COMPARATIVE POPULATION GENETICS OF *FUSARIUM GRAMINEARUM* AND NOVEL SOURCES OF RESISTANCE TO FUSARIUM HEAD BLIGHT IN SPRING WHEAT

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ABSTRACT

Fusarium graminearum is the major causal agent of Fusarium head blight (FHB) in cereal crops. The fungus produces several types of trichothecenes [Deoxynivalenol (DON) and its acetylated derivatives, 3-acetyldeoxynivalenol (3ADON) and 15-acetyldeoxynivalenol (15ADON), and nivalenol (NIV)]. Characterization of 123 isolates collected during 1980 to 2000 (old collection), and 43 isolates collected in 2008 (new collection) from North Dakota revealed that 15ADON producing isolates were predominant (98%) in the old collection while the 3ADON producing isolates accounted for 43% in the new collection. Further, evaluation showed that the 3ADON isolates caused a higher disease severity and accumulated more DON in spring wheat than the 15ADON isolates. The 3ADON also exhibited higher DON in rice culture, and produced more spores on agar media. Population genetic analyses revealed a significant genetic differentiation between the two populations. To elucidate the transcriptomic differences between the two populations *in vitro* and *in planta*, RNA-sequencing was used. The *in vitro* gene expression comparison identified 479 up- and 801 down- regulated genes in the 3ADON population compared to 15ADON population. The *in planta* pair-wise comparisons between the two populations revealed 185, 89, and 62 unique genes to 3ADON at 48, 96 and 144 hours after inoculation (HAI), respectively. In a different study, population genetic analysis was conducted on 160 isolates collected in 2008 and 2009 from a FHB disease nursery located in China. All isolates analyzed were *F. asiaticum* except one (*F. avenaceum*). Of the 159 isolates, 79% were NIV producing, 18% were 15ADON and 3% were 3ADON. The two populations grouped based on year of collection exhibited low genetic differentiation (F_{st} = 0.032). To identify new sources of FHB resistance, 71 wheat accessions of diverse origins were re-evaluated for FHB severity and haplotyped using seventeen DNA markers associated with known resistance quantitative

trait loci (QTL). Twenty two accessions had a haplotype different from all known sources used, suggesting that they may carry novel loci for FHB resistance. In conclusion, the information obtained in this study could have an impact on development of effective disease management measures and on improvement of FHB resistance in wheat.

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DEDICATION

This dissertation is dedicated to my loving parents: Tilu Puri and Dropati Puri

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CHAPTER 1. LITERATURE REVIEW

1.1. The Host: Spring Wheat

1.1.1. Evolution and economic importance

Wheat (*Triticum aestivum* L.) is one of the most important stable food crops grown across the world. In 2011, over 218.28 million hectors were planted with wheat, which yielded a total of 652.24 million metric tons of wheat grains globally (FAS/USDA, 2012). The single wheat crop contributes approximately 30% of the world's edible dry matter and 60% of the daily calorie intake in developing countries (FAOSTAT, 2008), along with the important supplement of proteins, vitamins and minerals. The wheat genus *Triticum* includes all species of diploid (2n = 14; Einkorn wheat, *T. monococcum*), tetraploid (2n = 28; durum or pasta wheat, *T. dicoccoides*) and hexaploid (2n = 42; bread wheat, *T. aestivum*) (Feuillet et al., 2008). The modern cultivated hexaploid wheat (AABBDD genome) was originated after an accidental crossing of domesticated tetraploid wheat *Triticum dicoccoides* (AABB genome) with the wild diploid species named goat grass (*Aegilops tauschii)* with DD genome, and followed by successive chromosome doubling (Salamini et al., 2002). The tetraploid (AABB) genome was evolved from a cross between *T. urartu* (AA genome) and an unknown species from *Sitopsis* selection (BB genome), that eventually became a species named as emmer wheat (*T. turgidum*) (Dvorak et al. 1998; Luo et al., 2007). The genome of hexaploid wheat contains a total of 17-gigabase-pair (Gb) with approximately 94,000 to 96,000 genes (Brenchley et al., 2012). The high-resolution synteny map indicates that the genome is highly dynamic, and has lost significant gene family members during the polyploidization and domestication process (Brenchley et al., 2012). The Fertile Crescent region of southwest Asia (Jordan, Israel, Iran, Iraq, Turkey, Syria etc.) is considered as the center of origin of modern hexaploid wheat (Brown et al., 2009).

Wheat is one of the widely adopted major cereal crops in the United States, which has an estimated production of total 60.1 million metric tons in 2011 (FAO, 2011). It includes several different classes, including hard red spring, hard red winter, soft red winter, and white wheat as well as durum wheat. These classes of wheat together occupy >23.9 million ha of growing areas in the USA, of which North Dakota (ND) alone accounts for 14.6% of the total area and produced a total of 10.26 million metric tons in 2009 (NASS, 2010). The major spring wheat cultivars grown in ND during 2009-2010 (with their area coverage percentage) were Glenn (25%), Faller (15%), Kelby (6.4%), RB07 (4.9%), Briggs (4.9%), Freyr (4.8%), Howard (4.7 %), Steele ND (3.6%), Alsen (3.0), Choteau (2.8), and Bernnan (1.9%) (NASS, 2010).

1.2. The Pathogen: *Fusarium graminearum*

1.2.1. Taxonomy and classification

The taxonomy of *Fusarium* is always a controversial issue. The genus *Fusarium* was first named by the German scientist JHF Link during 1809, based on morphological characteristics of macroconidia. The sexual state of the genus was described as *Gibberella* by Faries in 1821. Schwabe described the asexual stage as *F. graminearum*. Wollenweber and Reinking (1935) are the pioneers of taxonomy and systematics of *Fusarium*, and published the book *Die Fusarien*, which organized 1000 already described and named *Fusarium* species into 16 sections, 65 species, 55 sub-specific varieties, and 22 forms based on the mycological characters (presence or absence of microconidia and chlamydospores, and their shapes). During the 1940s and 1950s, Snyder and Hansen reduced the number of species and grouped them into nine species (*F. oxysporum, F. solani, F. moniliformae, F. roseum, F. lateritium, F. tricinctum, F. nivale, F. rifidiuscula* and *F. episphearia*). Leslie et al. (2001, 2003) introduced a distinct nomenclature and species concept on *Fusarium,* which includes morphological, biological and phylogenetic

species, respectively, based on morphology, cross fertility, and analysis of DNA sequences. Recently, an international nomenclature agreement has been made to discontinue the use of multiple names for the same fungus and therefore *Fusarium* has been proposed as the only name for this group of fungi for future publications (Hawksworth, 2011).

The filamentous fungus *Fusarium graminearum Schwabe* (telomorph: *Gibberella zeae* (Schwein) Petch belongs to Superkingdom- Eukaryota; Kingdom-Fungi; Phylum-Ascomycota; Subphylum-Pezizomycotina; Class-Sordariomycetidae; Subclass- Hypocreomycetidae; Order-Hypocreales; Family-Nectriaceae and Genus- Gibberella. Further in-depth analysis using DNA sequences of various genes for mating-types (*MAT1-1-3*, *MAT1-1-2*, *MAT1-1-1*, *MAT1-2-1*), Histone H3 reductase, Phosphate permase, *Tri101*, Ammonia ligase, Translation elongation factor 1α and β-tubulin, identified nine distinct and cryptic members within *F. graminearum* (commonly named as *F. graminearum* sensu lato) (O'Donnell et al., 2004). They were differentiated into nine distinct (lineage) species: (1) *F. austroamericanum,* (2) *F. meridionale,* (3) *F. boothii,* (4) *F. mesoamericanum,* (5) *F. acaciae-mearnsii,* (6) *F. asiaticum,* (7) *F. graminearum,* (8) *F. cortaderiae*, and (9) *F. brasilicum,* which were most commonly associated with *Fusarium* head blight. Starkey et al. (2007) described 16 phylogenetically divergent FHB isolates from a global collection of 2100 isolates based on phylogenetic analyses of multilocus DNA sequences (13 genes; 16.3 kb/strain), and identified two novel species (*F. vorosii* and *F. gerlachii*) into the *Fg* complex. Yli-Mattila et al. (2009) further added a novel Asian clade (*F. ussurianum*) into the species complex. More recently two new distinct species (*F. nepalense* and *F. louisianense*) have been identified, defining a total of 16 phylogenetically distinct species within the species complex (Sarver et al., 2011).

1.2.2. Biology

F. graminearum is the name for the anamorphic or conidial stage of a homothallic haploid fungus with the sexual stage name *Gibberella zeae*. It produces three types of asexual spores named macroconidia, microconidia, and chlamydospores (Leslie and Summerell, 2006). The asexual stage is commonly found in nature, and can be easily isolated from the infected plant host tissues. Macroconidia are produced on sporodochium, hyaline in color, septated (3-7 septa), with size ranging from 25-50×3-4 µm, generally sickle shaped, and often possess a well-developed pedicellate foot cell. Microconidia are absent in *F. graminearum*. Chlamydospores, if present, are globose, thick-walled with a slightly roughen outer surface, and present singly or on a chain. Colors varied from hyaline to pale-brown and sizes varied from 10-12µm in diameters (Leslie and Summerell, 2006).

The telomorphic phase *G. zeae* produces dark blue colored perithecia, which are generally ovoid, 140-250 µm in diameter, and composed with a rough tuberculate outer wall and thin walled inner cells. The sexual ascospores are produced in a sac like structure called an ascus. The ascus is 60-85×8-11 µm in size, clavate with a short stipe, and produces eight or occasionally 4-6 distichous or obliquely monostichous ascospores. Ascospores are 19-24×3-4 µm in size, hyaline to light-brown in color and with slightly rounded ends (Leslie and Summerell, 2006).

1.2.3. Genetics

F. graminearum contains four chromosomes, which can be visualized using cytological methods described by Taga et al. (2003). Gale et al. (2005) constructed a genetic map from the cross between PH-1 (NRRL 31084) and 00-676 (NRRL 34097), a closely related strain from Minnesota, USA. Genomic studies of *F. graminearum* were initiated in 1990s with the release of

databases for expressed sequence tags (ESTs). In 2002, Kruger et al. (2002) described an EST database having 4,838 sequences obtained from cDNA library that was created after of inoculation of *F. graminearum* on the resistant wheat variety Sumai 3. The whole genome shotgun (WGS) sequencing of *F. graminearum* strain NRRL 31084 (PH-1) was carried out at the Broad Institute, the Center for Genome Research (www.broad.mit.edu/annotation/fungi/*Fusarium*/). The whole genome sequence, generated by paired-end sequencing of plasmids, fosmids, and bacterial artificial chromosome (BAC) clones contains a total assembly of 36.1 Mb (Cuomo et al. 2007). Nearly all (99.8%) of the assembled sequences were anchored to the four chromosomes using 237 genetic markers (Gale et al., 2005). An initial set of 11,640 predicted genes were identified (Cuomo et al. 2007). The genome has a large number of genes predicted for transcription factors, transmembrane transporters and hydrolytic enzymes, and contains very few duplicated sequences (Cuomo et al., 2007). Reassembly of the *F. graminearum* genome (36.45 Mb) identified approximately 433 contigs, 13,321 genes and 37,516 exons (Broad Institute).

The alternative forms (idiomorphs) of the mating type (*MAT*) locus are located on a single chromosomal gene cluster on the genome in this homothallic fungus (Yun et al., 2000). The organization of the *MAT* region is quite different from that reported for other homothallic fungi such as *Neurospora* species. The *MAT*-2 idiomorph sequence has a single open reading frame (*MAT-2-1*), while the *MAT-1* idiomorph is more complicated and encodes for at least three transcripts (*MAT-1-1*, *MAT-1-2*, *MAT-1-3*) with a similar orientation as found in *Podospora* and *Neurospora* (O`Donnell et al., 2004). Deleting of one of the idiomorphs from *F. graminearum* can eliminate self-fertility, but retain the ability to outcross (Lee et al., 2003). All of these mating genes are subjected to a strong purifying selection in *F. graminearum* (O’Donnell et al., 2004).

Some out-crossings of the fungus have been made possible in laboratory using nitrate-non-utilizing mutants (Bowden and Leslie, 1999) or insertional mutagenesis of the mating type (MAT) locus (Lee et al., 2003). However, the outcrossing under natural conditions is very rare (O'Donnell et al. 2004).

Vegetative compatibility (VC) is a process in which two fungal isolates undergo a mutual hyphal anastomosis and result in a viable heterokaryon (Leslie and Summerell, 2006). In *F. graminearum,* VC and heterokaryon formation are governed by at least five or more vegetative incompatibility (*vic)* loci in a homogenic manner (Bowden and Leslie, 1999). Two fungal isolates can form a heterokaryon only when alleles at each of the corresponding *vic* loci are identical. Vegetatively compatible *F. graminearum* isolates can be identified using auxotrophic nitrate non-utilizing (*nit*) mutants (Bowden and Leslie, 1992) or assessing their inability to develop heterokaryons through a barrage formation (McCallum et al., 2004).

1.2.4. Population genetic study

Population genetics of the *F. graminearum* species complex has been studied in the major wheat growing regions across the world, including Asia (Gale et al. 2002, Zhang et al., 2007; Qu et al., 2008; Suga et al., 2008; Karugia et al., 2009; Lee et al., 2009, Puri et al., 2012), part of Europe (Gagkaeva and Yli-Mattila, 2004), Australia (Akinsanmi et al., 2006), the U.S. and Canada (Zeller et al., 2004; Gale et al., 2007, 2011; Ward et al., 2008; Puri and Zhong, 2010). Various molecular markers such as random amplified polymorphic DNA (RAPD) (Walker et al., 2001), restriction fragment length polymorphism (RFLP) (Gale et al., 2007), amplified fragment length polymorphism (AFLP) (Zeller et al., 2004), simple sequence repeat (SSR) (Mishra et al., 2004), sequence related amplified polymorphism (SRAP) (Fernando et al., 2007), variable

number tandem repeats (VNTR) (Suga et al., 2004), vegetative compatibility group (VCG) (Gilbert et al., 2001) were widely used for population genetic studies of the fungus.

Several *Fusarium* species such as *F. asiaticum*, *F. avenaceaum*, *F. culmorum*, *F. graminearum*, *F. nivale*, *F. poae*, *F. sporotrichioides* can cause Fusarium head blight (FHB) on wheat and barley (Osborne and Stein, 2007). Among them, *F. graminearum* is the predominant etiological agent for FHB in North America, though a recent study identified 41 *F. asiaticum* isolates from FHB samples in southern Louisiana (Gale et al., 2011). The population subdivision in *F. graminearum* often correlates with geographical differences and trichothecene types (Yang et al. 2008, Ward et al. 2008, Gale et al., 2002, 2007). Gale et al. (2003) first showed the population sub-division correlated with trichothecene chemotype in *F. graminearum*. Population subdivision based on chemotypes has also been reported in Canada (Ward et al. 2008) and other parts of the world.

Within the *F. graminearum* species complex, the isolates primarily producing DON and 15ADON (named 15ADON population) were predominant in North America (Miller et al., 1991; Abramson et al. 2001), however, more recent studies have indicated an increase of more aggressive isolates that produce DON and 3ADON (named 3ADON population) in North America (Guo et al. 2008; Ward et al., 2008, Puri and Zhong, 2010). Previously, Zeller et al. (2003, 2004) analyzed populations of *F. graminearum* from seven states of central and eastern U.S. and identified very low genetic differentiation among them. Based on their results, the local populations represented a subset of a larger panmictic population existing in the Northern Great Plains. However, the more recent studies revealed genetically divergent populations from the Northern Great Plains including the U.S. and Canada (Gale et al., 2007; Ward et al., 2008). Starkey et al. (2007) identified six *F. graminearum* isolates with the NIV or 3ADON chemotype

in Louisiana. Gale et al. (2007) also found highly divergent populations of *F. graminearum* in North Dakota and Minnesota and showed a significant genetic differentiation between the 3ADON and 15ADON chemotype populations. A significant increase of 3ADON isolates has been observed in the *F. graminearum* population in Canada in recent years (Ward et al. 2008; Guo et al. 2008). The newly emerging 3ADON population appeared to be more aggressive than the predominant 15ADON population (Ward et al. 2008, Puri and Zhong, 2010).

1.2.5. Trichothecenes produced by F. graminearum

Trichothecenes are toxic secondary metabolites produced by many species belonging to the *Fusarium* genus (Kimura et al. 2007). They include a family of over 200 toxins with a common core structure of tricyclic 12, 13-epoxytrichothec-9-ene (Grove, 2007). Deoxynivalenol (DON) and nivalenol (NIV) are primary trichothecenes found in *Fusarium* infected wheat and barley (McCormick, 2003). They are potential phytotoxins to many plant species, and can cause wilting, chlorosis, and necrosis (Cutler, 1988). They are toxic to human and animals mainly due to their ability to bind with the 60S ribosomal subunit of eukaryotes and successive inhibition of protein synthesis (Rocha et al., 2005). In wheat, the DON acts as a virulence factor that enables the fungus to spread beyond the infected spikelets into the rachis (Proctor and McCormick, 1995; Bai et al., 2002; Jansen et al., 2005, Maier et al., 2006).

Trichothecenes are produced via trichodiene on the farsesyl payrophosphate pathway (Michora et al., 2003). Structurally, they are classified as macrocyclic or nonmacrocyclic based on presence of a macrocyclic ester or an ester-ether bridge between C-4 and C-15 (Bennett and Klich, 2003). The nonmacrocyclic trichothecenes produced by *F. graminearum* can broadly categorize into two groups based on either presence or absence of oxygen atoms at carbon atoms 7 (C-7) and 8 (C-8) as type A or type B trichothecenes, respectively (Alexander et al., 2009;

Desjardins, 2006). Type A trichothecene has no hydroxyl group at C-7 but has a hydroxyl group at C-8 (e.g. neosolaniol) and ester group at C-8 (e.g. T-2 toxin) or no oxygen substitution at C-8 (e.g. trichodermin, 4,15-diacetoxyscirpenol, and harzianum A); whereas Type B trichothecenes has a hydroxyl group at C-7 and a keto (carbonyl) function at C-8 (e.g. DON, NIV, and its derivatives) (Desjardins et al. 1993; Ueno, 1980; McCormick et al., 2011). The DON and NIV have structural difference based on presence or absence of oxygen atom at C-4. DON and its acetylated forms (3A- or 15A- DON) lacks an oxygen atom in carbon C-4 but NIV and its C-4 acetyl derivatives have an oxygen atom at carbon C-4 (Alexander et al., 2009; Kimura et al., 2007).

Along with the structural differences, trichothecene biosynthesis in *Fusarium* involves a complex pathway of oxygenation, isomerization and esterification steps (Alexander et al., 2009). These biosynthetic enzymes are encoded by 15 different *TRI* genes located at three loci at different chromosomes (Gale et al. 2005; Alexander et al. 2009). These loci are 12 gene *TRI* core cluster (*Tri3*, *Tri4*, *Tri5*, *Tri6*, *Tri7*,*Tri8*, *Tri9*, *Tri10*, *Tri11*, *Tri12*, *Tri13*, and *Tri14*) (Brown et al., 2004), the two gene (*Tri1-Tri16*) locus (Brown et al., 2003; Meek et al., 2003, Peplow et al., 2003) and a single gene *Tri101* locus (Kimura et al., 1998). The genetic basis for production of DON versus NIV trichothecene is explained by the function of two genes namely *Tri13* (cytochrome P450 monooxygenase) and *Tri7* (acetyltransferase) (Lee et al., 2002). Both genes are required and functional in the NIV producers, while the DON producers have non-functional copies of these genes due to multiple deletion or insertions events (Lee et al., 2002; Brown et al., 2002). In the long pathway of *F. graminearum* NIV producers, 4ANIV is produced by the function of *FgTri1,* which is later converted to NIV with the function of *FgTri8* (McCormick et al. 2004). The exact genetic basis for 3ADON or 15ADON production and their biological

significance is still unclear. Alexander et al. (2011) indicated that the differential activity of *Tri8* (trichothecene C-3 esterase) regulates the production of 3ADON or 15ADON, and is required to convert the diacetylated 3- and 15- ADON intermediate into 3ADON or 15ADON. However, Kimura et al. (2003) previously showed that *Tri8* and *Tri3* are not necessary for 3ADON biosynthesis based on the comparative sequence and structure analysis of the *Tri5* gene cluster of 15ADON (strain H-11) and 4ANIV (strain 88-1) with 3ADON (strain F15). They emphasized the involvement of three pathway specific genes (*FgTri4*, *FgTri5* and *FgTri11*) in 3ADON biosynthesis.

Allelic polymorphisms at the trichothecene biosynthetic gene cluster are being used to identify chemotype differences using PCR (Ward et al., 2002, O'Donnell et al., 2000). Genetic markers at *Tri3*, *Tri12* and *Tri13* genes have been developed, which correlate with 3ADON, 15ADON or NIV chemotypes producers of *F. graminearum* (Starkey et al., 2007; Ward et al., 2008; Wang et al., 2008). Various chemical analysis methods such as gas chromatography (GC), spectrometry (MS) or GC-MS methods have been developed for the trichothecene quantification, and used for routine DON analysis in many FHB resistant breeding experiments (Mirocha et al., 2003). Other methods such as enzyme-linked immunosorbent assay (ELISA), thin layer chromatography (TLC) are also in use, although they are time consuming and not as reliable as GC-MS (Mirocha et al., 2003).

Several functional genomic studies have been conducted to understand host infection process of *F. graminearum* (Guenther and Trail, 2005; Miller et al., 2004), and a number of genes have been shown to be involved in pathogenicity, virulence, and DON accumulation (Kazan et al. 2012). The targeted deletion of various *Tri* genes has confirmed their role in virulence and DON accumulation on grains (Proctor et al., 1995). Deletion mutants of the core

DON biosynthetic gene *Tri5* lost DON accumulation on spikes and were unable to spread beyond inoculated points (Bai et al., 2002). Among genes within the *Tri* cluster, *Tri6* and *Tri10* are the global gene regulators. *Tri6* is a pathway-specific transcriptional regulator and required for trichothecene biosynthesis (Seong et al., 2009; Peplow et al., 2003). Further study indicated that it is involved in regulation of six *Tri* genes (*Tri1, Tri3, Tri6, Tri7, Tri12 and Tri14*) within cluster and additional 192 potential *F. graminearum* genes (Nasmith et al. 2011). *Tri10* has also been involved in trichothecene biosynthesis and regulates expression of several other *Tri* genes (*Tri3, Tri7, Tri8, Tri9, Tri11* and *Tri12*) in *F. sporotrichioides* (Peplow et al., 2003). Deletion mutants of both *Tri6* and *Tri8* had showed a reduced pathogenicity on wheat (Seong et al. 2009). Deletion mutant of another regulatory gene *Tri14* has also reduced pathogenicity and trichothecene accumulation on wheat heads (Dyer et al., 2005), although there was no difference from the wild type on toxin accumulation *in vitro* (Brown et al., 2002).

1.2.6. Transcriptome analysis by RNA-seq

RNA sequencing is a relatively new sequence based approach in transcriptomics for in-depth and robust assessments of transcript abundances and transcript structure and functional characterization of genes (Wang et al., 2009). This technology has several advantages over DNA hybridization based technologies such as microarray and tiling arrays (Marguerat and Bahler, 2010; Wang et al., 2009). The major advantages are that it requires a small amount of RNA, can detect transcripts beyond the existing genomic sequence, and has a very deep coverage with a high reproducibility from both technical and biological replications (Wang et al., 2009; Nagalajshmi et al., 2008). The steps for RNA sequencing procedure are easy and very straightforward. they include RNA isolation, purification of Poly-A containing transcripts, fragmentation of transcripts, conversion to cDNA, ligation of indexing adapters, and sequencing

of prepared libraries (Young et al., 2012; Marguerat and Bähler, 2010). Many high throughput sequencing technologies such as Roche 454 from Life Lcience (Barbazuk et al., 2007), SOLiD system from Applied Biosystem (Cloonan et al., 2008), Illumina HiSeq2000 (Glenn, 2011) from Illumina; Ion Torrent by Life Technologies (Rothberg et al., 2011) are increasingly being used for RNA sequencing, although other direct cDNA sequencing techniques such as serial analysis of gene expression (SAGE) (Velculescu et al. 1995), and massively parallel signature sequencing (MPSS) (Brenner et al., 2000) were in use.

The use of RNA-seq for comparative transcriptome study during host-pathogen interaction is emerging rapidly and used in identification of novel genes related to pathogenicity or virulence in plant pathogenic fungi. The infection related global gene regulation profiling of many fungal-host interactions such as *Pseuodperonospora cubensis* -cucumber (Savory et al., 2012), *Phytopthora phaseoli* - lima bean (Kungeti et al., 2012), *Magnaporthe oryzae*-rice (Bagnaresi et al., 2012), or defense transcriptome study in response to *Fusarium oxysporum*-infection in Arabidopsis has been widely studied using the RNA-Seq techniques.

In *F. graminearum*, the transcriptome profiling and their relationships with DON accumulation, virulence or aggressiveness during wheat and barley infection have been studied using DNA microarrays (Boddu et al. 2006, 2007; Gardiner et al. 2009, 2010; Lysoe et al. 2011) and several candidate genes for pathogenicity and DON were identified. The microarray analysis of deletion mutants for the two transcription factor genes *Tri6* and *Tri10* required for DON biosynthesis and pathogenicity showed their global regulation of over 200 genes during host infection (Seong et al., 2009) or during *in vitro* growth (Nasmith et al., 2011). Several secreted proteins identified during host infection by *F. graminearum* were proposed as the effector proteins for host infection or fungal growth on culture media (Paper et al., 2009). Using

microarrays, Lysoe et al. (2011) found that 43% of the *F. graminearum* expressed genes during wheat and barley infections had a signal peptide motif, and they might act as the effectors during host infection. Thus, the global gene profiling using microarrays or RNA-Seq technology will be a very powerful tool to provide new insights into mechanisms of host-pathogen interactions, pathogenicity and DON accumulation.

1.3. The Disease: Fusarium Head Blight (FHB)

Fusarium head blight (FHB) is a devastating and economically important disease of wheat (*Triticum aestivum* L) and barley (*Hordeum vulgare* L) in North America (McMullen et al. 1997; Jones and Mirocha, 1999; Gilbert and Tekauz, 2000; Windels, 2000) and worldwide (Parry et al., 1995; Goswami and Kirstler, 2004). The disease causes a significant reduction in yield and quality of harvested grains due to contamination with several mycotoxins including Deoxynivalenol (DON) and derivatives, oestrogenic mycotoxin, aurofusarin, and zearalenone (Scott, 1990; Bai and Shaner, 1996; McMullen et al., 1997; Trail, 2009). The disease has been listed as one of the major yield-limiting factors in wheat production throughout the world by CIMMYT (Dublin, 1997). The infected grains show low seed germination and may cause seedling blight with poor crop stand (Bai and Shaner, 1994). Further, the FHB infected scabby kernels have reduced grain test weight, lower market price or even without food and feed values. Consumption of mycotoxin-contaminated grains and their product may cause feed refusal, poor weight gain, diarrhoea, alimentary haemorrhaging and contact dermatitis on livestock. In humans, it may cause an acute or chronic health problems including alimentary toxic aleukia and akakabi toxicosis; nausea, vomiting, and anorexia; and neurological disorders and immunosuppression (Nelson et al., 1993, Desjardins, 2006, Bennett and Klich, 2003). The U.S.

food and drug administration (FDA) limits the level of DON to 1 μg/g on finished product for human consumption (Aakre et al., 2005).

1.3.1. Symptoms, disease cycle and epidemiology

Initial *Fusarium* infection appears as small water-soaked brownish spots on the rachis or on the bases of glumes, which later become bleached, giving a characteristic symptoms of partially white and partially green heads (Wiese, 1987; McMullen et al., 2008). Bleaching can extend in all directions from the point of infection and might lead to a complete dead head in susceptible cultivars (Wiese, 1987). Under prolonged wet conditions, a typical light pink to salmon-orange color fungal mass can be seen on infected tissues, and glumes can be covered with a white mycelial growth (McMullen et al., 2008; Trail, 2009). Infected grains at harvest might be shriveled, light weighted with a pinkish discoloration. Perithecia or sporodochia may be seen on exterior surface of wheat heads, peduncle or other tissues at the end of season in the high humid regions (Bushnell et al. 2003). Seeding blight might be obvious, if blighted seeds were planted (Stack, 2003). The generalized disease cycle of this monocyclic disease starts once the airborne ascospores land on flowering spikelets as a primary source of inoculum, which successively germinate and enters into the plant through natural opening or via the degenerating anther tissue (Trail, 2009, Bushnell et al., 2003). Initially, the pathogen grows inter-cellularly and asymptomatically via xylem and pith (Bushnell et al., 2003, Jansen et al., 2005). The detailed microscopic study of infection process has now showed no indication of necrotrophy at the initial stage of the infection. Once the fungus starts to grow intracellularly, the subsequent necrosis and cell death are prominent (Brown et al., 2010). Thus, *F. graminearum* has been classified as a hemibiotrophic pathogen (Kazan et al., 2012). This pathogen completes its life cycle in culture or in association with hosts (Trail, 2009). Perithecia are the sexual stage for

overwintering (Guenther and Trail, 2005). However, the fungus can also overwinter as mycelia on infested crop residues and seeds or on the wild plant hosts. Although ascospores are primary sources of inoculum, both ascospores and conidia have an important role in FHB epidemics (Markell and Francl, 2003). The rain-splashed conidia from infected tissue acts as a secondary source of inoculum to the newly flowering tiller heads. Crop rotation with corn in the preceding season and use of minimum tillage practices can also increase the risk of FHB on wheat and barley (Wilcoxson et al., 1988).

Fusarium head blight disease epidemics are favored by several factors. High humidity for a prolonged time (48-72h) coinciding with warm temperature (24-28°C) at anthesis is optimum for successful disease establishment and spread (Osborne and Stein 2007). The 25°C temperature favors optimal mycelial growth, while 32°C is optimum for sporulation (Osborne and Stein, 2007). However, infection might occur under the cooler temperature if high humidity persists for longer period of time (>72 h) (McMullen et al., 2008). Along with the high humidity and warm conditions, the level of host resistance, pathogen aggressiveness, abundance of inoculum, and agricultural practices also play a crucial role for disease epidemics (McMullen et. al. 1997, 2008, 2012).

1.3.2. FHB management

Integration of multiple management practices such as cultural practices, resistant cultivars, fungicide spray, proper tillage, and biological control, is recommended for reducing the yield loss due to FHB, rather than sole dependence on a single management tool (McMullen et al., 2008, 2012).

Cultural practices are targeted to reduce or avoid the exposure of wheat spikes to spore loads during anthesis to early milk fill stage. Many cultural practices such as crop rotation

(avoiding inoculum produced on cereal host residue); tillage practices such as deep burying or burning of infested residue; mechanical chopping of crop residue to enhance quick decomposition; proper fertilization; stagger planting date or use of cultivars that differed in maturity dates to reduce entire crop loss are effective measures to minimize the FHB incidence and severity (Parry et al., 1995, Pereyra and Dil-Macky, 2008, Pererya et al., 2004, McMullen et al., 1997, Pirgozliev et al., 2003). Additionally, FHB infected grains can be reduced using seed cleaning equipment after harvest. Increasing fan speeds and airflows can lower the number of Fusarium damaged kernels and DON level in grains (Salgado et al., 2011).

Biological control measures may be an important strategy of integrated FHB management, especially on organic farms where fungicides can not used (McMullen et al., 2012). Several fungal, bacterial and yeast species are reported to be potential biological control agents for *F. graminearum* (Yuen and Schoneweis, 2007, Gilbert et al, 2004, Jochum et al., 2006, Khan et al., 2004, 2008). Several bacterial species such as *Lysobactor* (induce localized resistance); *Bacillus* (produces antifungal metabolites), *Pseudomonas* and yeasts (*Cryptococcus*) are reported as potential biological control agents against *F. graminearum* (Yuen and Schoneweis, 2007). A significant reduction on disease and level of mycotoxin contamination has been obtained consistently from the greenhouse experiments, and occasionally from field experiments using biological control agents; however, successful commercialization of bio-control agents is still lacking and needs future research.

Fungicides, in integration with other management tools and a disease forecasting system, are and effective way of disease management (McMullen et al., 1997). Paul et al (2008) highlighted a few effective fungicides currently used for FHB management using multivariate analysis of over 100 uniform fungicide trials, and concluded that the combination of

tebuconazole and protihocnazole (Prosaro) is the most effective fungicide. This combination can suppress disease by 52% over the control. The same study further highlighted other effective fungicides with active ingredients of metconazole (50% reduction) (Caramba), protioconazole (Proline) (48% reduction), tebuconazole (40% reduction) (Folicur, Orius, Monsoon, Embrance, Emboss, TebuStar), and propiconazole (32% reduction) (PropiMax, Tilt) for FHB management. Also, some fungicides such as metconazole (45 % reduction) and prothioconazole (43% reduction) singly or in combination with other fungicides were found effective to reduce the level of DON on grains (Paul et al, 2008). In recent years, registrations of effective fungicides for disease management or to lower level of DON content have been increasing (Bradley, 2009). However, the higher costs associated with fungicide, lack of consistent disease control under field conditions, inappropriate fungicide application techniques, and lack of proper disease forecasting system are always the major pitfalls for effective fungicide application (McMullen et al, 1997). An integrated disease management approach using host resistance (discussed below), cultural practices and fungicide application are the most efficient and effective ways for FHB disease management (McMullen et al., 1997; Bai and Scanner, 2004, Wegulo et al., 2011).

1.3.3. Host resistance to FHB

Mesterhazy (1995) summarized various resistance mechanisms in wheat against Fusarium head blight, and categorized them as an active (physiological) or passive (morphological) resistance. The active resistance mechanisms include resistance to initial infection, resistance to pathogen spreading, resistance to kernel infection, resistance to toxins on grains, and tolerance (Schroeder and Christensen 1963, Miller and Young, 1985; Snijders and Perkowski, 1990). The passive resistance mechanism is disease escape or low disease due to plant height, flower opening time, heading date, ear compactness, and the presence/absence of

awns etc. (Mesterhazy 1995; Buerstmayr et al. 2009). For example, dwarf plants had higher chances of natural infection due to favorable microclimate and higher inoculum load from the ground compared to taller plants, or high spike density may create a favorable micro climate for the pathogen, or awned spikes can trap more spores and increase risk more than the awn-less spikes. In addition, presence of waxy glumes may exclude moisture from spikes and can act as a barrier to the fungal infection.

Although various types of active resistance mechanisms had been described on this pathosystem (Rudd et al., 2001), three types of resistance mechanisms known as Type I (resistance to initial infection), Type II resistance (resistance to spread), and resistance to DON accumulation (also known type III resistance) were well studied and documented (Schroder and Christensen 1963; Mesterhazy 1995; Somer et al., 2003). Among them, the type II resistance is the most studied, stable and widely used in many wheat-breeding program across the world (Bai and Shaner, 2004). In order to access these types of resistance, distinct inoculation methods are in use. The Type I resistance is measured by counting the number of diseased spikelets after whole spike spray inoculation, while Type II resistance is evaluated based on disease spread from the point of single floret inoculation at the middle of a spike during flowering (Bai and Shaner 2004; Buerstmayr et al. 2009). Measure of type III resistance needs a chemical analysis of harvested grain samples (Mirocha et al. 2003).

1.3.4. Sources of resistance to FHB

Fusarium head blight resistance sources in wheat can broadly be classified from four origins namely Europe, East Asia, South America, and North America (Bai and Shaner, 2004; Buerstmayr et al. 2009). None of these sources were identified as immune to the disease, but a very good level of resistance has been identified from Asian and European gene pools (Bai and

Shaner, 2004; Buerstmayr et al. 2009). The Chinese wheat cultivar Sumai 3 (Funo/Taiwan Xiaomai) or its derivatives and pedigrees are the most commonly used source of resistance (type II) and considered as the foundation source of FHB resistance in many U.S. wheat-breeding programs (Rudd et al., 2001). The resistance other than Sumai 3's has been identified from other sources such as 'Chokwang' from Korea (Shaner and Buechley, 2001), 'Fundulea 201R' from Romania (Shen et al. 2003a), and 'Ernie and Freedom' from the USA (Rudd et al. 2001). Other key spring wheat genotypes with a good type II resistance include Wangshuibai and several Ning selections from China, Nobeokabozu Komugi, Saikai 165 from Japan, Frontana and Encruzilhada from Brazil, and some CIMMYT genotypes (Wang and Miller, 1987; Snijders 1990; Ban, 2000; Anderson et al., 2001; Steiner et al., 2004; and Zhou et al, 2004). In addition, a few winter wheat cultivars (Renan, Arina, and Praag-8) were identified as a good source of resistance to FHB from the European gene pool (Snijders, 1990; Ruckenbauer et al., 2001; Gervais et al., 2003). Many alien species of wheat have FHB resistance (Cai et al. 2005). Wan et al. (1997) identified many grass species belonging to the genera *Hystrix, Psathyrostachys* and *Roegneria,* with a better type II resistance than Sumai 3. However, breeding for FHB resistance from these alien sources or grasses is very difficult because of their exotic origin, oligo or polygenic inheritance for resistance, and resistance genes closely linked with poor agronomic traits (Bai and Shaner, 2004). Further, the FHB screening program needs a significant amount of time and resources, as these traits are more severely subjected to genotype by environment interactions (Buerstmayr et al., 2002; Zwart et al., 2008,).

1.3.5. Inheritance, QTL mapping, and breeding of resistance to FHB

FHB resistance is quantitatively inherited and controlled by oligo or polygenic quantitative trait loci (QTL) (Mesterhazy, 1995; Buerstmayr et al. 2009). A few major QTLs and

several minor QTLs have been identified for FHB resistance across wide genetic backgrounds. The details of 52 QTLs mapped, their sources and the phenotypic variation governed by them are recently reviewed by Buerstmayr et al. (2009).

Molecular markers have been used in QTL mapping studies for decades. The restriction fragment length polymorphisms (RFLPs) (Grodzicker et al. 1974) were the first DNA based molecular markers used in linkage mapping. However, several drawbacks associated with RFLP markers such as low frequency of polymorphism, requiring relatively larger amount of DNA and the need for autoradiography, a long time-consuming procedure, have made them less popular. In recent years, several polymerase chain reaction (PCR) based markers such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) or various known sequences based markers such as simple sequence repeats (SSR) or microsatellite markers, sequence tagged sites (STS), sequence characterized amplified regions (SCAR), and single nucleotide polymorphisms (SNP) are becoming more popular and used in many mapping studies. Among them, the SSR markers are the common choice as they can detect variation from one to six bases, are highly polymorphic, mostly codominant and dispersed throughout the wheat genome (Röder et al. 1998). Somers et al. (2004) first developed a high-density microsatellite consensus map of bread wheat with 1,235 microsatellite loci covering 2569 cM. In recent years, the Diversity Arrays Technology (DarT) markers have also been in use for genetic mapping and fingerprinting studies in wheat (Akbari et al., 2006).

The molecular markers associated with known QTLs to FHB resistance have been mapped on almost all wheat chromosomes except 7D (Bai and Sanner, 2004, Buerstmayr et al. 2009). However, some QTLs were not detected in different genetic backgrounds. The inconsistent mapping result might be due to several reasons such as polygenic inheritance of

FHB resistance, difference in types of resistance evaluated, effect of genetic background, inoculation methods used or strong genotype×environmental interactions (Kolb et al. 2001).

Among the various QTLs mapped to date, those in Sumai 3 have been consistently shown to be consistent under wide environmental and genetic backgrounds (Anderson et al., 2001, Bai et al., 1999, Buerstmayr et al., 2002, 2003; Zhou et al., 2004, Yang et al., 2003). Sumai 3 has major QTLs on chromosome 3BS, 5AS, 6BS and minor QTLs on 6AS and 2AS (Waldron et al. 1999; Anderson et al. 2001; Buerstmayr et al. 2002; Zhou et al. 2002; Shen et al. 2003b; Yang et al. 2003). The resistance QTL known as *Fhb1* (*Qfhs.ndsu-3BS*) accounted up to 60% of the phenotypic variation for the type II resistance (Anderson et al., 2001, Buerstmayr et al., 2002) and it is also responsible for DON detoxification (Lemmens et al., 2005). Another major QTL from Sumai 3 has been mapped on the chromosome 6BS and named *Fhb2* (Cuthbert et al. 2006). Similarly, major QTLs governing a good resistance have been mapped on chromosome 3BS in Wangshuibai (Zhou et al. 2004), chromosomes 2DL and 4BS in Wuhan-1 (Somers et al. 2003), chromosome 5DL in Chokwang (Yang et al. 2005), chromosome 3AL in Frontana (Steiner et al. 2004), and chromosome 5A in PI 277012 (Chu et al. 2011). The European winter wheat Arina has major QTLs on chromosome 4AL, 5BL and 6DL (Paillard et al. 2004; Draeger et al. 2007; Semagn et al. 2007). Similarly, another cultivar Renan had QTLs on chromosomes 2BS and 5AL (Gervais et al. 2003). Several QTLs for Type I resistance have been mapped. The QTLs mapped on 3A and 5A of cultivar Frontana (Frontana×Remus) double haploid population explained 16% and 9% of the phenotypic variance for type I resistance, respectively (Steiner et al. 2004), while a QTL on 2BS in cultivar Goldfield explained 29% of the variation in FHB incidence (Gilsinger et al. 2005). Steed et al. (2005) also reported a QTL for Type I resistance on 4A using a double haploid population after spray inoculation with *F. culmorum*. The QTL on 5AS (*Qfhs.ifa-5A*) is

also the most frequently observed QTL from both Chinese and European resistant sources, which contribute towards type I resistance (Buerstmayr et al. 2003a, Lin et al. 2006) and also related to DON detoxification.

As the FHB resistance in wheat is controlled by polygenes and often associated with poor agronomic traits, it makes breeding a real difficult task and requires a long evaluation process (Mesterhazy, 1995, Rudd et al., 2001). In addition, various types of resistance (Type I, Type II, DON tolerance and so on) exist and it still not clear whether they are controlled by the same QTL or not (Bai and Shaner, 2004, Buerstmayr et al. 2009). In the past decades, the conventional breeding approach has succeeded in breeding and pyramiding of genes against FHB from various sources (Bai et al. 2003; Mergoum et al., 2007). Most of the breeding programs across the world, including the United States, Asia, Europe, and CIMMYT, have heavily relied on Sumai 3 or its derivatives as Type II resistance for wheat improvement (Rudd et al. 2001; Buerstmayr et al. 2002; Mesterházy, 2003). However, the continuous use of a single source of resistance might create a selection pressure on the pathogen, leading to breakdowns of existing resistance genes (Gervais et al. 2003). Thus, it is essential to identify new sources of resistance and integrate them into adapted cultivars with better agronomic traits. With the development of molecular markers linked to the QTL for FHB resistance, the breeding programs can greatly facilitate and hasten their transfer into wide generic backgrounds via marker-assisted selection (MAS). Several molecular markers tightly linked with FHB resistance have been identified and are being used to eliminate susceptible materials during the early breeding process. Thus, the use of conventional breeding in combination with MAS could be an effective way to obtain durable resistance to FHB in wheat (Bai and Shaner, 2004, Anderson et al., 2007).

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CHAPTER 2. THE 3ADON POPULATION OF *FUSARIUM GRAMINEARUM* FOUND IN NORTH DAKOTA IS MORE AGGRESSIVE AND PRODUCES A HIGHER LEVEL OF DON THAN THE PREVALENT 15ADON POPULATION IN SPRING WHEAT

2.1. Abstract

Fusarium head blight (FHB) is primarily caused by *Fusarium graminearum* in North America. Isolates of *F. graminearum* can be identified as one of three chemotypes, i.e., 3-acetyl-Deoxynivalenol (3ADON), 15-acetyl-Deoxynivalenol (15ADON) and nivalenol (NIV). In this study, we characterized *F. graminearum* isolates collected from 1980 to 2000 (old collection) and in 2008 (new collection) from North Dakota and found a 15-fold increase of 3ADON isolates in the new collection. Evaluation of randomly selected 3ADON isolates and 15ADON isolates on three spring wheat genotypes (Grandin, Steele-ND and ND 2710) by single-floret inoculation indicated that the 3ADON population caused a higher disease severity and produced more DON at a significant level than the 15ADON population on Grandin (susceptible to FHB) and ND 2710 (with FHB resistance from Sumai 3). However, no significant differences in disease severity and DON production were observed between the two populations on Steele-ND (with moderate resistance from *Triticum dicoccoides*). The 3ADON isolates also exhibited a higher DON production in rice culture and produced more spores on agar media than the 15ADON isolates, suggesting a fitness advantage of the newly emerging 3ADON population over the prevalent 15ADON population. Population genetic analyses using DNA markers revealed a significant genetic differentiation between the two populations. The information obtained in this study could have an impact on development of FHB resistant wheat cultivars and disease management.

2.2. Introduction

Fusarium head blight (FHB) or head scab is a devastating disease of wheat (*Triticum aestivum* L) and barley (*Hordeum vulgare* L) in North America (McMullen et al. 1997; Jones and Mirocha, 1999; Gilbert and Tekauz, 2000; Windels, 2000) and many other regions of the world (Parry et al., 1995; Goswami and Kirstler, 2004). The disease not only causes significant yield losses, but also reduces the quality of harvested grains due to contamination of mycotoxins produced by the *Fusarium* pathogens (Scott, 1990, Bai and Shaner, 1996). Consumption of mycotoxin-contaminated grains and their products poses acute or chronic health hazards to human and livestock (Nelson et al., 1993, Desjardins, 2006).

Several *Fusarium* species can cause FHB in barley and wheat, but *Fusarium graminearum* Schwable [teleomorph: *Gibberella zeae* (Schweinitz) Petch] is the primary causal agent of the disease in North America. The fungal pathogen belongs to the *F. graminearum* species complex (*Fg* complex), which has been separated into at least 11 distinct phylogenetic species (O'Donnell et al., 2004; Starkey et al., 2007). The *Fg* complex and the other five closely related species produce various B-trichothecene metabolites, including deoxynivalenol (DON) and its derivatives [3-acetyl deoxynivalenol (3ADON), 15-acetyl deoxynivalenol (15ADON)] and nivalenol (NIV) (O'Donnell et al. 2000). Based on the profile of trichothecenes produced, *F. graminearum* isolates can be grouped into one of three chemotypes, i.e., 3ADON, 15ADON and NIV (Miller et al., 1991, O'Donnell et al. 2004). A 3ADON chemotype produces DON and primarily 3ADON while a 15ADON chemotype generates DON and primarily 15ADON. Isolates with a NIV chemotype produce NIV and its acetylated derivatives. Previous studies indicated that isolates with a 15ADON chemotype in the *Fg* complex were predominant and largely responsible for FHB epidemics in North America (Miller et al., 1991;, Abramson et al.

2001). Zeller et al. (2003; 2004) analyzed and compared populations *of F. graminearum* from seven states of central and eastern United States (US) and found a low genetic differentiation among them. They suggested that these local populations represent a subset of a larger panmictic population in North America (Zeller et al., 2004). However, several recent studies indicated that genetically differentiated populations of *F. graminearum* have emerged in Canada and the US. In a global molecular surveillance of FHB pathogens, Starkey et al. (2007) identified six *F. graminearum* isolates with a NIV or 3ADON chemotype in Louisiana. Gale et al. (2007) identified highly divergent populations of *F. graminearum* in North Dakota and Minnesota where 3ADON chemotype isolates were identified, which showed a significant genetic differentiation from the 15ADON populations. More recently, Ward et al. (2008) and Guo et al. (2008) reported a significant increase of the *F. graminearum* isolates with the 3ADON chemotype in Canada. The newly emerging 3ADON population appears to be more aggressive than the 15ADON population based on growth rate and DON production *in vitro* (Ward et al. 2008). However, little information is available on the aggressiveness and DON production of the 3ADON population in spring wheat cultivars with different sources of FHB resistance. The objective of this study was to test the hypothesis that the increasing 3ADON population collected from spring wheat in North Dakota is more aggressive in disease development and DON production compared to the prevalent 15ADON population. We characterized *F. graminearum* isolates collected from 1980 to 2000 and those collected during 2008 from North Dakota spring wheat by multiplex PCR chemotyping, greenhouse inoculation on three wheat genotypes with different levels of FHB resistance, amplified fragment length polymorphism (AFLP) and variable number of tandem repeat (VNTR) analyses.

2.3. Materials and Methods

2.3.1. Fungal isolation and identification

A total collection of 165 isolates of *F. graminearum*, including 120 collected from 1980 to 2000, two collected in 2004, and 43 collected in 2008 from different counties of North Dakota, were used in this study. These fungal isolates were isolated from symptomatic wheat spikes collected from farmers' fields. Kernels showing FHB symptoms were surface sterilized with 6% sodium hypo-chloride for 2-3 minutes, rinsed 2-3 times with sterile distilled water, and kept on three layers of moist blotting paper. Visible fungal mycelia grown from kernel surfaces were transferred into half-strength (1/2) potato dextrose agar (PDA) (100g of potato, 10g of dextrose, 7.5g of bacto-agar per liter of water). After 5-6 days of growth, spore mass was picked with an inoculation loop and streaked onto a new 1/2-PDA plate. The individual single spores were allowed to germinate for 24 h. After that, a single-spore colony was picked and transferred into a new 1/2 PDA plate. After 7 days of growth, the single spore cultures were stored in 20% glycerol and kept at –80°C until needed. Morphological characters as well as the *Fg*-specific PCR primer sets, UBC85F410/ UBC85R410 (Schilling et al., 1996) and Fg16F/Fg16R (Nicholson et al., 1998), were used to confirm the identity of all isolates used in the study. To further identify the isolates in the *Fg* complex to the phylogenetic species level (O'Donnell et al. 2004), portions of the reductase and histone H3 genes were amplified by PCR from each of the isolates and sequenced according to the methods previously described (O'Donnell et al. 2000; 2004).

2.3.2. DNA extraction and PCR-based chemotyping

DNA was extracted from mycelia of the 6-7-day-old single-spore cultures grown over cellophane on 1/2 PDA using the FastDNA® Kit and FastPrep® instrument (MP Biomedicals,

Irvine, CA). For chemotype determination, the trichothecene primer 3CON (5-TGGCAAAGACTGGTTCAC-3) and the three chemotype-specific primers 3NA (5-GTGCACAGAATATACGAGC-3), 3D15A (5-ACTGACCCAAGCTGCCATC-3), and 3D3A (5-CGCATTGGCTAACACATG-3) were used for PCR amplification with the conditions described by Ward et al. (2002) and Starkey et al. (2007). These primer combinations amplify fragments of 840, 610, or 243 bp that correspond to the NIV, 15ADON, or 3ADON chemotypes, respectively (Ward et al. 2002).

2.3.3. Aggressiveness evaluation

A greenhouse experiment was conducted to test the aggressiveness of 25 randomly selected isolates of *F. graminearum* (twelves 15ADON and thirteen 3ADON isolates) (Table 2) on the spring wheat genotypes Grandin, Steele-ND, and ND 2710. Grandin is a spring wheat cultivar that is highly susceptible to FHB. Steele-ND exhibits a moderate level of resistance to FHB, which is thought to be derived from *Triticum dicoccoides* (Mergoum et al., 2007). ND 2710 is a spring wheat line with a high level of FHB resistance derived from Sumai 3 (Frohberg et al. 2004). A randomized complete block design (RCBD) was used with three replications and all isolates were randomized within each replication. For each wheat genotype, hill plots per replication were planted on a bed filled with sterilized topsoil, with 15 cm space between plots and 8-10 seeds/plot. The plants were grown at approximately 23±2°C with 16 h of supplemental lights until anthesis. Irrigation was done daily and plants were fertilized with Multicote®4 containing N:P:K (14:14:16) and minor nutrients (Heifa Biochemical, Israel).

To prepare spores for inoculation, each of the isolates was grown on mung bean agar (MBA) (Evans et al., 2000) plates at room temperature with a combination of fluorescent and near-UV light at 12- h light and dark cycle for 10-12 days. Then conidia were harvested from the

agar plates by adding sterilized distilled water, scraping the agar surface with a rubber policeman, then filtered through two layers of cheesecloth and quantified with a hemocytometer. Final inoculum at 1×10^5 macroconidia/ml in water was used to inoculate wheat spikes using the single-floret injection method (Rudd et al., 2001). The central floret of individual spikes at anthesis was inoculated with 10 µl of the inoculum using a micropipette. Inoculated heads were misted with the overhead misting nozzles for 30 seconds at 30-min intervals to maintain high humidity. Disease rating was done at 21 days after inoculation (dai) using the pictorial scale developed by Stack and McMullen (1995). The disease severity for each inoculated spike was measured based on the number of spikelets infected: no infection as 0%, one spikelet infected as 7%, two as 14%, three as 21%, four as 33%, ½ of the spike as 50%, 2/3 of the spike as 67%, ¾ of the spike as 80%, and the whole spike or dead rachis as 100%. At least eight spikes were inoculated and rated in each replication (hill plot) for each wheat genotype. The average disease severity was calculated according to Stack and McMullen (1995).

2.3.4. Analysis of trichothecenes from grains and rice cultures

For evaluation of trichothecene accumulation in grains, the inoculated heads of each cultivar from each replication were harvested and threshed. The grains were ground to a fine powder using a coffee grinder and sent to the Veterinary Diagnostic Laboratory, NDSU, for mycotoxin analyses. The amount of DON, its derivatives (3ADON, 15ADON) and NIV was quantified using the gas chromatography-mass spectrometry (GC-MS) method (Tanaka et al., 2000).

Mycotoxin production in rice cultures was measured according to Burlakoti et al. (2008) and Walker et al. (2001) with some modifications. Thirty grams of rice grains were soaked in deionized distilled water overnight (10 h) in a 250-ml Erlenmeyer flask. Excess water was

drained from the flask. After autoclaving, the rice grains were inoculated with three plugs of agar containing growing mycelia from a 4-5-days-old culture on PDA. Each flask was considered as one replication and three flasks for each isolate were prepared. To facilitate equal distribution of fungal growth, the culture was stirred with a sterile glass rod on the 2nd, 4th and 6th days. Then, the cultures were grown in the dark for 30 days at 23 ±1°C. Each rice culture was transferred into a 50-ml centrifuge tube and stored at -80^{0}C overnight and lyophilized for 6 days at -40°C. Then, the rice cultures were ground with a coffee grinder and five grams of each sample were analyzed for mycotoxins using the same method as used for the grain samples.

2.3.5. Growth rate and sporulation test

The growth rate and sporulation of isolates were evaluated *in vitro* on MBA plates in three replications. A small agar plug (5 mm in diameter) of each isolate was placed in the center of a 9-cm MBA plate and incubated the same way as that for inoculum preparation. Radial growth was measured at every 24 h until mycelia completely covered the plate surface. After 7 days of growth, conidia were harvested by adding one milliliter of distilled water and scraping the surface of the agar with a rubber policeman. The spores were counted under a compound microscope using a hemocytometer.

2.3.6. AFLP analysis

AFLP analysis was performed according to Vos et al. (1995) with some modifications. DNA templates for pre-selective amplification were prepared using the AFLP® Core Reagent Kit (Invitrogen Corporation Carlsbad, CA) according to the manufacturer’s protocol. Pre-amplification reactions contained 1:2 diluted DNA templates (approximately 62.5ng of DNA), 36ng of each *EcoRI* primer (E-A) and *Mse*I primer (M-C), 0.2 mM dNTPs, 1× buffer, 1.5mM $MgCl_2$, and 1 unit of *Taq* DNA polymerase and was performed for 20 cycles of 94°C for 30s,

56°C for 60s, and 72°C for 60s. Five sets of primer combinations (E-AC+M-CG, E-AT+M-CC, E-AA+M-CC, E-AA+M-CT, and E-AA+M-AT) were used in the selective amplification with the E- primers labeled with the fluorescent dye IRD 700 (Li-COR, Lincoln, NE). The selective amplification conditions were same as described by Vos et al. (1995). After selective amplification, the PCR reaction was mixed with 10μl of Formamide loading dye, heated at 95°C for 3 minutes and immediately chilled on ice, and loaded (0.5μl) on a polyacrylamide gel in the Li-COR system (Li-COR, Lincoln, NE). The gel was prepared by using 20ml of Rapid Gel-XL 6% Liquid Acrylamide (USB Corporation, Cleveland, OH), 15μl of Tetramethylethylenediamine (TEMED) and freshly prepared 10% Ammonium Persulfate. AFLP fragments of 90 to 600 bp were scored manually with reference to a 50-bp ladder (Li-COR, Lincoln, NE) and those fragments absent in at least one isolate but present in other isolates were considered as polymorphic. The presence and absence of fragments were scored as one and zero, respectively.

2.3.7. VNTR analysis

Nine VNTR markers (*HK913*, *HK917*, *HK957*, *HK965*, *HK967*, *HK977*, *HK1059, HK1073*, *HK1043*) developed by Suga et al. (2004) and one additional VNTR marker (*HK1003*) reported by Gale et al. (2005) were used to analyze the same set of isolates used in the AFLP analysis. To detect VNTR markers on Li-COR system (Li-COR, Lincoln, NE, USA), forward primers were synthesized with the M13 primer sequence (CACGACGTTGTAAAACGAC) added at the 5'-end and the M13 primer labeled with IRD-700 or -800 (Eurofins MWG Operon, AL, USA) was included in the PCR reactions. Each PCR reaction contained a total volume of 10 μl consisting of 1× buffer, 200 μM of dNTP, 1.0 μM of each primer (forward, reverse and IRD-700/800-labeled M13), 1 unit of *Taq* polymerase, and 25-30ng/μl of genomic DNA. PCR amplification was performed using a PTC-100 thermal cycler (MJ Research, USA).

Amplification was completed with initial denaturation at 94°C for 3 minutes followed by 3 cycles of 94°C for 30s, 56°C for 30s and 72°C for 1 minute and additional 25 cycles of 94°C for 30 s, 52°C for 30s, and 72°C for 45 s and final extension at 72°C for 5 minutes. PCR products were loaded on the Li-COR system (Li-COR, Lincoln, NE) following the same procedure used for detecting AFLP markers as described above. VNTR alleles were scored manually with reference to a 50bp ladder (Li-COR, Lincoln, NE). Amplicons with the same size were considered the same allele.

2.3.8. Statistical analyses

Analysis of variance (ANOVA) for disease severity and mycotoxin accumulation was performed on Arcsine and Log transformed data, respectively (Gomez and Gomez, 1984). ANOVA, least significance difference (LSD) for mean separation and Pearson Correlation Coefficients and t-test for population comparison were performed using SAS 9.1.3, (SAS Institute, Cary, NC). Standard deviation and standard error in disease severity and mycotoxin accumulation among isolates were calculated using Microsoft Excel 2003.

Genetic distance (*D*), Nei`s unbiased gene diversity (*H*) (Nei, M. 1973), and gene flow (N_m) (McDermott, and McDonald, 1993) were analyzed using POPGENE version 1.32 (Yeh et al., 1997). The software Multilocus 1.3 was used to calculate the genotype diversity (*GD*) as ($n/n-1$) ($1- \sum p_i^2$), where p_i is the frequency of the ith genotype and *n* is the number of individuals sampled (Agapow and Burt, 2001). Then the index of linkage disequilibrium (*LD*) was calculated to test non-random association of alleles at different gene loci based on the index of association (I_A). GenAlEx 6 was used for pair-wise population differentiation (F_{st}), gene flow (N_m) and analysis of molecular variance (AMOVA) (Peakall and Smouse, 2006).

2.4. Results

2.4.1. Fusarium species identification and trichothecene chemotyping

The primers UBC85F410 and UBC85R410 generated a *Fg*-complex-specific 332-bp amplicon, which was observed for all isolates analyzed except for R709. Phylogenetic analysis of the reductase and histone H3 gene sequences indicated that R709 is *F. culmorum* and all other isolates belong to the *F. graminearum* sensu stricto (data not shown).

Among the 120 isolates collected from 1980 to 2000 in ND, 93% were of the 15ADON chemotype and only a few isolates (3% and 4%) were of the 3ADON or NIV chemotypes, respectively (Fig. 2.1A). In contrast, 44% of the 43 isolates from the new collection (2008) were of the 3ADON chemotype, while 56% of the isolates were of the 15ADON chemotype and no NIV isolates were found (Fig. 2.1B).

2.4.2. Aggressiveness evaluation on spring wheat genotypes

The 25 isolates of *F. graminearum* s. str. evaluated on the three spring wheat genotypes (Grandin, Steele-ND and ND 2710) varied in aggressiveness. Analysis of variance indicated that interaction between fungal isolate and wheat genotype was not significant, but disease severity was significantly different among isolates or wheat genotypes (Table 2.1). The average disease severity caused by individual isolates pooled based on wheat genotype ranged from 13.5±3.6 to 55.6±5.4%. Isolate Fg08-001 had the highest disease severity but was not significantly different from some other isolates (Fg08-003, Fg08-004, Fg08-006, Fg08-008, Fg08-009, Fg08-010, Fg08-011, Fg08-012, R1700). The majority of the 3ADON isolates was highly aggressive causing a higher level of disease than the 15ADON isolates except for R1700 (Table 2.1).

Among the three wheat genotypes tested, ND 2710 showed the lowest disease severity (DS=21.2%) while no significant differences were observed between Grandin (DS=46.4%) and

Steele-ND (DS=42.4%). The two populations (3ADON and 15ADON) interacted differently with the wheat genotypes, with the 3ADON population causing a significantly higher DS than the 15ADON population on the susceptible Grandin (t=4.4; p<0.0001) and the moderately resistant ND 2710 (t=2.7; p=0.0087) (Fig. 2.2). However, no significant difference in DS was detected on Steele-ND between the two populations (t=1.9, p=0.065) (Fig. 2.2).

2.4.3. Trichothecene production in rice culture and grains

The total amount of DON, 15ADON and 3ADON produced by individual isolates in rice cultures varied, ranging from no detection to 398.5 ± 81.5 ppm (standard error) (Table 2.1). Two 15ADON isolates (R465 and R1247) had DON production below the detection level. All 3ADON isolates except for Fg08-002 and R459 produced a large amount of 3ADON (61.6 to 258.2 ppm), but the amount of 15ADON produced by the 15ADON isolates was relatively low (maximum of 19 ppm) (Table 2.1). Grouping of the isolates based on the chemotypes showed that the 3ADON population produced 1.5 times more DON and 86 times higher 3ADON but 6 times less 15ADON than the 15ADON population in rice cultures (Table 2.3).

The DON levels in grains harvested from the inoculated spikes were relatively low compared to those produced in rice cultures, ranging from 1.5±0.3 to 27.8±5.0 ppm (Table 2.1). The mean DON accumulated in grains from the susceptible cultivar Grandin (18.8 ppm) was significantly higher than that accumulated in those from ND 2710 (8.17 ppm) and Steele-ND (10.47 ppm). Although Steele-ND and ND 2710 showed significant differences in disease severity, the DON accumulation was similar between these two wheat genotypes, suggesting that different mechanisms may operate for resistance to FHB severity and DON accumulation. Comparison of the two populations (3ADON and 15ADON) on individual wheat genotypes indicated that the 3ADON population produced a significantly higher DON than the 15ADON

population on Grandin (*t*= 3.9; p=0.0006) and ND 2710 (*t*= 3.6; p=0.0016) (Fig. 2.3). Interestingly, the two populations did not show a significant difference in DON accumulation on Steele-ND (*t*= 1.7, p=0.1057) (Fig. 2.3).

2.4.4. Growth rate and sporulation

No significant differences in growth rates were observed between isolates of the 3ADON population and those of the 15ADON population (data not shown), but the 3ADON isolates produced a significantly (*t*=2.0, p=0.05) higher number of macroconidia (4.4±0.60 million conidia/ml) on MBA compared to the 15ADON isolates (3.3±0.74 million conidia/ml).

2.4.5. Correlation between trichothecene production and disease severity

A highly significant positive correlation existed between disease severity and DON production in both rice culture (*R*=0.58*, P*=<0.01) and grains (*R*=0.85*,* p=<0.01) (Fig. 2.4A and 1.4D). A significant positive correlation was also identified between disease severity and 3ADON (*R*= 0.57*,* p= <0.01) produced in rice culture (Fig. 2.4B). However, no significant correlation was found between disease severity and 15ADON amount (*R*=-0.05, p=0.64) (Fig. 2.4C).

2.4.6. Population genetic analyses

A total of 33 isolates (Table 2.1) were subjected to AFLP analysis. The five primer pairs (E-AC+M-CG, E-AT+M-CC, E-AA+M-CC, E-AA+M-CT, and E-AA+M-AT) generated 40, 39, 23, 38 and 32 polymorphic fragments, respectively. Among 172 AFLP loci scored, 70.9% were polymorphic in the 15ADON population compared to 64.5% in the 3ADON population. Both 15ADON and 3ADON populations showed a genotype diversity (*GD)* value of 1, indicating that all isolates had a unique genotype. The gene diversity (*H*) was 0.189 for the 15ADON population and 0.166 for the 3ADON population, respectively. The linkage disequilibrium (*LD*) value was

0.057 (p<0.001) for the 15ADON population and 0.075 (p<0.001) for the 3ADON population. There was significant population genetic differentiation between two population (F_{st}=0.065, p= 0.001) but two populations had relatively high gene flow (N_m= 7.1).

Analysis of the same set of isolates with the VNTR markers showed that 60.8% of the VNTR loci were polymorphic in the 15ADON population and 49.3% were polymorphic in the 3ADON population. The gene diversity was 0.161 and 0.148 for the 15ADON and 3ADON populations, respectively. The *LD* value was 0.001 (p=0.30) for the 15ADON population and 0.075 (p=0.001) for the 3ADON population. The Nei`s unbiased genetic distance (*D*) was 0.055 and the gene flow was relatively lower (N_m = 2.04) between the two populations. Analysis of molecular variance (AMOVA) showed 20% genetic variation between populations and 80% variation among individuals within population. There was significant population genetic differentiation (F_{st}= 0.197, p = 0.001) between 3ADON and 15ADON population.

2.5. Discussion

Our study showed that the frequency of *F. graminearum* isolates with a 3ADON chemotype was very low (3%) in the old collection, but increased by 15-fold in the new collection (Fig. 2.1A and 1.1B). This result is consistent with those reported previously (Gale et al. 2007; Ward et al. 2008; Guo et al. 2008), which demonstrated that a *F. graminearum* population with a 3ADON chemotype has emerged and increased since 1998. Gale et al. (2007) had reported genetically divergent populations of *F. graminearum* in North Dakota and Minnesota where thirty 3ADON isolates (9.4%) were identified among 587 isolates collected from 1999 to 2000. Ward et al. (2008) analyzed populations of *F. graminearum* in Canada and found that the 3ADON chemotype frequency in western Canada increased more than 14 fold between 1998 and 2004. They also demonstrated a dramatic longitudinal cline in which 3ADON

isolates were significantly more common in eastern Canada than in western provinces of Canada. In addition, Guo et al. (2008) analyzed *F. graminearum* samples collected from 15 locations of Manitoba from 2004 to 2005 and found that percentages of isolates with a 3ADON chemotype ranged from 0 to 95.7% depending on the locations sampled. These findings contradict earlier reports by Zeller et al. (2003, 2004), who suggested that the *F. graminearum* population in North America is homogeneous and can be represented by local inocula in breeding programs. Lack of significant differentiation or subdivision among the *F. graminearum* populations analyzed by Zeller et al. (2003, 2004) might be due to absence or very low frequency of the 3ADON isolates in their samples collected from 1999 and 2000. The dramatic population changes in *F. graminearum* and significant increase of the 3ADON isolates might have not occurred in the United States until 2000.

Based on the genetic variation derived from the AFLP and VNTR, we found higher gene diversity, polymorphic loci and significant linkage disequilibrium on 15ADON isolates compared to 3ADON isolates. Further, the significant population differentiation (F_{st}) and limited gene flow (N_m) between two populations showed that 3ADON population might be new origin. This result is consistent with the finding of Ward et al. (2008) which showed the significant level of genetic differentiation between 3ADON and 15ADON populations. However, to define the population genetic relation prevailing in North Dakota isolates, analysis of lager collection of isolates is necessary.

The origin of 3ADON isolates in the North American population is not known, but it was suggested that they were introduced from other continents through contaminated seed (Gale et al. 2007; Ward et al. 2008). Gale et al. (2007) compared 30 isolates of the 3ADON chemotype collected from North Dakota and Minnesota with a small sample of isolates collected from Italy,

and found significant genetic similarity between the two populations; therefore, they suggested that the 3ADON population in the Upper Midwest of the US might have originated from Europe. Ward et al. (2008) further indicated that Canadian 3ADON populations were more closely related to Italian populations than to the sympatric 15ADON populations and thus concluded that the 3ADON populations in North America were due to a transcontinental introduction. However, if the 3ADON populations did originate from other continent(s), the first introduction might not have occurred recently since three toxic and relatively aggressive isolates with a 3ADON chemotype were identified among the isolates collected between 1979 and 1981 from Ontario, Canada (Ouellet and Seifert, 1993; Gilbert et al. 2001) and two of these isolates were genetically connected to the 3ADON population (3P) from Prince Edward Island (Ward et al. 2008). We also identified three 3ADON isolates (R458, R459, and R1391) in the old collection during 1980s from North Dakota, suggesting that 3ADON isolates were present in the Upper Midwest of the US at least at the same time as they were in eastern Canada. It is not known where these old 3ADON isolates came from. Further studies are required to reveal their relationship to the new 3ADON isolates in ND and those previously reported in other regions of North America (Gale et al. 2007; Ward et al. 2008).

Our study indicated that 3ADON isolates accumulated significantly higher DON than 15ADON isolates in rice culture. This is consistent with the study of Ward et al. (2008), which demonstrated that mean trichothecene accumulation was significantly greater for isolates with a 3ADON chemotype than observed for the 15W population with a 15ADON chemotype *in vitro*. We inoculated three wheat genotypes with a subset of isolates from each of the two populations with a 3ADON or 15ADON chemotype and showed that the average trichothecene accumulation by 3ADON isolates inoculated on the susceptible wheat cultivar Grandin was greater than that

observed for 15ADON isolates. Mean trichothecene accumulation on the moderately resistant wheat line ND 2710 also was significantly greater for 3ADON isolates than for 15ADON isolates. However, no significant difference in trichothecene accumulation on Steele-ND was observed between the two chemotypes. These results suggest that the two chemotypes may interact differently with wheat genotypes having different levels or sources of FHB resistance. Similar results were observed in the study of Ward et al. (2008), which showed that the mean DON accumulation was significantly higher for the 3ADON population than for the 15ADON population on the susceptible cultivar Robin; however, this difference was not statistically significant on the moderately resistant cultivar 5062 HR.

Our study also showed that the 3ADON population caused a significantly higher level of disease than the 15ADON population on Grandin and ND 2710 although no significant difference in disease level was observed between the two chemotypes when inoculated on Steele-ND. This is in contrast to the result of Ward et al. (2008) who found no significant difference in pathogenicity between the two chemotypes on either of the two cultivars (Robin and 5062 HR) evaluated. The discrepancy between the results of Ward et al. (2008) and ours was probably due to the inoculation methods used. Ward et al. (2008) used spray inoculation while we used single-floret inoculation. Previous studies demonstrated that DON is not required for initial infection by *F. graminearum* to cause FHB, but it facilitates the disease spread from an infected floret into neighboring florets of a spike (Jansen et al., 2005; Proctor et al., 1995). Therefore, the spray-inoculation method might not be able to identify the differences in aggressiveness between isolates with different levels of DON production. In a field experiment, we also did not observe significant differences in disease severity on Trooper (susceptible to FHB) or Alsen (moderately resistant to FHB) when spray inoculated with the 3ADON and

15ADON isolates, although the DON levels were significantly higher for the 3ADON isolates than for the 15ADON isolates (Ali et al., unpublished data).

The spring wheat genotype ND 2710 with FHB resistance derived from Sumai3 (Frohberg et al. 2004) showed a significantly higher disease severity when inoculated with the 3ADON population than with the 15ADON population, but no significant disease severity difference was observed in Steele-ND (Fig. 2.2), a cultivar with FHB resistance from *T. diccocoides* (Mergoum et al. 2005), when inoculated with the two chemotype groups. Similarly, the 3ADON isolates accumulated higher DON in grains of ND 2710 and Steele-ND compared to the 15ADON isolates, but a significant difference in DON accumulation between the two chemotype populations were only observed on ND 2710 (Fig. 2.3). These results strongly suggest that differences in aggressiveness and DON production between the chemotype groups vary with host genotype used. In the Northern Great Plains, most recently released wheat cultivars such as 'Alsen', 'Faller' and 'Glenn' have FHB resistance derived from Sumai3 (Frohberg et al., 2004) while Steele-ND has resistance derived from *Triticum dicoccoides* (Mergoum et al. 2007). It remains to be investigated whether these wheat cultivars have different reactions to the two chemotypes and if FHB resistance plays any role in the population shift of *F. graminearum*.

Significantly high correlations between disease severity and total DON production were observed in three wheat genotypes inoculated with either of the two chemotypes (Fig. 2.4). This is consistent with the results of previous studies (Goswami and Kristler, 2005; Wang et al 2006; Carter et al., 2002). Mesterhazy et al. (1999), Bai et al. (1996), Paul et al. (2005), and Ma et al. (2009) showed a strong positive correlation between Fusarium-diseased kernels and total DON accumulation. The amount of total DON accumulation depends on the level of host resistance

and is generally higher in susceptible cultivars (Mesterha`zy et al. 1999; Mesterha`zy, 2002). However, other studies had shown a zero or negative correlation between FHB severity and amount of DON accumulation in grains (Martin and Johnston, 1982; Edwards et al., 2001; Alvarez et al., 2010). The contradictions among those studies could be due to the use of different hosts or isolates and/or inoculation methods.

In summary, our results indicate that *F. graminrarum* isolates with a 3ADON chemotype have increased very rapidly in recent years in North Dakota. They are more aggressive by causing more disease in wheat cultivars and produce a higher amount of total DON in grains harvested from inoculated spikes. This information has implications on development of FHB-resistant wheat cultivars and disease management. First, the *F. graminearum* population in North America consists of chemotypes with different aggressiveness and mycotoxin productivity. It is postulated that changes in agricultural practices such as use of host resistance and fungicides may drive the pathogen populations to shift to those with greater aggressiveness and DON production. Thus, sampling and monitoring of *F. graminearum* populations periodically on local and national scales are still necessary. In addition, screening of resistance for FHB should be made with inocula combining representative isolates with different trichothecene types in breeding programs. This will ensure the development of cereal crops with broad resistance to FHB pathogens. Finally, wheat genotypes with different sources of FHB resistance may react differentially to different chemotypes in disease severity and DON accumulation. This highlights the need and importance of using different sources of host resistance in combating the disease.

2.6. Acknowledgements

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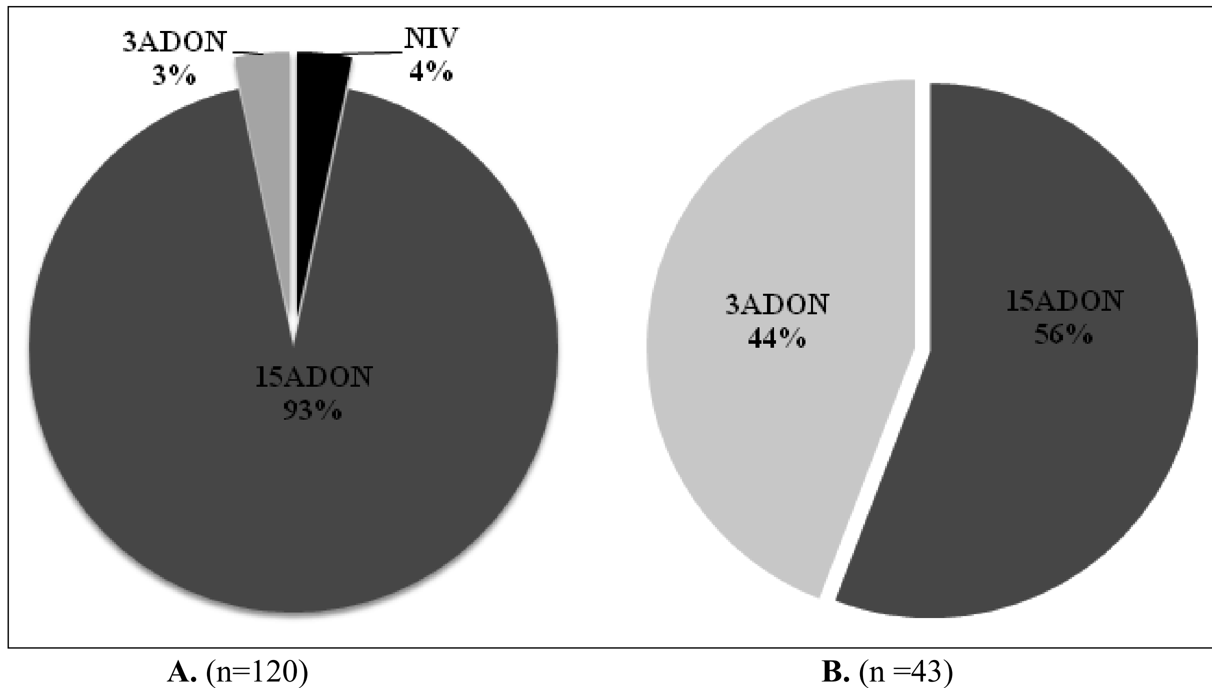

A. (n=120) **B.** (n =43)

Figure 2.1. Frequency distribution of chemotypes among the 120 *Fusarium graminearum* isolates collected during 1981-2000 (A) and the 43 isolates collected in 2008 (B) from different locations of North Dakota.

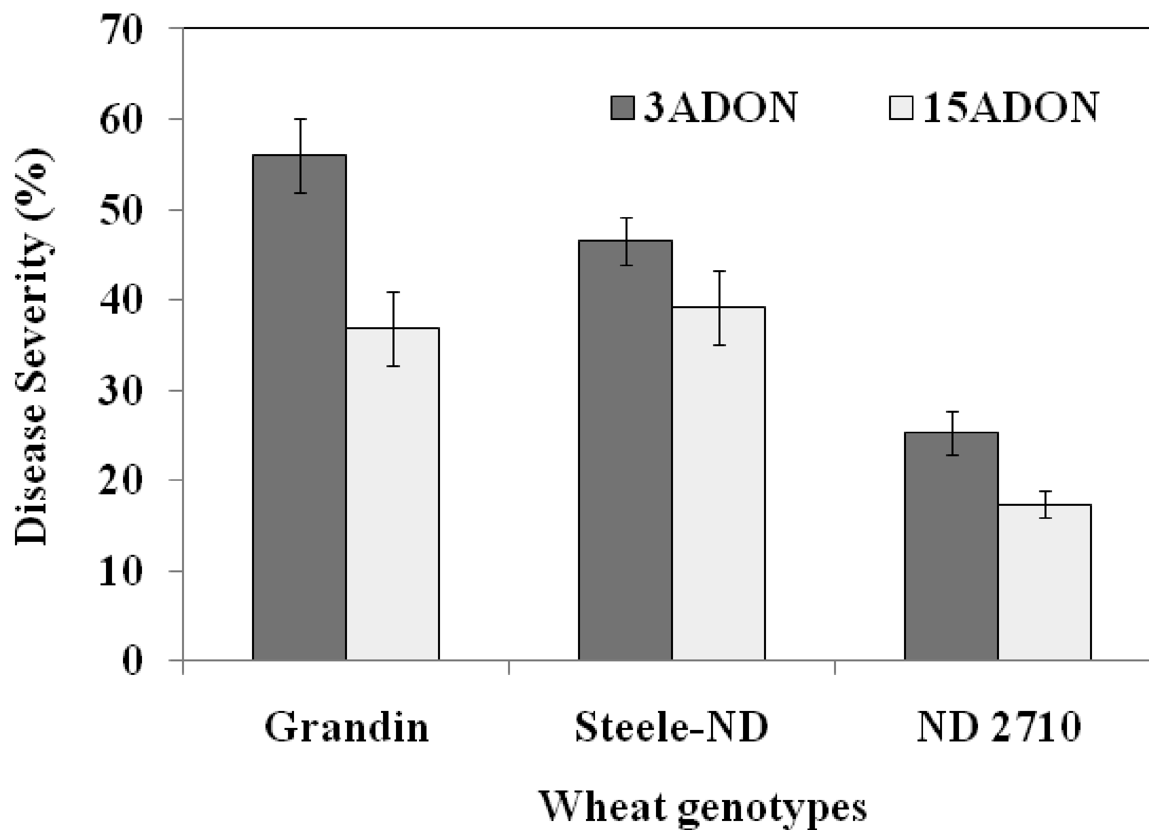

Figure 2.2. Disease severity (DS) on each of the three wheat genotypes (Grandin, Steele-ND, and ND 2710) averaged based on chemotype populations (3ADON or 15ADON). The 3ADON population caused a higher DS on Grandin and ND 2710 than the 15ADON population at a highly significant p<0.0001) and significant level p=0.0087), respectively. However, no significant difference in DS was detected on Steele-ND between the two populations (p=0.065).

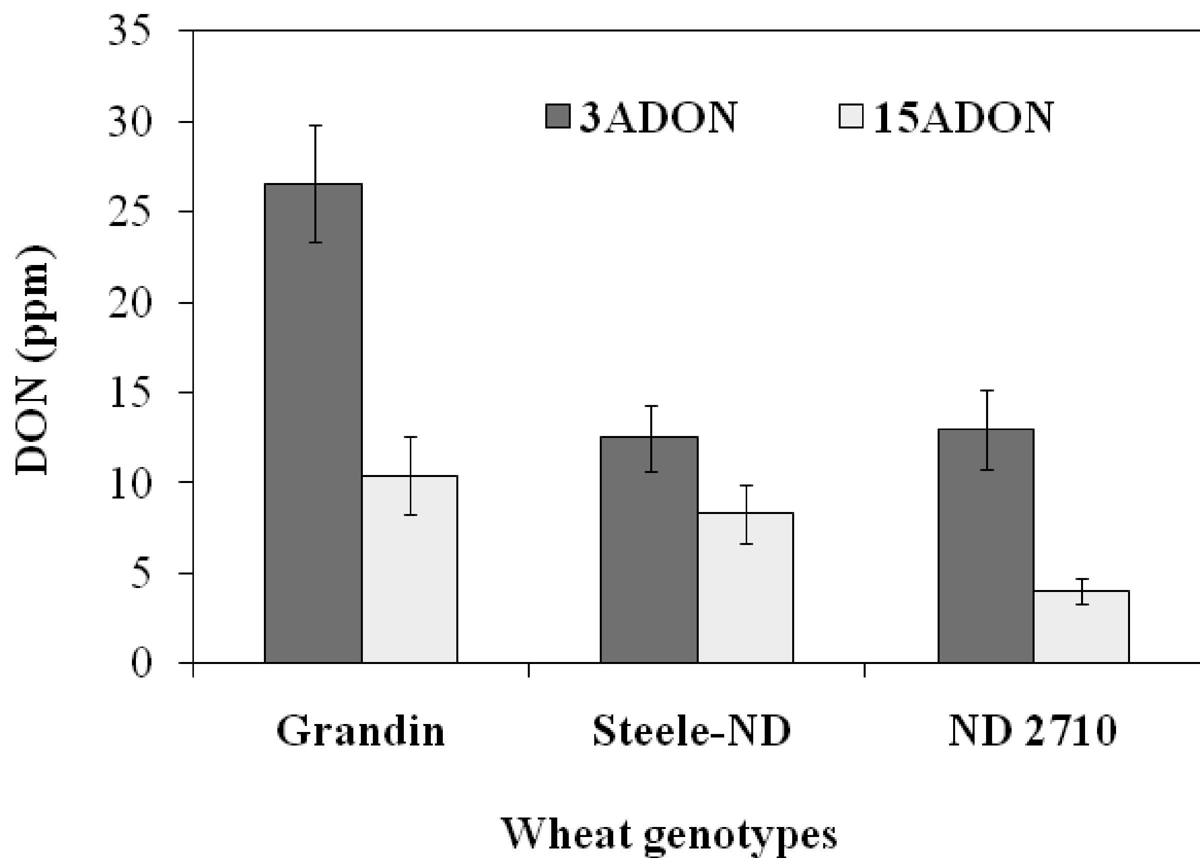

Figure 2.3. DON accumulation in harvested grains of the three wheat genotypes (Grandin, Steele-ND, and ND 2710) averaged based on chemotype population (3ADON or 15ADON). The 3ADON population accumulated significantly more DON on Grandin (p=0.0006) and ND 2710 (p=0.0016) than the 15ADON population. However, no significant difference (p=0.1057) in DON accumulation was detected on Steele-ND between the two populations.

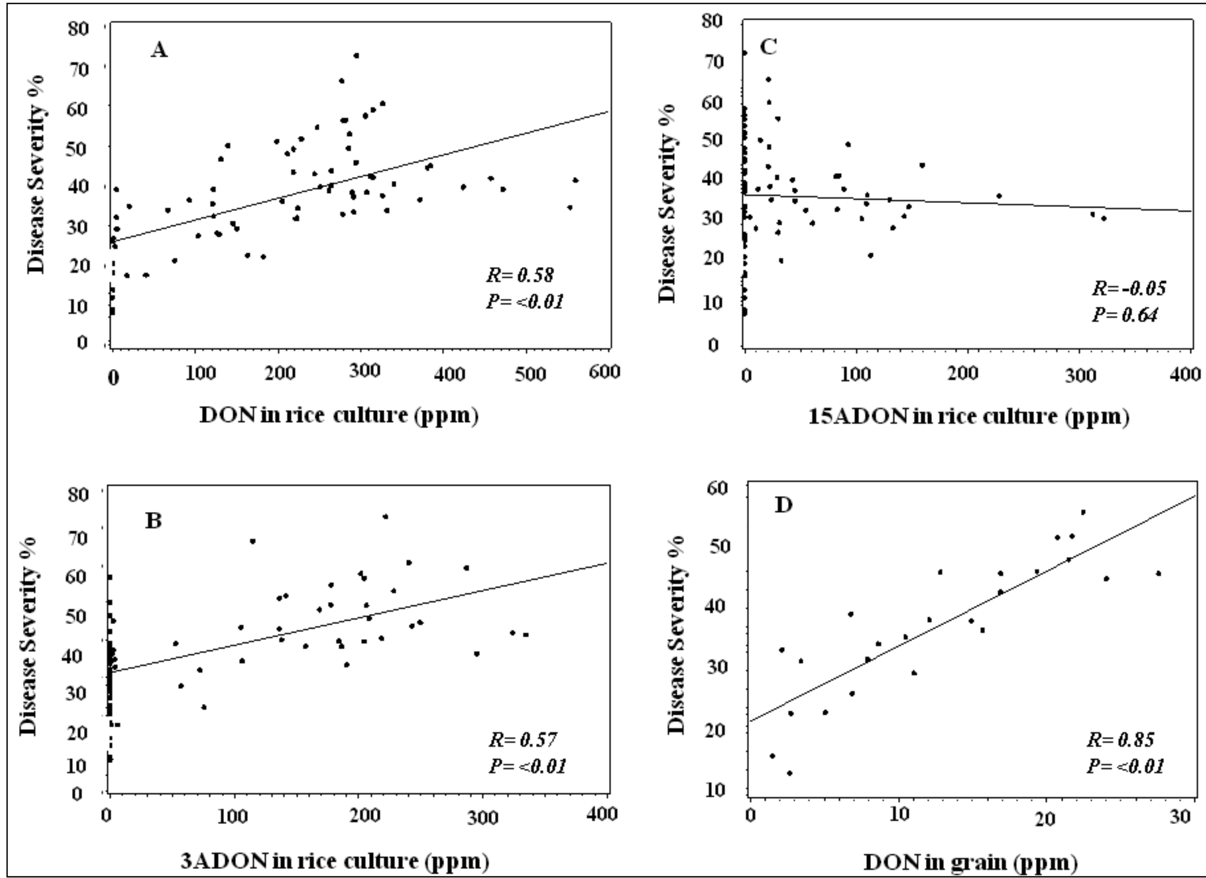

Figure 2.4. Correlation between disease severity (DS) and trichothecene production in rice culture and grains harvested from inoculated heads of the three wheat genotypes (Grandin, Steele-ND and ND 2710). A. DS with total DON in rice culture, B. DS with 3ADON in rice culture, C. DS with 15ADON in rice culture, and D. DS with DON in grains.

Table 2.1. Chemotype, aggressiveness based on disease severity (%), and trichothecenes (DON, 15ADON, 3ADON and NIV) (mg/kg = ppm) of the 33 *Fusarium graminearum* isolates used in the study.

Isolate	Year of collection	Origin	Chemotype[a]	DS[b]	Trichothecenes in rice culture (ppm)[c]				Trichothecenes in grains (ppm)[d]		
					DON	3ADON	15ADON	NIV	DON	3ADON	15ADON
Fg08-001	2008	Foster/ ND/USA	3ADON	55.6±5.4[a]	253.8 ± 27.6	142.9±18.3	*	*	22.5±8.0	1.2	-[e]
Fg08-002	2008	Barnes/ ND/USA	3ADON	23.2±5.8[ik]	25.7 ± 7.3	3.4±1.4	-	-	5.1±1.4	-	-
Fg08-003	2008	Steele / ND/USA	3ADON	44.8±3.9[af]	305.4 ± 44.3	182.6±33.8	*	*	24.0±4.0	0.6	-
Fg08-004	2008	Barnes/ ND/USA	3ADON	45.7±4.6[ae]	305.3 ± 59.5	172.8±19.9	2.1	*	16.9±10.7	0.9	-
Fg08-005	2008	Foster/ ND/USA	3ADON	36.5±3.5[dh]	221.8 ± 1.7	94.6±11.2	*	*	15.7±5.1	0.9	…
Fg08-006	2008	Steele/ ND/USA	3ADON	51.7±6.8[ab]	298.3 ±14.5	210.3±15.8	2.1	*	21.7±5.1	0.6	-
Fg08-007	2008	Foster/ ND/USA	15ADON	31.8±0.2[fi]	188.8 ± 33.5	*	19±6.7	*	7.9±2.2	-	0.6
Fg08-008	2008	Steele/ ND/USA	3ADON	46.0±7.4[af]	300.4 ± 7.3	228.6±29.7	*	*	19.3±3.1	0.7	-
Fg08-009	2008	Foster/ ND/USA	3ADON	51.4±10.6[ac]	398.5 ± 81.8	258.2±38.1	2.8±0.8	*	20.7±9.0	1.7	-
Fg08-010	2008	Barnes/ ND/USA	3ADON	42.7±3.4[ag]	282.5 ± 47.2	235.5±36.7	2.1	2.2	16.9±6.8	-	-
Fg08-011	2008	Foster/ ND/USA	3ADON	47.9±3.3[ad]	320.9 ± 71.1	252.7±36.2	2.3±0.3	*	21.5±7.5	0.7	-
Fg08-012	2008	Steele/ ND/USA	3ADON	45.7±5.9[ae]	273.5 ± 16.4	174.6±19.0	*	*	27.6±5.0	1.1	-
Fg08-013	2008	Steele/ ND/USA	15ADON	31.6±2.4[fi]	158.9 ± 23.7	*	3.5	*	3.4±1.2	-	-
Fg08-018	2008	Steele/ ND/USA	15ADON	39.2±3.2[bh]	396.2 ± 40.9	2.1±0.1	10.1±3.1	*	6.8±2.5	-	-
Fg08-019[f]	2008	Foster/ ND/USA	…	…	…	…	…	…	…	…	…
Fg08-020[f]	2008	Barnes/ ND/USA	…	…	…	…	…	…	…	…	…
Fg08-021[f]	2008	Barnes/ ND/USA	…	…	…	…	…	…	…	…	…
R010	1983	-/ ND/USA	15ADON	38.0±2.2[dh]	398.1 ± 77.7	*	11.3±1.9	*	14.9±4.0	-	0.6
R366	1981	- / ND/USA	15ADON	23.2±2.6[ik]	1.3 ± 0.7	-	…	…	2.8±1.3		-
R458[f]	1981	-/ ND/USA	…	…	…	…	…	…	…	…	…
R459	1981	- / ND/USA	3ADON	33.4±2.9[ei]	4.4 ± 0.2	-	*	-	2.3±1.4	-	-
R465	1981	- / ND/USA	15ADON	13.5±3.6[k]	*	*	*	*	2.7±0.9	-	-
R1171	1991	Cavalier/ ND/USA	15ADON	35.4±7.0[di]	195.1 ± 49.0	2.2±0.2	16.0	*	10.5±3.7	-	0.6
R1215[f]	1993	-/ ND/USA	…	…	…	…	…	…	…	…	…
R1247	1993	- / ND/USA	15ADON	16.3±5.2[jk]	*	*	*	*	1.5±0.3	-	-
R1265[f]	1994	- / ND/USA	…	…	…	…	…	…	…	…	…
R1310	1995	Benson/ ND/USA	15ADON	34.3±1.1[di]	145.9 ± 66.6	2.5±0.7	17.5±7.0	*	8.7±1.0	-	-
R1318	1995	Cass/ ND/USA	15ADON	38.2±6.2[ch]	136.8 ± 8.6	*	11.2±1.2	*	12.1±4.9	-	-
R1390	1990	-/ ND/USA	15ADON	26.4±2.7[ij]	107.8 ± 20.4	*	3.8±1.2	*	6.9±2.7	-	-
R1391	1990	- / ND/USA	3ADON	29.6±5.0[gi]	144.1 ± 19.0	61.6±7.1	*	*	11.1±3.0	-	-
R1628[f]	2001	Mountrail/ND/USA	…	…	…	…	…	…	…	…	…
R1694[f]	2004	Cass/ND/USA	…	…	…	…	…	…	…	…	…
R1700	2004	Cass/ ND/USA	15ADON	45.8±5.4[ae]	285.0 ± 14.4	*	5.3±1.6	*	12.8±5.1	-	0.9

[a]Chemotype was determined by the multiplex-PCR assay of Ward et al. (2002).

[b]The disease severity (DS) was recorded from Grandin, Steele-ND and ND 2710 at 21 days after inoculation and the averaged DS was calculated across the three wheat genotypes for each isolate. Least significant difference (LSD) analysis was done with the Arcsine transformed value of DS. DS values indicated with the same lowercase letter (s) were not significantly different at α= 0.05.
[c]Trichothecenes in rice culture were measured and averaged from three replications.
[d]Trichothecenes in grains was obtained from wheat heads inoculated in the greenhouse and averaged from the three wheat genotypes. ppm = part per million (mg/kg of samples), * =Not detected at <2ppm, -= not detected at <0.5ppm, ± = Standard Error of Mean
[e]– indicates the information is unknown.
[f]These isolates were only included in the genetic diversity analysis.

Table 2.2. Analysis of variance of disease severity (DS) on the three wheat genotypes (Grandin, Steele-ND and ND 2710) inoculated by 30 isolates of *Fusarium graminearum*.

Source of variation[a]	df	MS[b]	*F* value	P>*F*
Fungal isolate	24	444.5	5.47	<0.0001
Wheat genotype	2	5618.2	68.67	<0.0001
Fungal isolate × wheat genotype	48	83.5	1.12	0.45

df=degree of freedom.
A twenty fine isolates *Fusarium graminearum* and three wheat genotypes (Grandin, Steele-ND and ND 2710) were used in this study.
[b]Arcsine transformation of disease severity. Mean square (MS) derived from the type III sum of squares.

Table 2.3. Comparison of the 3ADON population with the 15ADON population of *Fusarium graminerum* in trichothecene production in rice culture and grains harvested from the inoculated wheat genotypes.

Population[a]	Trichothecene (ppm)[b]					
	In rice culture			In grains		
	DON	3ADON	15ADON	DON	3ADON	15ADON
3ADON	241.1±32.2	155.3±24.8	1.3±0.3	17.3±2	0.7	-[c]
15ADON	168.0±39.7	1.8±0.2	8.1±1.8	7.6±1.3	-	0.3

[a]The 3ADON population consisted of 13 isolates and the 15ADON population contained 12 isolates of *Fusarium graminearum*.
[b]Mean of DON or its derivatives was calculated among the isolates used in each population.
[c]- not detected.

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CHAPTER 3. RNA-SEQ REVEALED GENE EXPRESSION DIFFERENCES BETWEEN 3ADON AND 15ADON POPULATIONS OF *FUSARIUM GRAMINEARUM IN VITRO* AND *IN PLANTA*

3.1 Abstract

Fusarium graminearum is the major causal agent of Fusarium head blight (FHB) of wheat and barley worldwide. The fungus produces several trichothecenes [Deoxynivalenol (DON) and its acetylated derivatives, 3-acetyldeoxynivalenol (3ADON) and 15-acetyldeoxynivalenol (15ADON) as well as nivalenol (NIV)], which are harmful to humans and animals. Recent studies showed that the 3ADON-producing isolates dramatically increased in the fungal population and were more aggressive and accumulated more DON in wheat grains than the prevalent 15ADON-producing isolates in North America. To understand the genetic and molecular basis of the differences between these two populations, we compared transcriptomes of the 3ADON and 15ADON populations *in vitro* in culture media and *in planta* inoculation on susceptible host 'Briggs' using RNA-seq technology. The total number of reads generated from each sample (replicate) ranged from 26.4 to 49.5 million for the 3ADON population and 27.8 to 39.1 million for the 15ADON population. The *in vitro* gene expression comparison between the 3ADON and 15ADON population identified a total of 479 up-regulated and 801 down-regulated genes in the 3ADON population. Of the 479 up-regulated genes, 18.6% are involved in functions for C-compound and carbohydrate metabolism and 7.72% for polysaccharide metabolism, while 57.6% of them are under category of unclassified proteins. Further pair-wise gene expression comparisons between the two fungal populations *in planta* revealed 477, 451 and 303 differentially expressed genes at 48, 96, and 144 hours after inoculation (HAI), respectively. The 3ADON population had 185, 89, and 62 *in planta* up-regulated genes compared to the 15ADON

population, respectively. The *in planta* up regulated genes in 3ADON population were significantly enriched for categories related to non-vesicular cellular import, degradation of glutamine, C-compound and carbohydrate metabolism and transport, allantoin and allantoate transport at 48 HAI; genes for detoxification, cell rescue, defense and virulence at 96 HAI; and genes for metabolism of acetic acid derivatives, detoxification, and non-vesicular cellular import at 144 HAI. The comparative analyses of *in planta* versus *in vitro* gene expression profiles further revealed 2,159, 1,981 and 2,095 genes in 3ADON and 2,415, 2,059 and 1,777 genes in 15ADON were up-regulated *in planta* at three time points, respectively. Of these, 633, 526 and 668 genes were only up-regulated in the 3ADON population at the three time points. Our RNA-seq analyses provide a foundation for further understanding of molecular mechanisms contributing to the increased aggressiveness and DON production of the recently emerged 3ADON population.

3.2. Introduction

Fusarium graminearum (telomorph *Gibberrella zeae*) is the major causal agent of Fusarium head blight (FHB) in North America and other regions of the world. The pathogen not only causes direct yield losses, but also produces various types of trichothecenes [Deoxynivalenol (DON) and its acetylated forms (3-acetyl-4-deoxynivalenol=3ADON and 15-acetyl-4-deoxvevalenol=15ADON), nivalenol (NIV) and its acetylated form 4-acetylnevalenol] (Desjardins, 2006). DON is a eukaryotic protein synthesis inhibitor which acts as a virulence factor during disease development (Jansen et al., 2005; Proctor et al., 1995) and poses severe health hazards to human and animals (McLaughlin et al., 2009; Desjardins et al. 2000; Desjardins and Hohn, 1997). FHB and DON are primarily managed through an integrated approach that combines use of moderately resistant cultivars, reduction of sources of local

inoculum and a timely fungicide application (McMullen et al., 1997). However, sources of effective FHB resistance are limited and use of single source of resistance in commercial cultivars may create selection pressure on the pathogen and lead to outbreak of more virulent/aggressive pathogen population (Bai and Shaner, 2004; Buerstmayr et al., 2009).

In recent years, population genetics, global species structure and trichothecene chemotype diversity have been extensively studied to understand the FHB pathogen complex (Ward et al., 2008; Starkey et al., 2007, Gale et al., 2002, O'Donnell et al., 2004). Studies have shown that one chemotype is dominant in specific geographic regions along with co-existence of other types in small fractions. Dominance of NIV-type isolates along with low frequency of 15ADON- or 3ADON-type isolates is more common in Asian regions (Qu et al., 2008, Yang et al., 2008; Puri et al., 2012). In North America, dominance of 15ADON-type isolates along with presence of 3ADON- or NIV-type isolates was observed (Zeller et al. 2003; 2004; Puri and Zhong, 2010; Gale et al., 2011). However, recent studies have indicated that 3ADON-type isolates have been significantly increased in this region (Gale et al. 2007; Ward et al. 2008). More recently, significant increase of the *F. graminearum* isolates with the 3ADON chemotype has been reported in China (Zhang et al., 2010), Canada (Ward et al. 2008) and North Dakota (Puri and Zhong, 2010). The newly emerging 3ADON population appears to be more aggressive based on growth rate, disease severity on different cultivars with varied levels of resistance, and DON production *in vitro* (Ward et al. 2008; Puri and Zhong, 2010). Our data from two years of field experiments using susceptible and moderately resistant wheat cultivars also indicated that the 3ADON producers accumulated a higher level of DON on grains irrespective of host resistance after spray inoculation (unpublished). The recovery of *Fusarium* isolates from artificially inoculated heads with a mixture of both 3ADON- and 15ADON-type isolates

indicated the recovery frequencies were similar for both types of isolates; suggesting that the 3ADON isolates do not have advantage of outcompeting 15ADON isolates during infection. However, it is still not known why the 3ADON population produces a higher level of DON over the 15ADON population.

Trichothecenes produced by *F. graminearum* can be broadly categorized into two groups (type A or type B trichothecenes) based on presence or absence of oxygen atoms at carbon atoms 7 (C-7) and 8 (C-8) (Alexander et al., 2009; Desjardins, 2006). Type A trichothecene (T-2 toxin, HT-2 toxin, and 4,15-diacetoxyscirpenol) has no hydroxyl group at C-7 but has a hydroxyl group and ester group or no oxygen substitution at carbon atom C-8, whereas Type B trichothecenes (e.g. DON, NIV, and its derivatives) has a hydroxyl group at C-7 and a keto (carbonyl) group at C-8 (Desjardins et al. 1993; Ueno, 1980; McCormick et al., 2011). DON and NIV have structural differences; DON and its acetylated forms (3ADON or 15ADON) lack an oxygen atom at carbon C-4 while NIV and its C-4 acetyl derivatives having an oxygen atom at carbon C-4 (Alexander et al., 2009; Kimura et al., 2007).

Trichothecene biosynthesis in *Fusarium* involves a complex pathway consisting of oxygenation, isomerization and esterification steps (Alexander et al., 2009). The enzymes involved in these biosynthetic steps are encoded by 15 different *TRI* genes located at three loci on different chromosomes. These include the *TRI* core cluster with 12 genes (*Tri3*, *Tri4*, *Tri5, Tri6*, *Tri7 Tri8*, *Tri9, Tri10*, *Tri11*, *Tri12*, *Tri13*, and *Tri14*) (Brown et al., 2004), the two-gene (*Tri1-Tri16*) locus (Brown et al., 2003; Meek et al., 2003, Peplow et al., 2003), and a single gene (*Tri101*) locus (Kimura et al., 1998). Production of DON or NIV trichothecene depends on the function of the two genes, *Tri13* (cytochrome P450 monooxygenase) and *Tri7* (acetyltransferase) (Lee et al., 2002). Both genes are functional and required in the NIV producers, while the DON

producers carry non-functional copies of these genes due to multiple deletion or insertions events (Lee et al., 2002; Brown et al., 2002). The genetic basis of 3ADON and 15ADON production and their biological significance are still not clear. Recently, Alexander et al. (2011) indicated that *Tri8* (for trichothecene C-3 esterase) regulates the production of 3ADON or 15ADON and is required to convert diacetylated 3- and 15- ADON intermediate into 3ADON and 15ADON, respectively. However, Kimura et al. (2003) showed that *Tri3* and *Tri8* are not necessary for 3ADON biosynthesis based on comparative sequence and structure analysis of *Tri5* gene cluster of 15ADON (strain H-11) and 4ANIV (strain 88-1) with 3ADON (strain F15). They further highlighted the involvement of three pathway genes (*FgTri4*, *FgTri5* and *FgTri11*) in 3ADON biosynthesis. In addition, other research groups have identified genetic markers at *Tri3*, *Tri12* and *Tri13* (Starkey et al., 2007; Ward et al., 2008; Wang et al., 2008) which are correlated with the production of 3ADON, 15ADON or NIV. However, the causal relationship among these markers with 3A- or 15A- DON biosynthesis and their *in planta* expression have not been demonstrated yet (Alexander et al., 2009). Thus, a global gene regulation and transcript abundance study is required to understand the difference in DON production between the 3ADON populations and the existing 15ADON population during host infection (*in planta*) and under axenic culture (*in vitro*) conditions.

Gene expression profiles and their relationships with DON accumulation, virulence or aggressiveness during *F. graminearum*-wheat and barley interactions have been studied using DNA microarrays (Boddu et al. 2006, 2007; Gardiner et al. 2009, 2010; Lysøe et al. 2011). However, comparative studies of transcriptomes of the two trichothecene types (3ADON and 15ADON) during host infection have not been conducted and the mechanisms involved in higher DON production in 3ADON isolates are not known. With the development of next generation

sequencing technologies, new tools such as RNA-seq provide more effective approaches to study the gene expression profile changes of organisms under different conditions (Wang et al. 2009). The RNA-seq method is more sensitive than microarrays especially in detecting those transcripts that are rarely expressed (Wang et al. 2009). Wang et al. (2009) showed that 8 million reads are sufficient to reach RNA-Seq saturation for most samples with large genome sizes. More recently, Bashir et al. (2009) have demonstrated that more than 90% of the transcripts in human samples can be adequately covered with just a million sequence reads. The coverage is more than sufficient to reach the saturation needed in RNA-Seq for small genomes such as those of filamentous fungi, while costs are comparable to the DNA microarray approach. Thus, the overall goal of this study is to understand the molecular mechanisms that make the 3ADON population accumulate a higher DON on grains than the 15ADON population during infection on a susceptible cultivar with the following specific objectives: i) to compare the transcriptomes of the 3ADON- and 15ADON-type populations *in vitro* and *in planta* using the RNA-seq approach, and ii) to identify the candidate genes related to production of DON, and 3ADON or 15ADON in *F. graminearum*.

3.3. Materials and Methods

3.3.1. Fusarium graminearum isolates

Twenty *F. graminearum* isolates with ten each trichothecene (3ADON- and 15ADON-type) were collected during 2008 to 2010 from North Dakota and characterized for disease aggressiveness and DON accumulation (Puri and Zhong, 2010; and this study) (Table 3.1). Each isolates were tested on greenhouse experiments using point inoculation on three spring wheat genotypes with various levels of resistance to FHB, including Grandin (susceptible), Steele-ND (moderately resistant), and ND 2710 (resistant). Inoculated heads were harvested at maturity and

the amount of DON on grains was analyzed. The plant growth conditions, inoculum preparation, inoculation, disease scoring, data analysis, and DON evaluation were the same as previously described (Puri and Zhong, 2010).

3.3.2. Sample collection for RNA-seq

The *in vitro* samples were collected from axenic cultures of individual population (mixed spores) plated on the cellophane membrane overlaid on Mung Bean Agar (MBA) media. The fungal cultures were grown at 23±1°C with alternate 12 h dark and light cycles, and arranged in Completely Randomized Design (CRD) with two replicates. At fifth day after plating, mycelia were scrapped, frozen immediately in liquid nitrogen, grounded to a fine powder and stored at -80°C until use. In *in vitro* a toal four samples {two fungal populations (10 isolate mixtures from each population) with two replications} were used for RNA isolation.

The *in planta* samples were collected at various infection stages from the FHB susceptible wheat cultivar 'Briggs' (Devkota et al., 2007) inoculated with the 15ADON and 3ADON populations separately. Seven seeds were planted on 15cm plastic buckets. The buckets were filled with Sunshine pot mix (Sun Gro Horticulture Canada Ltd.) and kept under greenhouse with 16 h supplemental lights at 23±1°C. Plants were fertilized with slow releasing Osmocote^{+} (15:9:12) (Everris NA, Inc, Marysville OH) and Plantex 20-20-20 (Plant Products Co. Ltd, Ontario, Canada) at a two weeks interval. Two isolate populations were arranged on Randomized Complete Block Design with two replications.

Single central spikelet of individual head was inoculated during anthesis (Feekes growth stage 10.5) with 3ADON and 15ADON populations separately. For each population, 10 *F. graminearum* isolates were used. Inoculum was prepared as described by Puri and Zhong (2010). The central florets of eight to ten heads/reps were inoculated with 1000 macro-conidia per floret

and the florets adjacent to the inoculated floret were marked with sharpie markers. Then, the plants were kept in a humidity chamber for 48 h with temperature at 26-27°C and 18 h light under a high humidity condition (vapor misting run for 30 s on every 8 m). After 48 h, the plants were moved back to the greenhouse under normal conditions. The inoculated florets were collected from 8-10 heads per replicate at 48, 96 and 144 hours after inoculation (HAI), respectively, frozen immediately in liquid nitrogen, and stored at -80°C until use. Thus in total, 12 floret samples {three time points (48, 96, and 144 HAI), two treatments (15ADON and 3ADON), and two replications} were used for total RNA isolation.

3.3.3. RNA extraction, library preparation and RNA sequencing

Total RNA was extracted from approximately 30 mg of ground plant or mycelial tissue using the SV total RNA isolation system (Promega BioSciences LLC, CA, USA) following the manufacturer's instruction. RNA integrity, quantity and quality were determined using a 1.2% Agarose gel and the Bio-Analyzer 2100 (Agilent Technologies, San Diego, CA). Total RNA was diluted to 50 ng/µl and stored at -80°C for further use. Approximately 3µg of total RNA was used to prepare library using the TruSeq RNA Sample Preparation Kit (Illumuna, San Diago, CA) according to the manufacturer's protocol. Briefly, the poly-A containing mRNA was purified from the total RNA sample using the poly-T oligo attached to the magnetic beads. After purification, mRNA was fragmented into small pieces using divalent cations under elevated temperature. The fragmented mRNA was converted to first strand cDNA using reverse transcriptase and random primers. Single strand cDNA was further converted to double strand (ds) cDNA using DNA polymerase I and RNase H. Then, the ds cDNA was processed for end repair and added with single 'A' base to the 3' ends. The products were ligated with indexing adapters, purified and enriched with PCR to develop the final sequencing library. The prepared

libraries were sent to Huntsman Cancer Institute, University of Utah (Salt Lake City, UT) for sequencing. The Illumina HiSeq 2000 sequencing system was used for generating 50bp single-end reads for total 16 libraries (4 *in vitro* + 12 *in plnata*).

3.3.4. Mapping sequence reads to the reference genome and identification of differentially expressed genes

The sequence read mapping, transcript abundance, and differential gene expression analysis were performed as described by Trapnell et al. (2012). The assembled and annotated *F. graminearum* PH-1 genome sequence was downloaded from the Broad Institute (http://broadinstitute.org/annotation/genome/fusarium_group/MultiDownloads.html; files: supercontigs.fasta and transcripts.gtf) for estimation of transcript abundances and other analyses. All sequence reads were trimmed to remove the low-quality sequences (first 13 bases). The trimmed reads (37 bases) were then aligned to the *F. graminearum* reference genome (433 contigs, 13,321 genes and 37,516 exons) using the short DNA sequences aligner Bowtie v0.12.5 (http://bowtie-bio.sourceforge.net/index.shtml; Langmead et al., 2009) and TopHat v2.0.0 (http://tophat.cbcb.umd.edu/; Trapnell et al., 2009; 2012) with the default settings. The aligned reads were further processed for downstream analysis using Cufflinks v0.9.3 (http://cufflinks.cbcb.umd.edu/; Trapnell et al., 2010) which assembles individual transcript reads from RNA-Seq and map them into the genome. The normalized gene expression levels based on fragments per kilobase of transcript per million fragments mapped (FPKM) were calculated using all parameters on default settings (Trapnell et al. 2010). The transcript was considered as expressed when the FPKM value was greater than 0.1 and the lower boundary for FPKM value was greater than zero at 95% confidence interval.

Once the transcript abundance (e.g. FPKM) was calculated for individual sample files using Cufflinks, output files were merged pairwise for each comparison (*in vitro* comparison between two populations, *in planta* comparison between two populations and *in planta* versus *in vitro* for each population) using Cufflinks utility program, Cuffmerge, which gives a single annotation file for differential analysis (Trapnell et al, 2012). The pairwise comparisons of differential expression at gene level for each time point of infection or in culture media were done using the Cuffdiff program on the Cufflinks version 1.3.0 (Trapnell et al, 2010). The genes were considered to be significant in differential expression if Log2 FPKM (fold change) was ≥ 1.0 and false discovery rate (FDR, the adjusted P value) was <0.01. The q-value which is a positive FDR analogue of the p-value was set to <0.01 (Storey and Tibshirani, 2003).

In order to visualize the expression data from all samples into two dimensions, principal component analysis (PCA) was performed using JMP (JMP Genomics v 6.0 SAS Institute Inc., Cary, NC) for all genes (with novel transcripts excluded). The expression data were transformed using mean normalization prior to PCA. The expression data of individual conditions were divided by their mean values across all treatment conditions in order to neutralize the influence of hidden factors.

3.3.5. Functional categorization of differently expressed genes

The functional categorization analysis of differently expressed genes was done online for all pairwise comparisons using the Munich Information Center for Protein Sequences (MIPS) functional catalogue (Ruepp et al. 2004). The functional categories and subcategories were regarded as enriched in genome if an enrichment P- and FDR-value were below <0.05. The KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analyses were performed using

interface on Blast2GO (Blast2GO v2.6.0, http://www.blast2go.com/b2ghome) for all DEGs to identify gene enrichment on specific pathway.

3.4. Results

3.4.1. Aggressiveness in disease development and DON production of the 3ADON and 15ADON populations

Randomly selected *F. graminearum* isolates from both populations (3ADON and 15ADON) were evaluated for aggressiveness in disease development at 21 days after point inoculation, and for DON accumulation on grains using three spring wheat genotypes (Grandin, Steele-ND, and ND 2710). The results indicated that the differences in aggressiveness based on disease severity (DS) were not significant between the two populations in all genotypes evaluated (Grandin, $t=1.6$, $p=0.1939$; Steele ND, $t=0.58$, $p=0.5676$; ND 2710, $t=0.42$, $p=0.6825$). The average DS on the susceptible cultivar Grandin was 65.3±11.4% for the 3ADON population and 56.8±16.3% for the 15ADON populations. In the resistant genotype ND 2710, DS was 29.6±7.2% and 27.9±10.1% for the 3ADON and 15ADON populations, respectively.

However, DON levels on the harvested grains produced by the 3ADON isolates were higher than those produced by the 15ADON isolates on all genotype evaluated (Fig 2.1). The 3ADON population produced a significantly higher DON on the susceptible cultivar Grandin ($t=8.1$, $p< 0.0001$) and on the resistant genotype ND 2710 ($t=3.4$, $p=0.0034$), while no significant difference was found on the moderately resistant cultivar Steele ND ($t=0.8$, $p=0.44$) (Fig. 3.1). The DON derivaries i.e. (3ADON and 15ADON) were not detectable on grains from inoculated heads.

3.4.2. RNA-Seq read analyses

The Illumina sequencing of the 16 cDNA libraries generated 559,577,636 sequence reads, totaling 27.97 gigabase-pairs (Table 3.2). The numbers of sequence reads generated from each *in-vitro* sample (replicate) ranged from 32.2 to 40.9 million. For the *in planta* samples*,* the numbers of sequence reads varied from 26.4 to 49.5 million for the 3ADON population, and 27.8 to 39.1 million for the 15ADON population. No significant differences were found in the total numbers of sequence reads generated for the two populations (t=0.99, p=0.34).

Of the total reads generated from the *in vitro* samples, more than 80.3% of the reads were mapped to the reference genome of *F. graminearum* (PH-1) where at least 79.01% reads were uniquely matched and 1.07% reads were matched to multiple genomic locations. In *in planta* 5.3 to 13.3% of total reads in 3ADON and 6.5 to 8.2% of total reads in 15ADON populations were mapped to the reference genome, respectively. The unique matches (matched only one time in genomic location of reference genome) were 5.1-12.5 % in 3ADON, and 6.1-7.8% in 15ADON of the total reads, respectively. The total numbers of unmatched reads were significantly higher in the *in planta* samples (86.7-94.7% of total reads) than in the *in vitro* samples (17.6-19.9% of total reads) (Table 3.2). This is expected because most of the sequence reads were from the host (wheat) in the *in planta* samples.

3.4.3. Transcript abundance analyses

We examined transcript abundance, transcriptional changes, and differentially expressed genes between *in vitro* samples, and between two populations *in planta* at different infection stages, and by pair-wise comparisons between *in planta* conditions and corresponding *in vitro* conditions (Table 3.3). The total numbers of transcripts expressed at the estimated gene-level were higher in the *in vitro* samples than in the *in planta* samples. The numbers of transcripts

expressed ranged from 14,242 to 14,564 in *in vitro* samples and 12,163 to 13,586 in *in planta* samples (Fig. 3.2). A significant difference was found in the total number of transcripts expressed between the 3ADON and 15ADON populations *in planta* at 144 HAI (t=6.7, p=0.02) and under the *in vitro* (t=5.6, p=0.03) growth conditions, but the difference was not significant between the two populations at 48 HAI (t=1.6, p=0.26) and 96 HAI (t=2.9, p=0.12), respectively.

The PCA of transcript abundance, measured as the FPKM (fragments per kilobase pair of exon model per million fragments mapped) values, among all the 16 samples, identified the expression pattern into two major components. The principle component 1 (PC1) accounted for 21.6% variation, and clearly differentiated the *in vitro* samples from the *in planta* samples, while PC2 describes 14.6% variation and differentiates the early infection (48 HAI) from the late infection (96 and 144 HAI). However, no clear differentiation was observed between the 3ADON and 15ADON samples under both *in vitro* and *in planta* conditions based on the PCA result (Fig. 3.3).

The expression of genes involved in secondary metabolites production including genes required for biosynthesis of the trichothecene mycotoxin, polyketide synthases and non-ribosomal peptide synthetases in *F*. graminearum varied under both *in vitro* and *in planta* conditions (Fig.2. 4). The genes (*Tri1, Tri3, Tri5, Tri8, Tri9, Tri11, Tri12, Tri14, Tri15, Tri101* and *NPS9*) were up-regulated *in planta* compared to the *in vitro* growth conditions, while the genes (*NPS7, NPS11, PKS3, PKS4, PKS6, PKS11, PKS12,* and *PKS13)* had a higher expression *in vitro* than in *in planta.* At the early infection stage (48 HAI), the FPKM values of *Tri1, Tri4, Tri5, Tri9, Tri14* and *Tri101* were higher in both populations, and decreased successively afterwards (Fig. 3.4), indicating their crucial involvement in the establishment of early infection.

The higher expression of *Tri8* and *Tri12* in the 15ADON population might be specific for this chemotype only (Fig 2.4).

3.4.4. Differential expression and functional analysis of in vitro expressed genes between 3ADON and 15 ADON populations

The comparative gene expression analysis between 3ADON and 15ADON population during *in vitro* growth identified 479 up-regulated and 801 down-regulated genes in the 3ADON population. The gene and novel transcripts (here after called gene/s) were considered as differentially expressed in one population if the FPKM Log2 folds change value was ≥1 and false discovery rate (FDR) was 0.01 or less. Functional analysis identified the corresponding 454 and 723 genes in the MIPS database for two populations, respectively (Table 3.3). Among the up-regulated genes, categories in 'metabolism' ($p=1.8\times10^{09}$), 'cellular transport, transport facilities and transport routes' (p=0.033), and 'interaction with the environment' (p=0.0272) were enriched. The genes enriched within the 'metabolism' category include those related to metabolism of polysaccharide ($p=8.9\times10^{18}$), C-compound and carbohydrate (2.4×10^{16}), extracellular (5.0×10^{05}), secondary metabolism (0.0108), lipid, fatty acid, and isoprenoid metabolism (0.011), as well as polysaccharide and amino acid degradation. Further, the genes involved in non-vesicular cellular import, carbohydrate transport, heavy metal ion transport (Cu^{+}, Fe^{3+}), cation transport (H^{+}, Na^{+}, K^{+}, Ca^{2+}, NH_4^{+}), metabolism and FAD/FMN binding related genes were highly enriched. The genes for polyketide synthases [*PKS2* (FGSG_04694) and *PKS15* (FGSG_04588)], the non-ribosomal polyketide synthetase [*NPS9* (FGSG_10990)], putative C2H2 zinc finger transcription factor [*TRI15* (FGSG_11025)], galactose oxidase precursor [*GAOA* (FGSG_11032)] and oxidoreductase that involved in production of aurofusarin [*aurO* (FGSG_02321)] were up regulated exclusively in the 3ADON population.

Among the down-regulated genes, the most frequently observed gene category was unclassified proteins (586/723; 81%), in which genes were present in a significantly higher percentage ($p=2.9\times10^{22}$) than those found in the whole genome (Ruepp et al. 2004). An additional major group of enriched genes was within the metabolism category, with function for metabolism of many compounds such as melanins, membrane lipid, sugar alcohols, sesquiterpenes, glycolipid, alanine, primary metabolic sugar derivatives, and secondary metabolites, as well as genes related to NAD/NADP binding and virulence or disease related factors ($P>0.05$). The genes for trichothecene 3-O-esterase [*Tri8* (FGSG_03532)], *TOX4* (FGSG_10551), putative 3-hydroxyacyl-CoA-dehydrogenase [*OrfI* (FGSG_03546)], deacetylase [*OrfG* (FGSG_03544)], non-ribosomal peptide synthetase [*NPS1* (FGSG_11026)] and [*HETs* (FGSG_10600)] were exclusively down regulated in the 3ADON population.

KEGG pathway analysis of differently expressed genes under the *in vitro* growth conditions further identified a number of up-regulated genes involved in metabolism of starch, sucrose, methane, drugs, and required for inter-conversions of pentose and glucuronate, while down regulated genes were enriched on purine and thiamine metabolism (Fig. 3.5).

3.4.5. Differential gene expression between 3ADON and 15ADON population in-planta

We further analyzed differential gene expression between the 3ADON and 15ADON isolates *in planta* to determine the differences during host infection. By comparison, the numbers of up regulated genes in the 3ADON population were always lower than those of down regulated genes (up-regulated in 15ADON). We identified 185, 89, 62 up-regulated genes, and 292, 361, 241 down-regulated genes in the 3ADON population compared to the 15ADON population at 48, 96 and 144 HAI, respectively (Table 3.3). Four genes (FGSG_04621, FGSG_04694, FGSG_06540, and FGSG_10632) were found to be commonly up-regulated across all three time

points, while 167, 63 and 44 genes were up-regulated specifically at 48, 96 and 144 HAI respectively (Fig. 3.6).

Of the 717 genes for transcription factors (TF) identified by Ma et al. (2007), four genes (FGSG_00342, FGSG_08246, FGSG_11061, FGSG_13008) with C2H2 zinc finger domain at 48 HAI, three genes (FGSG_01214 with C2H2 zinc finger domain, and FGSG_04747 and FGSG_09177 with Zn2Cys6 domain) at 96 HAI, and three genes (FGSG_03881 with C2H2 zinc finger domain, FGSG_03695 with nucleic acid-binding with OB-fold domains, and FGSG_10277 with Zinc finger CCHC-type domain) at 144 HAI were exclusively up-regulated in the 3ADON population. Among the down regulated TFs, FGSG_04626 with GATA type zinc finger domain was common across all three time points, while additional two TFs (FGSG_07546 with *Myb* domain and FGSG_10508 with C2H2 zinc finger domain) were found at 96 and 144-HAI respectively. Besides these three TFs, additional twelve genes (FGSG_00196, FGSG_00725, FGSG_03292, FGSG_04083, FGSG_04293, FGSG_04747, FGSG_06436, FGSG_07116, FGSG_07482, FGSG_08617, FGSG_11271, FGSG_12134) at 48 HAI, and eight genes (FGSG_02874, FGSG_03399, FGSG_03649, FGSG_08064, FGSG_08954, FGSG_09368, FGSG_10030, FGSG_13314) with various protein domains were down-regulated at 96 HAI.

We further compared the expression of gene families encoding sugar-cleaving enzymes (carbohydrate active enzymes; CAZymes) at each time point and found that the glycoside hydrolase (GH) encoding genes (FGSG_00143, FGSG_01748, FGSG_02834, FGSG_04313, FGSG_04768, and FGSG_07593) were the most abundantly up-regulated in the 3ADON population during early infection, along with three GHs (FGSG_05401, FGSG_07351, FGSG_07639) at 96 HAI and three GHs (FGSG_00571, FGSG_03628, FGSG_03695) at 144 HAI. Uniquely, the genes encoding for glycosyltransferases (FGSG_01882, FGSG_08902, and

FGSG_11341), carbohydrate esterase (FGSG_03544, FGSG_11229, and FGSG_11578) and carbohydrate binding module (FGSG_11032) were up-regulated only at early infection stage (48 HAI), suggesting that these genes might be involved in the establishment of early infection. Of the 171 gene encoding the predicted proteins of *F. graminearum* involved in degradation of different cell components identified by Brown et al (2012), FGSG_04768 (required for degradation callose), FGSG_11032 and FGSG_11229 (required for degradation of hemi-cellulose) were up regulated at 48 HAI in the 3ADON population. Similarly, genes for hemicellulose degradation (FGSG_07639) and starch degradation (FGSG_04704) were up-regulated at 96 HAI. At the late infection stage (144 HAI), FGSG_03695 and FGSG_03628 for cellulose degradation, and FGSG_00028 for protein degradation were up-regulated.

Functional analysis of differently expressed genes among all infection stages were further classified based on functional catalogue (FunCat) annotation. More than 50% of the differently expressed genes were un-classified proteins with un-known functions. Among the functionally categorized genes, majority of the genes belonged to the functional category ‘metabolism’, in which maximum numbers of genes were either up- (20.9 %, of total 177), or down- regulated (31.2 % of the total 285) at 48 HAI (Table 3.4). Then, the numbers of both up and down regulated genes belonging to the metabolism category decreased at 96- and 144- HAI (Table 3.4). Among the gene sets for metabolism, those involved in energy, protein with binding functions, regulation of metabolism and protein function, cellular transport, involved in cell rescue, defense and virulence, and for interaction with environment were significantly enriched and down regulated at 48 HAI. Further, the genes required for cell rescue, defense and virulence were also significantly enriched and up-regulated at 96 HAI. Except them, none of the functional categories showed significant enrichment (Table 3.4). Further in-depth analysis of significant

sub-categories indicated that the genes up-regulated at 48 HAI in the 3ADON population were enriched for degradation of glutamine (p=0.0055); C-compound and carbohydrate metabolism (p=0.011); glutamine metabolism (p=0.0153); arginine biosynthesis (0.0241); metabolism of urea cycle, creatine and polyamines metabolism (0.036); lipid, fatty acid and isoprenoid metabolism (0.046); and assimilation of ammonia, metabolism of the glutamate group (0.0499). At the same time, genes required for non-vesicular and cellular import, transport of compounds such as C-compound and carbohydrate, allantoin and allantoate transport, vitamine/cofactor and sugar transport, and genes required for post-transcriptional control were enriched (p<0.05) at 48 HAI. At 96 HAI, the genes for detoxification by degradation (p= 0.0028) and modification (p=0.0062); cell rescue, defense and virulence (p=0.0059); and required for defense related proteins (p=0.0098) were highly enriched. Other enriched genes were involved in metabolism of secondary products, polyketides, acetic acid derivatives, and glycosides; sugar, glucoside, polyol and carboxylate catabolism, degradation exogenous compounds; required for oxidative stress and heat shock response; catalase reaction and NAD/NADP binding (P<0.05). At 144 HAI, genes enriched for secondary and polyketides metabolism, acetic acid derivatives metabolism, purine nucleotide catabolism, required for anaerobic respiration; somatic/mitotic recombination; DNA processing; detoxification by modification and degradation; required for degradation of ester compounds; non-vesicular cellular import; C-compound and carbohydrate transport; disease, virulence and defense; perception of nutrients and nutritional adaptation etc. were the most abundant (P<0.05).

We identified 36 genes that had at least 5 times expression changes *in planta* between the 3ADON and 15ADON populations. These genes may play important roles in contributing to the differences between the two chemotype populations. Seven genes (FGSG_02324, FGSG_02326,

FGSG_02327, FGSG_02329, FGSG_05805, FGSG_06540, and a novel transcript Supercontigs_3.7:2269192-2269685) had at least 10 fold greater expression, while 24 genes had at least 10 fold lower expression in 3ADON compared to 15ADON population *in planta* (Table 3.5). Two genes (FGSG_00032 and FGSG_04621) showed continuous up-regulation till 96 HAI. FGSG_05935 at 96 and 144 HAI, and FGSG_06540 at 48 and 144 HAI had at least five time greater expression in the 3ADON population. Three genes (FGSG_02672, FGSG_08961, and FGSG_10636) were continuously down regulated throughout all three infection stages evaluated.

3.4.6. Differential gene expression analysis of in planta vs in vitro samples

The comparative analysis of *in planta* and *in vitro* gene expression profiles identified a set of genes up-regulated during specific stages of infection *in planta*. A total of 2,159, 1,981 and 2,095 genes in the 3ADON isolates, and 2,415, 2,059 and 1,777 genes in the 15ADON isolates were *in planta* up-regulated compared to the corresponding *in vitro* conditions at 48, 96, and 144 HAI, respectively (Table 3.3). Further pairwise comparison considering only *in planta* up-regulated genes in both the 3ADON and 15ADON populations identified 1,526, 1,455 and 1,427 co-expressed genes between the two populations at the three respective time points (Fig. 3.7 A, B and C). This result indicates that majority of the up-regulated genes required for early infection and subsequent colonization are common between the two chemotype populations. Except for the commonly up-regulated genes, 633, 526, 668 genes in the 3ADON isolates, and 889, 604, 350 genes in the 15ADON population were uniquely up-regulated at 48-, 96-, and 144- HAI (Fig. 2.7 A, B, and C), respectively. Further analysis of exclusively up-regulated genes revealed a set of 503, 196, and 331 unique genes specific to the 3ADON population at 48, 96 and 144 HAI, respectively (Fig. 2.7D). The commonly up-regulated genes (301 genes) between 96 and 144 HAI were more than those between 48 and 96 HAI (94 genes) as well as between 48- 144

HAI (101 genes) (Fig 2.7D). Thus, our data clearly indicated that within 3ADON population, more specific genes were expressed at the early infection stage than at the late infection stage (Fig 2.7D). Among the up-regulated genes in 3ADON population, 65 genes were common at all three infection points.

3.4.7. Functional categorization of up-regulated genes in planta compared to in vitro

Functional categorization (FunCat) analysis of *in planta* up regulated genes further indicated a chemotype specific functional enrichment in both populations. We first combined all up-regulated genes at all three time points in each population and then focused on those unique genes for functional categorization. After removal of the duplicated copies of up-regulated genes, 1396 genes in the 3ADON population and 1398 genes in the 15ADON population were identified to be unique. Of them, 1257 genes in the 3ADON population and 1278 genes in the 15ADON population were found in the database of the Munich Information Centre for Protein Sequences (MIPS) functional catalogue (Ruepp et al. 2004) and were further categorized. The functional categories and subcategories of the genes exclusively up-regulated in 3ADON revealed significant enrichment in major categories, such as ‘metabolism’ (400 genes, p= 0), ‘protein synthesis’ (134 genes, $p = 1.2\times10^{38}$), and ‘protein with binding function or cofactor requirements (structural or catalytic) (339 genes, $p=1.7\times10^{5}$). In the 15ADON population, the major enriched categories were ‘metabolism’ (536 genes, p=0), ‘energy’ (77, p= 0.0017), and ‘cell rescue, defense and virulence’ (162 genes, p=0.0014) (Fig. 3.8). Majority of the genes from both 3ADON (552/1257, 43.9%) and 15ADON (516/1278, 40.3%) populations belonged to the ‘unclassified proteins’ category (Fig. 3.8). Major functional sub-categories significantly enriched among the genes expressed only in the 3ADON population were those involved in nitrogen, sulfur and selenium metabolism (p=0.0199), pentose-phosphate pathway (p=0.0085), RNA

processing (p=0.0002), ribosome biogenesis (p=$1.8\text{x}10^{46}$), ribosomal proteins (p=$6.8\text{x}10^{40}$), translation (p=$2.67\text{x}10^{26}$), translation initiation (p=0.0002), translation elongation (p=0.0012), protein (p=0.034), nucleic acid (p=$2.2\text{x}10^{05}$) and RNA binding (p=$5.1\text{x}10^{07}$), oxidative stress response (p=0.0243), detoxification by modification (p=0.0249), and related to nucleolus (p=0.0437).

Further analyses of the same gene sets using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway identified 205 genes involved in 65 metabolic pathways in the 3ADON population, and 178 genes involved in 72 metabolic pathways in the 15ADON population (Table 3.6). Table 3.6 highlights various metabolic pathways, in which genes from both 3ADON and 15ADON population were up-regulated *in planta* compared to *in vitro*; only the pathways consisting of at least two genes from either population are shown. Higher number of genes involved in pathways for metabolism of purine, arginine, proline and pyrimidine; citrate cycle (TCA cycle); valine, leucine and isoleucine biosynthesis, pentose phosphate pathway, carbon fixation pathways in prokaryotes, and carbon fixation in photosynthetic organisms were up-regulated in the 3ADON population (Table 3.6). In contrast, more genes involved in nitrogen metabolism, pentose and glucuronate inter-conversions, starch and sucrose metabolism, drug metabolism - cytochrome P450, N-Glycan biosynthesis, various types of N-glycan biosynthesis, benzoate degradation, chloroalkane and chloroalkene degradation, metabolism of xenobiotics by cytochrome P450 were up-regulated in the 15ADON population (Table 3.6).

Differential gene expression analysis also identified a set of known genes specifically enriched in the two chemotype populations. These include *PKS2* (FGSG_04694) for polyketide synthases, (*WC-2*) (FGSG_00710) for zinc finger protein white collar 2, *OrfJ* (FGSG_03547) for NADH cytb reductase, *OrfI* (FGSG_03546) for 3-hydroxyacyl-CoA-dehydrogenase, *NPS6*

(FGSG_03747) for AM-toxin synthetase (*AMT*), *HNM1* (FGSG_08347) for choline permease, *FGB1* (FGSG_09870) for CPC2 protein, and *EF1A* (FGSG_08811) for translation elongation factor alpha 1, which were up-regulated in the 3ADON population. In the 15ADON population, the genes encoding polyketide synthase [*PKS1* (FGSG_17387), *PKS7* (FGSG_08795), and *PKS15* (FGSG_04588)], non-ribosomal peptide synthetase [*NPS1* (FGSG_11026) and *NPS10* (FGSG_06507)], CHS-2 chitin synthase 2 (FGSG_16005), phosducin [*BDM1* (FGSG_04105)], extracellular matrix protein precursor [*FEM1* (FGSG_00523)], lysine permease [*LYP1* (FGSG_12440)], endo-1,4-beta-xylanase [*xylA* (FGSG_10999)] and regulatory protein alcR [*ZBC1* (FGSG_02874)] were up-regulated.

Interestingly, all known *TRI* genes (*Tri1, Tri3, Tri4, Tri5, Tri6, Tri8, Tri9, Tri10, Tri11, Tri12, Tri14,* and *Tri15*) involved in the biosynthesis or regulation of trichothecene production were up-regulated in both populations *in planta* compared to *in vitro*. These genes showed 10 to 1516 and 6.3 to 5782.1 fold expression increases in the 3ADON and 15ADON populations *in planta*, respectively (Fig 2.9, the values are shown in Log2 scale). The highest fold increase was observed for the *Tri3* gene in both populations followed by *Tri4, Tri5, Tri1* and others (Fig 2.9). Five *Tri* genes (*Tri1, Tri6, Tri10, Tri11,* and *Tri12*) showed at least 1.4 times higher expression in the 3ADON population compared to the 15ADON population.

Of the 65 genes commonly up-regulated within the 3ADON population across all infection time points, 61 were found in the MIPS database. However, 55.7% (35/61) of them belonged to the ‘unclassified proteins’ category. The remaining 27 genes were enriched in functions related to protein synthesis ($p=4.4\times10^{6}$), translation (p=0.0002), ribosome biogenesis (p=0.0003), translation, and amino acid metabolism (p=0.0177), as well as metabolism of the pyruvate family (alanine, isoleucine, leucine, valine) and D-alanine, DNA processing and

degradation; polyketides metabolism; peptide, antigen and GTP binding; and cation transport (H^+, Na^+, K^+, Ca^{2+}, NH^{4+} etc.). KEGG pathway analyses identified six genes involved in five metabolic pathways, including valine, leucine and isoleucine biosynthesis (FGSG_09589, FGSG_02056); inositol phosphate metabolism (FGSG_06735); phosphatidylinositol signaling system (FGSG_06735); glycine, serine and threonine metabolism (FGSG_10211), and pantothenate and CoA biosynthesis (FGSG_02056) pathway.

3.5. Discussion

In this study, we examined and compared aggressiveness and DON accumulation in grains, and gene expression in the 3ADON and 15ADON populations of *F. graminearum in vitro* and *in planta*. We found that the 3ADON population was more aggressive with a higher but non-significant disease severity than the 15ADON population. In our previous study, we showed the significant differences on disease severity (DS) between 3ADON and 15ADON on the resistant wheat line ND 2710 and the susceptible cultivar Grandin (Puri and Zhong, 2010). The discrepancy might be due to the different isolates used. In the present study, 80% (eight) of the 3ADON isolates were the same as used in the previous study (Puri and Zhong, 2010). However, in the 15ADON population used in this study, only two isolates were the same as used in the previous study and eight new isolates were used. All of the 15ADON isolates used in the present study were collected in 2008 and 2010 while 75% (9/12) of the isolates used in the previous study were sampled during 1980-2004. However, the results on DON accumulation in grains were consistent between the two independent studies. The 3ADON population produced a significantly higher DON on the resistant genotype ‘ND 2710’ and the susceptible cultivar Grandin, but no significant difference in DON amount was found on the moderately resistant cultivar ‘Steele ND’ (Puri and Zhong, 2010).

Our results showed that the 15ADON population had more expressed transcripts than the 13ADON population under *in-vitro* growth conditions (Fig 2.2). However, a different phenomenon was observed during *in planta* host infection. The 15ADON population had a higher but not significant number of transcripts in the first two infection stages (24 HAI and 96HAI), but a significantly lower number of transcripts at 144 HAI compared to the 3ADON population, indicating a difference in transcriptomes during different infection stages between the two populations. Noticeably, the number of transcripts expressed increased at 144 HAI compared to the early infection stages in the 3ADON population. The infection and colonization process by *F. graminearum* in wheat is well understood, which starts at anthesis, and the fungus grows along the epicarp to the endosperm (Jansen et al., 2005). A high concentration of DON is produced during infection of epicarp leading to early host cell death and fungal growth. DON acts as a virulence factor during disease development (Jansen et al., 2005; Proctor et al., 1995). The higher number of transcripts expressed at the late stage of infection by the 3ADON population correlates with its higher DON accumulation and virulence nature. Several gene knockout studies have shown that DON is required for fungal spread from floret to rachis, and DON deficient mutants are unable to spread beyond inoculated spikelets (Jansen et al., 2005, Maier et al., 2006). IIgen et al. (2009) found an intensive expression of a reporter gene (*TRI5*) on rachis node at 4-7 days post inoculation, which constitutes a formidable barrier for fungus spread, and thus higher DON biosynthesis is required to overcome this major obstacle for successful infection in wheat (Jansen et al., 2005, Maier et al., 2006). Thus, the higher DON biosynthesis and more aggressive nature of 3ADON population are prominent for vigorous infection establishment than the 15ADON population.

Lysøe et al. (2011) studied a global gene expression pattern of *F. graminearum* during infection of the wheat cultivar Bobwhite using microarrays and showed the number of expressed genes increased from 48 hai (>4000 genes) to 96 hai (>8000), but declined after 144 hai. In our study with RNA-seq, at least 12,100 gene transcripts were detected in all infection stages and more genes were expressed at the early stage than at the late stage. The significantly higher number of transcripts detected in our study might be due to the more sensitive nature of RNA-seq than the microarray technique in detecting those rarely expressed transcripts (Wang et al. 2009). A greater than 9,000-fold range was observed in transcriptional landscape of *Saccharomyces cerevisiae* using RNA-seq in comparison with the results obtained with microarrays (Nagalakshm et al., 2008). However, the higher number of genes expressed at the early stage than the late stage of infection observed in our study could be due to differences in plant inoculation and sampling methods used for RNA extraction. We used point inoculation and only collected inoculated spikelets at each time point for RNA extraction. In contrast, spray inoculation was used and whole spikes were used in the study of Lysøe et al. (2011). A different level of disease severity and DON content was also observed in spray versus point inoculated heads (Ward et al., 2008). The different hosts (‘Briggs’ in this study and ‘Bobwhite’ in the previous study) used may also contribute the differences in the number of expressed fungal genes at different infection stages. The number of *F. graminearum* transcripts expressed *in vitro* is consistent with those observed in the previous gene expression study using Affymetrix GeneChip (Güldener et al. 2006).

This is the first comprehensive transcriptional expression study on *F. graminearum* 3ADON and 15ADON chemotype populations during *in vitro* growth and *in planta* host-infection stages using RNA-sequencing. Our results identified highly conserved *in planta* up

regulated genes between two chemotype populations during infection (1526, 1455 and 1417 at 48, 96 and 144 HAI) compared to *in vitro* growth. In general, all known *Tri* genes required for trichothecene biosynthesis and genes involved in secondary metabolites production were expressed commonly in the two populations. Among them, *Tri5* which encodes trichodiene synthase (the first step in the trichothecene biosynthetic pathway) and required for DON synthesis (Hohn and Beremand, 1989), and *Tri8* which encodes C-15 esterase or C-3 esterase in 3ADON and 15ADON producers, respectively, and were responsible for production of 3ADON or 15ADON (Alexander et al., 2011), had a similar expression in the two populations, indicating that previously identified genes for 3ADON or 15ADON chemotype differentiation might be conserved in both populations (Alexander et al., 2011). However, three *Tri* genes (*Tri1, Tri6* and *Tri11*) had at least 2.6 times higher expression in the 3ADON population compared to the 15ADON population (Fig. 3.9). *Tri1* is located outside the *Tri*-cluster and encodes a P450 oxygenase. The deletion mutant of *Tri1* accumulated calonectrin (McCormick et al., 2004), an intermediate precursors for 3ADON biosynthesis in *F. culmorum* (Hesketh, et al., 1992). *Tri11* encodes cytochrome P-450 monooxygenase and is required for hydroxylation in trichothecene biosynthesis (McCormick et al., 2004). *Tri6* is a pathway-specific transcriptional regulator in trichothecene biosynthesis (Seong et al., 2009; Peplow et al., 2003). Nasmith et al. (2011) showed that *Tri6* regulates expression of six *Tri* genes (*Tri1, Tri3, Tri6, Tri7, Tri12* and *Tri14*) within the *Tri* cluster and additional 192 potential genes in *F. graminearum*. Similarly, *Tri10*, another regulatory gene which is required for trichothecene biosynthesis and regulates expression of six more trichothecene genes (*Tri3, Tri7, Tri8, Tri9, Tri11* and *Tri12*) in *F. sporotrichioides* (Peplow et al., 2003), had almost 1.4 times greater expression in the 3ADON population than the 15ADON population. The higher expression of these global regulating genes

might play an important role in the fitness advantage of the 3ADON isolates. The higher expression of *Tri3* gene in 15ADON isolates compared to 3ADON isolates can be explained by its role in biosynthesis of all three trichothecenes (3ADON, 15ADON and NIV) (Alexander et al. 2011) or in negative regulation of gene expression involved in biosynthesis pathway for 3ADON. Previous study showed that *Tri3* encodes 15-O-acetyltransferase in *F. sporotrichioides* and is required for conversion of 15-decalonectrin into calonectrin (McCormick et al., 1996).

A number of uniquely expressed genes were identified in each population at the three infection stages. At the early stages (48 HAI and 96 HAI), the two populations had a similar number of uniquely expressed genes. However, the uniquely expressed genes were double in the 3ADON population compared to those in the 15ADON population at the late infection stage (144 HAI) (Fig 2.7 A-C). IIgen et al. (2009) identified expression of *Tri5* gene on rachis node than the adjacent rachis at 4-7 days post inoculation, and required to overcome host resistance and successive spread of pathogen form inoculated to un-inoculated spikelets. Several other independent studies highlighted the role of DON to tackle host defense due to rachis node thickening in wheat (Jansen et al., 2005, Maier et al., 2008, Miller et al., 2011). Thus, the expression of higher number of genes at late stage of infection in 3ADON population might correlate with the need of specialized genes for successful disease establishment, spread and accumulation of higher DON on grains. However, the functional characterization of those stage specifically up-regulated genes is required to answer this question.

Functional analysis of uniquely expressed *in planta* genes than *in-vitro* in both 3ADON and 15ADON isolates identified a chemotype specific gene expression and the metabolic and potential molecular mechanisms during infection (Table 3.8). Among the genes found in the MIPS functional catalogue, 552 (43.9%) genes in 3ADON population and 516 (40.3%) genes in

the 15ADON population were unclassified proteins of unknown functions. Lysøe et al. (2011) studied the transcriptomes of *F. graminearum* during wheat infection stages and found that majorities of gene exclusively expressed in wheat were with unknown functions (72.6%). Seong et al. (2008) also noticed a similar overrepresentation of unclassified genes (52.3% to 76%) during spore germination of *F. graminearum* during 0 to 24 h. Major enriched functional categories identified in this study were consistent with those reported in the previous study (Lysøe et al. 2011), although some of the categories were specific to a particular chemotype population. For example, the functional category of nitrogen, sulfur and selenium metabolism was specific to the 3ADON population, and other categories (secondary metabolism, virulence factors, detoxification, degradation of lipids and polysaccharides, and allantoin and allantoate transport) were specific to the 15ADON population. Because of the high sensitivity of RNA-seq, we identified a very high number of genes on several significant functional categories. The highly specific functional categories exclusively enriched in the 3ADON population were protein synthesis, protein with binding function or cofactor requirements and sub-categories under metabolism. Within metabolism, genes involved in assimilation of ammonia, metabolism of the glutamate group, degradation and biosynthesis of amino acids such as glutamine, leucine and homocysteine; metabolism and biosynthesis of arginine, leucine, isoleucine, glycine, valine and glutamine; and metabolism and degradation of lysine were highly enriched. It is a well-established concept that nutrients availability and their acquisition by pathogens are pre-requisites for successful colonization and fungal establishment (Divon and Fluhr, 2007). The role of various nitrogen and carbon sources in trichothecene biosynthesis, secondary metabolite production, and virulence is well understood in many fungi including *F. graminearum* (Calvo et al., 2002; Gardiner et al., 2009, López-Berges et al., 2010). The nitrogenous compounds such as

ammonia, glutamine, glutamate, asparagine etc. are the primary nutrient sources for many fungi including *S. seeviceae*, *A. nidulans*, *N. crassa* etc. However, in case of lack or very low concentration of primary sources, fungi utilize many alternative nitrogen sources such as nitrate, nitrite, purines, amides, most amino acids and proteins after *de novo* secretion of pathway-specific catabolic enzymes and permeases (Marzluf, 1997, Crawford et al., 1993). In this study, genes involved in various metabolic pathways, and required for protein synthesis and processing complex were up-regulated in 3ADON (Table 3.4).

Members of the C2H2 (Cys-Cys-His-His) zinc finger transcription factor (TF) family were expressed more abundantly in the 3ADON population than in the 15ADON population. Transcription factors have a diverse role in signal transduction, respiration, nitrogen utilization, peroxisome proliferation, stress tolerance, drug resistance, gluconeogenesis, sugar and amino acid metabolism etc. (Shelest, 2008). Among the 76 TFs with C2H2 zinc finger domains identified in the *F. graminerum* genome (Ma et al., 2010), eight (FGSG_00764, FGSG_01298, FGSG_01350, FGSG_04288, FGSG_06701, FGSG_10350, FGSG_10470, FGSG_13964) were found to be exclusively up-regulated in the 3ADON population. The function of the C2H2 zinc finger proteins in *F. graminearum* is unknown, but they are required for calcium signaling in *Aspergillus nidulans* (*CrzA*) (Hagiwara et al., 2008), and for regulation of other biological processes such as sexual development (*SteA*) in *Aspergillus* (Vallim et al., 2000) and ustilagic acid biosynthesis (*Rua1*) in *Ustilago maydis* (Teichmann et al., 2010). Of the TFs up-regulated in the 3ADON population, two TFs (FGSG_00764 and FGSG_01298) with C2H2 zinc finger domain were found to be involved in regulating the virulence and DON (Son et al., 2011). Three TFs [(FGSG_09286 and FGSG_10142 with bZIP domain) and (FGSG_09871 with bromo domain)] along with additional two TFs (FGSG_09871 and FGSG_10142) were also found to be

involved in virulence and DON biosynthesis (Son et al. 2011). Of the 52 TFs up regulated in the 15ADON population, three were involved in virulence and none of them had effect on DON biosynthesis (Son et al. 2011). Thus, the up-regulated genes expressed specifically in the 3ADON population might be the cause that makes this population different from the 15ADON population more aggressiveness and DON accumulation during host infection.

Our gene expression analysis of *in vitro* samples further identified considerable differences in gene expression profiles, and the associated physiological and cellular metabolic processes between the two populations. Most remarkably, genes annotated for cellular transport, transport facilities and transport routes were significantly up-regulated in the 3ADON isolates. These include those required for ion (16 genes) and cation (H^+, Na^+, K^+, Ca^{2+}, NH_4^+) (15 genes) transport; sugars (10 genes) and carbohydrate transport (27 genes); and those involved in transported compounds (58 genes); as well as those required for cellular (31 genes) and non-vesicular (28 genes) import. Functional annotation of the up-regulated genes in the 3ADON isolates showed that they were inriched in genes those involved in active metabolism of internal metabolites and for uptake of additional nutrients from environments. This result is consistent with those of Seong et al. (2008) who identified a considerably high number (216 genes) of genes annotated for permeases or transporters during conidial germination (0-24h) and a higher number of genes enriched in permease and transporters in fresh spores and hyphae under the nutrient limiting condition. Further, Hallen et al. (2007) analyzed the changes in gene expression during perithecial development and compared them with those in vegetative mycelia (four days old) of the fungus. They identified 162 predicted ions transporter genes associated with perithecium development stages, which were considered to be needed for rapid change in protein profiles during development of sexual structures. In many fungi, nutrient deficiency is prominent

during their sporulation and early infection, and fungus requires transport of various nutrients (nitrogen and carbon sources) (Divon and Fluhr, 2007). We observed high sporulation of this fungus on mung bean agar media at five to seven days after plating. Thus, expression and enrichment of transporter genes in 3ADON population might be essential for uptake and metabolism of various carbon and nitrogen compounds during nutrient starvation period and for higher spore production. Of the 12 up-regulated TFs in the 3ADON population, FGSG_08403 (with bHLH domain) is involved in perithecial production (Son et al., 2011), indicating that some of genes up regulated in the 3ADON isolates are required for sexual development.

Polyketide synthases (PKS), non-ribosomal peptide synthetases (NRPs), and Terpenoid synthases (TS), are major enzymes responsible for fungal secondary metabolisms (Cuomo et al., 2007). The up-regulation of *PKS2* (FGSG_04694, 68.8 fold) and *PKS15* (FGSG_04588, 47.6 fold), *NPS9* (FGSG_10990, 22.4 fold), and *Tri15* (FGSG_11025, 2.8 fold) further provide evidence that they might play important roles in growth, development and trichothecene production of the 3ADON population. Although the function of *NPS9* and *PKS15* is not clear, *PKS2* was shown to be involved in mycelial growth (Gaffoor et al. 2005). *Tri15* is not required or negatively regulates trichothecene biosynthesis in *F. sporotrichioides* (Alexander et al., 2004).

We found that *NPS1* (FGSG_11026), *TOX4* (FGSG_10551, *KP4* killer toxin ortholog), *TRI8* (FGSG_03532, trichothecene 3-O-esterase), *OrfG* (FGSG_03544, deacetylase) and *OrfI* (FGSG_03546, putative 3-hydroxyacyl-CoA-dehydrogenase) located on the right side of the trichothecene gene cluster (Brown et al., 2004) were down regulated in the 3ADON isolates. Tobiasen et al. (2007) showed that *NPS1* is related to NPS hydroxamate siderophore biosynthesis and required for iron uptake, transport, and storage. They further demonstrated that *NPS1* is expressed only *in planta* and hypothesized that it plays a role in host infection in barley but not

in wheat, because no expression of *NPS1* was detected during wheat infection and the *in vitro* expression might be media specific. The gene expression studies of 12 *ORFs* along the 14-16 kb of up or downstream to the trichothecene core cluster indicated that none of them are required for trichothecene biosynthesis (Brown et al. 2004). Thus, the highly down regulated *ORFs* (*OrfG* and *OrfI*) found in the 3ADON isolates in this study might be involved in pathways other than trichothecene biosynthesis or might negatively regulate trichothecene biosynthesis. The down regulation of *Tri8* in the 3 ADON isolates supports the finding of Kimura et al (2003), who found that *FgTri3* and *FgTri8*, and pseudo *FgTri13* genes were apparently unnecessary in biosynthesis of 3ADON, but contradicts with the recent finding of Alexander et al. (2011), who showed that *Tri8* regulates the production of 3ADON and 15ADON, and is required to convert diacetylated 3- or 15- ADON intermediate into 3ADON and 15ADON, respectively. Kimura et al (2003) further highlighted involvement of three genes *FgTri4*, *FgTri5*, and *FgTri11* within the *FgTri5*-cluster for 3ADON production. However, in our study these genes were expressed equally on both populations.

Pairwise comparisons of gene expression profiles between the 3ADON and 15 ADON populations during host infection at three different time points identified a set of differently expressed and up-regulated genes in the 3ADON population. Tracking differential gene expression patterns in the host after subsequent pathogen infection provides an insight to understand mechanisms of host infection and pathogenicity (Huibers et al. 2009). Several studies on transcriptional changes of pathogens during host infection have been conducted to identify novel genes related to pathogenicity or virulence in many plant pathogenic fungi including *F. graminearum* (Lysøe et al., 2011), *Stagonospora nodorum* (Ipcho et al., 2012), *Pseuodperonospora cubensis* (Savory et al., 2012), *Phytopthora phaseoli* (Kunjeti et al., 2012).

When gene expression profiles were examined on a time course (Fig. 3.6), 185 genes of the 3ADON population were up-regulated only at 48 HAI, which were at least twice the genes expressed at 96 HAI or 144 HAI. Lysøe et al. (2011) identified at least 10 times more genes expressed at 96 HAI than at any other time points compared (from 24 to 192 HAI), and concluded that those expressed genes were required to overcome resistance governed by the rachis system to stop further disease spread (Jansen et al., 2005). However, the early expression of more genes at 48 HAI in this study might be explained as the differences in sampling method and tissues used (discussed above). Some of the genes up-regulated during early infection might acts as pathogenicity factors in this fungus. This result is further supported by the expression of a large number of gene families encoding sugar-cleaving enzymes (carbohydrate active enzymes; CAZymes) at 48 HAI as compared to the remaining infection stages. Of the 12 glycoside hydrolase encoding genes up regulated across all three infection time points, six were expressed at 48 HAI. Genes encoding different carbohydrate active enzymes such as glycosyl transferases, carbohydrate esterase and carbohydrate binding module were also expressed only at 48HAI, providing further evidence that CAZYs enzymes were required for early infection and had multiple functions such as host penetration, nutrient gaining and pathogen cell wall remodeling (Ma et al., 2010, Vincent et al. 2008). Up-regulation of genes for cell wall degrading enzymes (FGSG_04768 for callose degradation, FGSG_11032 and FGSG_11229 for hemi-cellulose degradation) also highlights their role in breakdown of complex cell substrates in the fungus (Brown et al. 2012).

Our study indicated that a set of transporter encoding genes were differently expressed, up-regulated and enriched in the 3ADON population. These include the genes involved in transport of carbohydrate, sugars, allantoin and allantoate, vitamine/cofactor transport and those

required for host invasion and utilization of nutrient sources such as carbohydrates, proteins, lipids and vitamins during early infection. While none of the transporter genes were enriched at 96 HAI, genes for carbohydrate transport and cellular import and required for transported substrate compound were enriched at 144 HAI. The allantoin and allantoate transport category, which is required to utilize uric acid, a host induced catabolic process with response to pathogen infection, was found specific to wheat infection under nutrient limiting conditions (Lysøe et al., 2011). The enrichment of this category among the up-regulated genes in the 3ADON population at 48 HAI signifies its importance in early infection and fitness advantage.

Metabolism is an important cellular function for degradation of nutrients and biosynthesis of cellular components. In this study, a large number of genes involved in nutrient metabolism were expressed and down regulated at 48 HAI than any other infection points. Similarly, genes in other categories for energy, protein with binding function, cellular transport, cell rescue and interaction with environment were also significantly enriched and down regulated. The genes involved in cell rescue, defense and virulence were up-regulated in 3ADON at 96 HAI only. At the late infection stage (144 HAI); genes for biosynthesis of secondary metabolites and detoxification of anti-microbial plant metabolism were significantly up regulated. In this study, genes for detoxification involving cytochrome P450 (four genes) and detoxification by degradation (five genes) were found significantly down regulated. Fungal cytochrome P450s is involved in oxygenation during secondary metabolite production and contributes to fungal virulence via detoxification of antimicrobial plant metabolites (van den Brick et al., 1998). Some of genes involved in secondary metabolite biosynthesis i.e. *Tri3, NPS11, Tri12*, or responsible for 3ADON or 15ADON inter conversion (*Tri8*) were down-regulated. The *Tri3* which is found functional among all three chemotype populations, was down regulated during early infection

(48-96 HAI), while the *Tri8* which is determinant for either 3ADON or 15ADON production (Alexgnder et al., 2011), was expressed across all infection time course and down regulated. Taken together, this finding suggests that virulence of *F. graminearum* is a function of timely expression of genes required for cell wall degradation, assisting mycotoxin production and metabolites that alter host resistance (Miedaner, 1997). In conclusion, this study has revealed a set of genes that were expressed differentially in the 3ADON and 15ADON populations during in *vitro* and *in planta* conditions. The future functional analysis (which is in progress) of the genes transcriptionally different between the two chemotype populations under both *in vitro* and *in planta* conditions might provide insights into the mechanisms involved in the higher virulence and DON production of the newly emerging 3ADON isolates.

3.6. Acknowledgements

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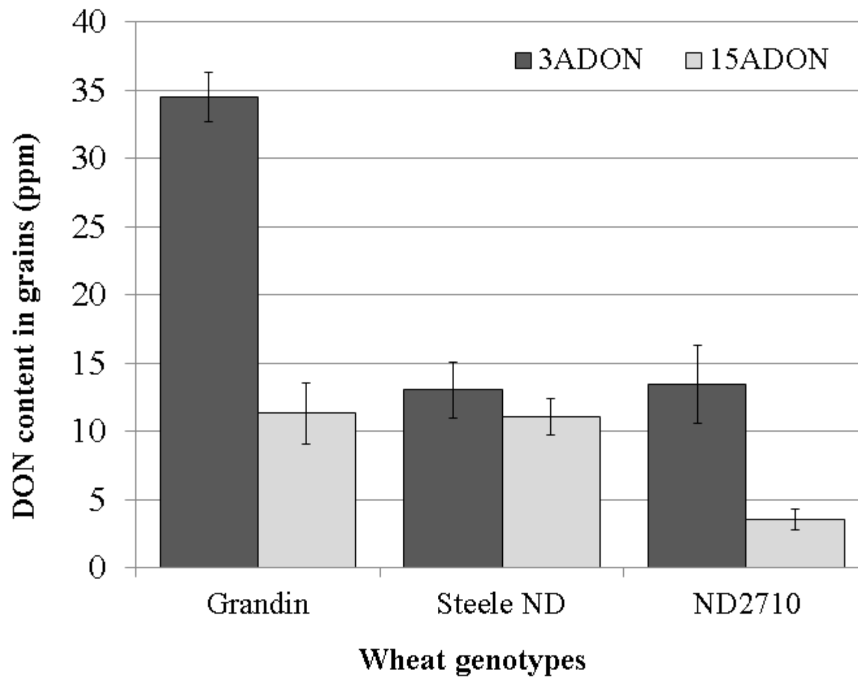


Figure 3.1. Total amount of Deoxynivalenol (DON) accumulated on grains obtained from inoculated spikes with 3ADON and 15ADON isolates. Three wheat genotypes namely Grandin (susceptible), Steele-ND (moderately resistant), and ND 2710 (high resistant) were inoculated separately. The 3ADON population accumulated a significantly high DON on Grandin (P <.0001) and ND 2710 (P=0.0034) than 15ADON. However, no significant difference (P=0.4366) was found in DON accumulation between two populations on Steele ND.

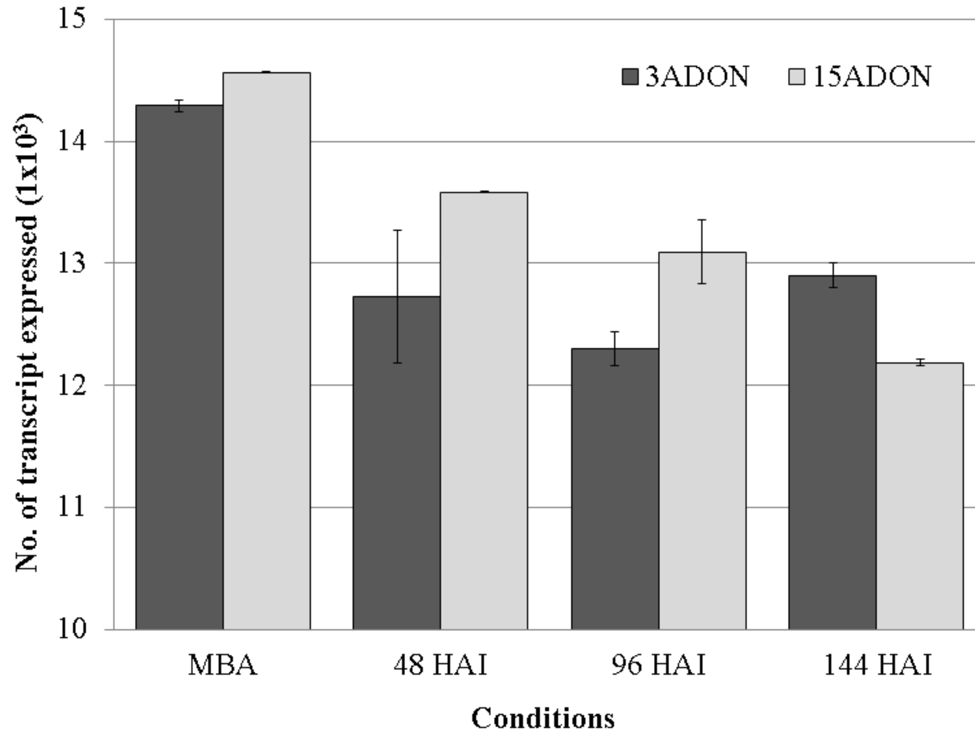


Figure 3.2. Total numbers of transcripts expressed both *in vitro* and *in planta*. Transcript-fragment reads form each samples were mapped to the *F. graminearum* PH-1 reference genome using Bowtie version 0.12.5 (Langmead et al., 2009) and TopHat version 2.0.0 (Trapnell et al., 2009). FPKM, fragments per kilobase of transcript per million fragments mapped, were calculated by Cufflinks version 0.9.3 (Trapnell et al., 2010). Each transcript was considered expressed when FPKM value was greater than 0.1, and lower boundary for FPKM value at 95% confidence interval was greater than zero. HAI: hours after inoculation, 3ADON: population producing 3-acetyl-deoxynivalenol and DON, 15ADON: population producing 15-acetyl-deoxynivalenol and DON, vertical bar represents standard error of means between replications; MBA: mung bean agar media (*in vitro*).

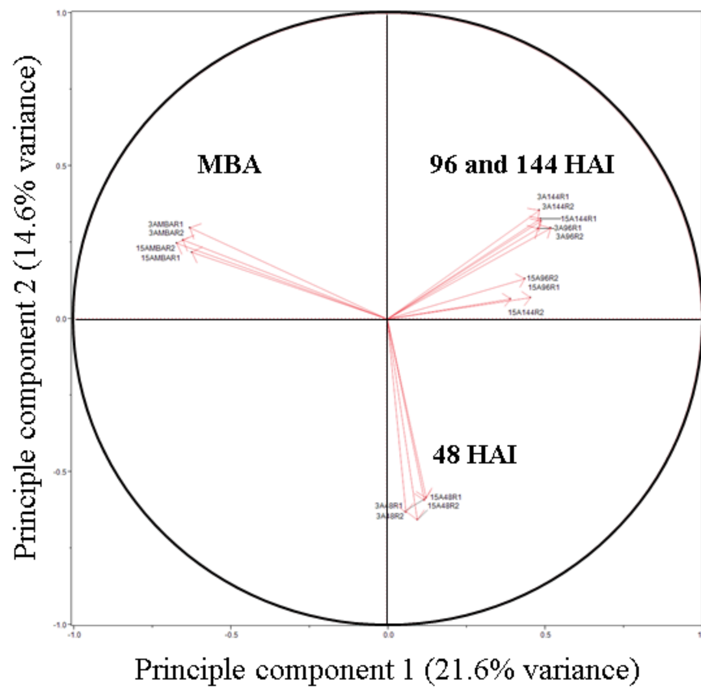


Figure 3.3. The principle component analysis of transcriptomes from all (16) samples. The principle component 1 (*PC1*) describes 21.6% variance, and differentiate *in vitro* samples from *in planta* samples, while principle component 2 (*PC2*) describes 14.6% variation and differentiate early infection stage (48 HAI) from the late infection stages (96 HAI and 144 HAI).

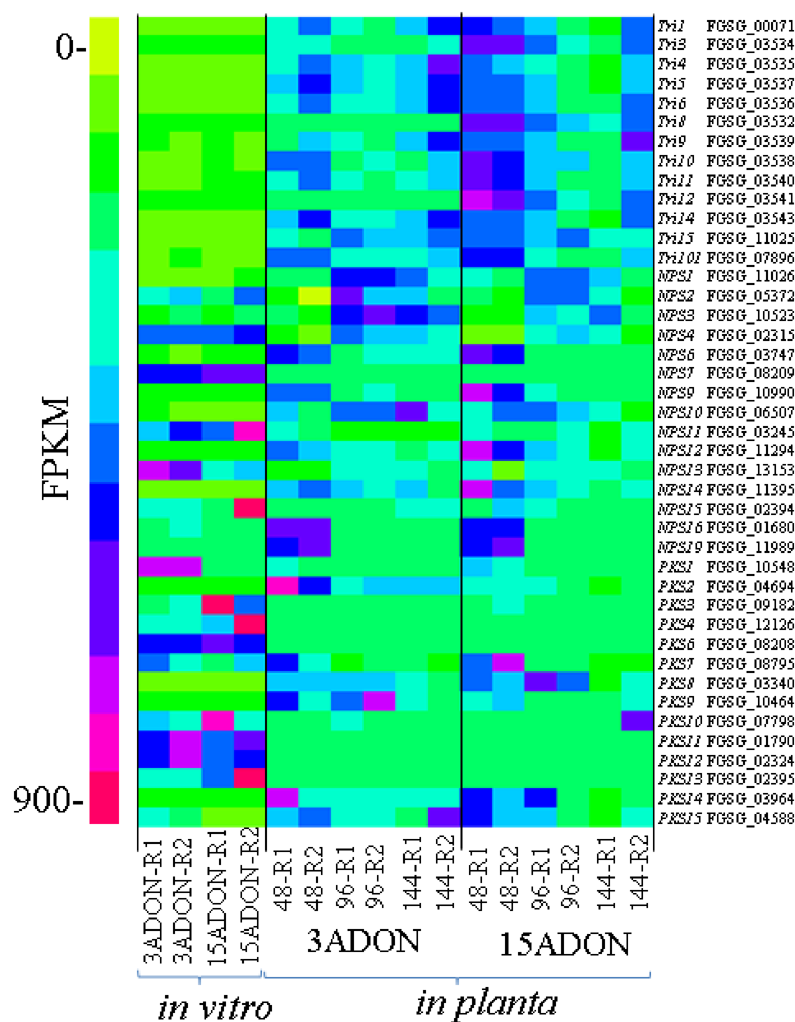


Figure 3.4. Expression of genes involved in biosynthesis of secondary metabolite. The genes (*Tri1, Tri3, Tri5, Tri8, Tri9, Tri11, Tri12, Tri14, Tri15, Tri101* and *NPS9*) were expressed higher *in planta* than *in vitro*, while genes (*NPS7, NPS11, PKS3, PKS4, PKS6, PKS11, PKS12,* and *PKS13)* had a higher expression *in vitro*.

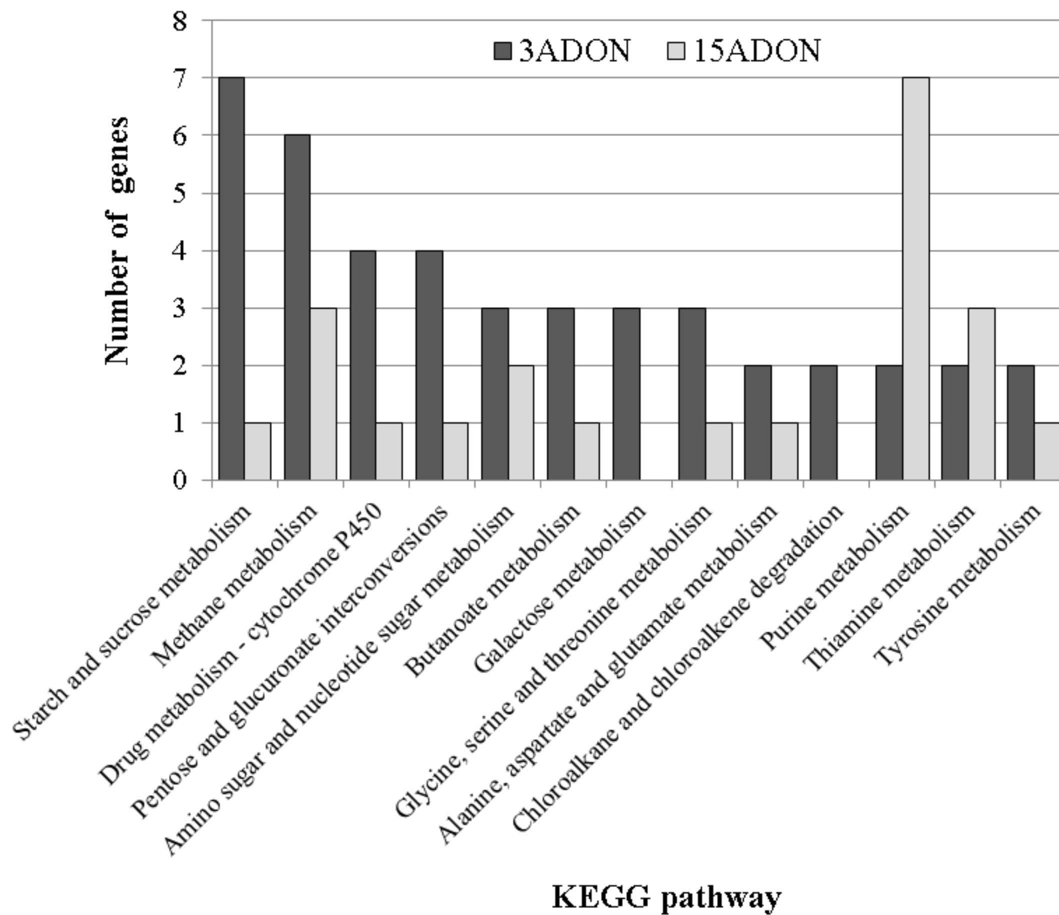
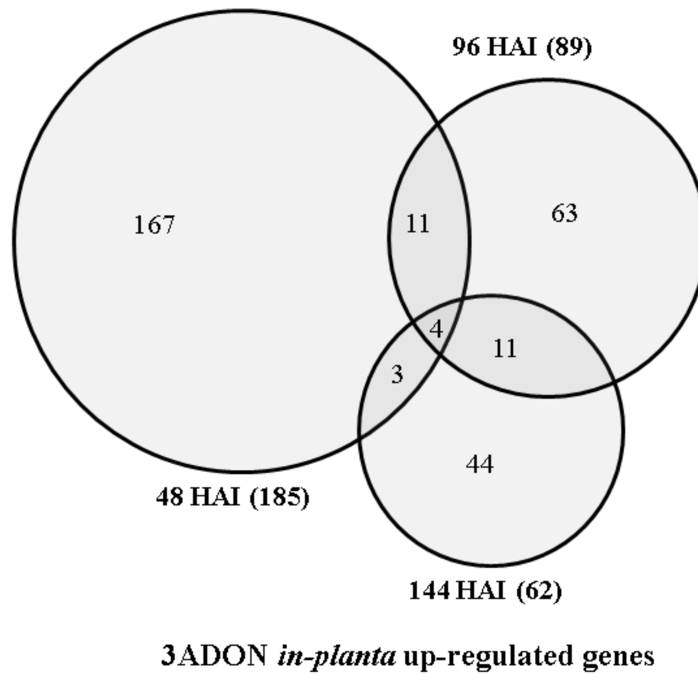


Figure 3.5. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of *in vitro* up-regulated genes. Values with log2 fold change >1 and false discovery rate (<0.01) were considered as differentially expressed. Only pathways having at least two genes up-regulated on either of population are shown.



3ADON *in-planta* up-regulated genes

Figure 3.6. Venn diagram of differentially expressed *in planta* genes in 3ADON population compared to 15ADON population. Among 185, 89, 62 up-regulated genes in 3ADON, a total 167, 63 and 44 genes were specific to three time points after inoculation, respectively. HAI: hours after inoculation, 3ADON: population producing 3-acetyl-deoxynivalenol and DON, 15ADON: population producing 15-acetyl-deoxynivalenol and DON.

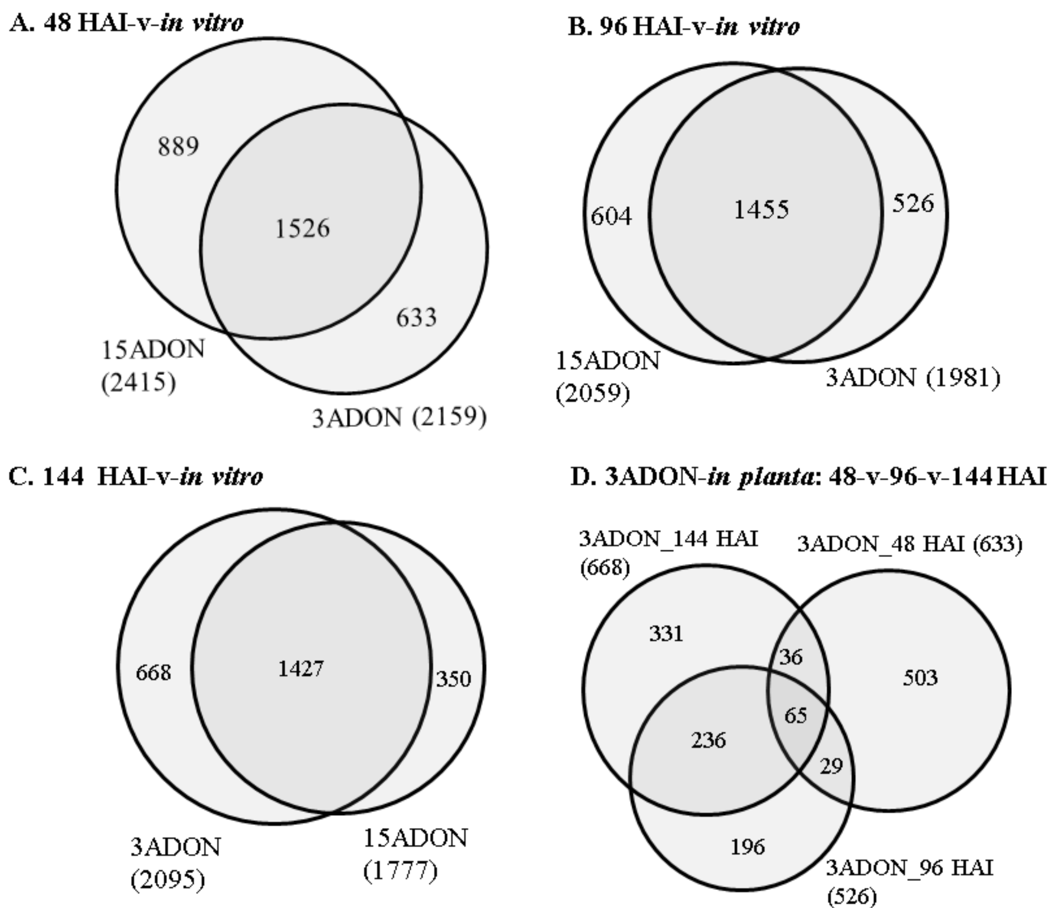


Figure 3.7. Venn diagram comparing exclusively up-regulated genes *in planta* vs *in vitro* in the two populations 3ADON and 15ADON at 48 (A), 96 (B), 144 (C) hours after inoculation (HAI). A total 633, 526, and 668 genes were up-regulated at three respective points in 3ADON). D). Comparison of genes upregulated within 3ADON population. Total 503, 196 and 331, genes were exclusively upregulated at 48, 96 and 144 HAI. Of which 65 genes were co-expressed among all three stages. 3ADON: population producing 3-acetyl-deoxynivalenol and DON, 15ADON: population producing 15-acetyl-deoxynivalenol and DON.

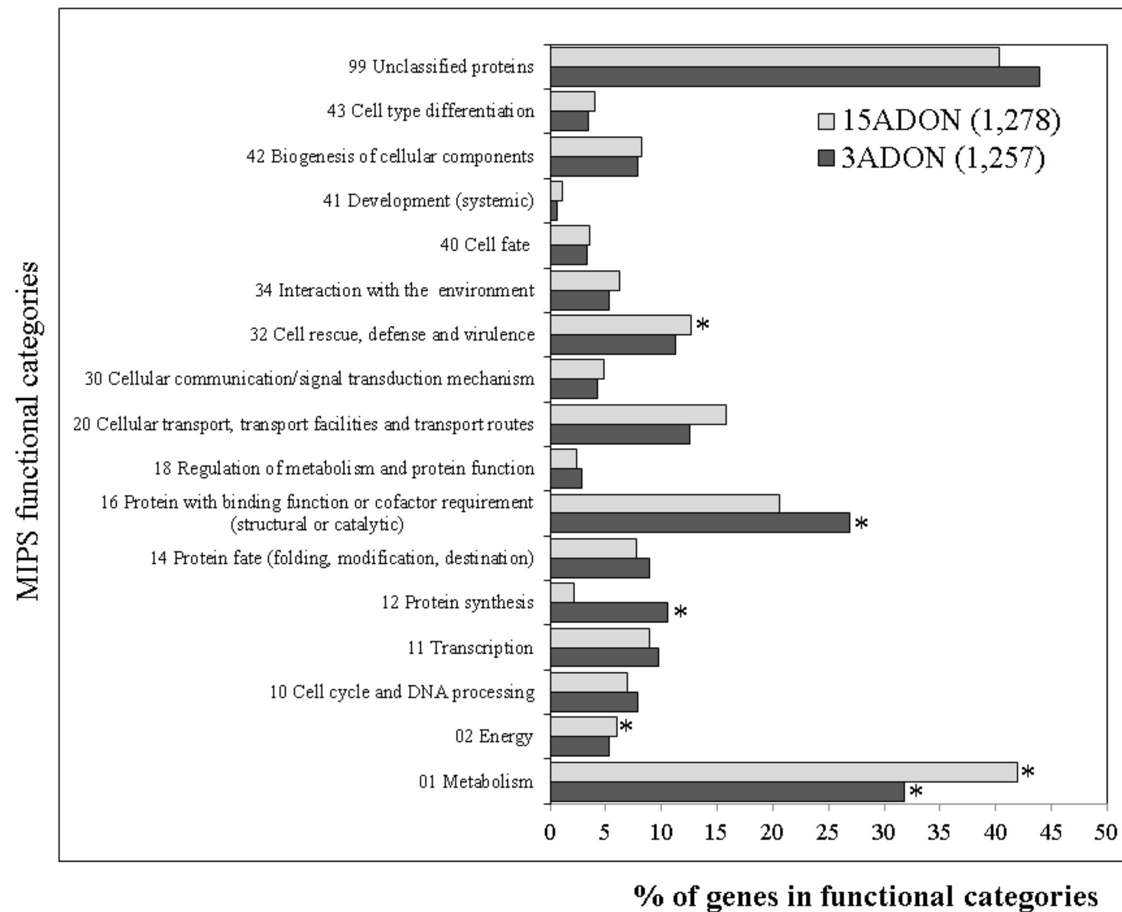


Figure 3.8. Functional analysis of *in planta* up-regulated genes in the 3ADON and 15ADON populations. Only genes up-regulated in 3ADON and 15ADON population were used for functional categorization. Total numbers of gene found in MIPS catalogue are listed in parenthesis. The functional categories in which members are significantly enriched than those found in the whole genome are marked with asterisks ($p < 0.05$, FDR < 0.05).

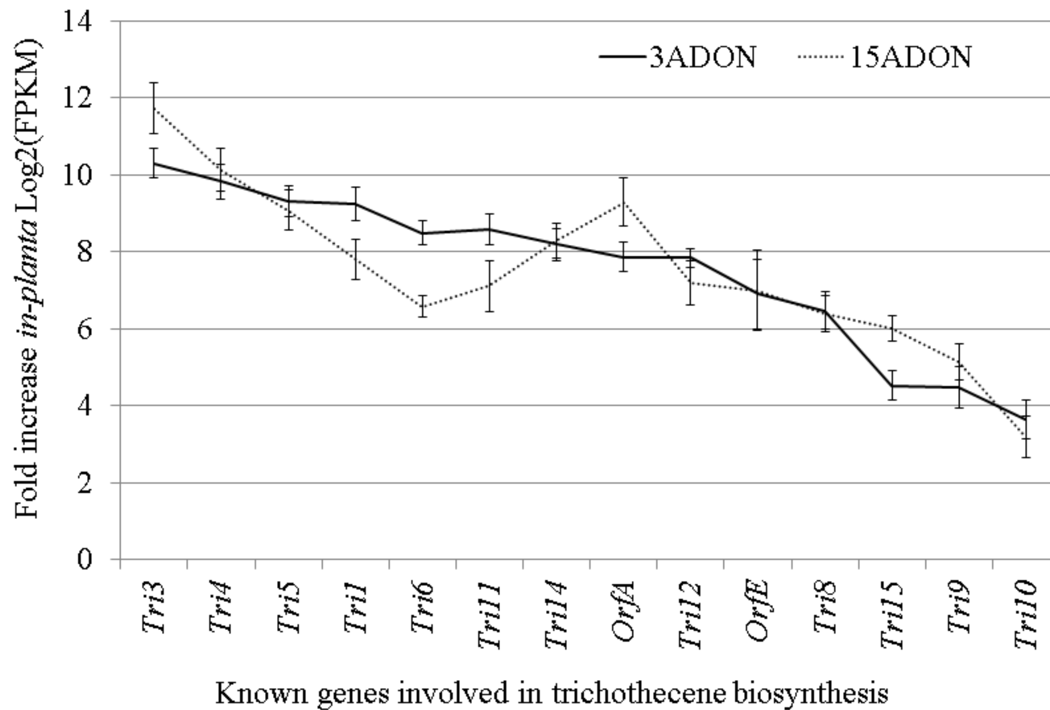


Figure 3.9. Fold change in expression of *TRI* genes during *in planta* infection compared to *in vitro.* The average of in planta fold-increase (Log2) across the all infection points were compared with corresponding *in vitro* expression in both populations. *Tri1, Tri6*, and *Tri11* were up-regulated in all infection points on 3ADON population. While *Tri3*, *OrfA* and *Tri15* were upregulated among all infection points on 15ADON population. Fold change in gene expression were measured using relative changes in FPKM values, and were statistically significant at FDR <0.01. Error bars represent the standard error of means.

Table 3.1. Name, origin, PCR based chemotype and collection information of 20 *Fusarium graminearum* isolates used in the study.

Isolate[a]	Origin	Cultivar	Year	Chemotype	Collected by
Fg 08-001	Foster, ND, USA	Reeder	2008	3ADON	S. Zhong
Fg 08-003	Steele , ND, USA	wheat	2008	3ADON	S. Zhong
Fg 08-004	Barnes, ND, USA	wheat	2008	3ADON	S. Zhong
Fg 08-005	Foster, ND, USA	Reeder	2008	3ADON	S. Zhong
Fg 08-006	Steele, ND, USA	wheat	2008	3ADON	S. Zhong
Fg 08-009	Foster, ND, USA	Steele ND	2008	3ADON	S. Zhong
Fg 08-010	Barnes, ND, USA	wheat	2008	3ADON	S. Zhong
Fg 08-012	Steele, ND, USA	wheat	2008	3ADON	S. Zhong
Fg 08-025	ND, USA	wheat	2008	3ADON	S. Zhong
Fg 08-029	ND, USA	wheat	2008	3ADON	S. Zhong
Fg 08-007	Foster, ND, USA	Vantage	2008	15ADON	S. Zhong
Fg 08-013	Steele, ND, USA	wheat	2008	15ADON	S. Zhong
Fg 08-026	ND, USA	wheat	2008	15ADON	S. Zhong
Fg 08-030	ND, USA	wheat	2008	15ADON	S. Zhong
Fg 08-034	ND, USA	wheat	2008	15ADON	S. Zhong
Fg 08-036	ND, USA	wheat	2008	15ADON	S. Zhong
Fg 08-037	Foster, ND, USA	wheat	2008	15ADON	S. Zhong
Fg 08-043	Foster, ND, USA	Durum	2008	15ADON	S. Zhong
Fg 08-057	Foster, ND, USA	Alsen	2008	15ADON	S. Zhong
09-1-H1-1	Dicky, ND, USA	wheat	2009	15ADON	S. Ali

[a]Ten isolates from each population (3ADON and 15ADON) were first separately evaluated for disease aggressiveness and amount of DON accumulation in grains using three spring wheat genotypes (Grandin, Steele ND and ND 2710) under the greenhouse conditions. While for transcriptome analysis, isolates form each population are mixed equally and inoculated on susceptible wheat cultivar ‘Briggs’ for *in planta* sample collectioan and grown for five days on mung bean agar media for *in vitro* sample collection.

Table 3.2. Summary of sequence reads (in millions) from each 16 RNA sequences library samples.

Summery[a]	*in vitro*				*in planta*_48 HAI				*in planta*_96 HAI				*in planta*_144 HAI			
	3A_R1	3A_R2	15A_R1	15A_R2	3A_R1	3A_R2	15A_R1	15A_R2	3A_R1	3A_R2	15A_R1	15A_R2	3A_R1	3A_R2	15A_R1	15A_R2
Total reads	40.92	34.87	34.58	32.23	34.42	30.27	27.81	34.50	33.43	26.48	39.18	36.94	49.56	41.21	29.17	34.01
Mapped reads	33.71	28.53	27.71	25.99	1.89	1.60	1.98	2.29	2.63	3.14	2.85	2.80	6.57	4.77	2.38	2.20
	82.4%	81.8%	80.1%	80.7%	5.5%	5.3%	7.1%	6.6%	7.9%	11.8%	7.3%	7.6%	13.3%	11.6%	8.2%	6.5%
Unique match	33.43	28.21	27.34	25.71	1.82	1.55	1.90	2.21	2.53	3.04	2.68	2.64	6.21	4.64	2.28	2.10
	81.7%	80.9%	79.1%	79.8%	5.3%	5.1%	6.8%	6.4%	7.6%	11.5%	6.9%	7.2%	12.5%	11.3%	7.8%	6.2%
Multi-position match	0.28	0.32	0.37	0.28	0.07	0.05	0.08	0.08	0.10	0.09	0.17	0.16	0.36	0.13	0.10	0.10
	0.68%	0.92%	1.07%	0.86%	0.22%	0.16%	0.30%	0.24%	0.31%	0.35%	0.42%	0.44%	0.73%	0.31%	0.35%	0.28%
Unmapped	7.21	6.33	6.87	6.24	32.53	28.68	25.83	32.21	30.80	23.35	36.33	34.14	42.99	36.45	26.78	31.81
	17.6%	18.2%	19.9%	19.4%	94.5%	94.7%	92.9%	93.4%	92.1%	88.2%	92.7%	92.4%	86.7%	88.4%	91.8%	93.5%

[a]Sequence reads from each treatment were mapped to the reference genome of *F. graminearum* (PH-1) using Bowtie v0.12.5 (Langmead et al., 2009) and TopHat v2.0.0 (Trapnell et al., 2009). HAI: hours after inoculation; 3A: 3ADON (3-acetyl-deoxynivalenol); 15A: 15ADON (15-acetyl-deoxynivalenol); R: Replicate.

Table 3.3. Pair-wise comparison of gene expression profiles on 3ADON and 15ADON populations.

Comparison[a]	significantly up-regulated genes[b]	significantly down-regulated genes
A. *In vitro* only		
3ADON-v-15ADON	479 (454)	801 (723)
B. *In-planta* only		
48 HAI: 3ADON-v-15ADON	185 (177)	292 (285)
96 HAI: 3ADON-v-15ADON	89 (85)	362 (339)
144 HAI: 3ADON-v-15ADON	62 (59)	241 (228)
C. *In planta* versus *in vitro*		
3ADON: 48 HAI-v-*in vitro*	2159	1631
3ADON: 96 HAI-v-*in vitro*	1981	2694
3ADON: 144 HAI-v-*in vitro*	2095	2632
15ADON: 48 HAI-v-*in vitro*	2415	1510
15ADON: 96 HAI-v-*in vitro*	2059	1975
15ADON: 144 HAI-v-*in vitro*	1777	2087

[a]Number of differentially expressed genes in each population were identified for three seaparte comparision A. compared within *in vitro*; B. compared within *in planta*; and among *in planta* all infection stages with corresponding *in vitro* condition for each 3ADON and 15ADON populations.
[b]Number of differentlyexpressed genes were calculated using Cuffdiff within Cufflinks v1.3.0 (Trapnell et al., 2010)]. Genes were considered significantly up-regulated or down-regulated if FPKM (fragments per kilobase of transcript per million fragments mapped) log2 (fold change) value was greater than one at the false discovery rate (q) of 1 % (<0.01). The numbers in parentheses denotes the numbers of genes found in MIPS functional catalogue (Ruepp et al., 2004). HAI, hours after inoculation; 3ADON, 3-acetyl-deoxynivalenol; 15ADON, 15-acetyl-deoxynivalenol.

Table 3.4. Functional annotation of *in planta* only up or down- regulated genes in 3ADON population at three respective infection points.

	Up-regulation			**Down-regulation**			**Whole genome**
Functional category	**48 HAI (177)**[a]	**96 HAI (85)**	**144 HAI (59)**	**48 HAI (285)**	**96 HAI (339)**	**144 HAI (228)**	
01 Metabolism	37 (20.9%)	13 (15.2%)	13 (22%)	**89** (31.2%)	63 (18.5%)	40 (17.5%)	2322 (16.7%)
02 Energy	3 (1.69%)	5 (5.88%)	2 (3.38%)	**18** (6.31%)	9 (2.65%)	6 (2.63%)	503 (3.63%)
10 Cell cycle and DNA processing	3 (1.69%)	2 (2.35%)	2 (3.38%)	9 (3.15%)	3 (0.88%)	1 (0.43%)	659 (4.76%)
11 Transcription	3 (1.69%)	-	1 (1.69%)	6 (2.1%)	1 (0.29%)	4 (1.75%)	718 (5.19%)
12 Protein synthesis	2 (1.12%)	-	1 (1.69%)	4 (1.4%)	1 (0.29%)	1 (0.43%)	370 (2.67%)
14 Protein fate	3 (1.69%)	1 (1.17%)	1 (1.69%)	19 (6.66%)	13 (3.83%)	5 (2.19%)	920 (6.65%)
16 Protein with binding function	12 (6.77%)	7 (8.23%)	5 (8.47%)	**46** (16.1%)	30 (8.84%)	19 (8.33%)	1714 (12.3%)
18 Regulation of metabolism and protein function	-	-	-	**11** (3.85%)	4 (1.17%)	1 (0.43%)	242 (1.75%)
20 Cellular transport	15 (8.47%)	10 (11.7%)	9 (15.2%)	**39** (13.6%)	41 (12%)	23 (10%)	1390 (10%)
30 Cellular communication	-	-	-	5 (1.75%)	4 (1.17%)	1 (0.43%)	312 (2.25%)
32 Cell rescue, defense and virulence	9 (5.08%)	**12 (14.1%)**	5 (8.47%)	**37** (12.9%)	22 (6.48%)	16 (7.01%)	856 (6.19%)
34 Interaction with the environment	5 (2.82%)	5 (5.88%)	4 (6.77%)	**25** (8.77%)	19 (5.6%)	10 (4.38%)	606 (4.38%)
36 Systemic interaction with the environment	-	-	-	1 (0.35%)	-	1 (0.43%)	12 (0.08%)
40 Cell fate	1 (0.56%)	1 (1.17%)	1 (1.69%)	7 (2.45%)	4 (1.17%)	1 (0.43%)	240 (1.73%)
42 Biogenesis of cellular components	3 (1.69%)	1 (1.17%)	1 (1.69%)	9 (3.15%)	8 (2.35%)	4 (1.75%)	617 (4.46%)
43 Cell type differentiation	-	-	-	6 (2.1%)	2 (0.58%)	1 (0.43%)	273 (1.97%)
99 Unclassified proteins	126 (71.1%)	61 (71.7%)	38 (64.4%)	148 (51.9%)	234 (69%)	**165** (72.3%)	9004 (65.1%)

[a]Numbers on parenthesis indicates number of genes found on MIPS FunCat.
[b]Number of genes present on whole in the specific functional category (retrieved from MIPS database).
[-]indicates genes that were not detected on specific functional category.
Numbers on bold letters are those significantly enriched at p= <0.05 and FDR>0.05.

Table 3.5. Differentially expressed *Fusarium graminearum* genes in 3ADON population showing at least 5-fold greater expression than 15ADON *in planta* expression or with corresponding *in vitro* expression.

Gene ID[a]	Condition	Gene description	Gene name	Compared to 15ADON[b]	Compared to *in vitro*
FGSG_00002	48HAI	Conserved hypothetical protein	..	6.5	22.7
FGSG_00032	48HAI	Related to non-heme chloroperoxidase	..	5.2	16.2
	96HAI	Related to non-heme chloroperoxidase	..	7.4	8.8
FGSG_00143	48HAI	Hypothetical protein	..	6.5	189.9
FGSG_02321	48HAI	Oxidoreductase that catalyses the conversion of dimeric 9-hydroxyrubrofusarin to aurofusarin	*aurO*	5.8	..
FGSG_02324	48HAI	Polyketide synthase that catalyse the condensation of one acetyl-coa and six malonyl-coa resulting in formation of nor-rubrofusarin	*PKS12*	35.0	..
FGSG_02325	48HAI	Conserved hypothetical protein		6.4	..
FGSG_02326	48HAI	O-methyltransferase that catalyse the methylation of nor-rubrofusarin resulting in formation of rubrofusarin	*aurJ*	10.4	..
FGSG_02327	48HAI	Flavin depend monooxygenase that catalyses the oxidation of rubrofusarin to 9-hydroxyrubrofusarin	*aurF*	10.2	..
FGSG_02328	48HAI	Laccase that catalyse the dimerization of two 9-hydroxyrubrofusarin in C7 positions	*gip1*	7.9	..
FGSG_02329	48HAI	Conserved hypothetical protein	..	10.0	..
FGSG_02833	48HAI	Probable alpha-glucoside transport protein	..	7.6	49.6
FGSG_02966	144 HAI	Conserved hypothetical protein	..	5.0	..
FGSG_03335	144 HAI	Conserved hypothetical protein	..	7.0	..
FGSG_03336	144 HAI	Related to integral membrane protein	..	6.6	..
FGSG_04599	48HAI	Related to peroxisomal short-chain alcohol dehydrogenase	..	5.1	76.7
FGSG_04621	48HAI	Related to monoamine oxidase N	..	8.4	4.2
	96HAI	Related to monoamine oxidase N	..	5.8	11.5
FGSG_04694	144 HAI	Polyketide synthase	*PKS2*	6.0	19.7
FGSG_04717	96HAI	Probable cytochrome P450 monooxygenase (*lova*)	..	5.2	194.7
FGSG_04787	144 HAI	Conserved hypothetical protein	..	5.1	..
FGSG_05322	48HAI	Probable fatty-acyl-coa synthase, beta subunit	..	5.1	..
FGSG_05805	96HAI	Related to aliphatic nitrilase	..	15.0	31.2
FGSG_05928	144 HAI	Conserved hypothetical protein	..	26	12.1
FGSG_05935	96HAI	Related to triacylglycerol lipase V precursor	..	6.6	2.1
	144 HAI	Related to triacylglycerol lipase V precursor	..	7.7	..
FGSG_06540	48HAI	Conserved hypothetical protein	..	17.3	9.1
	144 HAI	Conserved hypothetical protein	..	6.0	3.5
FGSG_06580	48HAI	Probable acetyl-coa carboxylase	..	5.7	..
FGSG_07666	144 HAI	Related to quinate transport protein	..	5.8	10.8
FGSG_08076	48HAI	Hypothetical protein	..	5.0	2.7
FGSG_09175	48HAI	Conserved hypothetical protein	..	6.3	..
FGSG_10326	48HAI	Conserved hypothetical protein	..	5.3	6.4

(continued)

Table 3.5. Differentially expressed *Fusarium graminearum* genes in 3ADON population showing at least 5-fold greater expression than 15ADON *in planta* expression or with corresponding *in vitro* expression. (continued)

Gene ID[a]	Condition	Gene description	Gene name	Compared to 15ADON[b]	Compared to *in vitro*
FGSG_11722	48HAI	Conserved hypothetical protein	..	6.3	4.6
FGSG_11723	48HAI	Conserved hypothetical protein	..	5.6	3.3
FGSG_12049	48HAI	Hypothetical protein	..	5.9	31.9
FGSG_12132	48HAI	Conserved hypothetical protein	..	5.2	2.1
SC_3.1:373085-373840	48HAI	..	..	5.2	..
SC_3.2:942339-943229	48HAI	..	..	5.2	..
SC_3.7:2269192-2269685	48HAI	..	..	14.1	93.5
	48 HAI	Probable cytochrome P450 monooxygenase (*lova*)	..	-72.3	**2.5**
FGSG_02672	96 HAI	Probable cytochrome P450 monooxygenase (*lova*)	..	-60.0	**5.3**
	144 HAI	Probable cytochrome P450 monooxygenase (*lova*)	..	-40.4	**6.5**
FGSG_03384	144 HAI	Probable exopolygalacturonase	..	-10.7	**..**
FGSG_04008	96 HAI	Conserved hypothetical protein	..	-13.5	**..**
	144 HAI	Conserved hypothetical protein	..	--13.1	**..**
FGSG_04679	144 HAI	Related to beta-mannosidase	..	-43.9	**51.0**
FGSG_04702	144 HAI	Related to dehydrogenase	..	-11.4	**..**
FGSG_04823	144 HAI	Hypothetical protein	..	-24.2	**..**
FGSG_04892	96 HAI	Conserved hypothetical protein	..	-12.5	**..**
	144 HAI	Conserved hypothetical protein	..	-41.7	**..**
FGSG_07205	96 HAI	Conserved hypothetical protein	..	-20.0	**..**
FGSG_07804	144 HAI	Hypothetical protein	..	-22.8	**..**
FGSG_08960	144 HAI	Related to kinesin light chain	..	-43.2	**..**
	48 HAI	Conserved hypothetical protein	..	-12.9	**..**
FGSG_08961	96 HAI	Conserved hypothetical protein	..	-59.1	**..**
	144 HAI	Conserved hypothetical protein	..	-87.8	**..**
FGSG_09072	96 HAI	Conserved hypothetical protein	..	-10.9	**13.5**
	144 HAI	Conserved hypothetical protein	..	-11.7	**12.3**
FGSG_09641	96 HAI	Conserved hypothetical protein	..	-37.8	**..**
	144 HAI	Conserved hypothetical protein	..	-33.5	**..**
FGSG_10085	48 HAI	Related to integral membrane protein	..	-10.7	**2.4**
	144 HAI	Related to integral membrane protein	..	-34.8	**..**
FGSG_10086	96 HAI	Conserved hypothetical protein	..	-22.1	**6.8**
	144 HAI	Conserved hypothetical protein	..	-18.1	**6.0**
FGSG_10603	144 HAI	Putative protein [EST hit]	..	-21.3	**..**
	48 HAI	Probable IgE -dependent histamine-r-factor	..	-10.2	**..**
FGSG_10636	96 HAI	Probable IgE -dependent histamine-r-factor	..	-12.7	**5.6**
	144 HAI	Probable IgE -dependent histamine-r-factor	..	-19.1	**6.3**
FGSG_10670	144 HAI	Probable acetylxylan esterase precursor	..	-79.0	**69.6**
FGSG_11009	96 HAI	Conserved hypothetical protein	..	-10.3	**117.9**
FGSG_11449	144 HAI	Conserved hypothetical protein	..	-13.8	**9.1**
FGSG_13464	48 HAI	Conserved hypothetical protein	..	-184.6	**2.8**
FGSG_13505	144 HAI	Conserved hypothetical protein	..	-12.7	**38.2**
SC_3.2:5039491-5040187	144 HAI	..	..	-61.7	**39.5**
SC_3.2:5039551-5040054	96 HAI	..	..	-95.8	..

[a]The differentially expressed genes were identified using Cuffdiff within Cufflinks interface (Trapnell et al., 2010). Genes were considered significantly up or down-regulated in expression

if the absolute value of FPKM (fragments per kilobase of transcript per million fragments mapped) Log2 (fold change) value was greater than one at the false discovery rate (q<0.01).
[b]Values indicated with (-) sign were fold down-regulated in 3ADON compared to 15ADON (*in planta* expression), and only genes with least 10 fold lower expression are shown. While, values indicated with bold letters are fold up-regulated in 15ADON condition as compared to *in vitro* expression.
..no information.

Table 3.6. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of *in planta* up-regulated genes in 3ADON and 15ADON populations compared to *in vitro*.

Pathway[a]	3ADON[b]	15ADON
Purine metabolism	15	10
Arginine and proline metabolism	11	2
Glycine, serine and threonine metabolism	8	6
Pyrimidine metabolism	8	4
Citrate cycle (TCA cycle)	7	2
Valine, leucine and isoleucine biosynthesis	7	1
Methane metabolism	6	9
Glyoxylate and dicarboxylate metabolism	6	4
Cysteine and methionine metabolism	6	3
Phenylalanine, tyrosine and tryptophan biosynthesis	6	3
Pentose phosphate pathway	6	1
Alanine, aspartate and glutamate metabolism	5	2
Pantothenate and CoA biosynthesis	5	2
Carbon fixation pathways in prokaryotes	5	1
Nitrogen metabolism	4	6
One carbon pool by folate	4	3
Glycolysis / Gluconeogenesis	4	2
Carbon fixation in photosynthetic organisms	4	-
Amino sugar and nucleotide sugar metabolism	3	5
Thiamine metabolism	3	4
Valine, leucine and isoleucine degradation	3	3
Butanoate metabolism	3	2
Fructose and mannose metabolism	3	2
Glutathione metabolism	3	2
Glycerolipid metabolism	3	2
Inositol phosphate metabolism	3	2
Lysine biosynthesis	3	2
Phenylalanine metabolism	3	2
Lysine degradation	3	1
Tryptophan metabolism	3	1
Phosphatidylinositol signaling system	3	-
Pyruvate metabolism	3	-
Pentose and glucuronate interconversions	2	8
Starch and sucrose metabolism	2	6
Tyrosine metabolism	2	4
beta-Alanine metabolism	2	2
Aminobenzoate degradation	2	1
Cyanoamino acid metabolism	2	1
Oxidative phosphorylation	2	1
Riboflavin metabolism	2	1
Steroid biosynthesis	2	1
C5-Branched dibasic acid metabolism	2	-

(continued)

Table 3.6. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of *in planta* up-regulated genes in 3ADON and 15ADON populations compared to *in vitro*. (continued)

Pathway[a]	3ADON[b]	15ADON
Glucosinolate biosynthesis	**2**	**-**
Isoquinoline alkaloid biosynthesis	2	-
Tropane, piperidine and pyridine alkaloid biosynthesis	2	-
Nicotinate and nicotinamide metabolism	1	3
Propanoate metabolism	1	3
Aminoacyl-tRNA biosynthesis	1	2
Porphyrin and chlorophyll metabolism	1	2
Toluene degradation	1	2
Drug metabolism - cytochrome P450	-	5
N-Glycan biosynthesis	-	5
Various types of N-glycan biosynthesis	-	4
Benzoate degradation	-	3
Chloroalkane and chloroalkene degradation	-	3
Metabolism of xenobiotics by cytochrome P450	-	3
Arachidonic acid metabolism	-	2
Chlorocyclohexane and chlorobenzene degradation	-	2
Fluorobenzoate degradation	-	2
Galactose metabolism	-	2
Other glycan degradation	-	2
Styrene degradation	-	2

[a]Only pathways that includes at least two genes up regulated on either population were listed. In 3ADON population, total 205 genes involved in 65 metabolic pathways were identified. While in 15ADON population, total 178 genes involved in 72 metabolic pathways were identified.
[b]Number of genes on specific pathway from each population.

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CHAPTER 4. MOLECULAR CHARACTERIZATION OF FUSARIUM HEAD BLIGHT PATHOGENS SAMPLED FROM A NATURALLY INFECTED DISEASE NURSERY USED FOR WHEAT BREEDING PROGRAMS IN CHINA

4.1. Abstract

Fusarium head blight (FHB) is an important disease of wheat and barley worldwide. The disease is primarily caused by members of the *Fusarium graminearum* species complex, consisting of at least 14 phylogenetically distinct species. To determine the population structure of the FHB pathogens in a naturally infected disease nursery located at Jianyang, Fujian province, China, 160 isolates of the *F. graminearum* complex were recovered from symptomatic wheat spike samples collected in two consecutive years (2008 and 2009) and characterized using species- and chemotype-specific polymerase chain reaction as well as variable number tandem repeat (VNTR) markers. All isolates analyzed were identified as *F. asiaticum* except for one isolate, which was identified as *F. avenaceum*. Among the 159 *F. asiaticum* isolates, 126 (79%) isolates were of the nivalenol (NIV) type while 29 (18%) isolates were of the 15-acetyl deoxynivalenol (15ADON) type and only 4 (3%) isolates were of the 3-acetyl deoxynivalenol (3ADON) type. The 10 VNTR markers revealed 124 distinct haplotypes and 76 polymorphic alleles across the whole population. The two subpopulations (FA-08 and FA-09) grouped based on the year of collection exhibited low genetic differentiation (F_{st} = 0.032) and high gene flow (N_m = 15.13). However, a significant genetic differentiation was found within the NIV-type isolates as revealed by the Structure software. The pairwise linkage disequilibrium tests did not support the hypothesis of random mating in the population because half (48.8%) of the locus pairs showed a linkage disequilibrium ($P > 0.05$). Our results suggest that FHB in this nursery was caused by a genetically homogenous and non-random mating population of *F. asiaticum* in

2008 and 2009, which consisted of all three trichothecene types with various levels of aggressiveness.

4.2. Introduction

Fusarium head blight (FHB), or scab, is a major disease of wheat and barley worldwide. The disease causes significant losses in yield and quality of the two crops (McMullen et al., 1997; Windels, 2000). In North America, more than $3 billion of losses were estimated after the FHB epidemics during the 1990s (McMullen et al., 1997). Frequent disease outbreaks also have been reported from Asia, Europe, and South America in recent years, posing a potential threat to the world's grain production and food supply (Goswami and Kistler, 2004). In China, the first FHB outbreak occurred in 1936 in Anhui province but epidemics were sporadic for almost a half century after that year (Xu and Chen, 1997). Since 1985, the disease has become more frequent and widespread in major wheat-growing areas of eastern and central China, especially along the lower and middle reaches of the Yangtze River Valley as well as the northeastern and central parts of China (Bai et al., 2003; Qu et al., 2008).

Several *Fusarium* spp. can cause FHB but the predominant pathogens vary with regions and may change over time. In many regions of the world, FHB is mainly caused by members of the *Fusarium graminearum* species complex, consisting of at least 14 phylogenic species (O’Donnell et al., 2000; O’Donnell et al., 2004; Starkey et al., 2007; Yli Mattila et al., 2009). In China, *F. graminearum* sensu lato was found in 94.5% of the samples recovered from 20 provinces along the Yangtze River Valley and 17 other *Fusarium* spp. were also isolated (Wang, 1997). Global studies of *Fusarium* spp. composition and population structure showed that *F. graminearum* sensu stricto, one member of the *F. graminearum* complex, is dominant in North America (O’Donnell et al., 2000; O’Donnell et al., 2004; Starkey et al., 2007), although other

species were also found in some other regions of the United States (Gale et al., 2011). In China, *F. asiaticum* and *F. graminearum* sensu stricto are the dominant species to initiate FHB in wheat (Gale et al., 2002; Qu et al., 2008; Zhang et al., 2007) and barley (Yang et al., 2008; Zhang et al., 2010a; Zhang et al., 2010b), with *F. asiaticum* being more common in southern China and *F. graminearum* sensu stricto being more frequent in the north (Qu et al., 2008). *F. asiaticum* and *F. graminearum* were also found to be the predominant etiological agents of FHB in Japan, although their distributions vary depending on the regions (Karugia et al., 2009a; Karugia et al., 2009b; Suga et al., 2008).

Population subdivision of *F. graminearum* correlates to geographical differences and trichothecene types worldwide (Gale et al., 2002; Gale et al., 2007; Ward et al., 2008; Yang et al., 2008). Previously, Karugia et al. (2009a) found 42.3% of isolates were of nivalenol (NIV) type and 57.7% were of 3-acetyl deoxynivalenol (3ADON) type but no 15-acetyl deoxynivalenol (15ADON)-type isolates were identified among 208 isolates collected from Zhejiang province in China. However, a recent study of 448 *F. asiaticum* isolates collected from barley at 18 sampling sites along the Yangtze River Valley identified all three trichothecene types (NIV = 109, 3ADON = 159, and 15ADON = 14) (Zhang et al., 2010). They also found significant genetic differentiation among populations originated from upper, middle, and lower regions of Yangtze River. Earlier studies on population genetics of *F. graminearum* indicated a relatively homogenous population in the United States (Zeller et al., 2003; Zeller et al., 2004), but more recent studies revealed genetically divergent populations in the Northern Great Plains of the United States and Canada (Gale et al., 2007; Puri and Zhong, 2010; Ward et al., 2008).

Although population genetics of FHB pathogens have been studied extensively on a global to regional scale (Gale et al., 2007; Qu et al., 2008; Ward et al., 2008; Yang et al., 2008;

Zeller et al., 2004; Zhang et al., 2010a; Zhang et al., 2010b), information on the species composition, trichothecene types, and genetic structure of the pathogen population from a smaller area or experimental unit is relatively limited. In Jianyang, Fujian province of China, where the weather is consistently warm and humid during and after wheat anthesis, FHB develops very well under natural infection conditions every year (Bai et al., 2003; Zhu and Fan, 1989). For this reason, a scab nursery was established in the early 1970s and has been used for screening breeding lines or other materials of wheat for FHB resistance ever since. However, the pathogen profile of the nursery is not known. Studies of the genetic structure of pathogen populations not only may provide important information regarding the epidemiology and evolutionary potential of the *F. graminearum* complex but also could lead to improved strategies for controlling this pathogen (Zeller et al., 2003). We attempted to answer several questions, including (i) how many species of the *F. graminearum* complex caused FHB in the nursery, (ii) what were the number and relative frequency of trichothecene types, and (iii) was there genetic differentiation in the pathogen population? Therefore, the major objectives of our study were to determine (i) species composition, (ii) trichothecene types, and (iii) genetic structure of FHB pathogens from this small (approximately 500 m^2) disease nursery under natural infection.

4.3. Materials and Methods

4.3.1. Fungal isolates

Symptomatic wheat heads were sampled from an FHB nursery (10 by 50 m^2 in size) located at Jianyang, Fujian province, China (Fig. 4.1), in two consecutive years (2008 and 2009). In 2008, wheat spikes showing FHB symptoms were randomly collected from representative plots of the nursery. In 2009, two to three scabby heads were sampled from each of 54 spots evenly distributed in the nursery, with a distance of 5 to 10 m between two neighboring spots.

Fungal isolation, growth, and storage were done as described by Puri and Zhong (2010). Each isolate was named after JY (Jianyang, the nursery location) followed by 08 or 09 (collection year) and serial numbers. For example, JY08-001 represents the isolate collected from Jianyang in 2008 with a serial number 001.

4.3.2. DNA extraction, Fusarium spp. identification, and trichothecene type determination

For DNA isolation, mycelia were harvested from a single spore-derived culture of each fungal isolate grown on cellophane layered over half-strength potato dextrose agar for 6 to 7 days. DNA was extracted using the Fast DNA Kit and Fast Prep instrument (MP Biomedical) according to the manufacturer's instructions, and then quantified using a Nano Drop (Nano Drop Technologies). To confirm the identity of the *Fusarium* spp., a portion of the histone *H3* gene was amplified using polymerase chain reaction (PCR) according to the method described by O'Donnell et al. (2000, 2004). The amplicons were purified by Exo SAP IT (USB Corporation) and sent to the University of Hawaii for sequencing. The isolates with identical DNA sequences were grouped together. The sequences (JQ435850 to JQ435858) from the representative isolates in each of the groups were used for BLASTN search against the National Center for Biotechnology Information GenBank database. The primers (6A3AF, 6A3AR, 6CNF, 6ANR, 6G3AF, 6G3AR, 3D15AF, and 3D15AR) developed and described by Suzuki et al. (2011) were used to differentiate *F. graminearum* and *F. asiaticum*. Trichothecene types were determined by PCR using the trichothecene-specific multiplex primers and conditions previously described (Starkey et al., 2007; Ward et al., 2002). The PCR products were separated on a 1% agarose gel and sized with reference to a 100-bp DNA ladder (NEB). The amplicon at 840, 610, and 243 bp corresponds to the NIV, 15ADON, or 3ADON type, respectively (Ward et al., 2002).

4.3.3. Analysis of variable number tandem repeats

Nine variable number tandem repeat (VNTR) markers (HK913, HK917, HK957, HK965, HK967, HK977, HK1043, HK1059, and HK1073) developed by Suga et al. (2004) and one additional VNTR marker HK1003, described by Gale et al. (2005) were used to analyze the population structure of the FHB pathogens. Information of the primer pairs used to detect the 10 VNTR markers is provided in Table 4.1. The forward primer of each primer pair was labeled with IRD700/800 at the 5′ end (Eurofins MWG Operon). PCR was performed as described by Suga et al. (2004), with some modifications. Each PCR reaction was run in a total volume of 10 µl containing 1× reaction buffer, 200 µM dNTP, 1 µM each primer (forward and reverse), 1 unit of *Taq* polymerase, and genomic DNA at 25 to 30ng/µl on a PTC-100 thermal cycler (MJ Research). Amplification profile consisted of initial denaturation at 94°C for 3 min; followed by 25 cycles of 94°C for 30 s, 52°C for 30 s, and 72°ºC for 45 s; and a final extension at 72°C for 5 min. The PCR products were detected on the Li-COR system (Li-COR) according to the method previously described by Puri and Zhong (2010). For each primer pair, amplicons with the same size were considered as the same allele and scored on size (bp). A binary system (i.e., 1 = present and 0 = absent) was also used as required for analysis.

4.3.4. Population genetic analysis

Initially, we used Structure 2.3.2 software based on a Bayesian-model-based clustering method (Falush et al., 2003; Pritchard et al., 2000) to assign each multilocus genotype to user-defined genetic *K* clusters. The analysis was performed with *K* = 1 to 5 for five iterations with 100,000 Markov Chain Monte Carlo reps after burn-in period of 10,000 (Pritchard et al., 2000). The exact number of clusters (*K*) was determined based on ln (P) curve for each subpopulation probability and verified with Structure Harvester, a web-based program to visualize Structure

output implementing the Evanno method (Earl and vonHoldt, 2011). To test whether there was a genetic differentiation between isolates collected from the 2 years, we also grouped isolates into populations according to the year of collection. Isolates collected from 2008 were grouped into one population (FA-08) and those from 2009 were grouped into another population (FA-09). The genotypic diversity (*GD*) and index of multilocus linkage disequilibrium (*rd)* were calculated using software MULTILOCUS 1.3 with the algorithm $G = (n/_{n-1})\{(1 - \Sigma p_i^2)\}$, where n is the number of individuals sampled and pi is the frequency of ith genotype (Agapow and Burt, 2001). The *GD* has a value of 0 if every individual is of the same genotype or 1 if every individual is different in the population. The index (*rd*) was used to test a hypothesis of random mating within a subpopulation after 1,000 permutations. The index (*rd*) gives a value of zero if there is no association among alleles on unlinked loci, as expected on null hypothesis of random mating. The significant linkage disequilibrium (LD) among VNTR loci and successive *P* values were generated using a Marcov chain length of 10^4 and dememorization of 10^4, as implemented in ARLEQUIN 3.1. The genetic differentiation and other population parameters were determined using different statistical tools and techniques. Analysis of molecular variance (AMOVA), population differentiation or fixation index (F_{st}), and number of migrants exchanged between two populations or gene flow (N_m) were calculated using ARLEQUIN 3.11 (Excoffier et al, 2005). The allele frequencies, percentage polymorphic loci, gene diversity (Nai, 1973, 1978), Nei's unbiased genetic distance (*D*) (Nei, 1978), and fixation index (G_{st}) for individual loci were calculated using POPGENE ver. 1.32 (Yeh et al., 1997). Molecular variance (AMOVA) was performed to test hierarchical partition of genetic variation between two populations and individuals within the whole population (Excoffier et al., 1992). The gene flow was calculated by

$N_m = (1 - F_{st})/2F_{st})$, where N is the effective population size and m is the migration rate per generation. The statistical significance of pairwise F_{st} was tested by 1,000 permutations.

4.3.5. Aggressiveness evaluation of representative F. asiaticum isolates

A greenhouse experiment was conducted to test the aggressiveness of 16 representative isolates of *F. asiaticum*, including 6 15ADON-type isolates, 4 3ADON-type isolates, and 6 NIV-type isolates, on the spring wheat 'Briggs' (susceptible to FHB). Seed were sown in plastic buckets (30 by 20 by 35 cm3) filled with Sunshine pot mix (Sun Gro Horticulture Canada Ltd.) with two rows (8 seeds/row) per bucket. For each isolate, three rows (replicates) of the plants were used for inoculation at the anthesis. Plant growth conditions, inoculum preparation, inoculation, and disease scoring were the same as previously described (Puri and Zhong, 2010), except that the inoculated plants were kept in a humidity chamber for 72 h instead of overhead misting.

4.4. Results

4.4.1. Fusarium spp. identification

In total, 160 *Fusarium* isolates (59 from 2008 and 101 from 2009) were first analyzed using the species-specific multiplex PCR (Suzuki et al., 2010). The result indicated that all isolates except isolate JY09-030 showed the amplicons unique to *F. asiaticum*. Further analysis of histone *H3* gene sequences indicated that JY09-030 (JQ435857) was *F. avenaceum* and the rest of isolates were identified as *F. asiaticum*. Compared with the histone *H3* gene sequence of the *F. asiaticum* strain (*F. graminearum* species complex number 9086), 92% of the *F. asiaticum* isolates analyzed in this study had no or one base difference and only 8% isolates had two to four base differences.

4.4.2 Trichothecene type determination

Trichothecene types were determined for the 159 *F. asiaticum* isolates using the multiplex PCR (Nei, 1972; O'Donnell et al., 2000). Among them, 126 (79%) of the isolates were of the NIV type while 29 (18%) and 4 (3%) isolates were of 15ADON and 3ADON types, respectively (Fig. 4.2C). In 2008, 45 (76%) isolates were of the NIV type and 14 (24%) isolates were of the 15ADON type but no 3ADON type was detected (Fig. 4.2A). In 2009, all three trichothecene types were identified, with 81 (81%) being of the NIV type, 15 (15%) being of the 15ADON type, and 4 (4%) being of the 3ADON type (Fig. 4.2B).

4.4.3. Population genetics analyses

In all, 144 *F. asiaticum* isolates were analyzed using the 10 VNTR markers and 76 polymorphic alleles were generated among them. The number of alleles generated by individual VNTR markers varied from 2 to 26 (Table 4.1). Gene diversity (*H*) also differed among the 10 VNTR markers, with HK1003 being the highest (0.94), followed by HK957 (0.82), HK1073 (0.78), and HK1059 (0.78). Marker HK967 and HK917 had the lowest *H* (0.02 and 0.13, respectively; Table 4.1). Using POPGENE, a fixation index (G_{st}) was generated for each VNTR marker. Marker HK917 had the highest G_{st} value (0.064) followed by marker HK965 (0.038). However, the three markers named HK917 (G_{st} = 0), HK1043 (G_{st} = 0.002), and HK967 (G_{st} = 0.005) had very low fixation indices (Table 4.1). Of the 144 isolates, 132 isolates remained after clone removal. All clonal isolates were found in 2008 and clones with the same haplotype belonged to the same trichothecene type. Using Structure, we assigned the 131 (excluding *F. avenaceum*) clone-corrected isolates into distinct clusters. Assignment of isolates into a specific genetic cluster was based on the average membership value *q* = 0.8 or above. Using this standard, 98 isolates, in total, were assigned into one of the three clusters (numbers 1, 2, and 3)

with the highest probability. However, 33 isolates did not fit to any of the clusters and, thus, were not assigned into any populations. Cluster 1 (*q* mean = 0.941, standard deviation [SD] = 0.036) had a total 45 isolates consisting of 44 NIV-type isolates and 1 15ADON-type isolate (*q* = 0.978), which was named as population NIV-1. Cluster 2 (*q* mean = 0.908, SD = 0.053) had 18 isolates comprising 16 NIV-type and 2 15ADON-type isolates and named population NIV-2. Because cluster 3 (*q* mean = 0.937, SD = 0.042) consisted of 18 NIV-type, 15 15ADON-type, and 2 3ADON-type isolates, we further divided them into two subpopulations, one named NIV-3, containing all NIV isolates, and the other named DON, consisting of the 3ADON-type and 15ADON-type isolates. AMOVA among the four populations (NIV-1, NIV-2, NIV-3, and DON) indicated that 17% of genetic variation was due to differences among populations and 83% of variation was from individuals within population. The three populations NIV-1, NIV-2, and DON had a similar level of gene diversity (0.41 to 0.46) whereas NIV-3 had relatively high gene diversity (0.54) across all loci. Population-wise comparison showed a significant differentiation among them, except between NIV-3 and DON (Table 4.2). A high gene flow (25.9) and a very low population differentiation (0.019 and $P = 0.139$) was found between NIV-3 and DON.

The genetic distance was low ($D = 0.05$) and genetic identity was high ($I = 0.95$) between the two populations (FA-08 and FA- 09) grouped according to the years of collection. The hierarchical AMOVA showed that genetic variation between the populations accounted for only 3.23% while most of the genetic variation (96.77%) was from individuals within population. Genetic differentiation was low ($F_{st} = 0.032$) and gene flow was high ($N_m = 15.13$) between FA-08 and FA-09. The r_d index was low in both populations (i.e., 0.073 for FA-08 and 0.050 for FA-09) but was significantly different from zero at $P < 0.001$. The index of association, another measure of linkage disequilibrium, was 0.575 and 0.424 for FA-08 and FA-09, respectively, and

was also significantly different from zero (*P* < 0.001). Analysis of linkage disequilibrium between loci pairs of the 10 VNTR loci showed that almost half (48.8%) of the 45 possible locus-pair combinations were in linkage disequilibrium (*P* > 0.05; Table 4.3), suggesting that out-crossing was not frequent in the population.

4.4.4. Aggressiveness evaluation

The average disease severity (DS) caused by individual isolates on Briggs ranged from 12.4% (JY08- 013) to 96.2% (JY09-45E). Analysis of variance indicated significant differences (*P* > 0.0001) in aggressiveness among the isolates based on the DS caused on Briggs. Isolate JY09-45E (a 3ADON type isolate) and JY08-043 (a 15ADON-type isolate) induced the highest DS, which was not significantly different from those induced by isolates JY08-009 and JY09-045 (Fig. 4.3). However, all NIV-type isolates evaluated were significantly less aggressive compared with the 3ADON and 15ADON isolates (Fig. 4.3).

4.5. Discussion

Our results indicated that *F. asiaticum* is the primary etiological agent prevalent in the FHB disease nursery. This is consistent with previous studies, which showed that *F. asiaticum* is the predominant and primary pathogen of FHB for all major cereals (wheat, barley, and rice) in warmer regions of Asian countries, including China, Korea, and Japan (Lee et al., 2009; Qu et al., 2008; Suga et al., 2008). The majority of Fusarium isolates collected from FHB-infected wheat and barley samples in eastern and central China along the Yangtze River were identified as *F. asiaticum* in several independent studies (Gale et al., 2002; Karugia et al., 2009a; Karugia et al., 2009b; Zhang et al., 2010a; Zhang et al., 2010b). Qu et al. (2008) reported that 79% of the 437 isolates collected from warmer (>15°C) regions of China were *F. asiaticum*. Karugia et al. (2010b) identified 179 *F. asiaticum* isolates among 183 isolates sampled in a small experimental

field in Japan. Lee et al. (2009) found that all of the 249 isolates collected from rice fields in southern provinces of Korea were *F. asiaticum* whereas isolates from eastern provinces of Korea were either *F. asiaticum* or *F. graminearum* sensu stricto. They hypothesize that *F. asiaticum* has a host preference and specificity to rice, and perithecium production typically favors rice straws under warmer conditions. The Jianyang nursery is located in the warmer region, and the field was planted with rice before wheat was grown for FHB evaluation (J. Huang, *personal communication*). Rice was the major cereal crop in Jianyang and no wheat, barley, and corn were grown around the region. Thus, initial infections might have come from inoculum sources originated from rice straws, as found in Korea (Lee et al., 2009). However, this hypothesis needs further investigation by comparing isolates originated from the nursery with those from the rice fields surrounding the nursery.

We found that a majority (79%) of the *F. asiaticum* isolates were of the NIV type although isolates of the 15ADON type (18%) and the 3ADON type (3%) were also identified. To our knowledge, this is the first time that all three trichothecene types were found in the same place in southeastern China. Karugia et al. (2009a) analyzed 208 isolates of *F. asiaticum* collected during 2000 by Gale et al. (2002) from four locations in Zhejiang province (approximately 500 km north of Jianyang) and showed that NIV and 3ADON isolates accounted for 42.3 and 57.7%, respectively, but no 15 ADON isolates were detected. Zhang et al. (2007) analyzed a 1999 collection of 299 isolates from 12 provinces along the middle and lower reaches of Yangtze River and found that 231 (77%) of the isolates were *F. asiaticum*. Of the 231 *F. asiaticum* isolates, 67% were of the 3ADON type and 23% were the NIV type but only 10% were 15ADON isolates (Zhang et al., 2007). The higher frequency of 15ADON isolates found in the present study may reflect a geographical difference between FHB epidemic regions in China

because Jianyang is the furthest place in southeast China where *F. asiaticum* isolates have been sampled and analyzed.

Our population genetics study suggested that the two *F. asiaticum* populations (FA-08 and FA-09) grouped based on the year of collection were genetically similar. This conclusion is supported by the high genetic identity (95%), high gene flow (N_m = 15.13), and very low genetic differentiation (F_{st} = 0.032) between FA-08 and FA-09. Gale et al. (2002) found a high gene flow (N_m = 7 to 30) and low F_{st} (0.01 to 0.07) among *F. asiaticum* populations collected from four fields located in Zhejiang province, China. Using the VNTR markers, Karugia et al. (2010b) analyzed *F. asiaticum* isolates collected in two successive years from a small wheat field (500 m^2) in Japan and failed to reveal distinct genetic differentiation between the populations sampled during the 2 years. Zeller et al. (2003) also observed a large number of migrants (approximately 70) and high genetic identity (99%) among *F. graminearum* isolates sampled in two small fields located in North Dakota and Kansas, respectively (Zeller et al., 2003). These results suggest that a pathogen population from a small, isolated disease nursery may keep stable for some period of time. However, population subdivisions based on trichothecene type, geographic difference, and temperature gradient within and outside China from a larger geographic area have been reported by several studies (Gale et al., 2007; Karugia et al., 2009a; Starkey et al., 2007; Ward et al., 2002; Yang et al., 2008; Zhang et al., 2010a; Zhang et al., 2007; Zhang et al., 2010b). Thus, information from a local population may not be applicable to a larger population across China or vice versa. Population studies on field, regional, and global scales are still necessary to have a better understanding of genetic variation and population structure of the pathogen.

Although little genetic differentiation was found between the two subpopulations (FA-08 and FA-09) grouped based on the years of collection, three distinct subpopulations existed (NIV-

1, NIV-2, and NIV-3) within the NIV-type isolates, as revealed by the membership assignment using the Structure software and further confirmed by the other genetic population analyses. Interestingly, the non-NIV-type isolates were clustered with one of the NIV-type subpopulations. This result suggests that a significant genetic differentiation occurred in the pathogen population of the disease nursery. Population subdivision has been reported for wheat and barley isolates of *F. asiaticum* collected along the Yangtze River in southern China (Zhang et al., 2010a) and for *F. graminearum* isolates sampled from the United States and Canada (Gale et al., 2011; Gale et al., 2007; Puri et al., 2010; Ward et al., 2008). The genetic differentiation found between the NIV-type populations (NIV-1, NIV- 2, and NIV-3) in our study likely suggests lack of enough sexual recombination between the NIV isolates (see below).

Out-crossing of *F. graminearum* was demonstrated in the laboratory by using nitrate non-utilizing mutants (Bowden and Leslie, 1999). Recently, Chen and Zhou (2009) reported out-crossing frequency of 5.7 to 20.9% in *F. graminearum* in field conditions in China. However, our LD analysis showed that almost half (48.8%) of the 45 possible locus pairs were in linkage disequilibrium. This result suggests that out-crossing among isolates of the *F. asiaticum* population in the Jianyang nursery might have been very rare. Previously, Gale et al. (2002) found 36% restriction fragment length polymorphism loci in linkage disequilibrium among the *F. asiaticum* isolates collected from Zhejiang province. Karagua et al. (2009b) also showed 10 to 12% of the VNTR loci in linkage disequilibrium condition in Japanese isolates. Thus, out-crossing might not be the major evolutional force for maintaining high genotype diversity of the population in the Jianyang nursery.

Our greenhouse inoculation experiments indicated that the DON-type isolates (15ADON- and 3ADON-type isolates) were similar in aggressiveness whereas they were more aggressive

than the NIV-type isolates on Briggs. This result agrees with the previous studies (Gale et al., 2011; Maier et al., 2006), which indicated that DON-type isolates are generally more aggressive on wheat compared with the NIV-type isolates. The aggressiveness difference between DON and NIV types may be due to different amounts or types of mycotoxins produced by the FHB pathogens (Alexander et al., 2011). DON-type isolates generally accumulated a higher level of trichothecene toxins than the NIV type isolates (Gale et al., 2011; Puri and Zhong et al. *unpublished*). Previous studies indicated that both DON and NIV are virulence factors, either of which is required for the *Fusarium* pathogen to cause disease spread in wheat spikes (Bai et al., 2001; Desjardins et al., 1996; Maier et al., 2006). However, the mechanism of how NIV is involved in pathogen infection and FHB development remains to be investigated.

Among trichothecenes, DON has attracted more attention than NIV (Pasquali et al., 2009), which may be due to the fact that DON contaminations were more common in cereal grain samples collected in North America, as is exemplified in the global analysis data of mycotoxin contamination reported by Placinta et al. (1999). However, in Asian countries, including China, Japan, Korea, and Vietnam, a high incidence and generally high concentrations of NIV have been reported earlier (Tanaka et al., 1988). In a more recent analysis of mycotoxin occurrence and concentrations in food and raw food materials from 12 European countries (Schothorst et al., 2004), 16% of the samples were also found to be positive for NIV. Although several studies suggested that NIV has higher cytotoxicity than DON (Eriksen et al., 2004; Minervini et al., 2004) in the experiments with animal or human cells and could be linked to a high incidence of cancer of the esophagus and gastric cardia in China (Hsia et al., 2004), there is limited information on the specific toxic effects of long-term NIV exposure in humans or the comparative toxicity of NIV and DON. Considering the fact that NIV-type *Fusarium* pathogens

are common in Asian countries, Europe, and the southern United States, and NIV-contaminated grains or food products were frequently detected in the FHB-affected samples; more attention should be paid to NIV and its impacts on cereal crop production as well as animal and human consumptions.

In summary, the FHB pathogen population from the small disease nursery was primarily composed of *F. asiaticum*, with the NIV type being predominant. However, isolates of *F. asiaticum* with 3ADON and 15ADON types were also present. Population subdivision based on year of collection was not significant, although a genetic differentiation was observed within the NIV-type isolates collected in the 2 years. This study provides important information about the population structure of *F. graminearum* complex in the nursery that has been used for FHB resistance evaluation for more than 25 years. The presence of all trichothecene types, which vary in aggressiveness and mycotoxin production, ensures the value of this particular nursery in FHB resistance evaluation and in breeding wheat cultivars with broad-spectrum FHB resistance.

4.6. Acknowledgments

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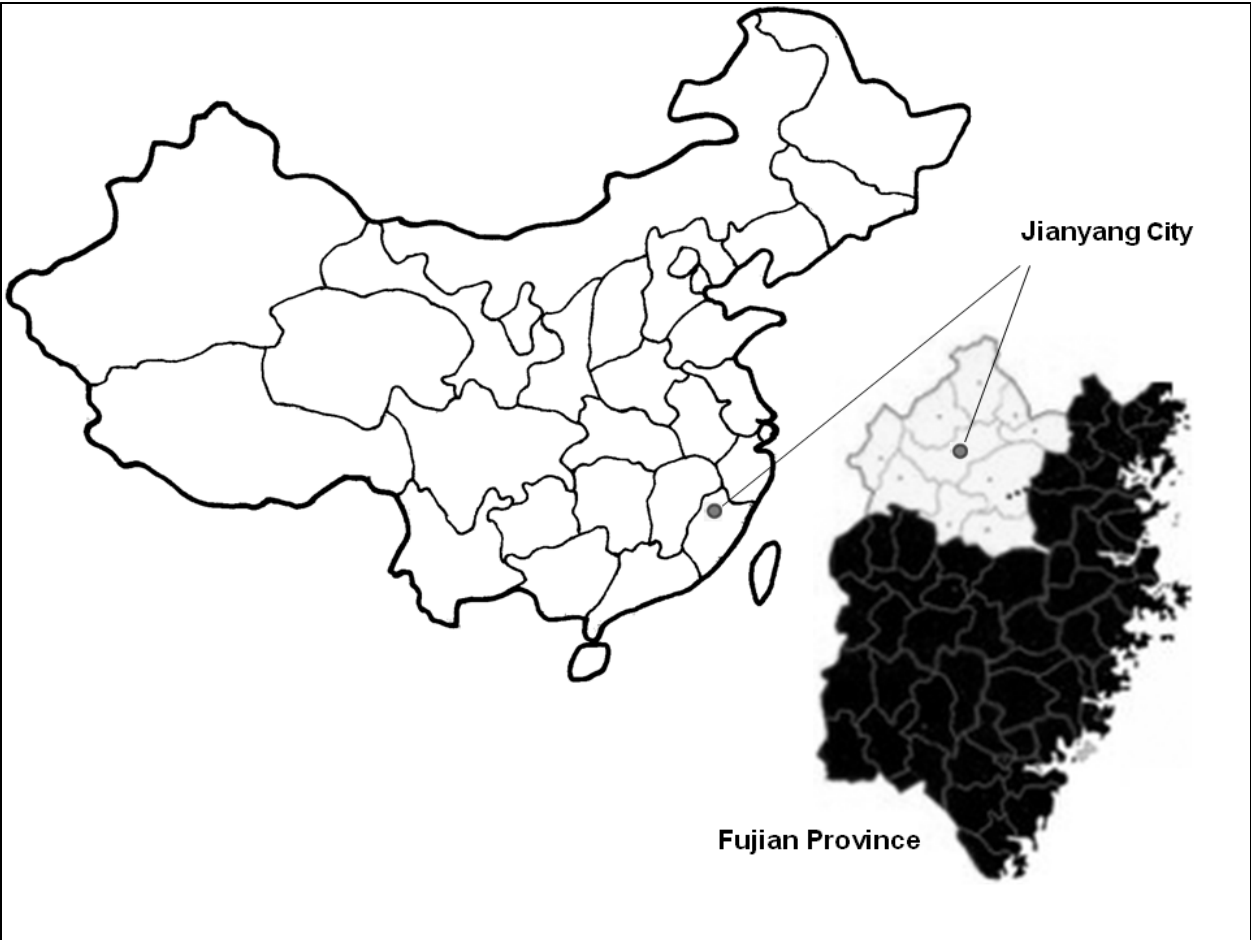
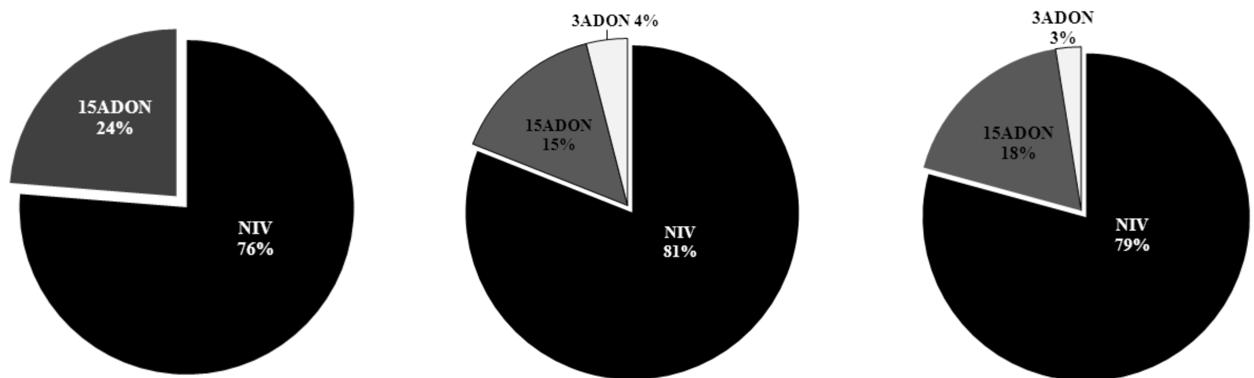


Figure 4.1. Map of Fujian province (low right), which is located in the southeast region of China (up). The naturally infected Fusarium head blight (FHB) disease nursery is at the city of Jianyang, north of Fujian province. Symptomatic wheat heads were randomly collected from a field ($10 \times 50\ m^2$) in the nursery for two consecutive years (2008 and 2009).



A. 2008 collection (59 isolates) B. 2009 collection (100 isolates) C. total collection (159 isolates)

Figure 4.2. Frequency distribution of chemotypes among *Fusarium asiaticum* isolates collected in 2008 (A), 2009 (B) and the two years combined (C).

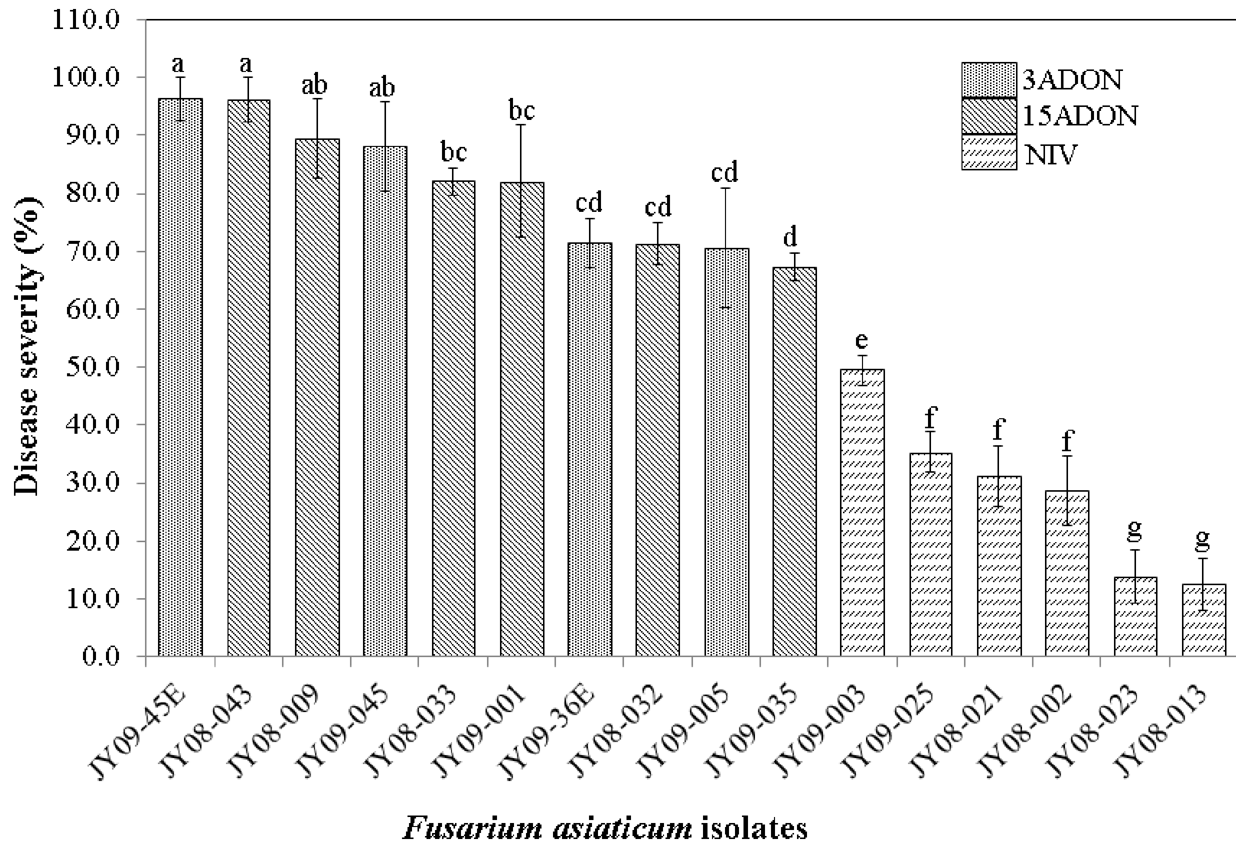


Figure 4.3. Average disease severity (DS) caused by each of the 16 representative isolates of *Fusarium asiaticum*: (six 15-acetyl deoxynivalenol [15ADON] isolates, four 3-acetyl deoxynivalenol [3ADON] isolates and six nivalenol [NIV] isolates) on the susceptible spring wheat cultivar Briggs in greenhouse. Single floret inoculation was used and infected spikelets in each of the inoculated spikes were counted at 21 days after inoculation. In general, the 3ADON-type and 15ADON-type isolates caused significantly higher DS than the NIV isolates ($P<0.0001$). Vertical bar represents standard error. Isolates followed by the same letters (indicated at the top of bar) were not significantly different at $P <0.0001$.

Table 4.1. Number of alleles, gene diversity and fixation index (G_{st}) of 10 variable number tandem repeat (VNTR) markers used to analyze the 144 *Fusarium asiaticum* isolates collected from the disease nursery at Jianyang, China.

VNTR[a]	**Location**[b]	**No of Alleles**	**Gene Diversity**[c]			*Gst*[d]
			2008	**2009**	**Overall**	
HK1043	Ch1/Ct1.52/41,839	3	0.38(0.39)	0.44(0.44)	0.42(0.42)	0.002
HK957	Ch1/Ct1.91/16,055	7	0.81(0.84)	0.80(0.81)	0.81(0.82)	0.012
HK967	Ch2/Ct1.154/53,868	2	0.00(0.00)	0.02(0.02)	0.02(0.02)	0.005
HK1003	Ch1/Ct437/6,986	26	0.90(0.93)	0.94(0.95)	0.94(0.95)	0.017
HK1073	Ch4/Ct1.398/70,812	12	0.75(0.77)	0.78(0.79)	0.78(0.79)	0.022
HK1059	Ch3/Ct1.196/164,228	6	0.74(0.76)	0.77(0.78)	0.78(0.78)	0.024
HK977	Ch3/Ct1.208/47,696	4	0.10(0.1)	0.29(0.29)	0.24(0.24)	0.020
HK917	Ch1/Ct1.82/2,471	3	0.15(0.15)	0.12(0.12)	0.13(0.13)	0.000
HK913	Ch1/Ct1.73/664	6	0.74(0.76)	0.63(0.63)	0.70(0.70)	0.064
HK965	Ch2/Ct1.154/51,671	7	0.64(0.66)	0.75(0.75)	0.74(0.75)	0.038
	Mean	7.6	0.52(0.53)	0.55(0.55)	0.56(0.56)	0.026

[a]HK1003 was described by Gale et al. (2005) and the others were reported by Suga et al. (2004).
[b]Marker location in the genome was indicated by Chromosome (Ch) No./Contig (Ct) No./Position in contig.
[c]Nei`s gene diversity (*H*) (Nei, 1973) within populations was calculated using POPGENE 1.32 (Yeh et al., 1997). Values in parenthesis were calculated by ARLEQUIN 3.1 (Excoffier et al., 2005).
[d]G_{st} fixation index (Nei, 1973) for individual loci were calculated for the 132 clone corrected isolates using POPGENE 1.32 (Yeh et al., 1997).

Table 4.2. Nei's genetic distance D (above diagonal) and *P* value from the exact tests for genetic differentiation *Φ*PT (below diagonal) between four populations of *Fusarium asiaticum* collected from the Fusarium head blight disease nursery in China.

Populations[a]	**NIV-1**	**NIV-2**	**NIV-3**	**DON**
NIV-1	---	0.240[b]	0.299	0.419
NIV-2	<0.001[c]	---	0.346	0.399
NIV-3	<0.001	<0.001	---	0.076
DON	<0.001	<0.001	0.139	---

[a]The four populations, NIV-1, NIV-2, NIV-3, and DON, are genetically defined and subdivided according to the member assignment by STRUCTURE and by taking into account the trichothecene types.

[b]Nei`s unbiased genetic distance (D) (Nei, 1972) was calculated using POPGENE (Yeh et al., 1997).

[c]The population differentiation ΦPT (analogous to *Fst*) was calculated as proportion of variance among and within population relative to the total variance (Excoffier et al., 1992) using GENALEX 6.4 (Peakall and Smouse, 2006). *Φ*PT = AP / (WP + AP) = AP/TOT, where AP = estimated variance among population, WP = estimated variance within population, and TOT = total variance. The probability of obtaining equal or lower Φvalue was determined by 1,000 random permutations.

Table 4.3. Significant linkage disequilibrium ($P < 0.05$) between 10 variable numbers of tandem repeat (VNTR) markers in 132 clone-corrected isolates of *Fusarium asiaticum*.

VNTR marker Pair[a]	P value	VNTR marker Pair[a]	P value
HK1043(1),HK957(1)	0.004	HK1003(1),HK1059(3)	0.000
HK1043(1),HK967(2)	0.000	HK1003(1),HK977(3)	0.037
HK1043(1),HK1073(4)	0.004	HK1003(1),HK913(1)	0.000
HK1043(1),HK1059(3)	0.025	HK1073(4),HK1059(3)	0.000
HK1043(1),HK913(1)	0.0003	HK1073(4),HK917(1)	0.004
HK957(1),HK1003(1)	0.007	HK1073(4),HK913(1)	0.004
HK957(1),HK1073(4)	0.009	HK1073(4),HK965(2)	0.036
HK957(1),HK1059(3)	0.024	HK1059(3),HK913(1)	0.010
HK957(1),HK913(1)	0.003	HK1059(3),HK965(2)	0.032
HK967(2),HK913(1)	0.013	HK977(3),HK917(1)	0.000
HK1003(1),HK1073(4)	0.000	HK913(1),HK965(2)	0.048

[a]Pair-wise estimate of linkage disequilibrium, based on Fisher's exact probability test by Slatkin (1994), was calculated for 45 possible marker pairs among 10 VNTR loci using Marcov chain length of 10^4 with 10^4 dememorization implemented in ARLEQUIN 3.1 (2005). Only significant loci combinations (P<0.05) were shown in the table. Number in parenthesis is the chromosome on which a particular VNTR locus resides.

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CHAPTER 5. VALIDATION AND HAPLOTYPING OF FUSARIUM HEAD BLIGHT RESISTANCE SOURCES IN A DIVERSE SPRING WHEAT GERMPLASM

5.1. Abstract

Fusarium head blight (FHB), primarily caused by *Fusarium graminearum* Schw., is the most destructive disease of wheat and barley in North America. Use of host resistance is one of the most efficient and economic strategies for disease management. However, sources of FHB resistance used in breeding programs are very limited. In this study, we re-evaluated and haplotyped 71 PI accessions from the National Small Grains Collections (NSGC), which were previously reported to show various levels of resistance to FHB. Seventeen DNA markers associated with FHB resistance QTLs on the chromosomes 2B (*Triticum carthlicum* 'Blackbird'), 3A (*T. aestivium* 'Frontana' and *T. dicoccoides* 'Israel A'), 3B (*T. aestivium* 'Sumai 3' and 'Wanshuibai'), 5A ('Sumai 3' and 'Frontana'), 6B ('Blackbird' and 'Wangshuibai'), and 7A (*T. dicoccoides*) were used in the haplotype analysis. Forty-nine of the PI accessions were different at the marker loci on 3B and 5A from Sumai 3, the most frequently used FHB resistance source in spring wheat breeding programs. Twenty two accessions had a haplotype different from all of the known resistance sources used in the study, suggesting that they may carry novel loci for FHB resistance. The novel FHB resistance sources could be utilized to develop wheat varieties with enhanced FHB resistance.

5.2. Introduction

Fusarium head blight (FHB), caused by *Fusarium graminearum* Schwabe (teleomorph: *Gibberella zeae*), is an important disease of wheat and barley in the United States and many other regions of the world (Parry et al., 1995; McMullen et al., 1997; Windels, 2000). Disease epidemics often occur when the flowering stage of the crops coincides with warm and humid

weather conditions (Parry et al., 1995; McMullen et al., 1997). During 1990s, economic losses of around $3 billion were reported in the United States (McMullen et al., 1997; Windels, 2000) and a similar loss occurred in eastern Canada due to the impacts of FHB on the cereal crops (Gilbert and Tecauz, 2000). The disease not only causes direct yield losses due to infected grains being shriveled, discolored, and reduced in weight (Snijders et al., 1990, Bai and Shaner, 2004), but also results in down grading the grain products due to mycotoxin contamination, which poses a severe health hazard to human and animals (Desjardins et al., 1993; Bai et al., 2001).

An integrated approach of combining host resistance, cultural practices and fungicide application is the most efficient and effective way for FHB management (Bai and Scanner, 2004; Wegulo et al., 2011; McMullen et al., 2013). Use of FHB resistant cultivars is a very important component in reducing the impacts of this disease on cereal production (Bai and Shaner, 1996). Several types of FHB resistance have been proposed, including Type I (resistance to initial infection), Type II (resistance to spread) and Type III (resistance to DON accumulation) (Schroder and Christensen 1963; Mesterhazy, 1995; Bai and Shaner, 2004). Among them, type II resistance is the most stable and widely used in wheat breeding programs in the world (Bai and Shaner, 2004).

Large efforts have been devoted in screening of various germplasm and integrating the identified resistance sources into adapted cultivars (Liu and Anderson, 2003; Somers et al., 2003; Zhang et al., 2008; Zwart et al., 2008). Key spring wheat genotypes with good type II resistance were identified, including Sumai 3, Wangshuibai, several Ning selections from China; Nobeokabozu Komugi, Saikai 165 etc. from Japan; Frontana and Encruzilhada from Brazil and some CIMMYT genotypes (Wang and Miller, 1987; Snijders, 1990; Anderson et al., 2001; Ban, 2000; Steiner et al., 2004; Zhou et al., 2004). In addition, a few winter wheat cultivars (Renan,

Arina, and Praag-8) were identified as a good source of resistance to FHB in the European gene pool (Snijders, 1990; Ruckenbauer et al., 2001; Gervais et al., 2003). Meanwhile, major and minor QTLs for FHB resistance have been identified across many genetic backgrounds (see review by Buerstmayr et al., 2009). These QTLs were mapped on all wheat chromosomes except 7D (Buerstmayr et al., 2009). The Asian spring wheat cultivar Sumai 3 has consistently showed a high level of type II resistance in a wide range of environments and genetic backgrounds (Bai et al., 1999; Anderson et al., 2001, Bai and Shanner, 2004; Buerstmayr et al., 2002, 2003; Yang et al., 2003; Zhou et al., 2004). It has major QTLs for FHB resistance on chromosome 3BS, 5AS, 6BS and minor QTLs for FHB resistance on 6AS and 2AS (Waldron et al., 1999; Anderson et al., 2001; Buerstmayr et al., 2002; Zhou et al., 2002; Shen et al. 2003; Yang et al., 2003). Similarly, major QTLs governing FHB resistance were mapped on chromosome 3BS of Wangshuibai (Zhou et al., 2004), chromosomes 2DL and 4BS of Wuhan-1 (Somers et al., 2003), chromosome 5DL of Chokwang (Yang et al., 2005), and chromosome 3AL of Frontana (Steiner et al., 2004). The European winter wheat cultivar Arina has major QTLs on chromosome 4AL, 5BL and 6DL (Paillard et al., 2004; Draeger et al., 2007; Semagn et al., 2007), while another cultivar Renan had QTLs on chromosomes 2BS and 5AL (Gervais et al., 2003).

Although the number of QTLs identified for FHB resistance is large, the Sumai 3 resistance gene known as *Fhb1* (*Qfhs.ndsu-3BS*) (Waldron et al., 1999; Cuthbert et al., 2006; Liu et al., 2006) is the most commonly used source for FHB resistance in most of wheat breeding programs worldwide. However, continuous reliance on a single source of resistance might create a selection pressure on the pathogen population and eventually make the existing resistance ineffective (Gervais et al., 2003). Thus, identification of new sources of resistance and integration of them into adapted cultivars are vital for combating FHB. Recently, Zhang et al.

(2008) screened 1035 wheat accessions from the USDA ARS, National Small Grain Collection (NSGC) Aberdeen, Idaho, USA, and identified 73 spring wheat PI accessions showing some resistance to FHB under field conditions. However, the novelty, type and source of FHB resistance in most of these accessions were not well understood. Therefore, the major objectives of this study were to i) haplotype the PI accessions (71) using SSR markers linked to QTLs for FHB resistance in known sources, and ii) validate the levels of FHB resistance of these accessions under field and greenhouse conditions in North Dakota.

5.3. Materials and Methods

5.3.1. Plant materials

A total of 71 spring wheat accessions (Table 5.1) previously reported to possess various levels of FHB resistance (Zhang et al., 2008) were selected and used in the study. Seeds were obtained from the USDA ARS, National Small Grain Collection (NSGC), Idaho. Additionalls six spring wheat genotypes (ND 2710, Sumai 3, PI 277012, Alsen, Steele ND and Choteau) were included as checks (Table 5.1). ND 2710 is an experimental breeding line with a high level of FHB registance derived from Sumai 3 (Frohberg et al., 2004). PI 277012 is from Spain and possesses a high level of FHB resistance (Chu et al., 2011). Alsen has a moderate resistance to FHB, which was derived from Sumai 3 (Frohberg et al., 2006). Steele-ND has a moderate resistance to FHB, which is derived from another source different from Sumai 3 (Mergoum et al., 2005). Choteau (PI 633397) is susceptible to FHB (Lanning et al., 2004) and has been routinely used as one of the most susceptible checks in FHB studies.

5.3.2. Haplotype analysis

Genomic DNA was extracted from leaves of one-week old seedling plants of each accession using the method described by Riede and Anderson (1996) with some modifications.

DNA was quantified using NanoDrop (Wilmington, DE) and diluted to 50ng/µl for further use. Seventeen SSR markers linked to nine different FHB resistance QTLs were used for haplotyping. These markers are localized on chromosomes 2B (*Triticum carthlicum* 'Blackbird' e.g. *wmc102*, *wmc441*, *gwm55*, *gwm388* (Somers et al., 2006), 3A {*T. aestivium* 'Frontana' e.g. *dupw227* (Eujayl et al., 2002), and *T. dicoccoides* 'Israel A' e.g. *gwm2* (Otto et al., 2002)}, 3B {*T. aestivium* 'Sumai 3' e.g. *umn8*, *gwm533*, *barc147* (Zhou et al., 2002)}, 5A {'Sumai 3' e.g. *barc180*, *barc186* (Somers et al., 2004) and 'Frontana' e.g. *gwm129* (Steiner et al., 2004)}, 6B {*Triticum carthlicum* 'Blackbird' e.g. *gwm193* (Somers et al., 2006) and 'Wangshuibai' e.g. *wmc397*, *gwm644*)}, and 7A- *T. dicoccoides* e.g. *wmc488*, *barc121* (Song et al., 2005). Haplotyping was conducted in the USDA-ARS Biosciences Research Lab, Fargo, ND, USA. The polymerase chain reaction (PCR) conditions were optimized for M13-tailing (e.g. 5'-CACGACGTTGTAAAACGAC+ microsatellite sequence-3') and fluorescent capillary electrophoresis was run on an Applied Biosystems 3130xl Genetic Analyzer. Individual PCR amplification reaction was performed in 10µl of total volume containing 1× PCR buffer (NEB), 0.125 mM each dNTPs, 3 pmols M13 fluorescently labeled FAM or VIC or NED or PET primer (Applied Biosystems), 0.4 pmols forward and reverse primer, 0.05U T*aq* DNA polymerase (NEB) and 50 ng template DNA. GeneAmp PCR 9700 system (Applied Biosystems) was used for thermal cycling with the following amplification conditions: 94°C for 2 min, then 40 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, with a final extension of 5 min at 72°C. PCR reactions for each dye were pooled and 3ul of each pooled aliquot were mixed with 7 µl of formamide plus size standards, denatured at 95°C for 5 min, ice chilled and run on the ABI system. Data analysis was performed on GeneMaper v3.7. The genetic relationships among PI accessions were analyzed using the NTSYSpc version 2.1 (Applied Biostatistics Inc., New York,

NY). The SHAN module was performed for cluster analysis using the unweighted pair group arithmetic mean (UPGMA). The polymorphism information content (PIC) of each molecular marker to identify their ability to detect polymorphism within a population was calculated using in build algorithm in SAS (v9.23; SAS Institute, Cary, NC).

5.3.3. FHB evaluation and DON testing in field experiments

Field experiments were conducted to evaluate FHB resistance of the PI accessions in 2009 and 2010 at two locations (Fargo and Prosper) in North Dakota. The numbers of wheat lines evaluated varied with different years and locations due to poor seed germination or lack of sufficient numbers of heads for disease scoring. In 2009, 73 and 76 lines (including checks) were evaluated at Fargo and Prosper, respectively. In 2010, 75 lines (including checks) were tested at both Fargo and Prosper locations. Individual lines were planted in hill plots on a randomized complete block design and replicated three times. Only two replications were planted in 2010 at the Prosper location. Four individual hills (12-15 seeds/hill) were planted 30 cm apart in each row spaced one meter.

Inoculum was prepared using mixture of 20 *F. graminearum* isolates (ten 3ADON type isolates and ten 15ADON type isolates). The fungal isolates were collected from ND in 2008 or in 2009 and characterized for chemotype and aggressiveness (Puri and Zhong, 2011; and unpublished). To produce spores (macroconidia), isolates were grown on Mung Bean Agar (MBA) media (Evans et al., 2000) for 3-4 days with a 12h alternate dark and 12h light cycles. Spores were recovered, counted and adjusted to 1×10^5 spores/ml. Spores prepared from each of the 20 isolates were mixed equally before inoculation. The infested corn kernels used as inoculum for field nurseries were prepared as described by Zhang et al. (2008). Briefly, clean yellow dent corn kernels were soaked in water for 24h, drained and transferred to steel trays

(46×30×6 cm^3). The corn trays were covered with tin foil and autoclaved for 2.5h, cooled overnight, and inoculated with 10-15ml spore solution (10^5 macro-conidia/ml) in the next morning. Inoculated corn kernels were incubated at room temperature until complete colonization by the fungus and then stored at 4ºC or frozen till needed for nursery inoculations.

To assure uniform disease pressure and proper ascospores production, infested corn kernels were applied to the nurseries at a rate of approximately 0.20kg/m^2 starting at the jointing stage (Feekes growth stage 5) of wheat, and repeated every two weeks until all wheat accessions completed anthesis (Feekes growth stage 10.5). During the inoculation period, plots were sprinkled with overhead misting for 30s to 2 min in every 2h interval depending on weather conditions. During flowering time, overhead misting was run for 10 seconds per hour during nights to ensure high humidity for uniform disease development. Fifteen to twenty heads/hill were rated at 21 days after flowering based on the scale developed by Stack and McMullen (1995). For DON testing, 10-15 randomly selected spikes from each accession were harvested and threshed manually. In each year and location, grains from all replications of the same accession were combined, ground to powder using a coffee grinder and sent to the Veterinary Diagnostic Laboratory, North Dakota State University, ND, for mycotoxin (DON, 15ADON and 3ADON) analysis.

5.3.4. FHB evaluation in greenhouse

The same sets of wheat accessions were evaluated in greenhouse to measure type II resistance (disease spread after single-floret inoculation) in 2009 and 2010. They were planted in plastic buckets (30×20×35cm^3) filled with Sunshine pot mix (Sun Gro Horticulture Canada Ltd). Seeds were sown in rows with eight to ten seeds/row and two rows per bucket. Plants were grown in greenhouse at 23±2°C with 16h supplemental lights, fertilized with Multicote 4

(N:P:K::14:14:16 plus minor nutrient) (Heifa Biochemical, Israel), and extra liquid fertilizers (20:20:20, J.R. Peters, Inc. Allentown, PA) once a week. Plants were arranged in a completely randomized block design (RCBD) with three replications. Four isolates mixture of *F. graminearum* {two 3ADON type (Fg08-001, Fg08-008) and two 15ADON type isolates (Fg08-013, Fg08-018)} were used for inoculation. Spores from each isolates were prepared as described above, adjusted to 1×10^5 spores/ml, and mixed equally before inoculation. At anthesis, the central spikelets of ten to twelve heads from each replicate were injected with 1000 macro-conidia. The plants were kept in a humidity chamber for 48 to 72 hours and then returned to the greenhouse with the same conditions as before inoculation. Disease rating was done at 21 days of inoculation using the scale described by Stack and McMullen (1995).

5.3.5. Statistical analysis of phenotypic data

Disease severity (DS) data were tested for homogeneity of variance using Brown and Forsythe`s test of homogeneity (hovtest=BF) using GLM procedure on SAS (v9.3; SAS Institute, Cary, NC). Due to lack of homogeneity among the field experiments, analysis of variance (ANOVA) for DS was calculated separately for individual years and locations using the GLM procedure on SAS. Mean separation was performed by least significance difference (LSD) test among the accessions. Logarithmic transformation (log+1) was done for total DON for further statistical analysis. Pearson correlation coefficient between disease severity and DON of each experiment and across all experiments were calculated using means across replications. Greenhouse data were combined after homogeneity test and analyzed for ANOVA, LSD and correlation between combined DON across all experiments.

5.4. Results

5.4.1. Haplotyping of wheat genotypes

A total of 77 wheat accessions (71 PI plus checks) were analyzed using seventeen microsatellite (SSR) markers linked to nine putative QTLs for FHB resistance. Among the 17 markers used, only 11 markers amplified the specific allele linked to resistant QTLs on Sumai 3 (3B and 5A), Frontana (3A and 5A), Wangshuibai (6B), or *T. dicoccoides* (*Td*) (7A), and were polymorphic among all accessions (Table 5.2). However, the specific alleles of SSR markers linked to resistant QTLs on 2B and 6B of *T. carthlicum* and 3A of *T. dicoccoides* (3A) were not detected on any genotypes used in this study. Among the 71 PI accessions genotyped, 49 had one or more alleles from one or more known sources for FHB resistance, while 22 had alleles different from any known sources (Table 5.1). Among the 49 accessions with at least one known resistant allele, six had alleles from Wangshuibai, 14 had at least one allele from Frontana, 24 had alleles from Sumai 3, and 33 had alleles from *T. dicoccoides.* Some accessions also carried multiple alleles from more than one sources (Table 5.1). Among check genotypes, Alsen and ND 2710 had alleles from Sumai 3. While other checks i.e. PI 277012, Steele ND and Choteau had resistant alleles different from Sumai 3, but they had at least one allele from *T. dicoccoides* (Table 5.2).

Cluster analysis using 13 SSR markers (*barc121*, *barc147*, *barc180*, *barc186*, *dupw227*, *gwm2*, *gwm129*, *gwm193*, *gwm644*, *umn8*, *wmc102*, *wmc397*, *wmc488*) linked to nine Fusarium head blight resistance QTLs on chromosomes 3A and 5A of Frontana, 2B and 6B of *T. carthlicum*, 3A and 7A of *T. dicoccoides*, 3B and 5A of Sumai 3, and 6B of Wangshuibai divided the 77 wheat accessions into four distinct clusters (Fig. 5.1). Groupings were primarily in accordance with country of origin and source of FHB resistance. The first group consisted of

three wheat accessions from South America and one from Europe. The second group consisted of a total of 27 accessions including 14 from Europe and 13 from South America. This group was further divided into two sub-clusters at a similarity level of 0.50 and grouped based on presence or absence of resistant alleles (Fig. 5.1). Fourteen accessions had no known alleles while five accessions had allele from *T. dicoccoides* (7A); three accessions had allele from Sumai 3 (5A) and the remaining three accessions had alleles from more than one sources. The third group consisted of 26 accessions that originated in South America (14), Europe (7), Asia (3) and the US (2). Accessions in this group had resistant alleles from Frontana (3A, 5A), Sumai 3 (5A), Wangshuibai (6B), *T. dicoccoides* (7A), or were without any known resistant alleles. The fourth group contained 18 accessions, including all of wheat genotypes of Sumai 3 origin. All of these accessions had resistant alleles from Sumai 3 (3B, 5A) and *T. dicoccoides* (7A) except one line PI 344454 which had only one allele for 3BS QTL of Sumai 3. Twelve wheat accessions of this group were originated from Asia and two each from Europe, South America and the US.

5.4.2. FHB resistance in greenhouse screening

The 71 wheat accessions along with the checks were evaluated for type II resistance by single floret inoculation in the greenhouse. Significant differences in FHB severity were observed among accessions ($p>0.001$) with mean severity ranging from 10.7 (PI 277012) to 77.2% (Choteau). The wheat accession PI 462151 (Shu Chou Wheat No. 3) carrying the same alleles as Sumai 3 had significantly lower disease (10.9%) than other accessions, but was similar to the highly resistant checks, ND 2710 (11.1%) or PI 277012 (10.7 %). Seven PI accessions [PI 344465 (Laah), PI 382153 (Nobeoka Bozu), PI 382154 (Nyu Bai), PI 382167, PI 644124 (Chuko), PI 644133 (Funo), and PI 644144 (Cluj 49-926)], which carries at least one resistance allele from known sources, showed a disease level (~26%) similar to those of Alsen, Sumai 3

and Steele ND (Table 5.3). Also, no significant difference in FHB severity was observed between these accessions and five PI accessions [PI 113948 (Kooperatorka), PI 185843 (Surpresa), PI 272348 (Lontoi), PI 644129 (Hatvani), and PI 644131(Prodigio Italiano)], had alleles different from any of the known resistance alleles.

5.4.3. FHB resistance in field conditions

In all four field experiments, wheat accessions with a low disease severity in greenhouse inoculations generally showed a similar or low level of disease severity in the field with some exceptions. Analysis of variance for individual years and locations showed a significant difference in disease severity ($P<0.01$) among the accessions. However, due to the highly variable nature of disease to the prevailing environmental conditions, we did not find the homogeneity of variance among experiments that were conducted in different field locations. Thus, we analyzed data separately.

At Fargo location in 2009, the disease severity varied from 7 % (PI 277012) to 45.4 % (Choteau). Two PI accessions e.g. Cltr 17427 (without known resistant allele) and PI 644135 (with known resistant allele) showed low DS (10.1% and 8.8% respectively), which were statistically different from those of the highly resistant checks (Sumai 3 and ND 2710). Eleven accessions had a disease severity below the resistant check Alsen (16.1%). These included six accessions [Cltr 12002 (Renacimiento), PI 83729 (Magyarovar 81), PI 104131 (Excelsior), PI 382167 (16-52-9), PI 462151 (Shu Chou Wheat No. 3), and PI 644148 (selection from Encruzilhada)], which carried at least one resistant allele of known sources, and five accessions [PI 113949 (Stepnjachka), PI 272348 (Lontoi), PI 644114 (selection from Belgrade 4), PI 644130 (selection from Iv C 390 ½ 10132), and PI 644140 (selection from 533b)] without any known resistant alleles. Other 17 accessions showed a disease severity below 20% but were

higher than Alsen (Table 5.3). In 2010 at the same location, the DS ranged from 11% (ND 2710) to 69% [PI 362437 (Iii/14-B], where the resistant check Alsen also had a high DS (41.4%). However, PI 462151 (Shu Chou Wheat No. 3) had a disease severity (11.2%) similar to those of the three resistant checks (Sumai 3, PI 277012, and ND 2710). Three PIs [PI 382153 (Nobeoka Bozu), PI 644119, PI 644123 (Norin 34)] and other 10 PIs had a similar DS compared to Sumai 3 (Table 5.3).

In Prosper, disease severity ranged from 12.1% [PI 382140) (Abura)] to 76.2% [PI 233207 (Odesskaja 13)] in 2009 and 8.9% [PI 434987 (Estanzuela Young)] to 77.9% (PI 644134, selection from Academia 48) in 2010. Two accessions [PI 382140 (Abura) and PI 434987 (Estanzuela Young)] had a significantly lower disease compared to the resistant checks in 2009 and 2010 respectively. In 2009, PI 182561 (Sin Chunaga) and PI 644124 (Chuko) had a disease level similar to Alsen and Sumai 3 (14.3%), while other twelve accessions showed a disease level lower than Steele ND (22 %) (Table 5.3). In 2010, the DS was relatively high for the resistant checks, Alsen (41.5%), PI 277012 (38.5%) and Sumai 3 (27.9 %). Twenty-eight accessions showed a disease severity lower than Sumai 3 and four accessions [PI 104131 (Excelsior), PI 344467 (Oncativo Inta), PI 382154 (Nyu Bai), and PI 519790 (274-1-118)] had a less disease than Steele ND (13.9 %). Two accessions [PI 434987 (Estanzuela Young) and PI 345731 (Tezanos Pintos Precoz)] had a disease less than ND 2710 (11.4%) (Table 5.3).

5.4.4. DON accumulation and correlation with DS

The amount of DON content in the harvested grains varied greatly among the wheat accessions. In the Fargo location, the average DON content ranged from 0.5 to 9.2 ppm in 2009 and 2.1 to 31.7 ppm in 2010. In general, DON accumulation was higher in 2010 compared to 2009. In 2010, only 9 lines had DON content below 5 ppm, whereas in 2009 71 accessions had

DON content below 5ppm. In the Prosper location, DON accumulation ranged from 0.5 to 21.6 ppm in 2009 and 0.7 to 14.3 ppm in 2010. There were 38 accessions with DON below 2 ppm in 2009, and 13 accessions with DON below 2 ppm in 2010. In general, wheat accessions with a low level of disease had a relatively low DON content (Table 5.3). The average DON content was lower in Prosper nursery compared to Fargo nursery in both years. The amount of DON in grains was positively correlated with disease severity (DS) in all experiments. In Fargo, DON concentration was highly significant ($p>0.0001$) and positively correlated with DS in both years ($r = 0.36$ in 2009 and $r = 0.30$ in 2010). However, in Prosper, significant and positive correlation was only observed in 2009 ($r = 0.32$, $p < 0.0001$). The correlation between DON and DS was positive but not significant in 2010 ($r= 0.21$, $p<0.075$).

Average DON content combined across all field experiments ranged from 1.2 ppm [PI 382161 (Tokai 66) to 17.7ppm [PI 362437(Iii/14-B)] (Table 5.3). The two resistant checks, ND 2710 and Sumai 3, had DON content below 2ppm. Twenty-three accessions with known resistant alleles (14 from Sumai 3 and 9 from non-Sumai 3 sources) and seven accessions without any known resistant alleles had a DON level below 5 ppm. Four accessions [PI 382153 (Nobeoka Bozu), PI 382154 (Nyu Bai), PI 382161 (Tokai 66), and PI 644124 (Chuko)] showed DON below 2 ppm (Table 5.3), three of which were among the four PIs identified to be very resistant (VR) to DON (Zhang et al. 2008). Significant positive correlation was observed between combined DON content on grain from field experiment with DS across all field experiments ($r = 0.36$, $P<0.0001$) (Fig. 5.2A) or with DS in greenhouse screening ($r = 0.39$, $P<0.0001$) (Fig. 5.2B). Overall the PI accessions with a combination of known resistance alleles linked to QTLs on Sumai 3 (3B, 5A) and *T. dicoccoides* (7A) had a low DS and DON content in both field and greenhouse.

5.5. Discussion

In this study, we evaluated 71 spring wheat PI accessions of the global origins using diverse molecular markers linked to the known sources of FHB resistance. The microsatellite marker alleles linked to the resistance QTLs from Sumai 3 (3B and 5A); Frontana (3A and 5A); Wangshuibai (6B); and *T. dicoccoides* (*Td*) (7A) were found in 69 % (49) of the accessions. However, a large number (24) of the accessions carried the haplotype of Sumai 3. This is due to the fact that Sumai 3 is the most commonly used resistance source in FHB breeding across the world (Parry et al., 1995; Bai et al., 2003). The majority of accessions with the Sumai 3 haplotype were originated from Asia (11 accessions, 46%), South America (eight accessions, 33%) and Europe (five accessions, 21%). Twenty-two PI accessions did not have resistance alleles from any known sources used and were from non-Asian in origin. The FHB resistance QTLs and resistance alleles present in these accessions might be novel and different from those currently used in breeding programs. In contrast, the resistance alleles linked to QTL of chromosome 3A in *T. dicoccoides*, and chromosomes 2B and 6B in *T. carthlicum* were not identified form wheat accessions used in this study. Sources of resistance to FHB in spring wheat has been reported from Asia, Europe and South America (Bai and Shaner, 2004; Buerstmayr et al., 2009). Independent to Asian sources, other resistant sources include those such as Brazilian line 'Frontana' (Steiner et al., 2004); European sources 'Arina' (Paillard et al., 2004); Dream (Schmolke et al., 2005) and US winter wheat lines Erine (Mckendry et al., 1995), Freedom (Gooding et al., 1997), Goldfield (Ohm et al., 2000), Roane (Griffey et al., 2001), Truman (McKendry et al., 2005) etc.

Buerstmayr et al. (2009) summarized 52 FHB resistant QTL distributed on all of wheat chromosomes except 7D. The major QTLs identified on 3BS locus (*Fhb1*) of Sumai 3 and its

derivatives are the most stable and consistent source of type II resistance to date (Buerstmayr et al., 2002, 2003, 2009). In our study, all of Sumai 3 type accessions were of Asian origin, except two PI lines [PI 382161 (Tokai 66) from Brazil and PI 351816 (Froment Du Japon) from Switzerland], which carry the same QTL alleles as Sumai 3 (3BS, 5A) and *T. dicoccoides* (7A). This result is consistent with Liu and Anderson (2003) who grouped these two PI lines into the same haplotype group. Using five SSR markers linked to *Qfhs.ndsu-3BS*, Liu and Anderson (2003) analyzed 74 wheat lines (54 from worldwide collection and 20 from North America) and found 26 accessions had the Sumai 3 alleles at least at one locus and 12 lines had the Sumai 3 alleles at least on four loci. They identified 42 lines as putative new sources for FHB resistance. The North American wheat line ND 2710 (ND2603/Grandin) had the same haplotype as Sumai 3 (3BS, 5AS) and *T. dicoccoides* (7A). Cultivar Alsen also carries Sumai 3 QTLs on chromosomes 3BS, 5AS and *T. dicoccoides* on 7A. Thus these cultivars might be very useful parents for introgression of resistance QTLs into other germplasm because these cultivars are well adapted in this growing region and have better agronomic quality than the exotic Sumai 3 source.

Most of the wheat accessions were clustered based on their geographic origin and haplotypes from different sources of resistance. Accessions carrying the haplotype of Sumai 3 related sources were separated from the non-Sumai 3 sources. All Asian accessions (14 in total) with the same haplotype as Sumai 3 were clustered together. This result is consistent with Yu et al. (2006) who reported that cultivar Funo (a Italian cultivar and a parent of Sumai 3) and Avrora (a Russian cultivar and a parent of Ning) were clustered separately from the Chinese or Japanese landraces. They used 25 SSR markers associated with FHB-resistance QTLs and amplified fragment length polymorphism (AFLP) markers using 24 primer combinations for genotyping and genetic clustering. However, three accessions [PI 382161 (Tokai 66), PI 344454 (Buck

Austral) and PI 382140 (Abura)] from South America and one accession PI 351816 (Froment Du Japon) from Europe were clustered with Asian accessions and they also carried the alleles from Sumai 3. Tokai 66 and Abura were originally from Japan and later introduced to Brazil (Zhang et al., 2008). Our study further indicated that these two lines might have originated from Japan. The close relationship between the Chinese and Japanese accessions was as expected, since several independent studies had also grouped Chinese and Japanese landraces together (Bai et al., 2003; Yu et al., 2006).

Resistance to Fusarium head blight is a quantitative trait and the disease severity can vary depending on environmental conditions such as temperature, humidity, and host growth stage (Parry et al., 1995). Thus, FHB screening needs to be repeated in multiple environmental conditions to test all of disease components, including initial infection, disease spread, DON content etc. (Campbell and Lipps, 1998; Fuentes et al., 2005; Zhang et al., 2008). Our re-evaluation of 71 accessions from four field and two greenhouse experiments identified potentially new sources of resistance to FHB. Three PI accessions, PI 382153 (Nobeoka Bozu), PI 382154 (Nyu Bai) and PI 382161 (Tokai), showed a low disease severity and DON content similar to or lower than the resistant checks (ND 2710, PI 277012 or Sumai 3), which had the same haplotype as Sumai 3 and carried resistant alleles from Sumai 3 (QTL on 3B, 5A) and *T. dicoccoides* (QTL on 7A). These lines were found very resistance (VR) to all components of FHB i.e. disease index, VSK and DON agreed with results of Zhang et al. (2008). PI 462151 (Shu Chou Wheat No. 3) which had the same haplotype as Sumai 3 (QTL on 3B, 5A) and *T. dicoccoides* (QTL on 7A), showed consistently low or similar DS and DON as resistant check ND 2710. This line will be a potentially new source for FHB breeding programs. This line was previously classified as resistant (R) based on index, VSK and DON (Zhang et al., 2008).

Our results indicated that 13 PI accessions previously identified as resistance (R) (Zhang et al. 2008) based on disease index showed varied disease responses in our field and greenhouse conditions. Four accessions PI 182561 (Sin Chunaga) (R allele from Sumai 3 3B, 5A, *Td* 7A), PI 382140 (Abura) (R allele from Sumai 3 3B, Frontana 5A; and *Td* 7A), two PIs without a resistant allele PI 644131 (selection from Prodigio Italiano), and PI 185843 (Surpresa) showed consistently low disease reaction in both field and greenhouse inoculations. However, six accessions including PI 644123 (selection from Norin 34), PI 644125 (selection from Norin 43) [R allele from Sumai 3 3B, 5A and *Td*], and those without resistant alleles, such as PI 519790 (274-1-118), PI 345731 (Tezanos Pintos Precoz), PI 644141 (Newthatch Selection), and PI 344467 (Oncativo Inta) showed very good resistance in the field condition but were susceptible after single floret inoculation in the greenhouse experiments. Among the PIs without a resistant allele, PI 644141 (Newthatch selection), PI 519790 (274-1-118), PI 113949 (Stepnjachka) and PI 344467 (Oncativo Inta) showed a very good resistance to DON content similar as in Zhang et al. (2008). Similarly, seven PIs without resistant alleles, namely PI 113948 (Kooperatorka), PI 113949 (Stepnjachka), PI 272348 (Lontoi), PI 644128 (selection from Prodigio Italiano), PI 644129 (selection from Hatvani), and PI 644144 (selection from Cluj 49-926 with R allele of *Td*), and PI 644133 (selection from Funo with R allele of Sumai 3), had very good resistance to disease spread in greenhouse but were susceptible in field conditions. However, these accessions had a mixed disease response to FHB in the previous report and were classified as susceptible to resistant (Zhang et al., 2008). These accession namely Cltr 5103 (274), PI 81791 (Sapporo Haru Komugi Jugo) and Cltr 12002 (Renacimiento), which classified previously as resistant (Zhang et al., 2008), were found susceptible in both field and greenhouse experiments in our study. The varied response of wheat accessions in greenhouse and field conditions might be due to a

different type of resistance mechanism existing in wheat or the influence of prevailing environmental conditions in different locations (Rudd et al., 2001; Xu, 2003; Osborne and Stein, 2007). The resistance from Sumai 3 or its derivatives and from Wangshuibai (3BS) are primarily type II, which prevents disease spread in spikes after initial infection (Buerstmayr et al., 2002, 2003; Zhou et al., 2004) or for DON accumulation (Somers et al., 2003), while resistance from Frontana (3A) and its derivative prevents initial infection (Singh et al., 1995, Steiner et al., 2004). Thus, FHB resistance present in these accessions might be novel and work for either type I or type II or DON resistance, and could be useful for gene pyramiding in wheat breeding programs or used for further genetic analysis.

Our result showed a moderate but significant positive correlation between disease severity (DS) and DON content. Previous studies also showed a consistent result and demonstrated a high positive correlation between DS and total DON (Mesterhazy, 2002; Paul et al., 2005; Puri and Zhong, 2010). However, some studies indicated no or negative correlation between these two components (Edwards et al., 2001; Champeil et al., 2004; Alvarez et al., 2010) or DON degradation on a resistant cultivar (Miller and Arnison, 1986). Paul et al. (2005) analyzed 163 different studies on correlation between DON and components of FHB (disease severity, disease index, disease intensity, visual scabby kernels etc.) and found that 65% of the studies had a correlation (r) values >0.50 and only 7% of the studies had correlation (r) below zero. Such contradictory results might be due to difference in inoculation methods (Yu et al., 2008), types of host resistance (Mesterhazy, 2002), difference in isolates and their trichothecene type (Puri and Zhong, 2010), or cropping system (Champeil et al., 2004). Differences in harvesting methods, blowing speed during threshing, DON analysis methods, weather conditions, and stage of crop infection can also fluctuate the results (Yu et al., 2008). In our

study, the low disease severity was always associated with low DON content. However, the same genotypes with similar disease level showed a varied amount of DON in two different experimental conditions. The non-significant correlation in 2010 Prosper field indicates the impact of weather conditions on DS and DON. Similar deviation on DS and DON had also been observed by Champeil et al. (2004).

In summary, we identified sources of FHB resistance, alternative to the predominant Asian source Sumai 3, among the NSGC spring wheat collections. Accessions with the FHB resistance haplotype of Sumai 3 sources were the most dominant. However, haplotype of Frontana, Wangshuibai, *T. dicoccoides* were also identified among the accessions. Approximately 30% of the accessions were without a haplotype of a known source. In general, accessions carrying haplotype from Sumai 3 showed a low disease severity and DON accumulation and were also genetically clustered together. Further, a close and positive correlation between FHB resistance and DON content indicates that the FHB resistant or moderately resistant accession might inhibit DON accumulation. Thus, the information on the haplotyping, FBH disease severity, DON content and genetic clustering among the accessions will permit breeders to select the most appropriate material for their breeding program to develop a new cultivar with high level of resistance to FHB and DON.

5.6. Acknowledgements

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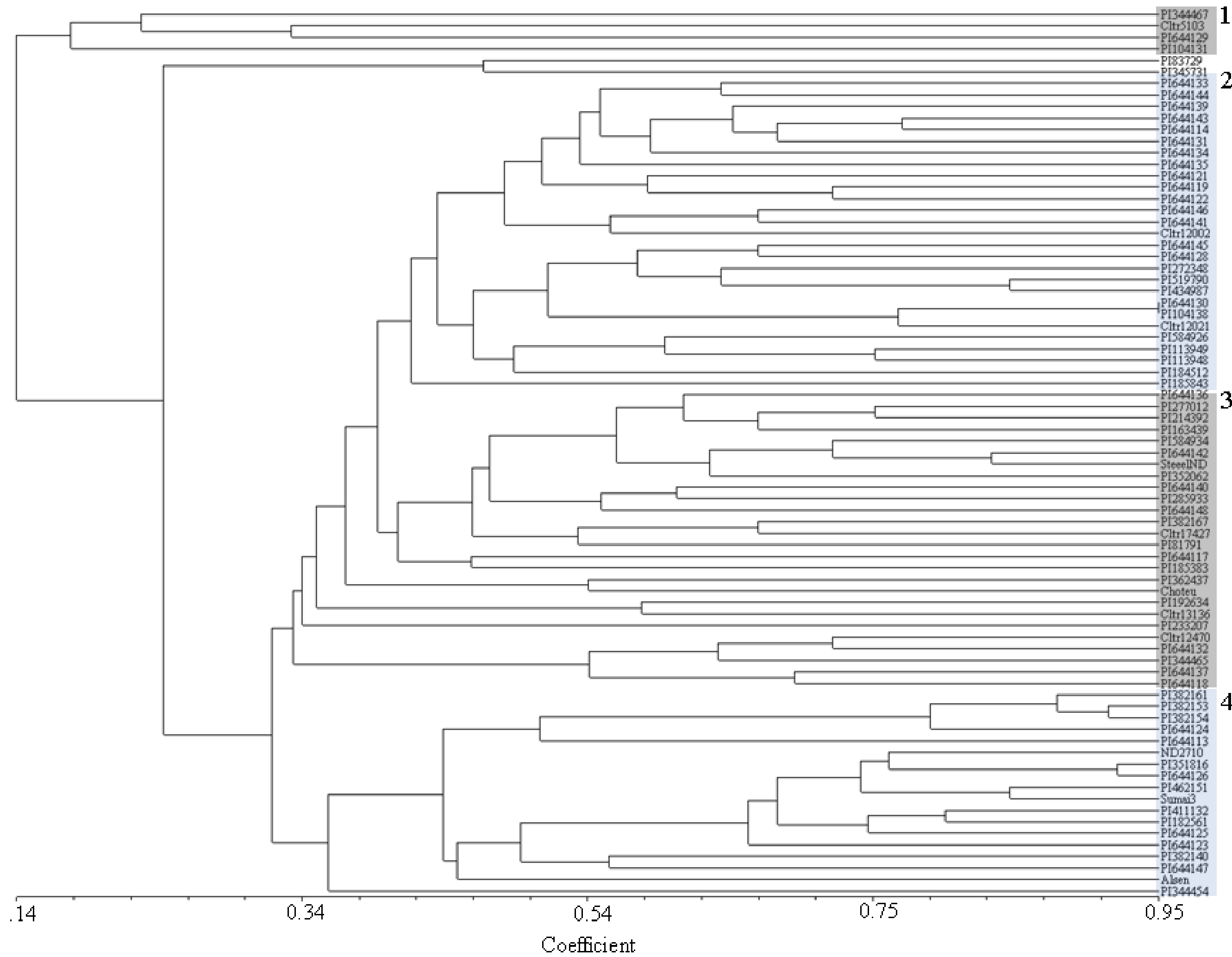

Figure 5.1. Dendrogram generated by cluster analysis using the unweighted pair group arithmetic mean (UPGMA) from thirteen SSR markers linked to nine Fusarium head blight resistance quantitate trait loci (QTLs) on chromosomes 3A, 5A (Frontana); 2B, 6B (*Triticum carthlicum*); 3A, 7A (*T. dicoccoides*); 3B, 5A (Sumai 3); and 6B (Wangshuibai) of 77 (including checks) wheat accessions of different origin and adaptability. A total of 98 polymorphic bands were used in analysis. The scale on the figure represents genetic similarity coefficient calculated according to Dice (1945). Shaded areas represent major genetic clusters.

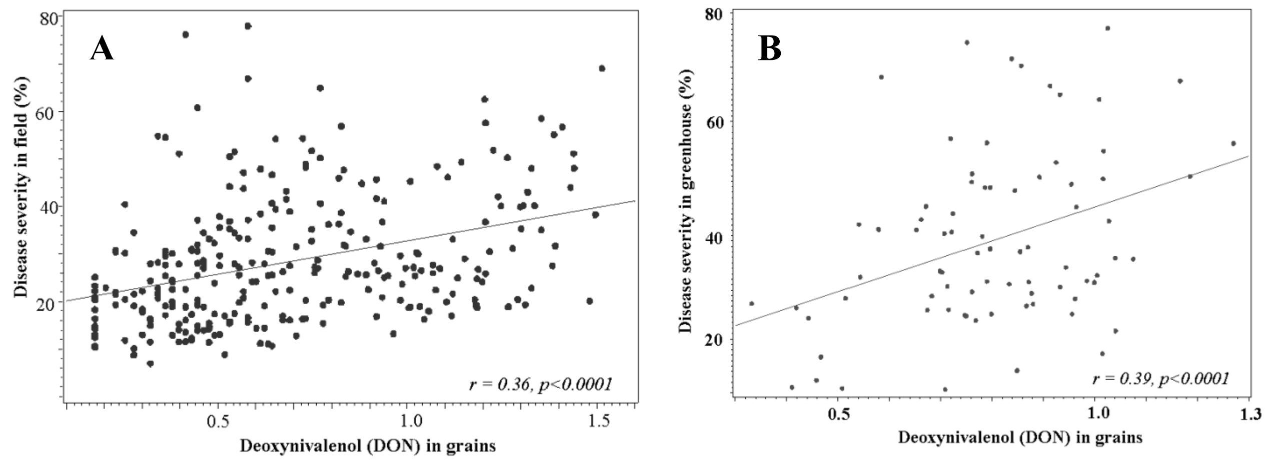

Figure 5.2. Correlation between disease severity (DS) and total deoxynivalenol (DON) production in grains harvested from field.A. Field disease severity with total DON in grains harvested from field B). Greenhouse disease severity with total DON on harvested grains in field.

Table 5.1. Origin and SSR allele of known resistant quantitative trait loci information of National Small Grains Collection (NSGC) wheat accessions and common checks used in the study[a].

NSGC Accession[b]	Allele[c]	New NSGC Accession	Name	Origin	Status	Pedigree
Cltr 12002	cd		Renacimiento	Uruguay	Cultivar	Americano 25C open pollinated
Cltr 12470	ade	PI 644116	Frontana	Brazil	Cultivar	Fronteira/Mentana
Cltr 13136	c		Rio Negro	Brazil	Cultivar	Supresa/Centenario
PI 104131	df		Excelsior	Argentina	Cultivar	Arminda/Virtue
PI 163439	e	PI 644120	-	Argentina	Cultivated	-
PI 182561	bcf		Sin Chunaga	Japan	Cultivated	-
PI 214392	ae		Colotana 266/51	Brazil	Breeding	Colonista/Frontana
PI 233207	f		Odesskaja 13	Ukraine	Cultivar	Erythrospermum 7623-1/Lutescens 62
PI 285933	f		Chudoskaja	Poland	Cultivated	-
PI 344454	c		Buck Austral	Argentina	Cultivar	Sola 50//Quivira/Guatrache/3/Massaux No.1/Buck Quequen 2-2-11
PI 344465	def		Laureano Alvarez Laah	Argentina	Cultivar	Benvenuto Inca/Klein 157
PI 351816	bcf		Froment Du Japon	Switzerland	Cultivated	-
PI 352062	f		Vivela Mar	Argentina	Cultivated	-
PI 362437	a		Iii/14-B	Yugoslavia	Landrace	-
PI 382140	bdf		Abura	Brazil	Cultivar	-
PI 382153	bcf		Nobeoka Bozu	Japan	Landrace	-
PI 382154	bcf		Nyu Bai	Japan	Landrace	-
PI 382161	bcf		Tokai 66	Brazil	Cultivar	-
PI 382167	a		16-52-9	Brazil	Breeding	Red Hart/PG 1
PI 411132	bcf		Gogatsu-Komugi	Japan	Cultivar	Gokuwase 2/Norin 61
PI 462151	bcf		Shu Chou Wheat No. 3	China	Cultivated	-
PI 584926	f		Pantaneiro	Brazil	Cultivar	Sonora 63*2/Lagoa Vermelha
PI 584934	f		Whestphalen	Brazil	Cultivar	CNT 10/Burgas 2//Jacui
Cltr 2492	bcef	PI 644113	White Russian	China	Landrace	-
PI 57364	f	PI 644117	CItr 7175	China	Landrace	Selection from PI 163429
PI 132856	d	PI 644118	Mentana	Italy	Cultivar	Rieti/Wilhelmina//Akagomughi
PI 163429	c	PI 644119	PI163429	Argentina	Cultivated	-
PI 168716	cdf	PI 644121	Klein Condor	Argentina	Cultivar	Standard F.C.S./Sud Oeste F.C.S.
PI 168727	c	PI 644122	Bahiense	Argentina	Cultivar	Klein Sinmarq/Eureka F.C.S
PI 182568	bcf	PI 644123	Norin 34	Japan	Cultivar	Shinchunaga/Eshimashinriki
PI 182583	bcf	PI 644124	Chuko	Japan	Landrace	-
PI 182586	bcf	PI 644125	Norin 43	Japan	Cultivar	Shiromansaku/Akakomugi 3//Shichunaga
PI 182591	bcf	PI 644126	Norin 61	Japan	Cultivar	Fukuoka 18/Shinchunaga
PI 203083	de	PI 644132	Wabian	Paraguay	Cultivated	-
PI 213833	c	PI 644133	Funo	Italy	Cultivar	Duecentodieci/Damiano
PI 256958	f	PI 644134	Academia 48	Romania	Cultivar	Selection from Romanian land variety
PI 264927	cf	PI 644135	220	Greece	Landrace	-
PI 264940	a	PI 644136	111a	Greece	Landrace	-
PI 264946	d	PI 644137	1032	Italy	Landrace	-
PI 294975	f	PI 644139	Artemowska	Bulgaria	Cultivated	-
PI 351256	f	PI 644142	Japon 2	Japan	Cultivated	-
PI 351256	f	PI 644143	Vaulion	Switzerland	Cultivar	-
PI 351743	f	PI 644144	Cluj 49-926	Romania	Cultivar	-
PI 351748	f	PI 644145	Jasi 10t	Romania	Cultivar	-
PI 351993	c	PI 644146	Z.88.54	Switzerland	Breeding	-
PI 360869	bcf	PI 644147	Fujimi Komugi	Japan	Cultivar	Norin 67/2*Norin 26
PI 382144	f	PI 644148	Encruzilhada	Brazil	Cultivar	Fortaleza/Kenya Farmer
PI 81791	f		Sapporo Haru Komugi Jugo	Japan	Cultivar	-
PI 83729	cf		Magyarovar 81	Hungary	Cultivar	-
Cltr 12021	*	PI 644115	Centenario	Uruguay	Cultivar	Selection from CItr 12021
Cltr 17427	*		16-52-2	Brazil	Breeding	-
Cltr 5103	*		274	Argentina	Landrace	-
PI 104138	*		Klein Triunfo	Argentina	Cultivar	Americano 25C/Pelon 33C1
PI 113948	*		Kooperatorka	Ukraine	Cultivar	Selection from Krymka
PI 113949	*		Stepnjachka	Ukraine	Cultivar	Selection from Banatka Khersonskaya
PI 184512	*	PI 644127	H 51	Argentina	Breeding	Americano 25e/Favorito//Universal
PI 185383	*		3084	Argentina	Cultivated	-
PI 185843	*		Surpresa	Brazil	Cultivar	Polyssu/Alfredo Chaves 6-21
PI 192634	*		Trintecinco	Brazil	Cultivar	Alfredo Chaves 3-21/Alfredo Chaves 4-21

(continued)

Table 5.1. Origin and SSR allele of known resistant quantitative trait loci information of National Small Grains Collection (NSGC) wheat accessions and common checks used in the study[a]. (continued)

NSGC Accession[b]	Allele[c]	New NSGC Accession	Name	Origin	Status	Pedigree
PI 272348	*		Lontoi	Hungary	Cultivar	-
PI 344467	*		Oncativo Inta	Argentina	Cultivar	Thatcher/Sinvalocho M.A.//Beckman 1971
PI 345731	*		Tezanos Pintos Precoz	Argentina	Cultivar	Frontana//Thatcher/Sinvalocho
PI 434987	*		Estanzuela Young	Uruguay	Cultivar	Bage/4/Thatcher/3/Frontana//Kenya 58/Newthatch
PI 519790	*		274-1-118	Uruguay	Breeding	Bage/Tehuacan/3/Frontana/Kenya 58/Newthatch/RL 4151
Cltr 11215	*	PI 644114	Belgrade 4	Yugoslavia	Cultivated	Selection from Cltr 11215
PI 185380	*	PI 644128	Prodigio Italiano	Italy	Cultivated	Selection from PI 185380
PI 192219	*	PI 644129	Hatvani	Hungary	Cultivar	-
PI 192498	*	PI 644130	Iv C 390 1/2 10132	Argentina	Cultivated	Selection from PI 192498
PI 192660	*	PI 644131	Prodigio Italiano	Italy	Cultivated	Selection from PI 192660
PI 349534	*	PI 644140	533b	Switzerland	Landrace	Selection from PI 349534
PI 351221	*	PI 644141	New thatch Selection	Switzerland	Cultivated	Selection from PI 351221
PI 615543	abc		Alsen	USA	Cultivated	ND674//ND2710 (PI 633976)/ND688
PI 633974	af		Choteau	USA	Cultivated	MT 9401/MT 9328
PI 633976	bcf		ND 2710	USA	Cultivated	Sumai3 / Wheaton // Grandin.
PI 277012	ef		I 826	Spain	Breeding	Extremo Sur / Argelino // *T.timopheevii*
PI 634981	F		Steele ND	USA	Cultivated	'Parshall' (PI 613587)/5/ 'Grandin'
PI 481542	bcf		Sumai 3	China	Cultivated	Funo / Taiwan Xiaomai

[a]Wheat accession origin, their original and new NSGC accession number, name, status and sources etc. is retrieved form Zhang et al. (2008) and online www.ars-grin.gov.

[b]Wheat accessions collected and maintained in National Small Grains Collection (NSGC), Aberdeen, Idaho, USA.

[c]Accession with one or more known resistant SSR alleles and their sources: a = Frontana (3A), b = Sumai 3 (3B), c = Sumai 3 (5A), d = Frontana (5A), e = Wangshuibai (6B) and f = *T. dicoccoides* (7A).

[-]information not available.

[*]Wheat accessions (separated by horizontal line) without a known alleles of known FHB resistance QTL on Frontana, Sumai 3, Wangshuibai and *T. dicoccoides* and common checks used in study.

Table 5.2. Distribution of microsatellite markers linked to the known FHB resistance QTLs on *Triticum carthlicum* (2B, 6B); *T. dicoccoides* (3A, 7A); Frontana (3A, 5A) and Sumai 3 (3B, 5A) among 71 NSGC wheat accessions and six checks.

Genotypes	***Triticum carthlicum***					***Triticum dicoccoides***				**Frontana**		**Sumai 3**					**Wangshuibai**	
	2B				**6B**	**3A**	**7A**			**3A**	**5A**	**3B**			**5A**		**6B**	
	wmc 102 [a]	***gwm 55***	***gwm 388***	***wmc 441***	***gwm 193***	***gwm 2***	***wmc 488***		***barc 121***	***dupw 227***	***gwm 129***	***umn 8***	*gwm 553*	*barc 147*	***barc 180***	***barc 186***	***wmc 397***	***gwm 644***
Cltr 12002	187	156	185	180	189	234	121	130	219	214	238	273	*nd*	126	201	211	184	184
Cltr 12470	187	151	183	182	191	221	104	130	219	206	238	273	135	143	193	214	174	*nd*
Cltr 13136	187	*nd*	183	182	201	*nd*	104	130	233	214	240	273	135	143	201	211	180	177
PI 104131	187	143	183	*nd*	*nd*	221	*nd*	*nd*	217	*nd*	238	*nd*	*nd*	145	190	*nd*	*nd*	188
PI 163439	187	155	191	168	191	221	104	130	219	214	241	273	*nd*	126	193	201	174	186
PI 182561	187	149	187	172	191	230	104	137	217	214	242	268	162	126	201	211	176	192
PI 214392	187	151	183	182	191	221	104	130	219	206	241	273	135	143	193	201	174	*nd*
PI 233207	187	155	185	169	203	230	104	137	233	214	240	273	170	128	*nd*	208	182	184
PI 285933	187	155	183	179	191	232	121	137	217	214	244	273	177	126	*nd*	201	194	179
PI 344454	187	145	*nd*	177	191	221	*nd*	*nd*	*nd*	*nd*	*nd*	281	*nd*	*nd*	196	211	*nd*	181
PI 344465	187	155	187	177	191	221	115	130	217	214	238	273	148	126	*nd*	214	174	196
PI 351816	187	147	187	172	191	230	104	140	217	214	242	268	162	126	201	211	176	192
PI 352062	187	145	191	193	191	221	121	137	217	214	240	273	135	126	196	201	180	196
PI 362437	187	155	187	199	189	232	104	*nd*	230	206	242	273	*nd*	157	193	201	182	*nd*
PI 382140	187	149	183	173	191	230	104	137	217	214	238	268	160	126	*nd*	201	178	188
PI 382153	187	151	*nd*	172	191	234	104	137	217	214	244	268	160	128	*nd*	211	178	177
PI 382154	187	152	183	172	191	234	104	137	217	214	244	268	160	128	*nd*	211	178	177
PI 382161	187	141	183	172	191	234	104	137	217	214	244	268	160	128	190	211	178	177
PI 382167	187	143	187	177	201	225	121	130	235	206	240	273	135	*nd*	193	201	178	192
PI 411132	187	149	187	172	191	230	104	140	217	214	242	268	160	126	*nd*	211	176	192
PI 462151	187	162	183	172	191	221	104	137	219	214	242	268	162	126	201	211	176	192
PI 584926	187	143	187	177	191	225	104	137	*nd*	214	242	273	135	143	196	201	180	196
PI 584934	187	143	187	177	191	236	104	137	219	214	240	273	135	143	193	201	190	196
PI 644113	187	*nd*	191	193	191	*nd*	104	137	217	214	242	268	*nd*	*nd*	190	211	174	184
PI 644117	187	*nd*	183	*nd*	*nd*	223	102	137	*nd*	*nd*	*nd*	*nd*	*nd*	126	178	201	*nd*	*nd*
PI 644118	187	152	185	172	191	221	104	130	219	214	238	281	*nd*	123	193	214	191	184
PI 644119	187	153	187	180	191	221	104	111	233	214	240	273	*nd*	126	201	211	184	186
PI 644121	187	147	189	*nd*	191	221	104	137	233	214	238	273	148	126	175	211	178	186

(continued)

Table 5.2. Distribution of microsatellite markers linked to the known FHB resistance QTLs on *Triticum carthlicum* (2B, 6B); *T. dicoccoides* (3A, 7A); Frontana (3A, 5A) and Sumai 3 (3B, 5A) among 71 NSGC wheat accessions and six checks. (continued)

Genotypes	***Triticum carthlicum***					***Triticum dicoccoides***				**Frontana**		**Sumai 3**					**Wangshuibai**	
	2B				**6B**	**3A**	**7A**			**3A**	**5A**	**3B**			**5A**		**6B**	
	wmc 102[a]	***gwm 55***	***gwm 388***	***wmc 441***	***gwm 193***	***gwm 2***	***wmc 488***		***barc 121***	***dupw 227***	***gwm 129***	***umn 8***	*gwm 553*	*barc 147*	***barc 180***	***barc 186***	***wmc 397***	***gwm 644***
PI 644122	187	*nd*	187	180	191	221	104	111	233	214	240	276	*nd*	*nd*	201	211	184	186
PI 644123	187	147	185	172	191	230	104	137	217	214	242	268	162	126	201	211	178	186
PI 644124	187	143	183	172	191	234	104	137	217	214	244	268	160	128	196	211	178	177
PI 644125	187	149	191	172	191	228	104	137	217	214	242	268	*nd*	126	199	211	176	192
PI 644126	187	*nd*	187	172	191	230	104	140	217	214	242	268	162	126	201	211	176	192
PI 644132	187	*nd*	183	182	191	221	104	130	219	214	238	273	135	143	193	214	174	184
PI 644133	187	132	183	172	191	221	104	130	219	214	240	273	135	126	*nd*	211	182	184
PI 644134	187	156	183	179	189	232	121	137	217	214	240	273	137	126	*nd*	208	184	186
PI 644135	187	145	183	*nd*	203	221	119	137	226	214	240	273	186	126	201	201	178	192
PI 644136	187	141	187	180	201	221	104	130	235	206	240	273	135	126	199	201	178	186
PI 644137	187	151	185	172	191	221	104	130	219	214	238	281	*nd*	123	193	214	176	184
PI 644139	187	147	191	179	191	221	121	137	228	214	240	273	150	126	*nd*	201	190	179
PI 644142	187	149	191	168	191	223	104	137	217	214	240	273	172	*nd*	196	201	184	181
PI 644143	187	150	191	193	191	232	119	137	226	214	240	273	*nd*	126	196	201	184	186
PI 644144	187	*nd*	191	193	191	221	119	137	235	214	242	273	135	126	178	201	176	184
PI 644145	187	143	191	193	191	219	121	137	217	214	242	273	135	126	*nd*	201	180	194
PI 644146	187	154	189	182	189	221	104	130	226	214	241	273	*nd*	126	*nd*	211	172	184
PI 644147	187	149	183	174	189	230	104	137	217	214	242	268	160	126	201	201	186	*nd*
PI 644148	187	159	187	177	201	236	104	137	235	214	241	273	*nd*	143	199	201	178	*nd*
PI 81791	187	155	*nd*	168	*nd*	223	*nd*	137	235	*nd*	240	273	*nd*	161	196	201	*nd*	181
PI 83729	182	145	*nd*	187	*nd*	223	*nd*	*nd*	217	*nd*	*nd*	273	*nd*	126	*nd*	211	*nd*	184
Cltr 12021	187	157	185	*nd*	201	221	115	130	230	214	243	273	*nd*	126	190	201	182	186
PI 17427	187	145	*nd*	177	*nd*	225	130	*nd*	233	*nd*	240	273	*nd*	143	196	201	*nd*	192
Cltr 5103	184	149	*nd*	168	*nd*	223	*nd*	*nd*	230	*nd*	241	*nd*	*nd*	145	*nd*	201	*nd*	188
PI 104138	187	155	191	168	201	234	104	130	228	214	*nd*	273	*nd*	126	190	201	182	186
PI 113948	187	156	187	*nd*	191	230	106	133	230	214	242	273	148	128	*nd*	201	176	186
PI 113949	187	155	187	*nd*	191	230	106	133	230	214	242	273	148	128	193	201	176	186
PI 184512	187	155	191	168	201	221	104	130	226	214	242	281	*nd*	*nd*	193	201	182	194
PI 185383	187	*nd*	183	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	214	*nd*	281	158	*nd*	*nd*	201	*nd*	*nd*
PI 185843	187	153	*nd*	182	203	221	104	130	235	214	241	273	135	143	199	201	182	177

(continued)

Table 5.2. Distribution of microsatellite markers linked to the known FHB resistance QTLs on *Triticum carthlicum* (2B, 6B); *T. dicoccoides* (3A, 7A); Frontana (3A, 5A) and Sumai 3 (3B, 5A) among 71 NSGC wheat accessions and six checks. (continued)

Genotypes	*Triticum carthlicum*					*Triticum dicoccoides*				Frontana		Sumai 3					Wangshuibai	
	2B				6B	3A	7A			3A	5A	3B			5A		6B	
	wmc 102[a]	***gwm 55***	***gwm 388***	***wmc 441***	***gwm 193***	***gwm 2***	***wmc 488***		***barc 121***	***dupw 227***	***gwm 129***	***umn 8***	*gwm 553*	*barc 147*	***barc 180***	***barc 186***	***wmc 397***	***gwm 644***
PI 192634	187	143	183	*nd*	201	234	115	130	237	214	240	281	158	*nd*	190	201	180	183
PI 272348	187	145	191	193	189	223	121	130	219	214	242	273	133	126	*nd*	201	180	186
PI 344467	177	*nd*	187	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	203	*nd*	*nd*	*nd*
PI 345731	*nd*	141	*nd*	182	191	221	*nd*	*nd*	235	214	*nd*	273	*nd*	143	*nd*	214	*nd*	184
PI 434987	187	*nd*	*nd*	182	176	221	115	130	230	214	242	273	135	126	196	201	180	186
PI 519790	187	151	183	182	176	221	115	130	230	214	242	273	135	126	196	201	180	186
PI 644114	187	*nd*	191	*nd*	191	232	121	133	221	214	240	273	*nd*	126	193	201	184	184
PI 644128	187	*nd*	189	184	191	221	119	128	226	214	242	273	137	126	196	201	182	*nd*
PI 644129	184	155	187	168	*nd*	232	133	*nd*	226	*nd*	242	273	135	167	196	201	*nd*	204
PI 644130	187	155	191	168	201	234	115	130	230	214	241	273	135	126	190	201	182	186
PI 644131	187	*nd*	189	184	191	*nd*	119	128	226	214	242	273	137	126	196	201	182	198
PI 644140	187	151	187	*nd*	199	232	104	133	226	214	241	273	135	126	*nd*	201	178	190
PI 644141	187	155	185	180	189	221	121	130	219	214	242	273	*nd*	126	184	201	184	184
Alsen	187	*nd*	191	193	191	223	115	130	230	206	242	268	162	126	201	211	184	198
Choteau	187	*nd*	*nd*	*nd*	191	221	115	137	230	206	242	273	135	*nd*	190	201	182	184
ND 2710	187	141	191	193	191	221	104	137	235	214	242	268	162	126	*nd*	211	176	196
PI 277012	187	145	191	*nd*	191	221	104	137	226	214	240	273	*nd*	147	193	201	174	*nd*
Steele ND	187	*nd*	191	193	191	223	115	137	230	214	242	273	137	143	*nd*	201	184	196
Sumai 3	187	*nd*	183	172	191	221	104	137	219	214	242	268	162	126	201	211	176	192
PIC[b]	0.10	0.88	0.71	0.85	0.48	0.72	0.79		0.80	0.17	0.69	0.43	0.77	0.55	0.78	0.49	0.84	0.84
NOA[c]	4	16	5	13	6	9	12		9	2	6	4	12	9	9	4	11	13

[a]Numeric value: the amplication size (base pairs) of the respective markers.
[b]PIC = polymorphism information content.
[c]NOC = number of alleles.
Color coding: green cell- accessions with the allele of same size as the *Triticum dicoccoides* linked to 7A FHB resistant QTL; yellow cell- accessions with the allele of same size as the Frontana linked to 3A or 5A FHB resistant QTLs; gold and blue cells- accessions with the allele of same size as the Sumai 3 linked to 3B and 5A FHB resistant QTLs respectively; and pink cell - accessions with the allele of same size as the Wangshuibai linked to 6B FHB resistant QTL. Accessions with the allele of same size as *Triticum carthlicum* linked to 2B and 6B or *T. dicoccoides* linked to 3A FHB resistant QTL were not detected in this study. *nd* = no data.

Table 5.3. Average Fusarium head blight disease severity (DS) and deoxynivalenol (DON) concentration of 71 wheat accessions and common checks used in the study.

Genotypes[a]	Allele[b]	Disease Severity (%)[c]					DON Concentration (ppm)[d]				
		Fargo		Prosper		Green-house	Fargo		Prosper		Combined DON[e]
		2009	2010	2009	2010		2009	2010	2009	2010	
Cltr 12002	cd	15.7	57.6	16.7	38.7	26.4	2.8	15.1	2.8	5.7	6.6
Cltr 12470	ade	-	36.8	-	41.7	49.5	0.5	11.2	2.0	7.6	9.4
Cltr 13136	c	35.8	31.1	47.2	46.0	52.5	3.4	18.0	2.7	5.6	7.4
PI 104131	df	11.5	26.5	20.9	12.0	30.6	1.5	9.9	7.6	1.7	5.2
PI 163439	e	26.6	-	48.9	-	71.6	7.4	20.0	4.4	-	5.9
PI 182561	bcf	17.2	22.4	14.1	15.9	27.5	1.3	3.9	1.5	2.4	2.3
PI 214392	ae	20.7	62.5	19.0	18.8	48.5	1.2	15.0	1.6	14.3	8.0
PI 233207	f	19.1	20.4	76.2	17.1	36.9	3.2	18.6	1.6	2.3	6.4
PI 285933	f	20.5	22.4	40.5	15.3	40.1	0.8	10.9	0.8	1.5	3.5
PI 344454	c	29.6	38.3	36.9	50.5	31.7	1.3	30.4	2.4	2.4	9.1
PI 344465	def	19.2	19.9	37.2	14.5	24.2	1.8	11.7	1.8	3.2	4.6
PI 351816	bcf	16.4	36.3	16.2	20.8	41.1	0.5	5.6	1.1	2.7	2.5
PI 352062	f	21.6	25.0	28.1	17.3	39.7	0.9	12.6	1.9	1.7	4.3
PI 362437	a	25.8	69.0	58.6	48.5	56.0	6.4	31.7	21.6	11.0	17.7
PI 382140	bdf	22.2	23.6	12.2	20.3	27.9	0.5	8.7	1.7	4.4	3.8
PI 382153	bcf	17.8	16.1	30.2	14.9	12.4	1.0	3.9	0.8	1.8	1.9
PI 382154	bcf	18.3	33.4	28.3	13.1	25.7	0.5	2.6	1.2	2.2	1.6
PI 382161	bcf	25.1	23.9	27.7	22.0	26.5	0.5	2.1	1.3	0.7	1.2
PI 382167	a	11.5	36.8	17.5	26.3	25.3	1.1	7.6	2.5	3.7	3.7
PI 411132	bcf	33.3	29.7	16.1	25.6	44.4	2.0	8.6	1.6	2.6	3.7
PI 462151	bcf	12.9	11.2	15.0	19.8	10.9	2.1	3.1	1.1	2.6	2.2
PI 584926	f	16.4	40.1	40.6	54.4	65.0	4.4	16.7	4.9	4.3	7.6
PI 584934	f	19.0	25.4	43.8	19.3	74.6	1.4	9.5	2.7	5.0	4.7
PI 644113	bcef	19.1	40.2	34.5	47.7	29.6	1.3	20.7	2.5	5.8	7.6
PI 644117	f	21.0	51.2	32.0	65.0	54.6	0.5	26.5	5.8	4.9	9.4
PI 644118	d	30.6	30.5	30.0	-	30.4	1.8	15.5	9.7	5.8	9.0
PI 644119	c	25.6	16.2	30.7	15.5	43.1	3.4	10.1	1.3	2.4	4.3
PI 644121	cdf	25.3	20.1	25.5	27.2	49.9	5.9	29.3	7.9	3.7	14.4
PI 644122	c	24.3	18.9	28.0	16.5	30.5	3.0	17.5	2.5	2.8	6.5
PI 644123	bcf	20.5	16.3	19.6	27.2	32.5	0.5	4.3	1.8	9.4	4.0
PI 644124	bcf	20.4	27.1	13.1	31.7	16.7	0.5	4.8	0.5	7.5	1.9
PI 644125	bcf	21.4	27.7	17.4	19.8	35.8	1.2	10.7	2.1	5.7	4.9
PI 644126	bcf	19.1	25.7	14.8	28.1	31.4	0.9	6.8	0.5	1.8	2.5
PI 644132	de	24.5	18.8	20.3	41.1	47.9	1.4	9.2	5.2	7.7	5.3
PI 644133	c	18.3	43.0	-	45.3	21.5	1.0	19.8	1.5	9.2	10.0
PI 644134	f	18.8	27.0	24.4	78.0	56.9	1.6	11.2	1.4	2.8	4.3
PI 644135	cf	10.1	49.3	15.5	-	36.6	0.9	12.9	2.0	1.8	5.3
PI 644136	a	22.1	31.7	27.2	25.1	41.7	2.7	23.6	4.7	7.7	9.7
PI 644137	d	20.0	27.1	22.6	28.2	28.7	1.4	8.9	1.6	7.2	4.8
PI 644139	f	27.1	24.1	33.2	43.3	49.8	1.9	14.8	6.8	3.8	6.8
PI 644142	f	-	21.3	28.9	20.1	48.9	0.7	6.5	1.4	6.4	4.8
PI 644143	f	30.1	35.3	32.0	44.9	66.6	1.4	19.0	1.8	6.6	7.2
PI 644144	f	19.4	18.0	35.6	39.5	25.4	0.7	10.4	2.2	3.5	4.2
PI 644145	f	30.5	22.8	24.2	44.3	70.3	0.7	20.3	1.4	2.4	6.2
PI 644146	c	34.5	19.0	37.9	33.3	29.7	1.9	9.8	2.2	2.8	4.2
PI 644147	bcf	19.0	20.4	22.6	46.7	47.9	1.6	14.1	1.4	3.4	5.1
PI 644148	f	14.7	36.8	31.0	36.2	47.3	2.0	15.1	1.7	5.2	6.0
PI 81791	f	24.4	40.2	22.2	16.9	33.2	1.1	19.3	3.5	7.3	7.8

(continued)

Table 5.3. Average Fusarium head blight disease severity (DS) and deoxynivalenol (DON) concentration of 71 wheat accessions and common checks used in the study. (continued)

Genotypes[a]	Allele[b]	Disease Severity (%)[c]					DON Concentration (ppm)[d]				
		Fargo		Prosper		Green-house	Fargo		Prosper		Combined DON[e]
		2009	2010	2009	2010		2009	2010	2009	2010	
PI 83729	cf	13.0	44.0	32.7	47.9	30.7	1.0	26.0	4.6	3.1	8.7
CItr 12021	*	30.9	29.0	30.7	14.5	38.9	0.7	13.2	3.3	3.0	5.1
Citr 17427	*	8.8	20.3	27.6	56.9	50.4	0.9	8.6	3.9	5.7	4.8
Citr 5103	*	23.3	33.2	35.3	54.6	39.4	0.5	12.2	2.4	1.3	4.1
PI 104138	*	-	35.1	34.2	22.6	67.5	1.7	29.8	9.0	2.3	13.7
PI 113948	*	25.5	39.9	37.2	51.5	26.1	1.3	18.9	2.8	2.5	6.4
PI 113949	*	12.9	46.1	41.7	67.0	30.1	1.4	11.8	7.3	2.8	5.8
PI 184512	*	27.2	19.3	28.7	26.4	44.3	2.6	19.5	4.8	6.0	8.2
PI 185383	*	31.5	27.5	27.6	45.7	34.7	-	23.2	2.2	7.3	10.9
PI 185843	*	18.8	24.7	28.2	32.3	24.6	1.9	14.3	2.9	2.1	5.3
PI 192634	*	28.0	42.1	30.3	26.0	34.9	3.2	16.4	9.6	10.7	10.0
PI 272348	*	13.9	56.7	29.7	54.2	24.6	1.8	24.7	2.2	3.5	8.1
PI 344467	*	16.4	22.6	25.5	13.6	68.2	1.1	6.9	1.8	1.6	2.9
PI 345731	*	22.2	25.9	22.8	10.7	56.1	1.0	15.1	1.2	3.4	5.2
PI 434987	*	20.4	18.7	54.9	8.9	42.0	1.8	9.1	1.2	2.3	3.6
PI 519790	*	21.5	17.0	30.8	12.3	32.3	0.7	12.1	1.7	1.7	4.1
PI 644114	*	11.9	51.9	34.7	30.5	28.4	0.8	15.9	6.1	3.4	6.6
PI 644128	*	27.9	35.0	26.0	50.3	27.4	1.5	21.6	4.7	4.9	8.2
PI 644129	*	20.8	48.0	28.7	31.7	17.3	-	20.3	1.9	5.9	9.4
PI 644130	*	16.1	19.3	39.0	24.2	36.1	3.7	14.2	3.9	2.9	6.2
PI 644131	*	22.9	21.7	25.5	40.7	23.4	0.6	12.2	3.3	3.4	4.9
PI 644140	*	11.6	48.0	31.6	51.8	64.1	1.6	26.6	4.1	4.6	9.2
PI 644141	*	-	19.1	23.9	21.1	40.2	0.5	6.5	2.0	2.2	2.8
Alsen	abc	16.4	50.3	14.2	41.6	14.2	1.1	17.4	2.0	3.8	6.1
Choteau	af	45.3	55.1	51.2	48.3	77.2	9.2	23.4	1.5	4.4	9.6
ND 2710	bcf	10.4	11.1	12.4	11.4	11.1	0.5	3.3	0.5	2.0	1.6
PI 277012	ef	7.0	13.3	12.8	38.5	10.7	1.1	8.2	4.1	3.1	4.1
Steele ND	f	34.6	26.8	22.0	14.0	24.4	0.9	14.4	1.2	1.9	4.6
Sumai 3	bcf	10.7	15.5	14.3	28.0	23.8	0.5	5.0	0.5	1.1	1.8
LSD		17.2	20.1	16.6	28.7	21.1					

[a]Wheat genotypes includes PI accession collected and maintained in National Small Grains Collection (NSGC) Aberdeen, Idaho, USA; PI carrying allele from known resistance source and checks used were separated by horizontal line.

[b]Accession with one or more known resistant SSR alleles and their sources: a = Frontana (3A), b = Sumai 3 (3B), c = Sumai 3 (5A), d = Frontana (5A), e = Wangshuibai (6B) and f = *T. dicoccoides* (7A) and * = accession without a known FHB resistance alleles that linked to resistant sources Frontana, Sumai 3, Wangshuibai or *T. dicoccoides*

[c]Disease severity (DS) % was recorded at 21 days after flowering in filed (corn inoculated) or 21 days after point inoculation in greenhouse. Field data were average of three replications. Prosper 2010 and all greenhouse data were average of two replications. The least significant difference (LSD) was calculated for individual years or locations using SAS 9.2.

[d]Deoxynivalenol (DON) in grains was obtained from wheat grains harvested in field. Samples were prepared combining grains from all replications and grounded. ppm = part per million (mg/kg of samples).

[e]DON value averaged from all four-field experiments; - Disease or DON data not available

5.7. Literature Cited

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