (*Tri1*, *Tri3*, *Tri4*, *Tri5*, *Tri8*, *Tri11*, *Tri12*, and *Tri14*) in the rachis samples of plants carrying either *Fhb1+* or *Fhb1–*. Notably, the expression levels of the trichothecene biosynthetic genes were higher in the resistant genotype than in the susceptible genotype. Hallen-Adams et al. (2011) also found that the relative expression of the DON biosynthetic gene *Tri5* was higher in a resistant wheat cultivar than in a susceptible cultivar. These results indicate that trichothecene biosynthesis occurs in the rachis but that the expression level is higher in the resistant genotype possibly in an attempt to overcome resistance. Additionally, in the resistant genotype, *F. graminearum* DEGs in rachis tissue were especially enriched for the MIPS category involved in disease, virulence, and defense including trichothecene synthesis genes. Taken together, our results are consistent with previous results that the rachis is a key site for mediating Type II resistance.

Few DEGs between the Resistant and Susceptible Genotype are Common to All Samples

Although we identified large numbers of DEGs between the resistant and susceptible genotypes for each treatment, we did not identify a large set of genes that were consistently differentially expressed in the different treatments. As expected, the water-inoculated samples exhibited the lowest number of DEGs, which are likely to be genes that differ constitutively between the NIL pair and which have a differing response to the mechanical stress of the inoculation technique. Only 25 genes were differentially expressed in both the *F. graminearum*- and DON-inoculated plants (Table 3, Fig. 4C,D). Taken together, our results indicate that *F. graminearum* infection and DON treatment resulted in detecting mostly distinct sets of DEGs; the DEGs in the rachis and spikelet during *F. graminearum* infection were also distinct.

Fhb1-specific Responses

To identify *Fhb1*-specific responses, we identified genes that were expressed in the resistant genotype but were not expressed in the susceptible genotype. We found 12 DEGs that were upregulated in the resistant genotype in the *F. graminearum*-inoculated spikelet, *F. graminearum*-inoculated rachis, and DON-inoculated samples (Fig. 4C; Table 3). In this group of genes, there were three genes that showed no expression in the susceptible genotype. These genes encode a MYB transcription factor, a receptor protein kinase, and a chaperone protein DnaJ. The receptor protein kinase and the chaperone protein DnaJ mapped to the *Fhb1* region on chromosome 3B. Interestingly, Boddu et al. (2006) and Steiner et al. (2009) both identified genes encoding a DnaJ-like protein during *F. graminearum* infection of barley and *F. graminearum* infection of FHB-resistant wheat, respectively. The sequences used for the mapping reference were derived from the cultivar Chinese Spring, which may not contain the *Fhb1* QTL. Thus the genes we have identified are probably not *Fhb1* but are genes that are involved in the resistance response associated with *Fhb1*.

Previous work by Jia et al. (2009) found 27 transcripts that were differentially expressed between the same NIL pair as used in this study. When compared to all of the DEGs, six of the transcripts from the Jia et al. (2009) study show sequence similarity to genes that were differentially expressed in at least one comparison. Genes encoding a Bowman–Birk trypsin inhibitor, a 3-isopropylmalate dehydrogenase, and a gene with an NB-ARC domain were found to be differentially expressed in both studies. Bowman–Birk trypsin inhibitors are a family of protease inhibitors that play a major role in the plant defense response and can be induced by wounding (Eckelkamp et al., 1993; Qi et al., 2005). Genes encoding NB-ARC domain proteins are resistance genes that are involved in pathogen detection and plant resistance (DeYoung and Innes, 2006). Taken together, these genes represent genes in the resistant genotype that exhibit an *Fhb1*-specific response.

F. graminearum Exhibits Different Expression Patterns during Infection of Resistant Compared to Susceptible Wheat Genotypes

Using the RNA-seq reads from the *F. graminearum*-inoculated spikelet and rachis tissues from the resistant and susceptible NILs sampled at 96 hai, we were able to map the reads to the *F. graminearum* CDS to identify genes that were differentially expressed by the pathogen during infection. The 96 hai time point was shown by Lysøe and associates (2011) to have the largest number of GeneChip probe sets expressed by *F. graminearum* after inoculation on wheat, indicating that this time point would allow us to capture the largest number of genes expressed by the fungus during infection. Comparing DEGs in the *F. graminearum*-inoculated spikelet and rachis samples, we saw very few genes that were differentially expressed in both tissues (Fig. 5A, Fig. 5B). We were also able to compare *F. graminearum* gene expression in the resistant and susceptible genotypes. Previous studies have used only susceptible genotypes when evaluating *F. graminearum* gene expression on wheat and barley (Güldener et al., 2006; Lysøe et al., 2011). More DEGs were upregulated in the resistant rachis sample than in the spikelet sample or were upregulated in the susceptible spikelet and rachis samples. We found a large set of genes relating to trichothecene biosynthesis that were upregulated in the resistant genotype of the rachis samples and a few genes relating to trichothecene biosynthesis upregulated in the resistant genotype of the spikelet samples. No known genes of the *TRI5* gene cluster were upregulated in the susceptible genotype. Taken together, our results show that *F. graminearum* expresses a different set of genes dependent on host resistance.

The categories of *Fusarium* DEGs enriched in the four treatments showed surprisingly different patterns, with plant genotype differences (resistant versus susceptible) having a greater influence than tissue type (i.e., spikelet versus rachis). Gene categories involved in secondary metabolism synthesis (especially toxins) as well as disease, virulence, and defense were enriched in resistant rachis and spikelet tissues. For example, the nonribosomal peptide synthase gene *NPS1* was upregulated in resistant spikelet and rachis tissue; *NPS1* is required for full expression of both extracellular and intracellular siderophores (Oide et al., 2007) that are themselves essential for full disease expression in wheat. On the other hand, gene categories that were enriched in susceptible tissue seemed more likely to be for specialized metabolic processes such as degradation of certain amino acids and polysaccharides. The most distinct treatment was in susceptible rachis tissue, where the category for "unclassified proteins" was most highly enriched. Taken together, our results show that *F. graminearum* expresses a different set of genes *in planta*, highly dependent on the host's resistance genotype.

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