Examining the Transcriptional Response of the Wheat *Fhb1* Gene to *Fusarium graminearum* infection and deoxynivalenol treatment

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Abstract

Fusarium Head Blight (FHB) is a disease caused by the fungal pathogen *Fusarium graminearum* that can lead to severe yield loss and reduction in grain quality in wheat. Trichothecene mycotoxins, such as deoxynivalenol (DON), accumulate during infection and increase pathogen virulence. The *Fhb1* locus on wheat chromosome 3BS confers type II resistance to FHB and is associated with DON resistance. To gain a better genetic understanding of the functional role of *Fhb1*, a near-isogenic line (NIL) pair carrying the resistant and susceptible alleles for *Fhb1* was examined for gene expression during *F. graminearum* infection and DON treatment. Post-infection DON concentration assays and transcriptomic results show that the rachis is a key location for conferring type II resistance. Additionally, the wheat transcriptome data reveal a set of *Fhb1*-responsive genes. Gene transcriptomic results of the pathogen show that the *F. graminearum* genome responds differently to the level of host resistance.

Supplemental Datasets

Supplemental Dataset 1. Differentially expressed wheat genes, upregulated in the resistant genotype, between the resistant (Fhb1+) and susceptible (Fhb1-) genotypes of: spikelet tissue 96 hours after *F. graminearum* inoculation (Spikelet), rachis tissue 96 hours after *F. graminearum* inoculation (Rachis), spikelet tissue 12 hours after DON inoculation (DON), and spikelet tissue 12 hours after water inoculation (Water).

Supplemental Dataset 2. Differentially expressed wheat genes, upregulated in the susceptible genotype, between the resistant (Fhb1+) and susceptible (Fhb1-) genotypes of: spikelet tissue 96 hours after *F. graminearum* inoculation (Spikelet), rachis tissue 96 hours after *F. graminearum* inoculation (Rachis), spikelet tissue 12 hours after DON inoculation (DON), and spikelet tissue 12 hours after water inoculation (Water).

Supplemental Dataset 3. Differentially expressed wheat genes, upregulated in the DON- or water-inoculated samples, between the resistant (Fhb1+) DON-inoculated (DON) and resistant water-inoculated (Water) samples of spikelet tissue 12 hours after inoculation, and between the susceptible (Fhb1-) DON-inoculated and susceptible water-inoculated samples of spikelet tissue 12 hours after inoculation.

Supplemental Dataset 4. Differentially expressed *Fusarium graminearum* genes, upregulated in the resistant or susceptible genotypes, between the resistant (Fhb1+) and susceptible (Fhb1-) genotypes of: spikelet tissue 96 hours after *F. graminearum* inoculation (Spikelet) and rachis tissue 96 hours after *F. graminearum* inoculation (Rachis).

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Chapter 1 Literature Review

Fusarium Head Blight

Fusarium head blight (FHB) is a fungal disease caused by the pathogen *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch) that affects small grains around the world, including wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). FHB infection causes bleaching or discoloration of the spike, necrotic lesions on the spikelet(s), and mycotoxin accumulation, which leads to decreased grain yield and quality (McMullen et al., 1997; Goswami and Kistler, 2004). The trichothecene mycotoxins produced during infection include deoxynivalenol (DON), nivalenol (NIV), 15-acetyldeoxynivalenol (15-ADON) and 3-acetyldeoxynivalenol (3-ADON); these are dangerous to the health of both humans and animals (Desjardins 2006; Miller and Ewen 1997). DON is the most common trichothecene found in cereal grains in the United States; NIV is found in higher levels in Europe where NIV-producing strains of *F. graminearum* are more prevalent (Desjardins 2006). The FDA recommends an advisory level of 1ppm DON for finished wheat products that may be consumed by humans (McMullen et al., 1997). The total direct economic losses caused by FHB in the United States from 1998 to 2000 for all classes of wheat and barley are estimated at $870.6 million (Nganje et al., 2004).

Fusarium graminearum Infection

Fusarium graminearum infection of wheat occurs between flowering and the soft dough stage, and symptoms can be seen three days after inoculation under favorable conditions (Leonard and Bushnell 2003). Infection occurs when fungal spores land on the spikelets and infect through the stomata, wounds, anthers, between the lemma and palea, or through areas with thinner cell wall surfaces such as the base of glumes (Leonard and Bushnell 2003). The fungus colonizes the spikelet by growing intercellularly through glumes and spreads via the xylem and pith to neighboring rachis nodes allowing infection to spread both up and down the spike (Brown et al., 2010; Leonard and Bushnell 2003; Pritsch et al., 2000). Symptoms of infection can be seen as the infection continues including necrosis, water soaked lesions, and bleached tissue (Kang and Buchenauer 2000b; Trail 2009). During infection and colonization of wheat spikes by a related pathogen that can cause FHB, *F. culmorum*, cell wall changes and degradation are observed, indicating the fungus produces cell-wall degrading enzymes including cellulose, xylanse, and pectinase (Kang and Buchenauer 2000a; Kang and Buchenauer 2000b; Kang and Buchenauer 2002). Plant cell wall-degrading enzymes are also produced by *F. graminearum* during infection (Brown et al., 2010; Güldener et al., 2006). Perithecia are formed in stomatal openings and in silica cells and allow the fungus to overwinter in the soil or on plant material left in the field (Guenther and Trail 2005).

During infection the fungus produces trichothecenes, including DON, which is a virulence factor that functions by suppressing cell wall thickening at the rachis nodes and inhibiting protein synthesis (Desjardins and Hohn, 1997; Ilgen et al., 2009; Jansen et al.,

2005). *F. graminearum* mutants in the *TRI5* gene, the first step in the trichothecene biosynthetic pathway, have been shown to develop more slowly, have reduced virulence, and cannot pass the thickened cell walls of the rachis node—leading ultimately to reduced spread of infection (Boddu et al., 2007; Desjardins et al., 2000; Jansen et al., 2005; Proctor et al., 1995a). Using a *F. graminearum* strain with a green fluorescence protein (GFP) gene fused to a TRI5 promoter resulted in high levels of GFP expression at the rachis node and lower levels in the rachis, indicating the rachis node is important in wheat defense (Ilgen et al., 2009). Both Jansen et al. (2005) and Ilgen et al. (2009) also demonstrated that DON is not a virulence factor in the fruit coat of wheat and barley.

FHB Resistance in Wheat

Five types of FHB resistance have been classified in wheat: resistance to initial infection (Type I), resistance to the spread of infection (Type II), resistance to kernel infection (Type III), tolerance to mycotoxins (Type IV), and resistance to mycotoxins accumulation (Type V) (Mesterhazy 1995; Miller et al., 1985; Schroeder and Christensen 1963). Type II resistance is the major resistance type that wheat breeders select for in their programs. However, barley exhibits a natural Type II resistance leading to more focus on increasing Type I resistance in barley (Bai and Shaner 2004; Leonard and Bushnell, 2003).

Numerous quantitative trait loci (QTL) mapping studies have been conducted in wheat to identify regions of the genome involved in FHB resistance. QTL for FHB resistance have been reported on all chromosomes except 7D (Buerstmayr et al., 2009;

Liu et al., 2009). The three major effect QTL are located on chromosomes 3BS, 6BS, and 5A (Bai et al., 1999; Buerstmayr et al., 2003; Lin et al., 2004; Shen et al., 2003; Waldron et al., 1999; Yang et al., 2005). The QTL on chromosome 3BS (*Fhb1*) is derived from the Chinese cultivar Sumai 3 (PI 481542) and its derivatives, and explains up to 60% of the phenotypic variation in FHB resistance (Anderson et al., 2001; Bai et al., 1999; Basnet et al., 2012; Buerstmayr et al., 2002; Liu et al., 2006; Pumphrey et al., 2007; Waldron et al., 1999; Zhou et al., 2002). Fine mapping of the 3BS resistance QTL region placed *Fhb1* in a 1.2 cM region between markers STS3B-189 and STS3B-206 (Liu et al., 2006). The codominant marker UMN10 has been developed for marker assisted selection (MAS) of *Fhb1* (Liu et al., 2008). *Fhb1* contributes to Type II resistance and is commonly used in wheat programs breeding for FHB resistance (Kolb et al., 2001). Sumai 3 is also the source of a QTL on chromosome 6BS, named *Fhb2*, which confers field resistance (Anderson et al., 2001; Basnet et al., 2012; Cuthbert et al., 2007; Waldron et al., 1999). Additionally, the QTL on chromosome 5A, *Qfhs.ifa-5A*, has been identified to play a role in Type I resistance (Buerstmayr et al., 2003). Noteworthy, Sumai 3 contains a QTL located on chromosome 2DS that increases susceptibility, indicating that selecting against this Sumai 3 allele may increase FHB resistance levels (Basnet et al., 2012).

Functional role of *Fhb1*

Fhb1 has been shown to be associated with resistance to DON accumulation and the conversion of DON to DON-3-*O*-glucoside (D3G) in resistant wheat plants carrying *Fhb1*, indicating that it may encode a UDP-glucosyltransferase or regulate a similar

enzyme (Lemmens et al., 2005). A UDP-glycosyltransferase (UGT) from *Arabidopsis thaliana*, DOGT1, converts DON to the less toxic DON-3-*O*-glucoside and confers resistance to DON in yeast. Moreover, overexpression of DOGT1 in *Arabidopsis* increases resistance to DON (Poppenberger et al., 2003). DON-treated barley also showed a conversion of DON to D3G and upregulation of UGTs (Gardiner et al., 2010). Barley UGTs are also induced by *F. graminearum* infection and trichothecene accumulation (Boddu et al., 2006; 2007). A UGT from barley, HvUGT13248, confers DON resistance in yeast and *Arabidopsis* (Schweiger et al., 2010; Shin et al., 2012). Two UGT gene clusters in *Brachypodium distachyon* contain UGTs that are induced by DON, of which two are homologous to HvUGT13248 and show resistance to DON in yeast (Schweiger et al., 2013a).

Isolating the *Fhb1* gene has been an active area of research and several candidate genes have been identified. Sequencing of BAC clones spanning the *Fhb1* region identified seven candidate genes for *Fhb1* in a 261 kb region (Liu et al., 2008). Cosmid clones containing the seven candidate genes were used to transform an FHB-susceptible line, and four of the five clones tested did not show increased resistance to FHB (Liu et al., 2008). Another candidate gene, *WFhb1_c1*, was identified as a cis-acting eQTL in the *Fhb1* region (Zhuang et al., 2013). *WFhb1_c1* is similar to an *Arabidopsis* methyl esterase inhibitor, and shows a slight increase in expression in resistant genotypes and a decrease in expression in susceptible genotypes after *F. graminearum* inoculation (Zhuang et al., 2013).

Response to FHB in Wheat

The host response of wheat during *F. graminearum* infection has been widely studied. Defense and stress response genes and pathogenesis-related (PR) genes are often reported to be expressed or upregulated in wheat during *F. graminearum* spikelet infection (Bernardo et al., 2007; Foroud et al., 2011; Golkari et al., 2007; Jia et al., 2009; Kong et al., 2005; 2007; Pritsch et al., 2000; 2001; Xiao et al., 2013; Yu and Muehlbauer 2001; Zhou et al., 2005). Defense response pathways may be regulated by jasmonic acid and ethylene signaling (Li and Yen, 2008).

Additional studies have compared the response to *F. graminearum* infection between resistant and susceptible wheat lines. Li and Yen (2008) identified 79 genes with expression patterns significantly changed in either a resistant line (Sumai 3) or susceptible line (Y1193-6). Gottwald et al. (2012) identified 2,169 differentially expressed genes between a moderately resistant and susceptible wheat cultivar after *F. graminearum* inoculation, including defense genes induced by jasmonate and ethylene signaling. Additionally, genes involved in the suppression of fungal virulence factors such as ABC transporters, UDP-glucosyltransferases, and protease inhibitor genes are seen in resistant wheat cultivars including Sumai 3 (Gottwald et al., 2012). The plant cytochrome P450 gene, *CYP709C1*, showed 7-fold greater accumulation in Sumai 3 than in an FHB susceptible line using quantitative real-time PCR (Li et al., 2010). These studies indicate that there are expression differences between resistant and susceptible genotypes. However, many of the differences between the genotypes may not be related to FHB resistance/susceptibility.

The use of near-isogenic lines (NILs) carrying the resistant or susceptible allele for a disease resistance locus can be a powerful genetic tool for dissecting the differences between resistant and susceptible genotypes. Using transcript-derived fragments (TDFs), Steiner et al. (2009) identified 5 TDFs with differential expression between wheat lines with or without the *Fhb1* and *Qfhs.ifa-5A* alleles. These TDFs showed homology with a UDP-glucosyltransferase, wheat phenylalanine ammonia-lyase, DnaJ-like protein, pathogenesis-related family protein, and a rice cDNA clone of unknown function (Steiner et al., 2009). A NIL pair carrying the *Fhb1* resistant or susceptible allele was studied for transcript accumulation under *F. graminearum* infection or mock inoculation at 48 and 96 hours after inoculation (hai) using the Affymetrix Wheat GeneChip (Jia et al., 2009). Jia et al. (2009) found 14 transcripts with differential expression in the resistant genotype compared to the susceptible genotype. Ten of these genes exhibited increased expression in the resistant genotype and encoded a proline-rich protein, an expansin, a NB-ARC, a putative band 7 protein, a Bowman-Birk trypsin inhibitor, and five unknown proteins. Four of the transcripts exhibited decreased expression in the resistant genotype and encoded a histidine-rich Ca^{2+}-binding protein and three unknown proteins. Metabolic profiling of wheat NILs carrying a resistant and susceptible allele for the *Fhb1* QTL identified 271 metabolites differentially accumulated in the rachis and 123 metabolites differentially accumulated in the spikelets under mock infection, and 1,309 and 2,412 metabolites differentially accumulated under *F. graminearum* infection in the rachis and spikelets, respectively (Gunnaiah et al., 2012). Proteomic profiling of *Fhb1* NILs identified 104 proteins induced in the resistant genotype in the rachis, including proteins

induced in response to biotic and abiotic stress, endogenous stimulus and signal transduction, and pathogenesis-related proteins (Gunnaiah et al., 2012). Based on these metabolic and proteomic profiles, Gunnaiah et al. (2012) showed evidence that rachis cell wall thickening contributes to reduced pathogen spread in lines carrying the *Fhb1* resistance allele.

Response to FHB in Barley

The response of barley to *F. graminearum* infection has also been studied and can help us understand the wheat response to *F. graminearum*. In the FHB susceptible barley cultivar Morex, 467 transcripts were found to be differentially accumulating between *F. graminearum* and water-inoculated spikelets using the Barley1 Affymetrix GeneChip (Boddu et al. 2006). The differentially accumulating transcripts included defense response proteins, oxidative burst-associated enzymes, phenylpropanoid pathway enzymes, trichothecene catabolic enzymes and transporters, and tryptophan biosynthetic and catabolic pathway enzymes (Boddu et al., 2006). *F. graminearum* infection on barley was defined by three stages: 0 to 48 hai with limited fungal development, low DON accumulation, and little change in transcript accumulation; 48 to 96 hai with increased fungal development and active infection, higher DON accumulation, and increased transcript accumulation; 96 to 144 hai with the development of hyphal mats, high DON accumulation, and a reduction in the number of transcripts (Boddu et al., 2006). Boddu et al. (2007) examined the response in barley to wild-type *F. graminearum* and loss-of-function *tri5* trichothecene nonproducing mutant. They found transcripts for putative

trichothecene detoxification, transport proteins, ubiquitination-related proteins, programmed cell death-related proteins, transcription factors, and cytochrome P450s induced during trichothecene accumulation (Boddu et al., 2007). Barley responds to trichothecene accumulation through two general responses proposed by Boddu et al. (2007). One response involves the induction of genes encoding trichothecene detoxification and transport activities that may reduce the impact of trichothecenes, and the second response induces genes encoding proteins associated with ubiquitination and cell death which may promote successful establishment of the disease (Boddu et al., 2007).

To more precisely define the response to DON accumulation, Gardiner and colleagues (2010) treated barley spikes from the FHB susceptible variety Morex with DON and water and examined gene expression over time. They found 255 genes that exhibited differential expression between the DON and water treatments. In addition, 40 genes were found that were in common with the genes that responded to *in planta* trichothecene accumulation in the Boddu et al. (2007) study. These genes represent a core set that respond to trichothecenes and are a rich source for further exploration of the host response to mycotoxin accumulation.

Transgenic Approaches to FHB Resistance

Several studies have identified genes that can improve FHB resistance in wheat using transgenic approaches. Enhanced FHB resistance and decreased or slowed FHB symptom development has been shown in transgenic wheat lines expressing *Arabidopsis*

thaliana NPR1 (AtNPR1), rice (*Oryza sativa*) thaumatin-like protein (TLP), barley class II chitinase, radish (*Raphanus sativus*) defensin (RsAFP2), bean (*Phaseolus vulgaris*) polygalacturonase-inhibiting protein (PvPGIP2), and maize (*Zea mays*) ribosome inactivating protein b-32 (Balconi et al., 2007; Chen et al., 1999; Ferrari et al., 2012; Li et al., 2011; Makandar et al., 2006; Shin et al., 2008). Transgenic durum wheat lines overexpressing *Taxi-III*, a wheat xylanase inhibitor, showed a reduction of FHB symptoms from 3 to 11 days after infection (Moscetti et al., 2013). Overexpressing defense response genes for a wheat α-1-purothionin, and a barley thaumautin-like protein 1 and β-1,3-glucanase also increased FHB resistance (Mackintosh et al., 2007). Taken together, although these lines exhibit reduced FHB severity the level of resistance is too low to be an effective control strategy.

Fusarium Genetics

A high quality whole genome sequence is available for *Fusarium graminearum* (Cuomo et al., 2007). The genome sequence is composed of 36.1 Mb that has been anchored to 4 chromosomes using a genetic map with 235 markers and an estimated 11,640 genes are predicted (Cuomo et al., 2007; Gale et al., 2005; Howson et al., 1963). The genome sequence enabled the development of an Affymetrix GeneChip designed to examine gene expression in *F. graminearum* (Güldener et al., 2006). In barley infected with *F. graminearum*, 7,132 fungal genes were detected at one or more timepoints (24, 48, 72, 96, and 144 hai) of infection (Güldener et al., 2006). Additionally, 408 genes were found to be expressed only on infected barley when compared to *F. graminearum* grown

on complete medium, minimal medium lacking carbon, and minimal medium lacking nitrogen; including 4 virulence factors and 32 plant cell wall degrading enzymes (Cuomo et al., 2007; Güldener et al., 2006). During infection on wheat, genes that encode proteins for plant cell wall degradation are expressed including β-D-galactosidase, alkaline proteinase, and cutinase binding proteins (Kruger et al., 2002). Using the *F. graminearum* Affymetrix microarray, 355 *F. graminearum* genes were expressed only during infection of wheat when compared against infection in barley and growth on complete, C, starvation and N starvation media (Lysøe et al., 2011). This set of 355 genes was enriched for genes involved in allantoin and allantoate transport, an effect not observed enrichment for these genes in barley (Lysøe et al., 2011). A similar microarray experiment showed 344 genes expressed at higher levels in wheat coleoptiles than *in vitro*, and this group showed enrichment for putative secreted proteins (Zhang et al., 2012).

F. graminearum is one of many *Fusarium* species that produce trichothecenes, sesquiterpene epoxide mycotoxins, which function in plants to inhibit ribosomal protein synthesis (Desjardins et al., 1993; Desjardins 2006). The trichothecene biosynthetic pathway is relatively well understood and many of the genes involved have been identified and functionally studied. The trichodiene synthase gene (TRI5) was the first discovered trichothecene biosynthesis gene and the center of a group of trichothecene biosynthesis genes found on chromosome 2; disruption of TRI5 causes a loss of trichothecene production (Desjardins 2006; Hohn and Beremand 1989; Hohn and Desjardins 1992; Jurgenson et al., 2002; Miller and MacKenzie 2000; Proctor et al.,

1995a). Other genes found in the cluster of trichothecene biosynthesis genes surrounding TRI5 include cytochrome P450 monooxygenases (TRI4, TRI11, TRI13), *O*-Acyl-transferases (TRI3, TRI7, TRI8), transcription factors (TRI6, TRI10), a transporter (TRI12), and two genes with currently unknown function (TRI9, TRI14) (Alexander et al., 1998; 1999; Brown et al., 2001; 2002; Desjardins 2006; Hohn et al., 1995; Lee et al., 2002; McCormick et al 1996; Peplow et al., 2003; Proctor et al., 1995b). These trichothecene biosynthesis cluster genes, all found in *F. graminearum*, are involved in the production of trichothecenes during infection (Desjardins 2006).

RNA-Seq is a Relatively New Approach to Examine Gene Expression

RNA-seq is a relatively new method of examining transcriptomes that has become more common with the development of high-throughput sequencing technologies. RNA-seq allows for transcriptome studies in species with or without genome sequence information. RNA-seq uses a set of RNAs that is converted to a cDNA library which is then high-throughput sequenced (Wang et al., 2009). The RNA-seq approach to studying transcriptomes has been used in a wide range of species and cell types (Marguerat et al., 2008). Using the Illumina sequencing platform to measure mRNA expression and identify differentially expressed genes is comparable to array-based platforms with the added benefit of detection of low-expressed genes, alternative splice variants, and novel transcripts (Marioni et al., 2008). RNA-seq has proven to be a useful tool for gene identification, SNP detection, transcript profiling, and identification of differentially expressed genes (e.g. Yang et al., 2011).

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Chapter 2 Examining the Transcriptional Response of the Wheat *Fhb1* Gene to *Fusarium graminearum* infection and deoxynivalenol treatment

Overview

Fusarium Head Blight (FHB) is a disease caused by the fungal pathogen *Fusarium graminearum* that affects wheat and other small grains 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, resistance to the spread of infection on the spike, and is associated with resistance to DON accumulation. To gain a better genetic understanding of the functional role of *Fhb1* and resistance/susceptibility to FHB, a near-isogenic line (NIL) pair carrying the resistant and susceptible alleles for *Fhb1* was examined for DON and ergosterol accumulation, FHB resistance, and gene expression during *F. graminearum* infection and DON treatment. The DON concentration and transcriptomic results show that the rachis is a key location for conferring type II resistance. In addition, the wheat transcriptome data revealed a set of *Fhb1*-responsive genes, one or more of which may possibly encode *Fhb1*. Gene 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 the *Fhb1* locus.

Introduction

Fusarium head blight (FHB), caused by the fungal pathogen *Fusarium graminearum*, affects small grains around the world. FHB 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 (DON), the most common trichothecene in *F. graminearum*-infected cereal grain in the United States, is dangerous to the health of humans and animals. The FDA advisory levels for DON in finished wheat products is 1 ppm (Desjardins 2006; McMullen et al., 1997; Miller and Ewen 1997). To date, protection of the wheat crop from FHB requires a combination of resistant varieties, agronomic practices, and fungicide treatments.

FHB infection occurs between flowering and the soft dough stages in wheat when *F. graminearum* spores penetrate through the stomata, wounds, anthers, 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 three days postinoculation and include necrosis, water soaked lesions, and bleached tissue (Kang and Buchenauer 2000b; Trail 2009; Leonard and Bushnell 2003). The trichothecene DON, which is a virulence factor, functions by suppressing cell wall thickening at the rachis nodes and inhibiting 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 have reduced virulence (Boddu et al., 2007; Desjardins et al., 2000;

Proctor et al., 1995a). *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 (Desjardins et al., 2000; Jansen et al., 2005; Proctor et al., 1995a). Using a *F. graminearum* strain with a 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 QTL have been identified for FHB resistance in wheat (Liu et al 2009). The major QTL on chromosome 3BS (*Fhb1*), derived from the Chinese cultivar Sumai 3, explains up to 60% of the phenotypic variation in FHB resistance, 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 is on chromosome 3BS and has been fine mapped to a 1.2 cM region between 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 (D3G); indicating *Fhb1* may encode a UDP-glucosyltransferase (UGT) or regulate a similar enzyme (Lemmens et al., 2005). Although *Fhb1* has received extensive study and the current data points to a trichothecene detoxification mechanism, to date, the gene that underlies *Fhb1* is unknown.

Identifying host resistance to trichothecenes may lead to designing novel resistance strategies. A UGT from *Arabidopsis thaliana*, DOGT1, converts DON to D3G, confers resistance to DON in yeast, and overexpression increases resistance to DON in

Arabidopsis (Poppenberger et al., 2003). DON treatment of barley showed a conversion of DON to D3G and upregulation of UGTs, and barley UGTs are induced under *F. graminearum* infection (Boddu et al., 2006; 2007; Gardiner et al., 2010). The barley UGT HvUGT13248 confers resistance to DON accumulation via conjugation to D3G in yeast and *Arabidopsis* (Schweiger et al., 2010; Shin et al., 2012). Two *Brachypodium distachyon* gene clusters containing UGTs are induced by DON, and two UGTs homologous to HvUGT13248 show resistance to DON in yeast (Schweiger et al., 2013a). Kluger et al. (2013) showed that DON can be detoxified via conjugation with glutathione, indicating that there are multiple plant host mechanisms of detoxifying DON. However, it is likely there are additional mechanisms that are undiscovered.

The host response in wheat to *F. graminearum* infection has been an active area of research. Defense, stress response, and pathogenesis-related (PR) genes are often reported to be expressed or upregulated in wheat during *F. graminearum* spikelet infection (Bernardo et al., 2007; Foroud et al., 2011; Golkari et al., 2007; Jia et al., 2009; Kong et al., 2005; 2007; Pritsch et al., 2000; 2001; Xiao et al., 2013; Yu and Muehlbauer 2001; Zhou et al., 2005). Genes involved in ethylene and jasmonate signaling have been implicated in the resistant response (Li and Yen, 2008). Xiao et al. (2013) identified eight genes upregulated during *F. graminearum* infection in the FHB resistant cultivar Wangshuibai including a receptor-like kinase, lipid transfer protein, CBL-interacting protein kinase, glycine-rich protein, NADP-dependent oxidoreductase, ARK protein, and two unknown proteins. A resistance pathway was proposed in which the spread of infection is blocked by the activation of the JA defense pathway, regulated by *Fhb1*

(Xiao et al., 2013). A near-isogenic line (NIL) pair carrying the *Fhb1* resistant or susceptible allele was studied for transcript accumulation during *F. graminearum* infection or mock inoculation using the Affymetrix Wheat GeneChip. Ten transcripts were observed with increased expression in the resistant genotype compared to the susceptible genotype, and 4 transcripts were observed with decreased expression in the resistant genotype compared to the susceptible genotype (Jia et al., 2009). The metabolites and proteins that accumulate in an *Fhb1* NIL pair carrying either the resistant or susceptible allele during infection have been examined (Gunnaiah et al., 2012). These authors showed increased accumulation of phenypropanoid metabolites and phenylpropanoid pathway enzymes in the resistant genotype, and proposed that this resulted in the increased cell wall thickening observed in the resistant genotype. A transcriptome study using the Affymetrix Wheat GeneChip identified 16 transcripts with differential expression in a NIL pair carrying either the resistant or susceptible allele for *Fhb1* during *F. graminearum* infection, including one transcript, an O-glucosyltransferase, likely involved in trichothecene detoxification and transport (Schweiger et al., 2013b). Until recently, these types of experiments were conducted with microarray technologies that are limited by the detection limit and by the probes that are on the array. The advent of RNA-seq technologies provides the opportunity to study all expressed genes. Recently, RNA-seq was used to study the host response in a NIL pair for the *Fhb1* QTL during *F. graminearum* infection (Kugler et al., 2013). These authors identified G-protein coupled receptor kinases and jasmonate and ethylene biosynthesis genes induced in lines carrying the resistance allele for *Fhb1*. Our research will add to

these results by specifically looking at the rachis during *F. graminearum* infection and examining the response of the *Fhb1* QTL to DON.

The response of *F. graminearum* during infection has been studied in barley and wheat using an Affymetrix GeneChip (Güldener et al., 2006). In barley, 7,132 gene transcripts were detected at one or more timepoints (24, 48, 72, 96, and 144 hai) after infection. These gene transcripts encoded cell wall degrading enzymes such as xylanases, mannanases, pectinases, glucanases, galactosidases, and cutinases; and trichothecene biosynthetic enzymes (Güldener et al., 2006). During infection on wheat, genes that encode proteins for plant cell wall degradation or modification and trichothecene biosynthesis are 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 approach in 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: 1) identify wheat genes that are differentially expressed in the *Fhb1* NIL pair during *F. graminearum* infection in the spikelets and rachis, and DON treatment; 2) identify *Fhb1* responsive genes; and 3) 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 near-isogenic line (NIL) pair 260-1-1-2 (Fhb1+) and 260-1-1-4 (Fhb1-) was used for this study. Fhb1+ carries the resistant allele for *Fhb1* and Fhb1- carries the susceptible allele of *Fhb1*. The FHB-resistant line Sumai 3 and moderately FHB-susceptible line Stoa were crossed resulting in the population RI 63. An FHB-resistant recombinant inbred line (RIL) from this population was crossed to an FHB-susceptible line, MN97448, and the NIL pair is derived from this cross (Liu et al., 2006; Pumphrey et al., 2007). The NILs were genotyped using four SSR markers in the 3BS *Fhb1* region (gwm533, gwm493, barc133, and barc87) and 20 additional randomly selected markers, one for each chromosome. The NILs were also genotyped using the Wheat 9K iSelect SNP assay (Cavanagh et al., 2013). The genotyping was conducted by Shiaoman Chao, USDA-ARS in Fargo, ND. The *F. graminearum* isolate Butte 86ADA-11 (supplied by R. Dill-Macky, University of Minnesota) was used for *F. graminearum* inoculations (Evans et al., 2000). This isolate has been shown to produce DON and 15-ADON in barley.

Growth Conditions

The NIL pairs were planted 5 seeds per 6-inch pot in Sunshine MVP (SunGro Horticulture, Agawam, MA) and grown in the growth chamber under 16 hours of light at 20ºC and 8 hours of darkness at 18ºC. Light intensity of the growth chamber at pot level

was 170 ± 20 µE $m^{-2}s^{-1}$. Osmocote Plus 15-9-12 (5ml) (Scotts Company, Marysville, OH) was added 1 week after planting. Plants were watered daily until sampling.

Experimental Design

For the spikelet sampled plants, at anthesis four central spikelets on 10 spikes per genotype were inoculated with 10µl *F. graminearum* inoculum (100,000 macroconidia per ml in water) or 10µl sterile water. Spikes were covered with a small clear plastic bag until sampling. Inoculated spikelets and associated rachis were sampled at 96 hai. For the rachis sampled plants, at anthesis four central spikelets on 10 spikes per genotype per timepoint were inoculated with 10µl *F. graminearum* inoculum (100,000 macroconidia per ml in water). Spikes were covered with a small clear plastic bag until sampling. At 48 and 96 hai, inoculated spikelets were removed and the exposed rachis was sampled. For DON and water inoculated plants, at anthesis four central spikelets on 10 spikes per genotype per treatment were inoculated with 2µg DON/10µl or 10µl sterile water. Spikes were covered with a small clear plastic bag until sampling. Inoculated spikelets and the associated rachis were sampled at 12 hai. Each experiment had three biological replications with a completely random design. These experiments and the samples from each experiment are summarized in Table 1.

Phenotype/Disease Severity

At anthesis, four central spikelets on 5 spikes per genotype were inoculated with 10µl *F. graminearum* inoculum (100,000 macroconidia per ml in water). Spikes were covered with a small clear plastic bag for 48 hours. Infected spikelets were counted at 7, 14, and 21 dai on five plants per genotype with three replications. Disease severity was scored by dividing the number of infected spikelets by the total number of spikelets (% infected spikelets).

DON/Ergosterol Analysis

Analysis of DON 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. Analysis of 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. Additional DON analysis was determined on spikelet tissue inoculated with *F. graminearum* or water and sampled at 1, 2, 4, and 6 days after inoculation. All DON and ergosterol analyses were performed by Yanhong Dong (University of Minnesota). DON and ergosterol concentration were measured by gas chromatography/mass spectrometry (Dong et al., 2006; Mirocha et al., 1998)

RNA-seq

RNA was extracted for sequencing using the RNeasy Plant Mini Kit (QIAGEN, Valencia, CA) from each replication of the *F. graminearum*-inoculated spikelets sampled at 96 hai, *F. graminearum*-inoculated rachis tissue sampled at 96 hai, DON-inoculated spikelets sampled at 12 hai, and water-inoculated spikelets sampled at 12 hai. RNA from the 3 replications of the *F. graminearum*-inoculated spikelet samples at 96 hai were sequenced separately. RNA from the 3 replications of the *F. graminearum*-inoculated rachis samples at 96 hai, 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 base pair paired-end reads with approximately 200 base pair insertion size. The average Q-score for all pass filtered reads was above Q30 for all samples.

RNA-seq reads from each sample were mapped on the WCS contigs (IWGSC, unpublished results) that served as mapping reference to obtain gene models based on 42 chromosome arms of cultivar Chinese spring (Figure 1). For mapping, the Cufflinks package was used (v2.0.2, PMID:22383036) and additional programs necessary for mapping used under following versions: bowtie (v2.0.6, doi:10.1186/gb-2009-10-3-r25), SAMtools (v0.1.18, PMID: 19505943) and tophat (v2.0.7, doi:10.1093/bioinformatics/btp120). Reported gene models were merged using 'cuffcompare' and reported transcripts compared against the published gene models of wheat genes. Differentially expressed genes (DEGs) of individual comparisons (i.e.

resistant vs. susceptible genotype) were calculated with Cuffdiff (v.2.1.1). A q-value (adjusted p-value) of less than 0.05 and a two-fold change in RPKM value were used to call differentially expressed genes. Figure 1 shows a schematic of the analysis pipeline. Annotations were assigned by the IWGSC (Klaus Mayer, unpublished results).

RNA-seq reads for *F. graminearum*-inoculated spikelet and rachis samples were mapped using Bowtie (bowtie-0.12.8) with default parameters against the *F. graminearum* coding sequences (CDSFGDB_v32.orf), a collection of 13,826 sequences. For the mapping, Bowtie (bowtie-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* CDS. A q-value (adjusted p-value) of less than 0.05, a two-fold change in FPKM value, and an FPKM value of at least ten in one of the samples were used to call differentially expressed genes. Venn diagrams were constructed using the online tool Venny to compare DEGs between samples (Oliveros 2007).

Gene Ontology

GOs were taken from the IWGSC bread wheat annotation. To test for an enrichment the R package GOstats was applied by considering the GO graph structure (conditional=TRUE) and keeping overrepresented terms below a p-value of 0.05. In addition, the GO terms were compressed by using GO slim (as provided by GOSlimViewer), conditional was set to FALSE and BH-adjusted p-values below 0.05 were kept.

Results

Graphical genotype of *Fhb1* NIL pair

To assess the extent of the introgressed *Fhb1* region, the NIL pair carrying either the *Fhb1* resistant or susceptible allele was genotyped with SSRs and SNPs. The NIL pair was genotyped using four SSR markers in the 3BS *Fhb1* region (gwm533, gwm493, barc133, and barc87) and 20 additional randomly selected 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 containing 6,293 SNPs (Cavanagh et al., 2013; Figure 2). Of the reported 6,293 SNPs, 5,934 were placed on the wheat map and 74 were polymorphic between the NILs (1.2%). 18.9% of the polymorphic SNPs map to chromosome 3BS. The polymorphic SNPs on chromosome 3BS span an approximately 31.5 cM region containing the *Fhb1* QTL (Liu et al., 2008). Small blocks of polymorphic SNPs were also seen on chromosomes 1A, 1B, and 7D. The polymorphic regions on chromosomes 1A and 1B span approximately 9 and 18 cM, respectively, and do not correspond to the QTL for FHB severity and FHB spread that have been mapped to chromosomes 1A and 1B (Buerstmayr et al., 2009). Additional major QTL regions on chromosomes 5A and 6BS (*Fhb2*) are not polymorphic between the NILs.

Phenotypic characterization of the *Fhb1* NIL pair

The wheat *Fhb1* NIL pair with either the resistant (Fhb1+) or susceptible (Fhb1-) allele at the *Fhb1* locus was analyzed for DON and ergosterol concentration on spikelet or rachis tissue inoculated with *F. graminearum*, DON, or water at 12, 48, or 96 hai (Table 2). Additional *F. graminearum*-inoculated samples were taken at 1, 2, 4, and 6 days after inoculation (dai) and analyzed for DON and ergosterol concentration (Figure 2). DON concentration was similar between resistant and susceptible genotypes for rachis tissue after *F. graminearum* inoculation at 48 hai but significantly different at 96 hai ($P \leq 0.05$) and the concentration increased from 48 to 96 hai in both genotypes. Consistent with the study by Jia et al. (2009), DON concentration was not significantly different between the genotypes for spikelet samples after *F. graminearum* inoculation at 96 hai. DON concentration was also not significantly different between the genotypes for DON inoculations at 12 hai. Water-inoculated samples at both 12 and 96 hai had levels of DON below the threshold value (less than 5 ng/sample) and were not reported. In the *F. graminearum*-inoculated plants, DON was also below the threshold value and not reported for 1 dai in both genotypes. DON concentration increased over time in both genotypes point inoculated with *F. graminearum* with the highest DON levels in the susceptible genotype after 6 days. DON concentration was significantly different between the resistant and susceptible genotype at 6 dai ($P \leq 0.05$).

Ergosterol concentration was not significantly different between the resistant and susceptible genotypes for any of the tissue x genotype x treatment x timepoint

combinations; however ergosterol concentration increased in the rachis samples from 48 to 96 hai, and increased in the spike over time from 1 to 6 dai.

Disease severity was measured by calculating the percent infected spikelets at 7, 14, and 21 days after inoculation (dai) (Table 3). Percent-infected spikelets was significantly different between the resistant and susceptible genotypes at 7 dai ($P \leq 0.05$), 14 dai ($P \leq 0.001$), and 21 dai ($P \leq 0.001$) with over 80% of the susceptible spikelets infected at 21 dai. These results clearly show the Type II resistance contributed by the *Fhb1* resistant allele (Kolb et al., 2001). These results are similar to other studies with lines containing *Fhb1* (Jia et al., 2009, Liu et al., 2006).

Wheat response to *F. graminearum* infection

To compare the host response in plants carrying the resistant allele to 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 from three experiments: *F. graminearum*-inoculated spikelet samples at 96 hai; *F. graminearum*-inoculated rachis samples at 96 hai; and DON and water spikelet samples at 12 hai, were paired-end sequenced using the Illumina HiSeq 2000 (Illumina, Inc., San Diego, CA) resulting in 314.1 million, 111.8 million, 83.6 million, and 45.3 million 100 base pair paired-end reads for the resistant genotype per tissue sample and 301.9 million, 92.6 million, 130.6 million, and 50.4 million reads for the susceptible genotype, respectively. The RNA-seq reads were mapped to the WCS arm sorted contigs (IWGSC, 2013), RPKM values obtained for each transcript, and differential expression analysis was performed between

the resistant and susceptible line for each sample. Genes were called as differentially expressed with a q-value of 0.05 and a 2-fold change in expression.

The 96 hai timepoint was chosen for the *F. graminearum*-inoculated samples to capture gene expression differences during the period of high levels of infection. Jia et al. (2009) identified a greater number of genes with increased or decreased transcript accumulation at 96 hai than 48 hai in wheat NILs carrying either the resistant or susceptible allele for *Fhb1*. We found that the 96 hai timepoint showed significant differences in DON concentration in the rachis after *F. graminearum* inoculation (Table 2) and is just prior to significant differences in DON in *F. graminearum*-inoculated spikelets (Figure 3). Additionally, Ilgen et al. (2009) showed *F. graminearum* completely colonized the inoculated wheat spikelet at 4 dai. The 12 hai timepoint was chosen for the DON-inoculated samples based on results from Gardiner et al. (2010), which showed gene expression values for DON-inoculated barley florets peak at 12 hai. This study also showed DON movement in barley florets from the DON-treated floret to neighboring florets from 1 to 72 hai.

For the differentially expressed genes (DEGs) between the resistant and susceptible lines in the *F. graminearum*-inoculated spikelet samples at 96 hai, 2909 genes exhibited increased expression in the resistant genotype with 480 of those genes showing no expression in the susceptible genotype. Alternatively, 3629 genes exhibited increased expression in the susceptible line with 698 of those genes showing no expression in the resistant genotype. For the *F. graminearum*-inoculated rachis samples at 96 hai, there are 949 DEGs. In the resistant genotype, 398 genes exhibited increased expression with 318

showing no expression in the susceptible genotype. In contrast, in the susceptible genotype 551 genes exhibited increased expression with 118 showing no expression in the resistant genotype. For the DON-inoculated spikelet samples at 12 hai, there were 700 DEGs between the resistant and susceptible genotypes. In the resistant genotype, 230 DEGs exhibited increased expression and 85 showed no expression in the susceptible genotype. In the susceptible genotype, 470 exhibited increased expression with 139 that showed no expression in the resistant genotype. For the water-inoculated spikelet samples at 12 hai there were a total of 78 DEGs, 14 exhibited increased expression in the resistant genotype and four of those DEGs showed no expression in the susceptible genotype. Sixty-four genes exhibited increased expression in the susceptible genotype, with 51 that showed no expression in the resistant genotype. Differentially expressed genes for all of the experiments are summarized in Table 4.

Differentially Expressed Genes Found in All of the Samples

To identify gene expression similarities in the various treatments, we examined DEGs that were significantly upregulated in the resistant and susceptible genotypes in each experiment. A Venn diagram of the DEGs upregulated in the resistant genotype from all of the samples (Figure 4A) showed four genes differentially expressed in all of the samples. These genes encode a DnaJ homolog subfamily C member, 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein, a ubiquitin-conjugating enzyme 22, and an unknown protein (Table 5). Two of the four genes map to chromosome 3B and the other two genes map to chromosomes 1BL and 7AS. A Venn

diagram of the DEGs upregulated in the susceptible genotype from all of the samples showed five DEGs in all of the samples (Figure 4B). These five genes encode a tetratricopeptide repeat (TPR)-like superfamily protein, dephospho-CoA kinase, alanine-tRNA ligase, WD repeat-containing protein-like protein, and an unknown protein (Table 5). Four of these five genes show no expression in the resistant genotype. Three of the five genes map to chromosome 3DS and the other two genes map to 3B and 7AS.

Differentially Expressed Genes after F. graminearum inoculation or DON Treatment

Comparing DEGs in the *F. graminearum*-inoculated spikelet, *F. graminearum*-inoculated rachis, and DON-treated samples, we found 14 DEGs upregulated in the resistant genotype in all of the samples (Figure 4C). The 14 genes upregulated in the resistant genotype in all samples include a 2-oxoglutarate and Fe(II)-dependent oxygenase superfamily protein, tetratricopeptide repeat protein, WD-repeat protein, and receptor protein kinases (Table 6). Eleven of the 14 genes are found on chromosome 3B and the other three genes on 1BL, 7AS, and 7DL. Additionally there are five genes in this group that do not show expression in the susceptible genotype in all three samples and three genes that show no expression in the susceptible in two of the three samples. We also found 29 DEGs upregulated in the susceptible genotype in the *F. graminearum*-inoculated spikelet, *F. graminearum*-inoculated rachis, and DON-treated samples (Figure 4D). This group of genes contains three O-methyltransferases, a UDP-D-glucose epimerase, and three WRKY transcription factors. The 29 genes are found on chromosomes 1AL (1), 1BL (6), 3B (5), 3DL (1), 3DS (7), 5BL (3), 5DL (2), 6BS (1),

7AS (1), 7DL (1), and 7DS (1). Nine of the 29 genes show no expression in the resistant genotype in all of the samples.

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

The rachis is thought to be a major determinant of resistance to FHB, thus we compared the DEGs found in the *F. graminearum*-inoculated spikelet and rachis. We expected to see a reasonable level of genes in common due to the sampling of the spikelets including the associated rachis in the sampled tissue. We found 60 DEGs upregulated in the resistant genotype in common between the *F. graminearum* inoculated spikelet and rachis samples. This is approximately 2.0% of the spikelet DEGs upregulated in the resistant genotype also found in the rachis and 15.0% of the rachis DEGs upregulated in the resistant also found in the spikelet. We found 181 DEGs upregulated in the susceptible genotype in common between the *F. graminearum*-inoculated spikelet and rachis samples. This is 4.9% of the spikelet susceptible upregulated DEGs also found in the rachis and 32.8% of the rachis susceptible upregulated DEGs also found in the spikelet sample. It is possible the number of overlapping DEGs is lower than expected due to the spikelet tissue comprising a larger amount of the total sample than rachis tissue in the spikelet samples. The DEGs upregulated in the resistant genotype in common between the spikelet and rachis samples include ABC transporter, cytochrome P450, and DnaJ homolog subfamily member. Many genes often found associated with *F. graminearum* infection are found upregulated

in the susceptible genotype in both the spikelet and rachis samples. These genes include ABC transporters, cytochrome P450 family proteins, ethylene responsive transcription factors, glutathione transferases, O-methyltransferases, and WRKY transcription factors. Genes with the same annotations are upregulated in the resistant genotype of the spikelet and rachis samples but the specific genes are found only in either the spikelet or the rachis 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). Several genes annotated as pectin methyl esterase inhibitors are found in the DEGs from the spikelet sample; however, they are upregulated in the susceptible genotype. Genes annotated as a pectin methyl esterase inhibitor were not found in other samples.

Differentially expressed genes in DON and water-inoculated samples

To study the effect of DON inoculation on gene expression, we compared the DEGs from the DON-inoculated spikelets to the DEGs from water-inoculated spikelets both at 12 hai. We found five genes upregulated in the resistant genotype of both samples: a Bowman-Birk trypsin inhibitor and 2-oxoglutarate and Fe(II)-dependent oxygenase superfamily protein (both with no expression in the susceptible genotype), a DnaJ homolog, ubiquitin-conjugating enzyme, and an unknown protein. There were also 34 DEGs upregulated in the susceptible genotype in both samples, and 30 of the DEGs showed no expression in the resistant genotype. There were 255 genes upregulated in the resistant genotype of the DON-inoculated samples that were not found in the water-inoculated samples, and 83 DEGs showed no expression in the susceptible genotype.

These genes were found on all of the chromosomes, with the majority on chromosome 3B (52 DEGs). Many of the DEGs uniquely upregulated in the resistant genotype of the DON-inoculated samples compared to the water-inoculated samples may be involved in DON detoxification including multiple genes encoding glutathione S-transferases, cytochrome P450s, ABC transporters, glycosyltransferases, an O-methyltransferase, and a UDP-glucosyltransferase.

DON specific gene expression

Loss-of-function mutants for the trichothecene biosynthesis gene *TRI5* do not produce trichothecenes and exhibit reduced virulence on wheat and barley (Boddu et al., 2007; Desjardins et al., 2000; Proctor et al., 1995a). Gardiner et al. (2010) found 255 barley transcripts with increased accumulation and three with decreased accumulation after DON inoculation. When the DEGs between DON and water-inoculated samples from the resistant genotype and DEGs between the DON and water-inoculated samples from the susceptible genotypes are compared against the Gardiner et al. (2010) transcripts, there are 22 wheat genes that show sequence similarity to 16 of the 255 barley transcripts. These include genes containing WRKY and poly(ADP-ribose) polymerase catalytic domains. Proteins containing WRKY domains function in response to pathogen or other stress (Eulgem et al., 2000). Additionally, Gardiner and associates (2010) found 40 transcripts which also responded to trichothecene-producing *F. graminearum* in barley from Boddu et al (2007). Four of these transcripts which show trichothecene specific responses have sequence similarity to DEGs in our study. These transcripts were

annotated by Gardiner et al. (2010) as a serine/threonine phosphatase, multidrug resistance, mitochondrial chaperone, and protein ATP8.

Additionally, we compared the DON-inoculated resistant and susceptible samples to the water-inoculated resistant and susceptible samples. We found 1428 DEGs between the DON and water-inoculated resistant genotype and 1164 DEGs between the DON and water-inoculated susceptible genotype. There were 1154 genes upregulated in the DON-inoculated samples of the resistant genotype and 869 genes upregulated in the DON-inoculated samples of the susceptible genotype. Of these genes, 518 are upregulated in the DON-inoculated samples of both genotypes, and 351 of the 518 genes are expressed only in the DON-inoculated samples. These genes are induced by DON inoculation in both the resistant and susceptible genotypes and include detoxification and transport genes such as glutathione S-transferases, UDP-glycosyltransferases, ABC transporters, and cytochrome P450 genes.

GO Enrichment Analysis

To gain a genome-wide understanding of the gene expression differences between the resistant and susceptible genotypes, we calculated gene ontology (GO) enrichments based on the DEGs. GO terms were called significant with a p-value of less than 0.001. DEGs between resistant and susceptible in *F. graminearum*-inoculated spikelets sampled at 96 hai have 40 terms called significant but are most highly enriched for DNA binding and defense response genes. *F. graminearum*-inoculated rachis samples at 96 hai DEGs have 20 terms called significant and are most highly enriched for copper ion binding and

response to oxidative stress. DEGs for the DON-inoculated samples have 37 GO terms called significant and are enriched for 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase activity and sequence-specific DNA binding. The water-inoculated samples have five genes annotated as a carbonate dehydratase activity, adenyl nucleotide binding, nucleotide binding, organic cyclic compound binding, and purine ribonucleotide binding.

Pathogen response during infection

To examine the *F. graminearum* transcriptome during infection, Illumina RNA-seq reads from the rachis and spikelet sampled tissues at 96 hai were mapped to the *F. graminearum* CDS and the number of DEGs between the resistant and susceptible genotypes was determined. Genes were considered differentially expressed with a q-value ≤ 0.05, 2-fold change in expression values, and at least one sample expression value ≥ 10. We found 245 and 409 DEGs in the spikelet and rachis samples, respectively. In the spikelet samples 112 DEGs were upregulated in the resistant genotype and 133 DEGs were upregulated in the susceptible genotype. In the rachis samples, 277 and 132 DEGs were upregulated in the resistant and susceptible genotypes, respectively. Of the DEGs upregulated in the resistant genotype, 9 were found in both sample comparisons (Figure 5A). Of the DEGs upregulated in the susceptible genotype, 5 were found in both sample comparisons (Figure 5B). Higher expression levels are seen in the same genotype for the spikelet and rachis samples in 14 of the 20 common DEGs (Table 7). The DEGs differing in direction of differential expression show higher expression in the susceptible line in the spikelet samples and higher expression in the resistant line in the rachis

samples. NPS14 (nonribosomal peptide synthetase) is expressed at higher levels in the resistant genotype of both the spikelet and rachis samples and is related to the AM-toxin synthetase gene, which is a major virulence factor in *Alternaria alternata* which causes Alternaria blotch on apple (Johnson et al., 2000).

Of the 245 DEGs in the spikelet samples, 57 showed no expression in the resistant genotype and 41 showed no expression in the susceptible genotype. Of the 409 DEGs in the rachis samples, 9 showed no expression in the resistant genotype and 7 showed no expression in the susceptible genotype. Trichothecene biosynthesis genes are found differentially expressed in both tissue samples. TRI9 and a gene related to trichodiene oxygenase cytochrome P450 are upregulated in the resistant genotype in the spikelet tissue. In the rachis samples, a large set of genes related to trichothecene biosynthesis were upregulated in the resistant genotype including: TRI1, TRI3, TRI4, TRI5, TRI8, TRI11, TRI12, TRI14, TRI7-like, and a trichothecene gene cluster gene.

Discussion

This study examined gene expression in a wheat NIL pair carrying either the resistant or susceptible allele for the *Fhb1* QTL after *F. graminearum*, DON, and water-inoculation. We used RNA-seq to examine gene expression to expand upon and validate the results of the Jia et al. (2009) study that used the same NIL pair, but was limited by the GeneChip platform to identify differential transcript accumulation. Our results provided the opportunity to address our original three main objectives: 1) identify wheat genes that are differentially expressed in the *Fhb1* NIL pair during *F. graminearum* infection in the spikelets and rachis, and DON treatment, 2) identify *Fhb1*-responsive genes, and 3) identify *F. graminearum* genes that are differentially expressed during *F. graminearum* infection of the *Fhb1* NIL pair. The results also provide the opportunity to address several key features of the function of the *Fhb1* locus.

Fhb1 exhibits Type II resistance mediated at the rachis node

The rachis node is an important site of resistance in the wheat defense response to *F. graminearum* (Ilgen et al., 2009). *F. graminearum* mutants in the *TRI5* gene cannot pass through the cell walls of the rachis node to enter the rachis and spread to neighboring spikelets (Jansen et al., 2005). We observed a significantly higher concentration of DON in the susceptible rachis compared to the resistant rachis at 96 hai, indicating that the rachis is a key structure that differentiates Type II resistance from susceptibility. We found 949 genes differentially expressed in rachis tissue 96 hours after

inoculation of the adjacent spikelets with *F. graminearum*. Of the 318 genes with expression only in the resistant genotype we found five genes annotated as cytochrome P450 genes, one of which is highly expressed and located on chromosome 1BL. Other genes expressed only in the resistant genotype include three genes encoding NBS-LRR disease resistance proteins, two located on chromosome 3B. A gene annotated as a Bowman-Birk type trypsin inhibitor, mapped to 3B, was found expressed only in the resistant genotype. This gene is also only expressed in the resistant genotype in the DON and water-inoculated spikelet experiment samples. Other Bowman-Birk trypsin inhibitors are seen differentially expressed, with increased expression in the resistant genotype, in the *F. graminearum*-inoculated spikelet samples.

There are many groups of genes found in the DEGs of the *F. graminearum*-inoculated spikelet and rachis samples either upregulated in the resistant or susceptible genotypes. Genes annotated as cytochrome P450s, glutathione S-transferases, glycosyltransferases, methyltransferases, and NBS-LRR genes are found upregulated in both genotypes and in both tissue samples. The genes are expressed uniquely in each tissue and genotype, but the same annotations are found in both genotypes and tissues. The differentially expressed NBS-LRR genes are often expressed only in one of the genotypes or samples, indicating the different tissues types and genotypes express unique genes in this gene family during infection. Similarly, in *Arabidopsis*, NBS-LRR-encoding and related genes exhibited tissue-specific expression patterns (Tan et al., 2007).

To examine the gene expression patterns in the pathogen during infection, we mapped the RNA-seq reads to the *F. graminearum* coding sequences and found DEGs

between the fungus infecting the FHB resistant and susceptible lines. We found 225 DEGs only in the spikelet tissue sample and 389 DEGs only in the rachis sample. Both tissues showed DEGs in the trichothecene biosynthesis pathway; however the rachis sample showed a much higher number of genes involved in the trichothecene biosynthesis pathway. Savard et al. (2000) found that the rachis has a higher concentration of DON than the spikelets when measured from 4 to 25 days after *F. graminearum* inoculation, although we did not see a higher concentration of DON in the rachis than spikelets at 96 hai. These results indicate that trichothecene production may be increased in the rachis tissue during infection on wheat. Differentially expressed in both the spikelet and rachis samples and upregulated in the resistant genotype was the gene NPS14 which is related to the AM-toxin synthetase gene (AMT). AMT is important in the production of AM-toxin which is required for pathogenicity of *Alternaria alternata* on susceptible apples (Johnson et al., 2000). NPS gene products may function as virulence factors in *F. graminearum* (Tobiasen et al., 2007). NPS14 was found by Lysøe et al. (2011) to be expressed in *F. graminearum* during infection of wheat and barley. Additionally, Lysøe et al. (2011) found 404 probe sets expressed only during infection of wheat when compared to probe sets expressed during infection of barley and on complete and starvation media. We found 142 genes from this set also expressed in both the spikelet and rachis samples. This includes a polyketide synthase, PKS8, which is one of four polyketide synthases Lysøe and associates (2011) identified expressed only in wheat, and TRI12, a trichothecene efflux pump.

Few genes differentially expressed between the resistant and susceptible genotype are common in all samples

Each of the four tissue samples: *F. graminearum*-inoculated spikelet sample 96 hai, *F. graminearum*-inoculated rachis tissue sample 96 hai, DON-inoculated spikelet sample 12 hai, and water-inoculated spikelet sample 12 hai, show a different number of DEGs between the resistant and susceptible alleles. There are 6538, 949, 700, and 78 DEGs in the *F. graminearum* spikelet, *F. graminearum* rachis, DON spikelet, and water spikelet samples, respectively. The water-inoculated samples exhibited the lowest number of DEGs, likely genes differing constitutively between the NIL pair and differing response to the mechanical stress of the inoculation technique. Nine genes are differentially expressed in all of the samples in this study, four genes upregulated in the resistant genotype and five genes upregulated in the susceptible genotype (Table 5). These genes may represent genes that are constitutively differentially expressed between the two genotypes and not responding to any of the *F. graminearum*, DON, or water treatments. It is possible these genes are differentially expressed in response to the stress or physical damage caused during inoculation. Seven of the nine genes are on chromosomes that have regions polymorphic between the genotypes from the SNP genotyping (one on 1B, three on 3B, and 3 on 3D), indicating these genes may be differentially expressed due to allelic differences between the genotypes.

Previous work by Jia et al. (2009) found 27 transcripts differentially expressed between the same NIL pair used in this study. One of the 9 DEGs found in all of the samples of this study has sequence similarity to an Affymetrix transcript differentially

expressed in the Jia et al. (2009) study. This unknown protein shows similarity to Ta.6873.1.S1. When compared to all of the DEGs, 14 of the transcripts from the Jia et al. (2009) study show sequence similarity to 24 genes differentially expressed in at least one sample. Genes encoding a Bowman-Birk trypsin inhibitor, a glutathione S-transferase, and a gene with an NB-ARC domain are found 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). Glutathione S-transferases (GSTs) are involved in detoxification, cell-signaling, and stress responses including pathogen attack in plants (Marrs, 1996). A wheat GST, *GstA1*, is induced by fungal pathogen infection and shows a large increase in expression after fungal inoculation (Dudler et al., 1991; Mauch and Dudler, 1993). Conjugation of DON to glutathione has been shown in wheat and may represent a mechanism for detoxification of DON (Kluger et al., 2013). Genes encoding NB-ARC domain proteins are resistance (R) genes that are involved in pathogen detection and plant resistance (DeYoung and Innes, 2006). Taken together, these genes may function to provide an increased level of resistance to FHB seen in the resistant genotype.

***Fhb1*-specific responses**

To identify candidate genes for *Fhb1*, we reasoned that genes that are expressed in the resistant genotype, not expressed in the susceptible genotype, and mapped to the *Fhb1* region on chromosome 3BS were good candidates for *Fhb1*. We found 14 DEGs upregulated in the resistant genotype in the *F. graminearum*-inoculated spikelet, *F.*

graminearum-inoculated rachis, and DON-inoculated samples (Figure 4C). In this group of genes there are four genes that showed no expression in the susceptible genotype and are found on chromosome 3B. These genes encode two receptor protein kinases, an inositol monophosphate family protein, and a chaperone protein DnaJ. 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. Therefore, the genes encoding these various proteins are good candidates for *Fhb1* or genes possibly regulating or involved in the pathway of resistance associated with *Fhb1*. Of particular importance, the susceptible line used in this study does not contain the *Fhb1* resistance allele, making these genes that show no expression in the susceptible genotype the best candidates for the *Fhb1* gene. It is thought that *Fhb1* encodes a DON-glucosyltransferase that functions to detoxify DON by converting DON to DON-3-glucoside or as a gene that regulates a DON-glucosyltransferase (Lemmens et al., 2005). Noteworthy, we do not observe any glucosyltransferases in this set of *Fhb1* candidate genes that we expected to find. The sequences used for the mapping reference were derived from the cultivar Chinese Spring which may not contain the *Fhb1* QTL. Chromosome 3B in Chinese Spring has been shown to carry resistance genes to FHB; however, Chinese Spring carries a different allele at the *Fhb1* locus than Ning 7840, a derivative of Sumai 3 (Bernardo et al., 2012; Grausgruber et al., 2004; Ma et al., 2006). Thus, the candidate genes we have identified may only be involved in the resistance pathway associated with *Fhb1*.

Additionally, three genes show no expression in the susceptible genotype of the *F. graminearum*-inoculated rachis and DON-inoculated samples, but show a low level of expression in the susceptible genotype of *F. graminearum*-inoculated spikelet samples. Two of these genes, a tetratricopeptide repeat (TPR) protein and receptor-like protein kinase, are on chromosome 3B. Their location on chromosome 3B and lack of expression in the susceptible genotype of the *F. graminearum*-inoculated rachis and DON-inoculated samples makes these genes possible candidates for *Fhb1*. TPR proteins are involved in many functions including stress response, and TPR-containing proteins have been identified to play a role in disease resistance in *Arabidopsis* and barley (Azevedo et al., 2002; Tör et al., 2002). The third gene in this group is a 2-oxoglutarate and Fe(II)-dependent oxygenase superfamily protein located on chromosome 7AS.

F. graminearum exhibits a different expression pattern during infection of resistant compared to susceptible wheat genotypes

Using the RNAseq 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 differentially expressed by the pathogen during infection. The 96 hai timepoint 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 this time point allowed 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 see very few genes

differentially expressed in both tissues (Figure 5A, 5B). We were also able to compare the *F. graminearum* gene expression on the resistant and susceptible genotype. 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). There are a larger number of DEGs upregulated in the resistant rachis sample than spikelet sample, or upregulated in the susceptible spikelet and rachis samples. We found a large set of genes related to trichothecene biosynthesis upregulated in the resistant genotype of the rachis samples and a few genes related to trichothecene biosynthesis upregulated in the resistant genotype of the spikelet samples. No known genes of the TRI5 gene cluster are upregulated in the susceptible genotype.

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Table 1. Samples collected for analysis and sequencing.

Treatment	Timepoint	Tissue	Sequenced	DON	Ergosterol
F. graminearum	96 hai	Spikelets	Yes	Yes	Yes
Water	96 hai	Spikelets	No	Yes	Yes
F. graminearum	48 hai	Rachis	No	Yes	Yes
F. graminearum	96 hai	Rachis	Yes	Yes	Yes
DON	12 hai	Spikelets	Yes	Yes	No
Water	12 hai	Spikelets	Yes	Yes	No

Each sample taken is listed according to the treatment, timepoint sampled, and tissue collected. The table indicates if the sample was used for sequencing and analyzed for deoxynivalenol (DON) or ergosterol concentration.

Table 2. DON concentration and ergosterol concentration in deoxynivalenol and *F. graminearum* infected wheat spikes for the *Fhb1* NIL pair carrying either the resistant or susceptible allele.

Sample		DON (ppm)					Ergosterol (ppm)				
Genotype	Tissue	F48[a]	F96[b]	W96[c]	D12[d]	W12[e]	F48	F96	W96	D12	W12
Resistant	Spikelet	NA[f]	89.90±56.68	nd[g]	53.03±8.70	nd	NA	39.95±8.12	0.31±0.22	NA	NA
Susceptible	Spikelet	NA	79.30±10.84	nd	58.1±4.65	nd	NA	37.38±7.19	0.22±0.09	NA	NA
Resistant	Rachis	0.71±0.75	21.94±4.93	NA	NA	NA	1.14±0.42	49.72±23.36	NA	NA	NA
Susceptible	Rachis	0.68±0.06	38.91±9.04	NA	NA	NA	1.15±0.35	61.75±26.63	NA	NA	NA

[a] *F. graminearum*-inoculated, sampled 48 hai

[b] *F. graminearum*-inoculated, sampled 96 hai

[c] Water-inoculated, sampled 96 hai

[d] DON-inoculated, sampled 12 hai

[e] Water-inoculated, sampled 12 hai

[f] Samples were not taken or measured at this genotype x tissue x treatment x timepoint

[g] Value fell below the threshold and was not reported

Deoxynivalenol and ergosterol concentrations in parts per million (ppm) ± standard error were determined by averaging 3 replications of 10 spikelets per replicate.

Table 3. Disease severity in *F. graminearum* infected wheat spikes for the *Fhb1* NIL pair carrying either the resistant or susceptible allele.

Sample	% Infected Spikelets		
Genotype	7dai	14dai	21dai
Resistant	14.40±2.17[a]	16.05±3.21	16.59±4.66
Susceptible	17.32±4.12	32.57±12.02	82.47±20.05

[a] Infected spikelet percentage was determined by averaging 3 replications of 5 plants per genotype per replicate.

Table 4. Differentially expressed genes (DEGs) between the *Fhb1* NIL pair carrying either the resistant or susceptible allele.

Sample			# of DEGs			
Tissue	Inoculum	Timepoint	Up Fhb1+[a]	Only Fhb1+[b]	Up Fhb1-[c]	Only Fhb1-[d]
Spikelet	*F. graminearum*	96 hai	2909	480	3629	698
Rachis	*F. graminearum*	96 hai	398	318	551	118
Spikelet	DON	12 hai	230	85	470	139
Spikelet	Water	12 hai	14	4	64	51

[a] DEGs upregulated in the resistant genotype.

[b] DEGs that show expression only in the resistant genotype.

[c] DEGs upregualted in the susceptible genotype.

[d] DEGs that show expression only in the susceptible genotype.

Table 5. Differentially expressed genes common in all comparisons.

Gene	CHRM[a]	Spikelet Fhb1+ FPKM[b]	Spikelet Fhb1- FPKM	Rachis Fhb1+ FPKM	Rachis Fhb1- FPKM	DON Fhb1+ FPKM	DON Fhb1- FPKM	Water Fhb1+ FPKM	Water Fhb1- FPKM	Annotation
Ta1blLoc006658	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	7AS	86.10	2.28	39.43	0.00	38.42	0.00	143.22	0.00	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
Ta3bLoc032788	3B	14.65	2.78	9.05	1.79	18.05	4.76	16.67	4.82	Ubiquitin-conjugating enzyme 22
Ta3dsLoc006576	3DS	0.00	11.63	0.00	1.79	0.00	1.86	0.00	4.39	Tetratricopeptide repeat (TPR)-like superfamily protein
Ta3dsLoc008571	3DS	0.00	2.27	0.00	4.88	0.00	12.39	0.00	26.99	Dephospho-CoA kinase, putative, expressed
Ta3bLoc024435	3B	0.00	2.42	0.00	1.21	0.00	1.75	0.00	6.10	Alanine--tRNA ligase
Ta7asLoc002401	7AS	1.50	15.13	1.07	9.10	1.93	18.60	1.68	20.08	WD repeat-containing protein-like protein
Ta3dsLoc008200	3DS	0.00	3.30	0.00	2.84	0.00	28.39	0.00	19.70	Unknown protein

[a]CHRM = chromosome

[b]FPKM = fragments per kilobase of transcript per million mapped reads

Samples represented in the table are *F. graminearum*-inoculated spikelets sampled 96 hai, *F. graminearum*-inoculated rachis sampled 96 hai, DON-inoculated spikelets sampled 12 hai, and water-inoculated spikelets sampled 12 hai for the resistant (*Fhb1*+) and susceptible (*Fhb1*-) genotypes for each treatment.

FPKM values are colored from green (low values) to red (high values).

Table 6. Differentially expressed genes common in all samples except the water-inoculated samples.

Gene	CHRM[a]	Spikelet Fhb1+ FPKM[b]	Spikelet Fhb1- FPKM	Rachis Fhb1+ FPKM	Rachis Fhb1- FPKM	DON Fhb1+ FPKM	DON Fhb1- FPKM	Annotation
Ta1blLoc006658	1BL	61.52	12.97	41.69	11.62	45.34	3.23	Unknown protein
Ta3bLoc003627	3B	8.94	0.73	5.13	0.71	4.88	0.89	WD-repeat protein, putative
Ta3bLoc011967	3B	47.62	3.13	17.12	0.85	2.88	0.36	DnaJ homolog subfamily C member 25 homolog
Ta3bLoc028859	3B	23.30	0.00	2.05	0.00	4.34	0.00	Receptor-like protein kinase
Ta3bLoc032788	3B	14.65	2.78	9.05	1.79	18.05	4.76	Ubiquitin-conjugating enzyme 22
Ta3bLoc039717	3B	8.23	2.75	11.48	1.47	7.66	1.20	Metal tolerance protein C3
Ta3bLoc042226	3B	14.85	0.26	4.92	0.00	2.08	0.00	Tetratricopeptide repeat protein, tpr, putative
Ta3bLoc044326	3B	3.88	0.00	1.35	0.00	24.36	0.00	Inositol monophosphatase family protein
Ta3bLoc047534	3B	1.69	0.00	0.93	0.00	0.85	0.00	Receptor kinase
Ta3bLoc047728	3B	20.60	0.35	13.09	0.40	2.28	0.40	Subtilase
Ta3bLoc047729	3B	9.24	0.00	3.68	0.00	3.17	0.00	Chaperone protein DnaJ, putative
Ta3bLoc048370	3B	29.33	0.05	14.13	0.00	11.36	0.00	Receptor-like protein kinase
Ta7asLoc012784	7AS	86.10	2.28	39.43	0.00	38.42	0.00	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
Ta7dlLoc025634	7DL	9.06	0.00	7.71	0.00	2.45	0.00	Myb transcription factor
Ta1alLoc010167	1AL	9.95	13.92	7.64	42.13	10.92	33.56	unknown protein
Ta1blLoc001983	1BL	2.60	175.60	0.00	128.38	0.00	212.23	O-methyltransferase ZRP4
Ta1blLoc010801	1BL	55.17	156.94	19.89	156.18	35.12	90.34	UDP-D-glucose epimerase 1
Ta1blLoc018184	1BL	0.00	3182.10	21.37	447.42	1.23	7.28	ATP-dependent zinc metalloprotease FTSH 5, mitochondrial
Ta1blLoc018757	1BL	0.00	18.14	0.00	13.98	0.00	6.74	O-methyltransferase
Ta1blLoc020932	1BL	0.24	26.28	0.00	15.28	0.00	12.10	O-methyltransferase

Ta1blLoc027134	1BL	0.00	5.43	0.00	1.14	0.00	20.42	Calmodulin, putative
Ta3bLoc016873	3B	0.72	40.13	0.68	25.18	0.18	12.82	Protein C20orf11, putative
Ta3bLoc024435	3B	0.00	2.42	0.00	1.21	0.00	1.75	Alanine--tRNA ligase
Ta3bLoc028058	3B	42.02	58.22	9.66	47.01	84.47	150.76	WRKY transcription factor 21
Ta3bLoc043552	3B	1.07	79.55	0.73	127.75	0.00	76.05	Sarcoplasmic reticulum histidine-rich calcium-binding protein, putative, expressed
Ta3bLoc050198	3B	4.39	6.35	4.27	13.83	24.55	46.29	unknown protein
Ta3dlLoc013155	3DL	3.83	7.44	4.30	26.01	22.31	74.45	unknown protein
Ta3dsLoc004114	3DS	0.00	2.68	0.00	2.18	0.00	1.98	1-deoxy-D-xylulose 5-phosphate reductoisomerase
Ta3dsLoc006576	3DS	0.00	11.63	0.00	1.79	0.00	1.86	Tetratricopeptide repeat (TPR)-like superfamily protein
Ta3dsLoc008200	3DS	0.00	3.30	0.00	2.84	0.00	28.39	Unknown protein
Ta3dsLoc008571	3DS	0.00	2.27	0.00	4.88	0.00	12.39	Dephospho-CoA kinase, putative, expressed
Ta3dsLoc008798	3DS	0.00	6.53	0.00	14.03	0.00	39.35	60S ribosomal protein l9
Ta3dsLoc012385	3DS	0.00	1.53	0.00	1.90	0.13	18.19	unknown protein
Ta3dsLoc012499	3DS	0.00	1.24	0.00	1.17	0.00	6.05	Sucrose-phosphate synthase 2
Ta5blLoc013404	5BL	15.14	22.42	11.45	72.50	24.49	50.12	WRKY transcription factor 3
Ta5blLoc014338	5BL	4.99	6.93	5.24	28.71	2.04	10.48	redox responsive transcription factor 1
Ta5blLoc036986	5BL	11.33	19.65	1.46	3.81	39.61	85.91	Tryptophan synthase alpha chain
Ta5dlLoc003506	5DL	7.37	17.39	2.40	12.70	20.84	35.06	Chymotrypsin inhibitor-2
Ta5dlLoc005223	5DL	21.67	29.00	15.48	91.67	61.05	121.42	WRKY transcription factor 3
Ta6bsLoc019663	6BS	3.99	17.24	2.07	9.57	0.42	7.22	Unknown protein
Ta7asLoc002401	7AS	1.50	15.13	1.07	9.10	1.93	18.60	WD repeat-containing protein-like protein
Ta7dlLoc017283	7DL	13.73	23.05	5.27	24.01	31.13	51.17	Receptor-kinase, putative
Ta7dsLoc011242	7DS	2.56	56.78	2.36	82.64	0.54	13.95	Heat-shock protein, putative

[a]CHRM = chromosome

[b]FPKM = fragments per kilobase of transcript per million mapped reads

Genes above the line in the table are upregulated in the resistant genotype and genes below the line are upregulated in the susceptible genotype.

Samples represented in the table are *F. graminearum*-inoculated spikelets sampled 96 hai, *F. graminearum*-inoculated rachis sampled 96 hai, and DON-inoculated spikelets sampled 12 hai for the resistant (*Fhb1*+) and susceptible (*Fhb1*-) genotypes for each treatment.

FPKM values are colored from green (low values) to red (high values).

Table 7. *F. graminearum* genes differentially expressed in both *F. graminearum*-inoculated spikelet and rachis samples.

Gene Code	Spikelet Fhb1+ FPKM[a]	Spikelet Fhb1- FPKM	Rachis Fhb1+ FPKM	Rachis Fhb1- FPKM	Gene	Annotation
FGSG_01770	9.57	19.58	19.15	6.29		conserved hypothetical protein
FGSG_03213	2.64	17.80	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	56.80	18.27	NPS14	related to AM-toxin synthetase (AMT)
FGSG_00799	19.40	415.69	530.69	113.45		conserved hypothetical protein
FGSG_04581	372.14	185.59	606.72	219.00		conserved hypothetical protein
FGSG_07386	46.68	21.17	55.85	27.07		conserved hypothetical protein
FGSG_07597	27.50	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	200.70	52.44		conserved hypothetical protein
FGSG_10593	11.38	27.87	7.40	21.65		conserved hypothetical protein
FGSG_10754	517.29	1435.32	40.01	1457.30		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	25.30		hypothetical protein

[a]FPKM = fragments per kilobase of transcript per million mapped reads

Samples represented in the table are *F. graminearum*-inoculated spikelets sampled 96 hai and *F. graminearum*-inoculated rachis sampled 96 hai for the resistant (*Fhb1*+) and susceptible (*Fhb1*-) genotypes for each treatment.

FPKM values are colored from green (low values) to red (high values).

Figure 1. RNA-seq Analysis Pipeline.

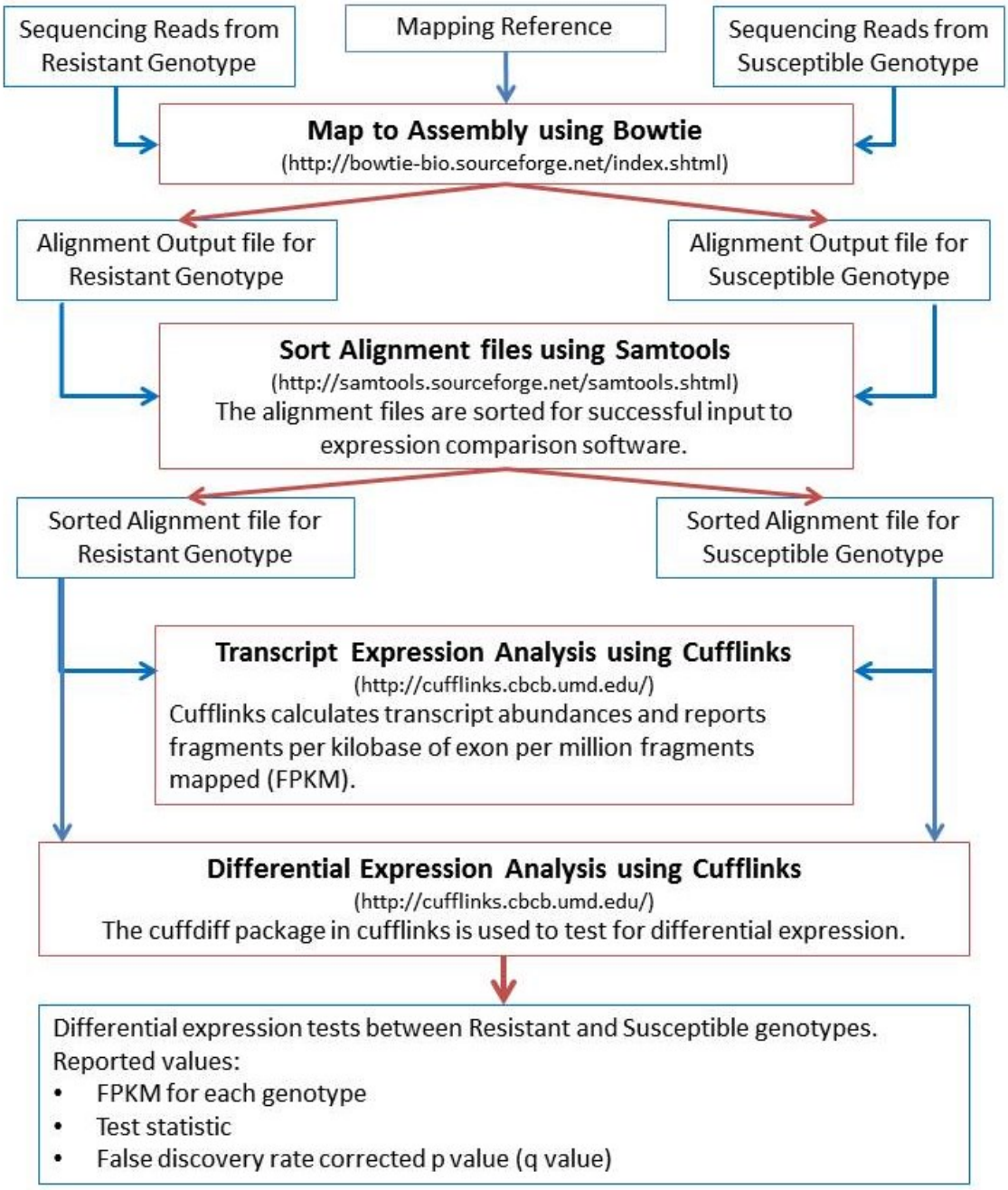


Figure 2. Genetic characterization of the Fhb1 NIL pair. 9K iSelect SNP data for near-isogenic line pair Fhb1+ and Fhb1-. SNPs shown in blue are the same in the NILs and SNPs shown in red are polymorphic between the NILs.

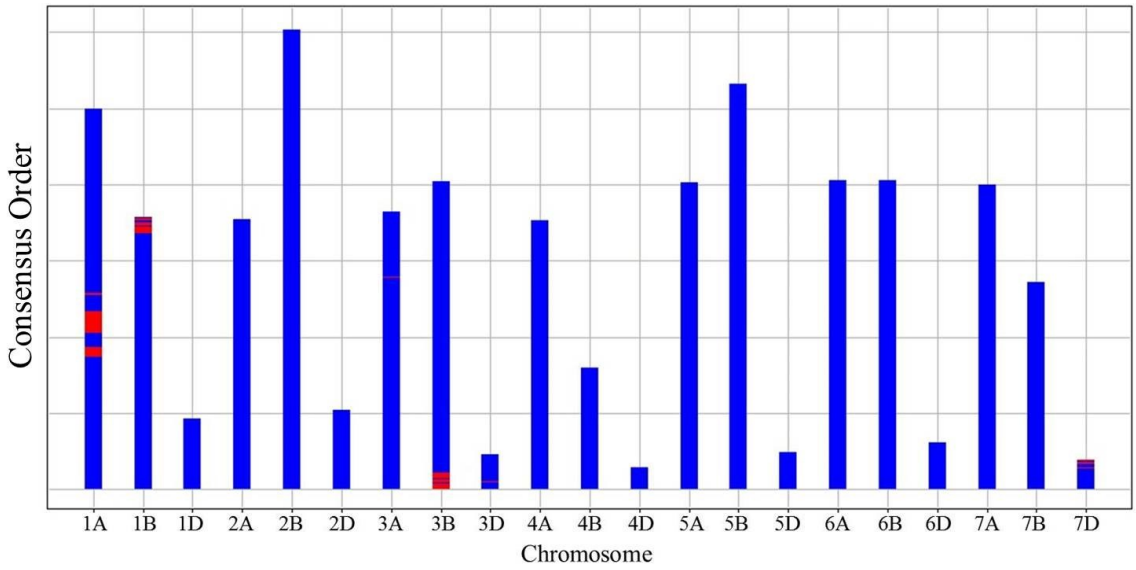


Figure 3. DON and ergosterol concentration in the spikelets of the NIL pair 1, 2, 4, and 6 days after *F. graminearum* inoculation.

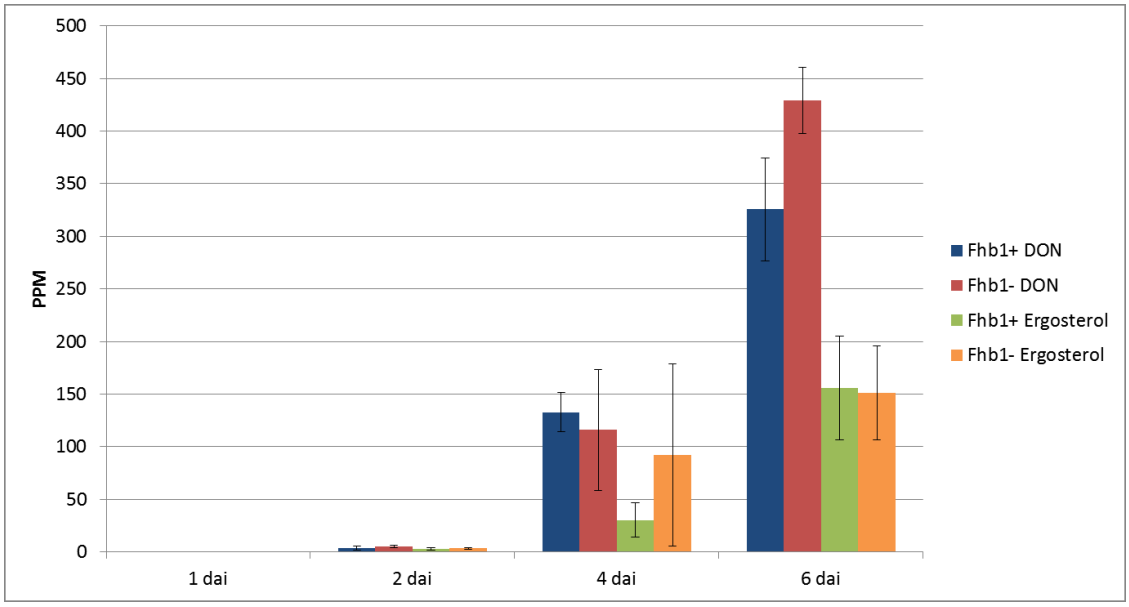
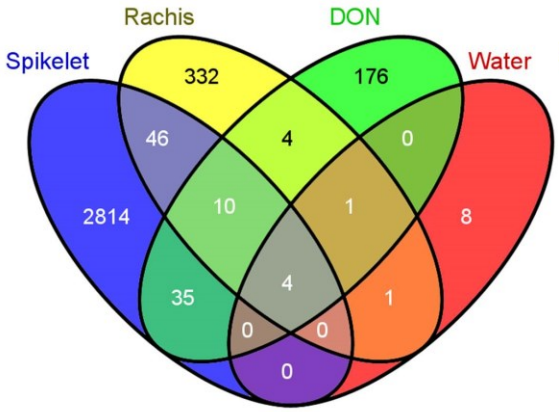


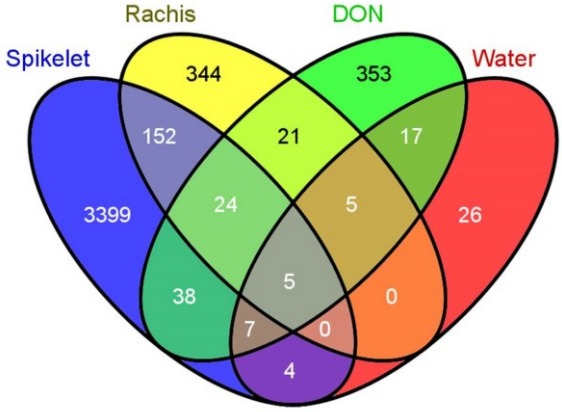
Figure 4. A. Venn diagram of differentially expressed genes (DEGs) upregulated in the resistant genotype for the *F. graminearum*-inoculated spikelet and rachis samples at 96 hai and the DON and water-inoculated spikelet samples at 12 hai. **B.** Venn diagram of DEGs upregulated in the susceptible genotype 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 upregulated in the resistant genotype between the resistant and susceptible genotypes 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 upregulated in the susceptible genotype between the resistant and susceptible genotypes for the *F. graminearum*-inoculated spikelet and rachis samples at 96 hai and DON-inoculated spikelet samples at 12 hai.

A.



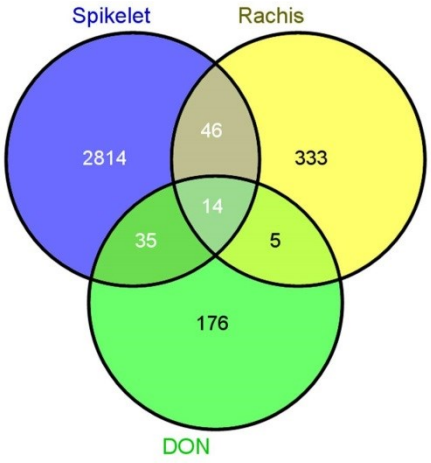
DEGs upregulated in the resistant genotype.

B.



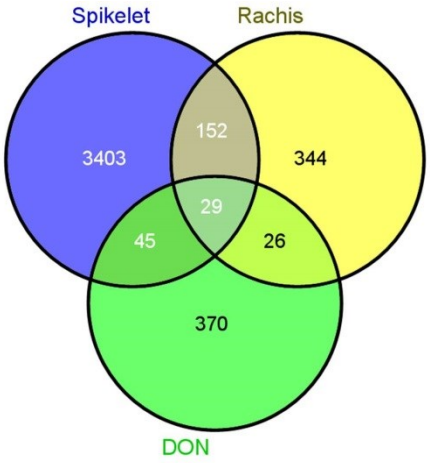
DEGs upregulated in the susceptible genotype.

C.



DEGs upregulated in the resistant genotype.

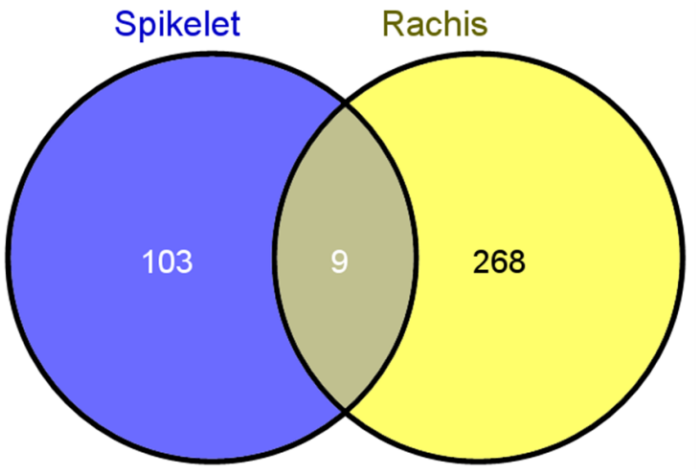
D.



DEGs upregulated in the susceptible genotype.

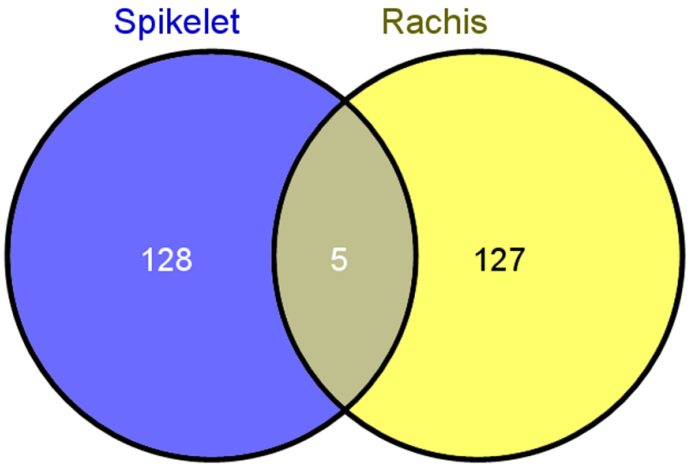
Figure 5. A. Two-way Venn diagram of *F. graminearum* genes differentially expressed between the resistant and susceptible NILs in *F. graminearum*-inoculated spikelets sampled at 96 hai and rachis sampled at 96 hai, upregulated in the resistant genotype. **B.** Two-way Venn diagram of *F. graminearum* genes differentially expressed between the resistant and susceptible NILs in *F. graminearum*-inoculated spikelets sampled at 96 hai and rachis sampled at 96 hai, upregulated in the susceptible genotype.

A.



DEGs upregulated in the resistant genotype.

B.



DEGs upregulated in the susceptible genotype.

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