UNDERSTANDING HOST RESISTANCE AND PATHOGEN BIOLOGY IN THE WHEAT-*FUSARIUM GRAMINEARUM* PATHOSYSTEM

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Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

SUPERVISORY COMMITTEE:

Dr. Shaobin Zhong
Chair

Dr. Luis Del Río Mendoza

Dr. Zhaohui Liu

Dr. Steven Xu

Dr. Xuehui Li

Dr. Gideon Francois Marais

Approved:

April 27, 2020
Date

Jack Rasmussen
Department Chair

ABSTRACT

Fusarium head blight (FHB) is a major challenge in global wheat production. In the United States, the disease is predominantly caused by the fungus *Fusarium graminearum*. Utilization of FHB-resistant wheat cultivars integrated with other measures such as fungicide application is the most effective approach for the management of this disease. This study aimed to 1) identify novel quantitative trait loci (QTL) for resistance to FHB in a Brazilian spring wheat cultivar 'Surpresa' through bi-parental mapping, 2) detect QTL for FHB resistance in a global panel of 233 spring wheat accessions by genome-wide association analysis (GWAS), and 3) localize genomic regions governing traits associated with virulence in *Fusarium graminearum*. Using phenotypic and genotypic data from 187 recombinant inbred lines derived from the cross between Surpresa and a susceptible spring wheat cultivar 'Wheaton', four QTL (*Qfhb.ndwp-2AS, Qfhb.ndwp-2AL, Qfhb.ndwp-3B,* and *Qfhb.ndwp-4D*) were mapped on chromosomes 2A, 3B, and 4D of Surpresa, respectively. *Qfhb.ndwp-2AS, Qfhb.ndwp-2AL, and Qfhb.ndwp-3B* were found to be novel based on physical locations of the markers tightly linked to these QTL. Two significant marker-trait associations (*Qfhb.ndwp-3A* and *Qfhb.ndwp-2BL*) were detected by GWAS of 233 spring wheat accessions, which conferred type II and type III FHB resistance and mapped on chromosomes 3A and 2B, respectively. Both QTL were novel based on the physical locations of markers tightly linked to them. GWAS using 183 *F. graminearum* isolates collected from North Dakota identified two significant marker-trait associations in chromosomes 1 and 3 for mycotoxin production, and one for fungicide sensitivity to each of the fungicides, respectively. The genes detected in this study associated with mycotoxin production were not previously reported. Identification of these novel genes in metabolic pathways of *F. graminearum* could help to develop new strategies for the management FHB.

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DEDICATION

This work is humbly dedicated to all the valuable treasures in my life:

To,

Grandparents: Dina Nath Poudel, Nanda Maya Poudel, Badri Maya Karanjit

Parents: Moti Ram Paudel, Bimala Kumari Poudel, Laxmi Poudel, Hema Laxmi Karanjit

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In-laws: Late Mr. Hom Nath Bhattarai, Saraswati Bhattarai, Regan Bhattarai, Sneha Bhattarai, Biswas Bhattarai, Anita Khadka, Ronal Bhattarai, Surabhi Timilsina

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For shaping me in a way that I am today.

And,

Kanchan Bhattarai,

my better half,

for your love, patience, faith, and inspiration. You are always beside me even for better for worse

Bikash Poudel

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LITERATURE REVIEW

The Host: Spring Wheat

Wheat (*Triticum* spp.) is one of the major food crops grown in more than 122 countries in the world (FAOSTAT 2015). It ranks third, after maize and rice, as the most-produced cereal in the world, contributing around 700 million metric tons annually and is grown on more land area (215 million hectares) in the world than any other commercial crop (Golan et al. 2015). Wheat is an important food staple for 35% of the world population and contributes around 20% of the total caloric intake (Gao et al. 2012; Tzarfati et al. 2014).

To feed the predicted world population of between 9.4 and 10.2 billion by 2050, agricultural production in 2050 needs to be at 150% of what it was in 2012 (FAO 2017). A surplus of global wheat production over increasing consumption has been observed over the last five years and is expected to continue in 2019/20 (https://apps.fas.usda.gov/psdonline/circulars/grain-wheat.pdf). Further rise in wheat consumption is projected over time based on population growth trends, rising food use, changing tastes and preferences, rising incomes and increased urbanization. In 2017/18, a record world wheat production of 763 million metric tons was achieved, which is approximately 116% of the world total wheat production in 2012. Considering the constant rise in global wheat consumption in coming years, production must continue to increase to meet global demand by 2050 (http://www.fao.org/worldfoodsituation/csdb/en/). While it is necessary to increase the production, the quality of the crop produced also needs to be improved. Limited availability of arable land, changing weather patterns, and unpredictable biotic and abiotic stresses are key challenges to producing high yielding, quality crops (Asseng et al. 2011; Figueroa et al. 2018). Therefore, it is very essential to cope with these issues and meet the need of food security.

Despite producing only 7% of the total world wheat crop, the United States manages to be a major player in international wheat trade as one of the top three wheat exporters. In the U.S., wheat is the principal food grain commercially grown in almost all states and is the third major field crop behind corn and soybean in terms of production, acreage and gross farm receipts (https://www.ers.usda.gov/topics/crops/wheat/wheat-sector-at-a-glance/). Three primary categories, based on time of planting, of wheat: winter, spring, and durum are grown in the U.S. (https://www.ers.usda.gov/topics/crops/wheat/wheat-sector-at-a-glance/#classes). Winter wheat is the largest produced category followed by spring and durum. As the name suggests, winter wheat is planted in the fall and harvested in summer while spring and durum wheat are planted in spring and harvested in late summer or fall of the same year. The three wheat categories are further classified into five major classes based on hardness, color and shape of wheat kernel, and sowing seasons as: hard red winter (HRW), hard red spring (HRS), soft red winter (SRW), white (both winter and spring) and durum wheat. Each of these classes have somewhat different end-use purposes, related to milling and baking, and are grown in different regions of the U.S.

Evolution and Domestication of Wheat

Common wheat (*Triticum aestivum* L. 2n = 6x = 42, AABBDD genomes) and durum (*Triticum turgidum* ssp. *durum* L., 2n = 4x = 28, AABB genomes) are the two primary cultivated forms of modern wheat, contributing about 95% and 5% of the global wheat crop, respectively (Faris et al. 2014). These wheat forms evolved from the same evolutionary lineage as a result of domestication, natural hybridization and allopolyploid speciation events. Wheat and its wild relatives belong to the genus *Triticum* in the tribe Triticeae and comprises six biological species at diploid, tetraploid, and hexaploid levels ; *T. monococcum* (AA genome), *T. urartu* Tumanian ex Gandilyan (AA genome), *T. turgidum* L. (AABB genomes), *T. timopheevii* (Zhuk.) Zhuk.

(AAGG genomes), *T. aestivum* L. (AABBDD genomes) and *T. zhukovskyi* Menabde & Ericz. (AAAAGG genomes) (Matsuoka 2011). Different ploidy levels in wheat are the result of hybridization among different genera within the tribe (Nevo 2014). The tetraploid species of wheat, AABB and AAGG, are believed to originate from at least two independent hybridization events between *T. urartu* and *Aegilops speltoides* Tausch (SS genome). The hexaploid wheat species referred to as common wheat, *T. aestivum* (AABBDD), is thought to have emerged as a result of natural hybridization between *T. turgidum* and *Aegilops tauschii* Coss. (DD genome) (Matsuoka 2011).

Domestication of wheat started in the "Fertile Crescent" of the Middle East around 10,500 years ago in both tetraploids and hexaploids (Berkman et al. 2013; Golan et al. 2015). During the domestication process, some genetic changes occurred in morphology, physiology and phenology in domesticated varieties. For example, transition from brittle rachis to non-brittle rachis and evolution of soft glumes which can be threshed easily are some of the traits which were developed during domestication in tetraploid populations. These traits are important for harvesting and post-harvest processing (Tzarfati et al. 2014).

Wheat Genome and Sequencing

With 95% of cultivated wheat being bread wheat, it provides roughly 1/5 of the world's food (Montenegro et al. 2017). A reason for the global success of wheat as a food crop is its adaptability to a wide range of climatic conditions which is in part attributable to its allohexaploid genome structure (Matsuoka et al. 2011; IWGSC 2014). Increase in chromosome numbers and genome size during its evolution led to the massive, complicated genome of modern day hexaploid wheat. The size of its genome is estimated at ~ 17Gb, composed of three closely-related and independently maintained genomes obtained from its progenitor genomes, *T.*

urartu (A-genome donor), an unknown grass thought to be related to *Aegilops speltoides* (B-genome donor) and the goat grass species *Aegilops tauschii* (D-genome donor) (https://plants.ensembl.org/Triticum_aestivum/Info/Annotation) . Besides its huge genome size, the repetitive nature of the genome (>80% highly repetitive) and polyploid complexity are serious barriers in genome sequencing (Brenchley et al. 2012). Despite this complexity, several early efforts were made to sequence the genome using advanced next generation sequencing technologies with primary focus on its diploid progenitors (Jia et al. 2013; Ling et al. 2013). Initial efforts to sequence the bread wheat genome were directed towards individual chromosomes or chromosome arms rather than the entire genome (Shi and Ling 2018). The International Wheat Genome Sequencing Consortium (IWGSC) adopted a chromosome-based approach to overcome the genome complexity in sequencing the entire genome. IWGSC initiated the construction of bacterial artificial chromosome (BAC) libraries and physical maps of individual chromosomes or chromosome arms followed by BAC sequencing. The largest chromosome (3B ~1 Gb) was the first to be successfully flow-sorted and a physical map was constructed in 2008 (Paux et al. 2008). Using this physical map, a reference sequence of chromosome 3B was published later in 2014 (Choulet et al. 2014). Many chromosomes or arms of wheat variety - Chinese spring, have since been flow-sorted, physical maps constructed, and sequences made publicly available (Shi and Ling 2018).

The first report of whole genome sequencing of bread wheat (variety – Chinese Spring CS42) appeared in 2012 using Roche 454 pyrosequencing technology and assembled around 5.42 billion bases (Gb), approximately 1/3 of the entire genome size (Brenchley et al. 2012). This method identified about 95,000 genes that were assigned to its constituent sub genomes (A, B, and D). IWGSC (2014) published a chromosome-based draft sequence of Chinese spring that

generated a genome assembly of 10.2 Gb, approximately 2/3 of the genome size. This genome assembly was divided into hundreds of thousands of contigs with N50 sizes ranging from 1.7 to 8.9 Kb for different chromosome arms (Zimin et al. 2017). Zimin et al. (2017) later reported Triticum 3.0, a near complete assembly of bread wheat, using a combination of Illumina and Pacific Biosciences technologies with a genome assembly of approximately 15.34 Gb with a weighted average N50 contig size of 232 Kb. This assembly was also the first one to separate the D genome from the rest of the sub genomes. Most recently, ISWGSC reported yet another annotated reference sequence of the hexaploid wheat genome, IWGSC RefSeq v1.0, in the form of 21 pseudomolecule sequence assemblies with 107,891 annotated genes. This reference genome assembly is 14.5 Gb in size and has N50 contig size of 52 Kb (IWGSC 2018). Availability of a complete reference genome sequence could further enhance breeding innovations in wheat to achieve goals of food safety and sustainability.

The Disease: Fusarium Head Blight

Fusarium head blight (FHB) is a destructive disease of wheat worldwide with a devastating impact on both yield and grain quality of the crop (Del Ponte et al. 2017). The disease was first described in 1884 by W.G. Smith in England as “wheat scab” and has since been referred to as both “scab” and “head blight” (Stack 2003). Members of the *Fusarium graminearum* species complex (FGSC) are the primary etiological agents of this disease worldwide (Aoki et al. 2012; Starkey et al. 2007; Ward et al. 2008).

FHB causes two-fold damage in the infected plants: 1) reduces yield by causing shriveled, low test-weight kernels and 2) contaminates the grain with mycotoxin that has a potential of both acute and chronic toxicity in human and animals (Bertero et al. 2018). Yield reduction due to FHB is attributed to sterility of the infected spikelet, reduced kernel size and

production of ‘tombstone’ kernels (Parry et al. 1995; Wegulo et al. 2015). The mycotoxins are chemically and thermally stable thereby occurring in the entire food supply chain, from the field to end-users, causing problems to all stakeholders (Miedaner et al. 2017). Mycotoxins, especially deoxynivalenol (DON) and its derivatives; contaminated food and feed grains are subject to FDA advisory limits leading to steep price discounts, increased cost of management and growers switching to less risky crops (Dahl and Wilson 2018). Overall, yield reductions of up to 74% have been reported from studies on natural disease epidemics, fungicide trials, and inoculation studies (Wegulo et al. 2015).

History of Disease Epidemics

Wheat scab or FHB is considered a major threat to wheat and barley production worldwide. Increasing reports of disease outbreaks have been made worldwide from the time it was first described. Major wheat producers such as the European Union, China, India, Russia and the United States, which contribute more than 70% of the global wheat production (FAO 2019), have reported outbreaks of FHB making it a global issue (Brown et al. 2010). Epidemics of FHB are considered an outcome of changing weather conditions coupled with agronomic practices, pathogen virulence and lack of effective genetic resistance (Osborne and Stein 2007; Rudd et al. 2001).

The first FHB outbreak in the United States, caused by the fungus *Fusarium graminearum* Schwabe, was documented by J.C. Arthur in 1890 in Indiana (Bai and Shaner 1994; Ireta et al. 1994). Gradually, reports of the disease started to appear from Ohio (1890, 1892), Delaware (1890), Indiana (1891), Iowa (1892), Pennsylvania (1892) and Nebraska (1898) (Stack 2003).

In the United States, five major epidemics occurred between late 1910s to early 1930s (Stack 2003). The earliest records of losses due to an FHB epidemic dated 1917 showed a loss of 288,000 metric tons (10.6 million bushels) of wheat with highest losses occurring in Ohio, Indiana and Illinois (Atanasoff 1920). Estimated losses of wheat increased to 2.18 million metric tons (80 million bushels) in the disease epidemic of 1919 (Dickson and Mains 1929). Between 1928 and 1935, average annual losses for the eastern half of the U.S. (except in the extreme southern states) was estimated at 1.9 million bushels (Dickson 1942). The disease re-emerged in the 1980s in several states of the U.S. and is believed to be driven by changes in climate and agronomic practices (McMullen et al. 1997).

The disease began to occur sporadically in 1880s but by 1990's, re-emerged as an economically significant disease in wheat and barley following a series of disease epidemics (Freije and Wise 2015). Recurring epidemics in North America during the 1990s incurred over $3 billion losses in wheat and barley production (Windels 2000). Since 1997, the disease continued to have economic implications in one or more wheat market classes in most years (McMullen et al. 2012).

In China, the largest wheat producing country in the world, the first serious outbreak of FHB was reported as early as 1936 and several severe and moderate epidemics have occurred since then (Wang 1997). The disease was more frequent in the wheat growing areas along the Yangtze River valley and mountainous areas in the southwest but since 1985 it expanded to the north, southwest, and northwest winter wheat growing areas (Ma et al. 2008; Zhang et al. 2012). Between 1991 and 2007, two severe and seven moderate epidemics occurred causing large yield losses in wheat (Ma et al. 2008). Wheat grown in rotation with rice and corn is more prone to

FHB outbreaks in the wheat growing areas of China (Qu et al. 2008). In addition, climate change is believed to be responsible for more frequent epidemics in recent decades (Yi et al. 2018).

Symptoms, Disease Cycle, and Epidemiology

As the name "head blight" suggests, following infection the disease produces notable blighting symptoms on infected wheat heads of a susceptible variety. During anthesis (stage at which anthers rupture and shed pollen during flowering), wheat is most susceptible to FHB infection with susceptibility decreasing strongly after the dough stage (Champeil et al. 2004). Severe damage can result from infection during anthesis (Wegulo et al. 2013).

Initial infection appears as small, 2-3 mm water-soaked spots with dark brown margins and clear centers (bleaching) on glumes, or on the rachis of centrally flowering spikelets, or anywhere on the head (Parry et al. 1995; Wegulo et al. 2015). Warm temperatures (25°C to 30°C) with moist conditions can produce these symptoms within 2 to 4 days of infection (Wegulo et al. 2013). The bleaching symptom then spreads throughout the spike to most or all the spikelets, in all direction from the point of infection, causing the entire head to be bleached (Shaner 2003). These symptoms are confined to the wheat head, kernels and may occasionally be observed immediately below the head (peduncle), causing a brown to purplish discoloration of the stem tissue (McMullen and Stack 2008; Woloshuk and Freije 2015).

Symptomatic heads would appear as ripe wheat heads in color at a very early stage while healthy heads are still green (Atanasoff 1920). Under prolonged warm and moist conditions, light pink or salmon-orange colored spore masses, the diagnostic feature of FHB, appear on the rachis and glumes of infected spikelets (Wegulo et al. 2013). Such conditions often result in sterile spikelets or spikes that contain shrunken, lightweight, shriveled, and chalky white/pink grains, referred to as "tombstones". These symptoms often appear together with signs of the

pathogen on the glumes such as purple black perithecia and/or pink sporodochia (Osborne and Stein 2007).

In the absence of host plants in winter, the pathogen survives as chlamydospores or mycelia in the soil or on/within host crop residues. Corn, wheat and barley residues are specifically suitable for pathogen survival and proliferation, which is further enhanced with limited tillage systems (Osborne and Stein 2007; Wegulo et al. 2015). Inocula, primarily sexual ascospores and asexual conidia, are produced in the following spring when conditions are mild and dispersed by wind or rain splash. Jin et al. (2001) observed that ascospores produced in the laboratory when applied into These ascospores and conidia may remain viable for several days on leaves under field conditions (Osborne and Stein 2007). Prolonged conditions of high humidity coupled with warm temperatures during wheat flowering ensures infection in the host. FHB is a monocyclic disease, with rare secondary infections from asexual conidia (referring to the number of infection cycles in a cropping season). A short critical period (period of greatest host susceptibility to infection) in the host limits the chances of multiple infection cycles (Dill-Macky and Jones 2000). Susceptible and resistant varieties of wheat undergo very similar infection processes, however, differ in incubation period, rate of subsequent disease development and maximum disease severity (Champeil et al. 2004; Ribichich et al. 2000). The infection biology of *Fusarium graminearum* on hexaploid wheat entails: germinated fungal spores enter individual spikelets through natural openings (stomata) or by penetration; the fungus sporulates within the florets; invades the ovary and floral brackets; colonizes the rachis, and spread into the vascular system and the cortex both inter- and intracellularly, eventually collapsing the invaded phloem, chlorenchyma and occasionally parenchyma host cells (Brown et al. 2010). The incubation period varies with the fungal species associated with infection along with climatic

conditions and results in development of more conidia and perithecia on the surface of the spikelet and rachis (Champeil et al. 2004).

Integrated Disease Management

Losses due to FHB encompass two-fold damage: loss of yield (quantitative) and contamination of grains with DON (qualitative). Both losses are of major concern and therefore strategies to manage the disease require a holistic approach. Several options, such as improved cultural practices, improved cultivar resistance, chemical measures, biological control, disease forecasting models, and improved harvesting strategies are available to growers to minimize losses due to FHB. Each of these measures have varying efficacy depending upon environmental conditions during the growing season (Wegulo et al. 2015). A single control measure, however, does not provide enough reduction in disease and DON, especialy under favorable environmental conditions for the disease (McMullen et al. 2012). In the decade of 2000, Wegulo et al. (2013) conducted studies to assess the effectiveness of combining two or more strategies to manage FHB and DON, with greater emphasis on fungicide and cultivar resistance. Salgado et al. (2015) concluded that the most effective management programs integrated multiple strategies in the field, in combination with harvesting and post-harvesting procedures.

Agronomic/Cultural practices

Practices such as crop rotation, deep tillage operations, and residue management are aimed at reducing inoculum for disease initiation. Reducing or eliminating inoculum is an important aspect of FHB management as a single infection cycle could cause a disease epidemic under favorable environmental conditions. Crop rotation with broad leaf crops such as soybean has been shown to slightly reduce FHB and DON as compared to rotation with wheat or corn regardless of tillage practices (Dill-Macky and Jones 2000). Despite its significance in

minimizing FHB and DON, the economic importance of corn and cereal crops renders crop rotation impractical in the United States (Yuen and Schoneweis 2007). Efficacy of crop rotation is compromised, even in the absence of inoculum within the field, by long distance dispersion of spores, especially ascospores (McMullen et al. 2012). No-tillage and conservation-tillage practices further aggravate the disease and DON accumulation under favorable environmental conditions indicating the relative importance of deep tillage (Koch et al. 2006). Wheat grown in rotation with corn or wheat showed reduced incidence and severity of FHB with mold board plowing; however, the reduction was not significant for wheat grown in rotation with a non-host crop such as soybean (Dill-Macky and Jones 2000). In addition to crop rotation and tillage practices, residue management is also important as the pathogen can survive and produce inoculum on crop residues (wheat, barley, corn, and gramineous species) (Pereyra and Dill-Macky 2008). Mechanical chopping of crop residues could increase microbial activity thereby reducing nutritional resources for pathogen growth and hasten their decomposition rates (Yuen and Schoneweis 2007). Growers also seem to adopt strategies such as staggering planting dates or planting cultivars with differing maturing period to minimize the risk of entire crop damage due to weather favoring FHB during the critical period in wheat (McMullen et al. 2012). Overall, these cultural practices are very dependent upon weather and thus requires integration with other strategies to obtain the best control.

Chemical measures

Chemical control is an important aspect in FHB management. In the year following re-emergence of FHB in 1990s, concerted efforts were made to find effective fungicides to minimize losses due to FHB. The United States Wheat and Barley Scab Initiative (USWBSI) initiated multi-state “Uniform Fungicide Trials” (UFT) in 1997 to develop fungicides for

effectively managing FHB (McMullen et al. 2012). Fungicides of the demethylation inhibitor (DMI) class have been effective by reducing FHB and DON when applied at early anthesis (Willyerd et al. 2012). Commonly used DMI fungicides include metconazole, prothioconazole, tebuconazole and a combination of tebuconazole and prothioconazole. The efficacy of DMI fungicides however vary with environmental conditions and can reduce FHB and DON by around 50% on an average. Prothioconazole and metconazole, either alone or in combination with tebuconazole, were consistently superior than tebuconazole and propiconazole in terms of efficacy in reducing FHB and DON in both spring and winter wheat (Paul et al. 2008). Besides the role of the active ingredient, time of fungicide application also differentially affects FHB and DON (Yoshida et al. 2012). Thiophanate-methyl fungicides applied early in the anthesis were crucial in reducing FHB while fungicide applied 20 days after anthesis (DAA) was effective in reducing DON with no effect in reducing FHB. It is important here to note that significant accumulation of DON occurs later in the grain development process. In a similar study conducted with application of DMI fungicides 0-6 DAA in soft red winter wheat, significantly lower FHB and DON levels were recorded for both anthesis and post-anthesis application as compared to the untreated control (D'Angelo et al. 2014). No significant difference was observed in FHB and DON between 0 and 6 DAA application of DMI fungicide. In a more recent study conducted to evaluate the effect of infection timing on secondary metabolite accumulation in grain, inoculations done 3 DAA produced higher level of all secondary metabolites as compared to inoculation on the day of anthesis (Beccari et al. 2019). All these factors need to be considered in disease management programs to obtain the best results.

Biological measures

Besides cultural, chemical and genetic measures, strategies to utilize antagonistic microbes to manage FHB and DON have also been pursued. Potential strategies for use of bio-control agents to intervene in the fungal life cycle were aimed either at reducing inoculum production or at preventing infection, spread, and blight development (da Luz et al. 2003). Application of fungal, bacterial, and yeast strains such as *Trichoderma harzianum, Microsphaeropsis sp, Bacillus subtilis, Lysobacter enzymogenes, Cryptococcus* species and their efficacy on limiting FHB and mycotoxin production have been well documented (Fernandez 1992; Inch and Gilbert 2007; Khan et al. 2004; Khan et al 2001; Xue et al. 2009).

Trichoderma harzianum, known to have high antagonist potential against many plant pathogens, was evaluated for its ability to decrease incidence of *F. graminearum* in field grown wheat residues in Brazil (Fernandez 1992). A significantly lower incidence of *F. graminearum* in *Trichoderma*-treated wheat straw as compared to treatment control indicated the potential of *T. harzianum* in reducing saprophytic growth of *F. graminearum*. Furthermore, *T. harzianum* was shown to reduce perithecia and ascospore production under greenhouse as well as field conditions (Inch and Gilbert 2007). Similar approach of limiting saprophytic growth of *F. graminearum* was adopted using *Microsphaeropsis* species, a saprophyte well adapted to winter conditions (Bujold et al. 2001). *Microsphaeropsis* species demonstrated the capacity to decrease perithecia production, however, the reduction was not significant enough.

In a study involving in-vitro screening of numerous microorganisms collected from wheat anthers to control FHB, seven antagonists including *Bacillus* strains and *Cryptococcus* species were found to be effective depending upon the genotype of the pathogen (Khan et al. 2001). Field testing of these seven antagonists on hexaploid wheat indicated enough levels of

FHB reduction (50-60%) with yeast strains of *Cryptococcus* species (Khan et al. 2004). Greenhouse and field evaluation of antagonists identified by Khan et al. 2001 in two durum wheat cultivars indicated that *Bacillus subtilis* strains were effective against FHB in greenhouse conditions regardless of durum cultivar while yeast strains were more effective in field conditions (Schisler et al. 2002).

Host resistance

The most preferred, effective, economic, and environmentally friendly measure of FHB management is the use of resistant cultivars (Bai et al. 2018). The use of heritable resistance to manage FHB has been recognized since J.C. Arthur observed variation in susceptibility to the disease in wheat cultivars as early as 1891 (Rudd et al. 2001). Breeding for resistance to FHB in commercial wheat cultivars, however, relied solely upon phenotypic assessments for a long time until the advent of molecular markers (Steiner et al. 2017). Breeding efforts led by the USWBSI initially focused on finding resistance in new and adapted wheat germplasm and later shifted to also incorporate resistance genes from other sources into adapted material (McMullen et al. 2012). Despite these investments, no cultivar that is immune or highly resistant to FHB has been bred so far. FHB resistance has complex inheritance as it is governed by multiple genes with usually small effects and is strongly affected by environmental conditions with significant genotype-by-environment interactions (Steiner et al. 2017). Considering these complexities, the goal in breeding programs for FHB resistance has been for the development of cultivars with low disease severities and mycotoxin contamination under high disease pressure (Buerstmayr et al. 2009).

Resistance to FHB has been classified into active, passive and/or tolerance mechanisms (Mesterhazy 1995). Active resistance includes physiological mechanisms that are referred to as

type I (resistance to initial infection), type II (resistance to disease spreading), type III (resistance to DON accumulation or degradation), type IV (resistance to kernel infection), and type V (tolerance) (Mesterhazy et al. 1999). Resistance mechanisms type I, and II, as described by Schroeder and Christensen, and type III, as described by J.D. Miller, are widely accepted; however, only type II is extensively characterized as well as used in breeding programs owing to its stability and simplicity in assessment (Bai et al. 2018; Miller et al. 1985; Schroeder and Christensen 1963). Assessment of type II resistance in wheat involves inoculation of a single central spikelet in a spike with fungal conidia and estimating the percentage of infected spikelets showing head blight symptoms under controlled conditions (Bai and Shaner 2004). Screening for type I resistance on the other hand is usually done either by spraying fungal conidia over spikes or corn-spawn inoculating the field for creating natural infection, and then assessing FHB incidence (percentage of spikes infected per hill plot) (Bai and Shaner 1994). Assessment of type I resistance is considered painstaking as its accurate assessment is affected by several factors including inoculum concentration, timing of inoculation, plant height, and anther extrusion (Bai and Shaner 1994; Bai et al. 2018; He et al. 2016). More recently, type III and IV resistances have been increasingly investigated pertaining to the economic and food safety concerns (Liu et al. 2009; McMullen et al. 2012).

Passive resistance to FHB involves numerous morphological and phenological traits such as plant height, anther extrusion, days to heading, length of last internode, presence/absence of awn, and spikelet density that aid disease escape or provide some form of resistance to FHB and DON (He et al. 2016; Mesterhazy 1995). Plant height and the extent to which anther is retained after flowering were shown to have a major role in FHB resistance (Lu et al. 2013; Steiner et al. 2017). In general, shorter plants show more severe FHB symptoms (Steiner et al. 2017). Among

the semi-dwarfing alleles, *Rht-D1b* and *Rht-B1b*, are widely deployed in wheat breeding to reduce plant height. The *Rht-D1b* allele specifically, was found to be associated with high FHB severity as compared to *Rht-B1b* (Buerstmayr and Buerstmayr 2016).

Sources of FHB resistance

Resistance to FHB has been found in hexaploid wheat, but no immunity to FHB (Rudd et al. 2001). Highly resistant spring wheat accessions from Asia (China, Japan and several other countries) and Latin America and resistant winter wheat accessions from Japan, countries in Europe and the United States have been identified through routine screening of large numbers of wheat accessions (Bai and Shaner 2004; Buerstmayr et al. 2009). Introgression of resistance from these highly resistant materials into commercial cultivars has been a problem owing to linkage drag of undesired agronomic traits (Bai and Shaner 2004). 'Sumai 3', a Chinese spring wheat cultivar (Funo/Taiwanxiaomai, transgressive segregant), is reported to be an excellent source of FHB resistance with high yieldpotential (Bai and Shaner 1994). With 'Sumai 3' as a parent, Chinese breeders at Jiangsu Academy of Agricultural Science selected 'Ning 7840'; another wheat cultivar with resistance equivalent to 'Sumai 3' but better agronomic traits and additional genes for resistance to rust and powdery mildew (Bai et al. 2003). 'Sumai 3' and some of its' derivatives such as 'Ning 7840' are commonly used in many breeding programs as resistance sources due to the high heritability, stability and consistency of its FHB resistance across environments (Bai et al. 2003; Rudd et al. 2001). Several other spring wheat cultivars besides 'Sumai 3' exhibit strong resistance to FHB including 'Wangshuibai' from China, 'Shinchunaga', 'Nobeokabouzu', and 'Nyu Bai' from Japan, 'Frontana', and 'Encruzilhada' from Brazil, 'Chokwang' from Korea, and 'Fundulea 201R' from Romania (Bai and Shaner 2004; Gilbert and Tekauz 2000). Winter wheat cultivars like 'Praag 8', 'Renan', and 'Novokrumka' from Europe and cultivars 'Ernie', and 'Freedom' from the United States also

exhibit resistance to FHB and are used as parents in many breeding programs (Bai and Shaner 2004).

The fact that most of the FHB resistance could be traced back to only a few varieties urged wheat breeders to broaden the search for scab resistance to also include the wild relatives (Chen et al. 1997). High levels of FHB resistance were found in *Leymus racemosus*, *Elymus tsukushiensis*, *Thinopyrum ponticum*, *Roegneria kamoji*, and *R. ciliaris* (Cainong et al. 2015; Chen et al. 1997; Qi et al. 2008; Shen et al. 2004). The resistance conferring chromosome segments have been successfully transferred to wheat through cytogenetic techniques to achieve elevated resistance to FHB. Wheat lines with alien chromosome additions, *T. aestivum-L. racemosus*, *T. aestivum-R. kamoji*, and *T. aestivum-R. ciliaris*, even showed better FHB resistance than 'Sumai 3' (Chen et al. 1997).

Breeding for resistance to FHB in tetraploid wheat has been less successful due to limited within species genetic variation and most elite cultivars being susceptible to FHB (Steiner et al. 2019a). Durum wheat, used exclusively for human consumption, is very susceptible to this devastating disease. As a result, durum breeding programs are now trying to find resistance in related diploid and tetraploid species or to introgress resistance from hexaploid wheat (Somers et al. 2006). Some success has been achieved in identifying resistance in wild and cultivated emmer and other tetraploid wheat species (Buerstmayr 2014; Oliver et al. 2008).

Inheritance of FHB resistance

Inheritance of FHB resistance in wheat has been extensively studied and shown to be quantitative with a few major and several minor QTL across wide wheat genetic backgrounds (Buerstmayr et al. 2009). Phenotypic expression of resistance to FHB is often governed by quantitative genes with strong genotype x environment interaction (Buerstmayr et al. 2014). This significant role of environment in the expression of resistance results in reduced heritability of

the trait, thus making it hard to phenotypically assess the resistance reaction. Besides the role of the environment and the additive effects of polygenes, dominance and epistatic effectsare also observed, further complicating the inheritance and expression of resistance (Bai et al. 2000; Miedaner 1997). Mapping resistance to FHB therefore mandates high quality of phenotypic resistance data by incorporating appropriate inoculation techniques and replicated experiments across environments to identify stable QTL (Buerstmayr et al. 2014).

Molecular markers

Phenotypic markers do not always provide a precise genetic estimate as expression of a morphological trait is greatly dependent on the environment and could be altered by epistatic and pleiotropic interactions (Kumar 1999). Few phenotypic markers are available, they are less polymorphic and not associated with important economic traits which limits their use (Jiang 2013).

Cytological and biochemical markers are other classical markers besides morphological markers. Cytological markers are based on variations present in chromosomes, such as numbers, banding patterns, size, shape, order, and position while biochemical markers are based on variation in proteins produced as a result of gene expression (Nadeem et al. 2018). These markers too, are less in number, less polymorphic, are influenced by other factors including plant growth stages, and require technical knowledge thus making them less popular in breeding programs (Nadeem et al. 2018).

Molecular markers, on the other hand, are fragments of DNA in specific genomic location that serves as signposts in genome analysis. They are used for creating genetic maps showing the relative locations of genes or QTL governing the traits of our interest. Molecular markers co-segregating with the traits of interest are used to detect polymorphisms and to select genotypes with the desired trait in marker-assisted selection and breeding applications. Features

such as abundance, high level of polymorphism, even distribution across the genome, low cost, less technical knowledge requirement, non-pleiotropism, and ease of detection, depending upon the type of marker and species involved, make these markers ideal for efficient use in marker-assisted selection (MAS) (Jiang 2013). Molecular markers have been used for genetic improvement of major crops as early as the 1990s (Tanksley et al. 1989). In wheat, the use of molecular markers started with the development of a linkage map of homoeologous group 7 chromosomes in 1989 (Chao et al. 1989). Molecular markers coupled with the technique of marker-assisted selection have been successfully practiced in many parts of the world to supplement conventional wheat breeding in significantly improving wheat production and productivity (Gupta et al. 2010).

Molecular markers can be classified into three types based on the method of polymorphism detection; i) hybridization-based (non-PCR), ii) polymerase chain reaction (PCR) based, and iii) sequence based (Nadeem et al. 2018). The first DNA-based and most widely used hybridization-based molecular marker is restriction fragment length polymorphism (RFLP), first used in 1975 to identify temperature sensitive mutants of Adenovirus (Nadeem et al. 2018; Semagn et al. 2006a). Despite being highly reproducible and primarily used in wheat genetics, factors such as it being technically demanding, requirements of high quantity and quality of DNA, low level of polymorphisms, low genotyping throughput, and need for radio-active autoradiography in genotyping made the RFLP analysis approach less useful (Jiang 2013; Kim et al. 2017; Semagn et al. 2006a).

The development of the PCR technique by Kary B. Mullis in 1983 led to the development of several PCR-based molecular markers: randomly amplified polymorphic DNA (RAPD) (Welsh and McClelland 1990; Williams et al. 1990), amplified fragment length polymorphisms

(AFLPs) (Vos et al. 1995), Inter simple sequence repeats (ISSRs), microsatellites or simple sequence repeats (SSRs), cleaved amplified polymorphic sequence (CAPS), sequence characterized amplified region (SCAR), and sequence tagged site (STS) (Gupta et al. 1999; Semagn et al. 2006a). SSRs or microsatellites have been most frequently used in wheat breeding programs considering the abundance, ubiquity, high level of polymorphism, specificity, and codominance (Roder et al. 1995). The first genetic map of wheat based on 279 microsatellite markers that were evenly distributed among the linkage-groups initiated mapping in thewheat genome with better resolution for important traits (Roder et al. 1998). Later, a high-density microsatellite consensus map based on 1,235 microsatellite loci covering 2,569 cM of the bread wheat genome was made publicly available to enhance development of new molecular breeding and genomic strategies (Somers et al. 2004). More recently, Han et al. 2015 reported a total of 295,267 SSR markers (approx. 36/Mb) in Chinese Spring wheat cultivar with 224,703 (76.1%) being polymorphic indicating abundance and high level of polymorphism of SSR markers in wheat.

Low-cost DNA sequencing technologies from Sanger to next-generation sequencing (NGS) and beyond have played a great role in revolutionizing molecular approaches in plant breeding through single nucleotide polymorphisms (SNPs) (Nadeem et al. 2018). A SNP represents single base transition or transversion in the genome sequence. Unlike other markers, SNPs are more abundantly present in plants and animals, with SNP frequency ranging between 1 SNP in every 100-300bp in plants (Xu 2010). Abundance and high polymorphism levels make SNPs the most robust marker type in analyzing genomic variation and increase the probability of finding a marker close to or in the gene of interest (Xu and Crouch 2008). In addition, easy automation and amenability to high throughput make them even more attractive in genetic study

and breeding (Jiang 2013). Several different high-throughput platforms for SNP genotyping of various crop species are available with cost and objective being the deciding factor in choosing the platform for sequencing (Rasheed and Xia 2019).

Genetic Map and QTL Mapping

With the advent of molecular markers in the 1980s, screening for resistance to FHB in wheat breeding programs changed drastically to also incorporate marker-aided (genotypic) selection in addition to resistance phenotyping. Genotypic selection is based on specific markers that closely co-segregate with the trait of interest.

A genetic map (also referred to as a linkage map), gives the relative positions and genetic distances between coding and non-coding loci and landmarks such as the centromere, satellite, and telomeres on chromosomes. The process involved in determining the relative positions of loci on a chromosome is referred to as genetic mapping. Genetic mapping is based on the principle that loci in proximity on the same chromosome may segregate following crossing over between them during the meiotic phase of sexual reproduction (Semagn et al. 2006b). Thus, polymorphic markers can be used to generate genetic maps for a mapping population based on their recombination frequency to show their relative position in chromosomes. Generation of a genetic linkage map requires the use of an appropriate mapping population, which usually is a population of divergent parents and segregating progenies for the trait of interest. Bi-parental populations, including doubled haploid populations (DHs), backcrossed populations (BCs), progenies from an F_2 generation, and advanced generations of recombinant inbred lines (RILs) can be used for this purpose. Genetic/linkage maps generated based on different mapping populations may vary in terms of positions and distances for the same markers used. For a

precise and more accurate reference of markers and QTL position, genetic maps based on different populations can be combined to produce a consensus map.

Almost 500 QTL associated with FHB resistance types I- IV have been mapped on all 21 chromosomes of hexaploid wheat in different studies from North America, South America, Asia, and Europe (Buerstmayr et al. 2019). Despite being detected in individual studies, only a few are stable across several studies and successfully employed in breeding programs (Buerstmayr et al. 2009). This inconsistency could be explained in part by the nature of plant material used, such as genetic background, magnitude of difference in resistance between the parents, and sources of resistances utilized; the species of pathogen used in the study, such as *F. graminearum or F. culmorum*; the type of resistance being evaluated, such as type I or type II; the variation in techniques employed, such as spray inoculation, spikelet injection; and finally the evaluation of strong genotype x environment interaction in the phenotypic expression (Kolb et al. 2001).

Of the numerous QTL genetically mapped, seven major QTL have been formally assigned gene symbols, i.e. *Fhb1*, *Fhb2*, *Fhb3*, *Fhb4*, *Fhb5*, *Fhb6*, and *Fhb7* where QTL *Fhb3*, *Fhb6*, and *Fhb7* are derived from alien species (Bai et al. 2018).

Fhb1, derived from 'Sumai3', is the most stable QTL with the largest effect among all for type II resistance and was mapped on the short arm of chromosome 3B (Cuthbert et al. 2006; Su et al. 2019; Waldron et al. 1999). Previously designated as *Qfhs.ndsu-3BS*, *Fhb1* was mapped as a single Mendelian locus between two STS markers spanning a genetic distance of 1.2 cM (Liu et al. 2006). The critical region was further narrowed to 0.08 cM between STS markers STS3B-355 and STS3B-334 (Liu et al. 2008). Owing to its large effect and stability across a wide range of genetic backgrounds and environments, *Fhb1* has by-far been the most extensively utilized QTL for type II resistance in most wheat breeding programs and was targeted for fine mapping

and map-based cloning of the underlying genes (Rawat et al. 2016; Schweiger et al. 2016). A candidate gene in the QTL region, *PFT*, was characterized and thought to be *Fhb1* (Rawat et al. 2016). Further studies to characterize *PFT* in 348 wheat accessions, however, only partially confirmed its role in FHB resistance as the same gene also existed in susceptible wheat accessions (He et al. 2018).

Two recent studies focusing on map-based cloning of *Fhb1* have led to the identification of a histidine-rich calcium-binding gene '*His'* (syn: *TaHRC*) that is believed to confer FHB resistance (Li et al. 2019; Su et al. 2019). '*His'* is a conserved gene in grass species with a nuclear localization signal. In both studies, a large deletion in the '*His'* gene encoding a histidine-rich calcium-binding protein was shown as the causative mutation giving rise to resistance to FHB. Despite the predicted involvement in resistance conferred by *Fhb1*, the presence of a deletion in the resistance allele is contradictory. (Lagudah and Krattinger 2019).

Apart from *Fhb1,* three other named genes for FHB resistance have been reported in wheat germplasm: *Fhb2, Fhb4,* and *Fhb5.* These genes have been fine mapped, and markers tightly linked to them have been identified (Cuthbert et al. 2007; Liu et al. 2010; Steiner et al. 2019b; Xue et al. 2010; Xue et al. 2011). *Fhb2,* mainly conferring type II resistance, is located on the short arm of chromosome 6B and has been confirmed in numerous experiments along with *Fhb1* (Bai et al. 2018; Buerstmayr et al. 2009; Cuthbert et al. 2007). *Fhb4* and *Fhb5,* located on chromosomes 4B and 5A respectively, were identified from 'Wangshuibai' conferring type I resistance against FHB (Lin et al. 2006; Xue et al. 2010; Xue et al. 2011). *Fhb2* along with *Fhb4* and *Fhb5* identified from Asian spring wheat resources have also been detected in resistant resources from other geographic origins and were shown to exhibit varying levels of resistance to

FHB (Bai et al. 2018; Buerstmayr et al. 2009). These three genes, however, have been associated with type I or II resistance depending upon the resistant resources used (Bai et al. 2018).

The remaining designated genes, *Fhb3, Fhb6,* and *Fhb7*, are derived from wild relative species of wheat. *Fhb3*, conferring type II resistance, was identified in the tetraploid species *Leymus racemosus* and successfully transferred to chromosome 7A of hexaploid wheat through cytogenetic manipulation (Qi et al. 2008). *Fhb6*, also conferring type II resistance, was transferred to wheat chromosome 1A through homoeologous recombination involving another distant wild relative of bread wheat, *Elymus tsukushiensis* (Cainong et al. 2015). Like *Fhb3* and *Fhb6*, *Fhb7* confers FHB type II resistance and was derived from wheat grass *Thinopyrum ponticum* chromosome $7el_2$ (Shen et al. 2004; Shen and Ohm 2007). Recently, Wang et al. (2020) cloned and characterized the gene *Fhb7* that encodes a glutathione S-transferae (GST) based on genome assembly of *Thinopyrum elongatum*. The enzyme GST was shown to be involved in biochemical detoxification of deoxynivalenol, thereby conferring FHB resistance. Interestingly, the *Fhb7* GST homologs were absent in plants and instead shared 97% identity in the genome of *Epichloë* species, an endophytic fungus of temperate grasses, thereby suggesting a fungus-to-plant horizontal gene transfer of *Fhb7*.

Despite the high level of resistance to FHB in the alien species, these resistance genes when used in conjunction with major QTL *Fhb1* failed to raise resistance and therefore needs to be evaluated more before their use in breeding programs (Bai et al. 2018; Guo et al. 2015; Salameh et al. 2010).

Breeding for Resistance to FHB

Several QTL for FHB resistance have been identified from diverse gene pools and were subsequently shown to give additive effects implying that gene pyramiding to achieve a high

level of resistance is feasible (Kolb et al. 2001; Rudd et al. 2001). Conventional as well as marker-assisted (MAS) breeding strategies have been adopted to efficiently introgress such loci in wheat breeding.

MAS for *Fhb1* has been successfully applied in locally adapted spring and winter wheat breeding programs since the introduction of 'Sumai3' (Rudd et al. 2001). Before 'Sumai3', cultivars 'Frontana' and 'Nobeokabouzu' from Brazil and Japan, respectively, were extensively used (Rudd et al. 2001; Zhu et al. 2019). 'Frontana' confers a moderate type I resistance, as compared to type II resistance conferred by 'Sumai3' with no known large-effect QTL (Buerstmayr et al. 2009). Besides the exotic sources of resistances, several locally adapted winter wheat cultivars with moderate resistance to FHB have also been identified. Soft winter wheat (SWW) cultivars such as 'Ernie', 'Massey', 'Freedom', 'Truman', and 'Roane', and hard winter wheat (HWW) cultivars such as 'Everest', 'Overland', 'Lyman', 'Heyne', and 'Hondo' contribute to this list (Bai et al. 2018; Liu et al. 2013; Rudd et al. 2001). Use of QTL from these resources together with exotic sources could further enhance resistance to FHB.

Transgenic/genetically engineered wheat expressing genes to target the virulence factor 'DON' and its metabolic pathways or defense-response genes have also been tested as an alternative approach to confer resistance to FHB. An experiment aimed at DON metabolism in wheat using a trichothecene acetyltransferase gene from *Fusarium sporotrichioides* conferred moderate levels of resistance to the disease in greenhouse tests but was ineffective in the field (Okubara et al. 2002). In another study, a transgenic wheat line expressing high levels of a combination of two defense response genes: β-1,3-glucanase and chitinase, was linked to a significant increase in scab resistance while other lines expressing both transgenes or a line expressing only β-1,3-glucanase at lower-levels showed no improvement in disease resistance

(Anand et al. 2003). In a later study, however, transgenic wheat lines expressing either of these defense response genes showed enhanced resistance to FHB (Mackintosh et al. 2007; Shin et al. 2008). Di et al. (2010) used transgenic wheat lines expressing a truncated yeast L3 ribosomal protein, a target of DON to inhibit protein translation, to show improved FHB resistance. Since then, several studies to demonstrate the role of transgenic wheat in improving resistance against FHB have been documented (Cheng et al. 2015; Li et al. 2017; Li et al. 2015). Despite these studies, transgenic wheat for improved resistance to FHB is still not available for breeding (Bai et al. 2018).

The Pathogen: *Fusarium graminearum*

History, Taxonomy, and Classification

The ascomycete genus *Fusarium* (Nectriaceae, Hypocreales, Sordariomycetes) includes many species that are pathogenic to many agriculturally important crops. Many of them produce toxic metabolites that have detrimental effect on humans and animals. The generic concept of *Fusarium* was first described by Johann H. F. Link, the director of the Botanic Garden in Berlin, as having the characteristic fusiform macroconidia produced by the anamorphic stage of the fungus in 1809 (Desjardins 2006). Later in 1821, Swedish mycologist/botanist Elias M. Fries described the sexual stage (teleomorph) *Gibberella* and validated the genus in terms of the International Code of Botanical Nomenclature (ICBN). The fungus *Fusarium graminearum* was isolated from *Zea mays* by the American mycologist Lewis David von Schweinitz in 1822 (Desjardins 2006). *Fusarium graminearum* was described as the asexual stage of *Gibberella zeae* by Schwabe in 1838 (Stack 2003).

The taxonomy of *Fusarium* has been unstable with a long and complicated history. The observation of key characteristics of fusiform macro-conidia associated with different hosts led

to the identification of over 1000 species of *Fusarium* with no clear distinction between each other until the end of 19th century (Stack 2003: Toussoun and Nelson 1975). In 1935, Wollenweber and Reinking published a remarkable book in taxonomic studies of *Fusarium*, "*Die Fusarien",* and organized over 1,100 published species of *Fusarium* into 65 species, 55 varieities, and 22 forms (Aoki et al. 2014; Lidell 2003). This organization was based on morphological features such as the presence/absence of microconidia, chlamydospores, intercalary/terminal chlamydospores, shape of macroconidia and the shape of basal foot cells on macroconidia (Nelson 1991). Despite the comprehensive description, this system did not receive much attention outside of Europe as it demanded microscopic study involving detailed measurements of conidia and additional morphological features (Aoki et al. 2014). Snyder and Hansen (1945) further reduced the number of *Fusarium* species to nine, solely based on the morphology of the macro-conidia using single-spore derived cultures (Nelson 1991). These two systems continued to operate in parallel to each other in defining species of *Fusarium* until mid-1980s when more than just morphological features were used to discern *Fusarium* species (Summerell 2019).

Biological species recognition, based on cross fertility between isolates, began to appear in *Fusarium solani* and *Fusarium moniliforme* as early as 1970. The biological species concept however holds little significance in *Fusarium* taxonomy as it could not be applied to the homothallic or asexual species (Leslie et al. 2001). The phylogenetic species recognition approach, that followed the biological species concept, usually utilizes DNA sequence data to identify differences between species, which are unresolved by morphological and biological methods (Aoki et al. 2014; O'Donnell et al. 1998).

Fusarium graminearum, the causal agent of Fusarium head blight in wheat, was formerly classified into two groups namely Group 1 and Group 2 by Burgess et al. (1975). Group 2 consisting of the *Fusarium graminearum* population, is different from Group 1, the *Fusarium pseudograminearum* population, in terms of their ability to produce perithecia on carnation leaf agar media (Aoki and O'Donnell 1999). Until 2000, the *Fusarium graminearum* population was considered a single species causing FHB worldwide with variation in terms of virulence and type of trichothecene produced. However, genealogical concordance phylogenetic species recognition (GCPSR) using DNA sequences from portions of 12 genes revealed 16 phylogenetically distinct homothallic species associated with geographic isolation associated with *Fusarium graminearum* (O'Donnell et al. 2000; O'Donnell et al. 2004; Sarver et al. 2011; Starkey et al. 2007; Yli-Mattila et al. 2009). GCPSR has been applied successfully to several fungal species including *Gibberella fujikuroi* and proven to be effective in detecting species not differentiated by the morphological or biological species recognition criteria (Taylor et al. 2000). The 16 phylogenetically related species are *Fusarium austroamericanum* (lineage 1), *Fusarium meridionale* (lineage 2), *Fusarium boothi* (lineage 3), *Fusarium mesoamericanum* (lineage 4), *Fusarium acacia-mearnsii* (lineage 5), *Fusarium asiaticum* (lineage 6), *Fusarium graminearum* sensu stricto (lineage 7), *Fusarium cortaderiae* (lineage 8), *Fusarium brasilicum, Fusarium aethiopicum, Fusarium gerlachii, Fusarium vorosii, Fusarium ussurianum, Fusarium louisianense,* and *Fusarium nepalense* (van Der Lee et al. 2015). These 16 species all together are now referred to as the Fusarium Graminearum Species Complex (FGSC).

Prior to 2011, the International Code of Botanical Nomenclature (ICBN) Article 59 allowed the use of two names for one species of the fungus with a priority to the use of the sexual stage name. However, changes adopted in the new International Code of Nomenclature

for algae, fungi, and plants (ICN, Melbourne Code) as of January 1, 2013 dictated unification of pre-existing anamorph and teleomorph names. The use of *Fusarium* over other teleomorph names have been supported by plant pathologists and other applied biologists.

Pathogen Biology

Fusarium graminearum (teleomorph *Gibberella zeae*) is a haploid ascomycete fungus that has both sexual and asexual life cycles.

Sexual reproduction

Fusarium graminearum is a haploid ascomycete fungus in the sordariomycetes class characterized by a flask-shaped fruiting body (usually perithecium) that bears a sac like structure (ascus) in which sexual spores (ascospores) are formed. As a homothallic ascomycete, *Fusarium graminearum* is self-fertile meaning each isolate possesses both mating type idiomorphs (*MAT1-1* and *MAT1-2*) in its haploid genome allowing production of ascospores. Outcrossing within strains is uncommon in nature but outcrosses have been made in culture within strains or between species belonging to the species complex (Goswami and Kistler 2004).

The fungus survives saprophytically on crop debris of wheat and corn giving rise to purplish-black perithecia that plays an important role in the survival of the pathogen (Sutton 1982). The formation of perithecia has been observed at temperatures between 2°C and 20°C while the optimal temperature range for maturation of perithecia is between 15°C and 28.5°C even if the fungus survives at temperatures below freezing point (Leplat et al. 2013). Curved, fusiform, and hyaline ascospores form within sacs called asci and are forcibly discharged through a pore at ascus tip (ostiole) due to the development of turgor pressure within the ascus (Khonga and Sutton 1988). The ascospores are septate, hyaline, uniform in size (17-25.5 x 3-5

μm) and constitute the primary inoculum that causes primary infection in flowering wheat heads (Sutton 1982).

Asexual reproduction

Asexual reproduction in *Fusarium graminearum* is facilitated by macro-conidia produced from mycelium on the surface of infected plants or crop residues during moist periods (Trail 2009). Unlike other species of *Fusarium*, micro-conidia are absent while chlamydospores are rare and may form in the macro-conidia. Macro-conidia are canoe shaped, hyaline in color, and septate (3-7) with size ranging from 25-50 x 3-4 μm borne on pedicellate foot cells called phialides clustered together in masses known as sporodochia. They constitute the primary source of inoculum and serve as inoculum for secondary infections.

Mycotoxins

Mycotoxins, which are toxic compounds, are secondary metabolites produced by some fungi, including *Fusarium*, that have no direct involvement in growth, development, or reproduction of the organism. *Fusarium* species produce a range of mycotoxins that have detrimental effects on human and animal health (Summerell 2019; Desjardins et al. 2007). Of all, trichothecenes are most strongly associated with chronic and fatal toxicity in humans and animals (Amarasinghe et al. 2019; Desjardins et al. 1993). The toxicity due to trichothecenes result from inhibition of protein translation in eukaryotes (Kimura et al. 2007).

The members of the Fusarium Graminearum Species Complex (FGSC) can produce diverse group of mycotoxins, including type B trichothecenes and zearalenones. The zearalenones show estrogenic activity, causing infertility, abortion or other breeding problems primarily in swine. The type B trichothecenes and its variants produced by the members of FGSC, such as deoxynivalenol (DON) and its acetylated forms, are of the greatest concern in

wheat and barley growing regions of the world (Desjardins 2006). Besides its toxicity in humans and animals, DON acts as a virulence factor in causing FHB and enables the fungus to spread further into the rachis (McCormick et al. 2011; Proctor 1995). Based on the strain-specific type B trichothecene primarily produced, the members of the FGSC complex are divided into three genotypes (chemotypes) namely: nivalenol (NIV), 3acetyl-deoxynivalenol (3ADON), and 15acetyl-deoxynivalenol (15ADON) (Ward et al. 2008).

The biosynthesis of type B trichothecenes in *Fusarium graminearum* involves a complex pathway beginning at cyclization of farnesyl pyrophosphate and culminates in the formation of nivalenol, deoxynivalenol, or their acetyl derivatives (Brown et al. 2002). The pathway involves a series of enzymatic and non-enzymatic oxygenation, isomerization, hydroxylation, acetylation, and esterification reactions to form nivalenol, deoxynivalenol and their acetyl derivatives (McCormick et al. 2011). The enzymatic reactions are catalyzed by enzymes produced by multiple genes at three independently segregating loci in the *Fusarium graminearum* genome (Brown et al. 2003; Desjardins 2006). The largest contiguous sequence involved in the biosynthetic pathway, referred to as the *Tri* gene cluster or *Tri5* gene cluster, spans 26 Kb and harbors 12 coordinately expressed genes (Brown et al. 2002). The second locus consist of two genes, *Tri1* and *Tri16,* located adjacent to each other in a 2-gene mini-cluster while the third locus consists of a single gene *Tri101* (Brown et al. 2003; Kimura et al. 1998).

Fusarium graminearum Genome

The genome of the filamentous fungus *Fusarium graminearum* strain NRRL 31084 (PH-1, originally isolated from Michigan) was originally sequenced using Sanger technology at the Broad Institute, Center for Genome Research (www.broad.mit.edu/annotation/fungi/*Fusarium/*) (Cuomo et al. 2007). With the help of the genetic map, the genome assembly was anchored to

four large chromosomes with an approximate whole genome size of 36.1 Mb. However, this genome assembly was not complete with over 212,843 unknown bases and missing centromeric and telomeric sequences (King et al. 2015). Despite being incomplete, this assembly served as a basis for aligning and assembling genomes of four other plant pathogenic species of *Fusarium*: *F. fujikuroi* B14, *F. verticilloides*, *F. oxysporum* f. sp. *Lycopersici*, and *F. pseudograminearum* (CS3096 & CS3005) (King et al. 2015). The Munich Information Services for Protein Sequences (MIPS) later produced a comprehensive *Fusarium graminearum* Genome Database (FGDB) after manually curating the 11,640 gene models predicted by the Broad Institute (Güldener et al. 2006; Wong et al. 2011). This improvement reduced most gaps due to unknown bases; however, four centromeres still had no sequence and over 99,000 bases remained unassembled in small contigs (King et al. 2015).

To provide a more complete version of *Fusarium graminearum* genome, King et al. 2015 resequenced the PH-1 strain using whole genome shotgun re-sequencing to 85-fold coverage. This re-sequencing produced a nearly complete *Fusarium graminearum* genome (RRes v4.0) of 36,563,796 bp assembled into four chromosomes along with a mitochondrial genome. This version of the reference genome narrowed down the gaps to two and resolved the telomeres and centromeres (King et al. 2017). King et al. 2017 updated the RRes v4.0 with significant improvements to the annotation resulting in 14,145 genes with predicted protein function.

Pathogenicity and Virulence Factors in *Fusarium graminearum*

Pathogenicity is defined as the ability of a pathogen to cause disease while virulence is the degree of pathogenicity of a given pathogen (Agrios 2005). Mutagenic studies in *Fusarium graminearum* have led to the identification and confirmation of several virulence factors.

Trichothecenes are known to play a crucial role in virulence as shown in several studies conducted using trichothecene-deficient mutants (Bai et al. 2002; Desjardins 1996). When the *TRI5* mutant of fungal strain GZ3639 was evaluated for virulence on wheat, its presence was limited to the inoculated spikelet and it did not produce DON in the inoculated wheat spike (Bai et al. 2002). Results like that obtained with *TRI5* deletion mutants were also reported with the *FGL1* deletion mutant of *F. graminearum* following infection of wheat (Ilgen et al. 2008; Voigt et al. 2005). *FGL1* encodes a secreted lipase enzyme and is induced by lipid-containing substrates during plant infection. Wild-type conidia supplemented with a known lipase inhibitor showed significantly reduced virulence in wheat (Voigt et al. 2005). Jansen et al. (2005) showed that the development of strong cell wall fortifications in the rachis node, a defense inhibited by the mycotoxin, prevented the movement of *TRI5* mutants into adjacent spikelets indicating the role of *TRI5* in virulence. Apart from genes involved in trichothecene biosynthesis, *TRI14*, another gene in the core cluster, was also shown to be required for high virulence on wheat (Dyer et al. 2005). However, unlike *TRI5* deletion mutants, the FHB symptoms generated by *TRI14* deletion mutants were more likely observed in the neighboring spikelets.

Mitogen-activated protein (MAP) kinase genes mediate signal transduction in eukaryotic organisms and has been shown to regulate various biological processes such as mating and filamentation, hyphal growth, conidiation, and conidial germination including pathogenesis in fungal pathogens (Xu 2000). In *F. graminearum* strain 8/1, MAP kinase gene deletion mutants (Δ*gpmk1*) were unable to produce FHB symptoms on inoculated wheat spikes and cause the kernels to degenerate as the wild-type strain does, indicating that *Gpmk1* is a pathogenicity factor on wheat (Jenczmionka et al. 2003). In addition, the *Gpmk1* deletion mutants showed reduced conidial production and were sexually sterile.

Urban et al. (2003) used six independent *MAP1* deletion mutants of *F. graminearum,* homologous to pathogenicity MAP kinase gene (PMK1) in *Magnaporthe grisea,* to demonstrate that inactivation of the MAP1 dependent signaling pathway inhibits pathogenicity on wheat. The *MAP1* mutants were still able to produce toxin; however, the amount of toxin recovered was two-fold lower than from wild-type infected wheat. This adds to the fact that *MAP1* mutants are unable to cause infection not because of lack of toxin production (Urban et al. 2003). No effect was observed in the ability of mutants to produce asexual spores.

With increasing interest in the roles of protein kinase (PK) genes in various developmental and pathogenicity traits of fungal pathogens, Wang et al. (2011) generated deletion mutants for 96 PK genes in the *F. graminearum* genome. Forty two of the 96 PK genes, including *Gpmk1,* were found to have significant roles in virulence or pathogenicity.

Seong et al. (2005) used restriction enzyme-mediated integration approach to generate random insertional mutants, of which 11 were associated with pathogenicity of the *F. graminearum* PH-1 strain. Mutants with disrupted *CBL1* and *MSY1* genes, encoding cystathionine β-lyase and methionine synthase respectively, were severely impaired in terms of virulence on flowering wheat heads. Both mutants displayed a methionine auxotrophic phenotype indicating that methionine synthesis is an important virulence factor in *F. graminearum.* Furthermore, putative b-ZIP transcription factor *ZIF1,* unique to filamentous fungi and tranducing beta-subunit-like protein gene *TBL1,* highly conserved among filamentous fungi, were also identified as virulence factors in *F. graminearum* based on gene complementation studies.

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MOLECULAR MAPPING OF QUANTITATIVE TRAIT LOCI FOR FUSARIUM HEAD BLIGHT RESISTANCE IN BRAZILIAN SPRING WHEAT CULTIVAR 'SURPRESA'

Abstract

Fusarium head blight (FHB) is a devastating disease in wheat and use of resistant germplasm from diverse sources can significantly improve resistance to the disease. 'Surpresa' is a Brazilian spring wheat cultivar, which exhibits moderate resistance to FHB and is of origin different from currently used sources. To identify quantitative trait loci (QTL) for resistance to disease spread within a spike (type II resistance) and deoxynivalenol (DON) accumulation (type III resistance) in Surpresa, 187 recombinant inbred lines (RILs) were developed from the cross between Surpresa and a susceptible wheat cultivar 'Wheaton'. The population was evaluated by point-inoculation method in three field and three greenhouse experiments. Mean disease severity for Surpresa and Wheaton were 41.2% and 84.90%, ranging between 30.30-59.09% and 74.32-91.37% respectively. Mean FHB severity of the population was 57% with an overall range between 7 to 100%, suggesting transgressive segregation in the population. The population was genotyped using a two-enzyme genotyping-by-sequencing approach and a genetic map was constructed with 5,431 single nucleotide polymorphisms (SNPs). Four QTL for type II resistance were detected on chromosomes 2A (2 loci), 3B, and 4D, respectively, explaining 11-15.8% of the phenotypic variation. No significant QTL were detected for DON accumulation. The largest effect QTL was mapped on chromosome 4D and explained 15.8% phenotypic variation, however, it co-localized with a QTL conferring plant height. The QTL detected on chromosome 3B is different from *Fhb1* and other previously detected loci and may be novel. FHB resistance identified in Surpresa may diversify the FHB resistance gene pool and increase overall resistance to the disease.

Introduction

Fusarium head blight (FHB) is a destructive disease of wheat worldwide. It is primarily caused by the fungus *Fusarium graminearum* in North America, with a devastating impact on grain yield and quality (Del Ponte et al. 2017). FHB outbreaks occur when warm and moist conditions persist at wheat anthesis, resulting in sterile spikelets or shrunken, lightweight, shriveled, and chalky white/pink grains, referred to as 'tombstones' (Wegulo et al. 2013). Besides the impact on yield, DON contamination of the grains poses the risk of both acute and chronic toxicity in humans and animals (Bertero et al. 2018). DON contaminated food and feed grains are subject to FDA advisory limits and contaminated grains lead to steep price discounts and increased cost of disease management causing growers to switch to a less risky crop (Dahl and Wilson 2018). Yield reductions due to FHB were estimated to be as high as 74% based on natural disease epidemics, fungicide trials and inoculation studies (Wegulo et al. 2015). Integrated pest management incorporating two or more control strategies with greater emphasis on the use of cultivar resistance and effective fungicides has been evaluated since the early 2000s. The most effective management program was one that integrated multiple strategies in the field, harvesting and post-harvesting procedures (Salgado et al. 2014; Wegulo et al. 2013). Individual strategies have varying levels of efficacy in reduction of disease severity and DON accumulation especially when the environmental conditions are favorable for disease development (McMullen et al. 2012; Wegulo et al. 2015).

Host resistance to FHB is a complex quantitative trait that is usually governed by small effect quantitative trait loci (QTL) and is strongly affected by environmental conditions (Steiner et al. 2017). No source of immunity to FHB has been discovered so far, although sources conferring partial FHB resistance have been identified through extensive germplasm evaluations.

Host resistance to FHB has been classified into active, passive and/or tolerance mechanisms (Mesterhazy 1995). Active resistance mechanism includes physiological processes and five types of active mechanisms have been described: type I (resistance to initial infection), type II (resistance to fungal spread within spike), type III (resistance to toxin accumulation / ability to degrade toxin), type IV (resistance to kernel infection), and type V (tolerance to yield loss) (Mesterhazy et al. 1999; Miller et al. 1985; Schroeder and Christensen 1963). However, only type II resistance is extensively characterized as well as used in breeding programs owing to its stability and easy assessment. Passive resistance to FHB involves numerous morphological and phenological traits such as plant height, days to heading, anther extrusion, length of last internode etc. that aid in disease escape or some form of resistance to FHB and DON (He et al. 2016; Mesterhazy 1995). Plant height and extent of anther retention after flowering are primarily shown to have a major role in resistance to FHB (Lu et al. 2013; Steiner et al. 2017). In general, shorter plants are observed to incur more severe FHB infection (Steiner et al. 2017). The semi-dwarfing alleles, *Rht-D1b* and *Rht-B1b,* are widely deployed in wheat breeding to reduce plant height. The *Rht-D1b* allele specifically was found to be associated with more severe FHB symptoms as compared to *Rht-B1b* (Buerstmayr and Buerstmayr 2016).

More than 250 QTL distributed on the 21 chromosomes of wheat have been reported to contribute different types of FHB resistance (Buerstmayr et al. 2009; Liu et al. 2009). Genetic variation in wheat gene pools from diverse geographic regions has been a valuable resource to detect such resistances and create locally adapted cultivars with elevated resistance to FHB (Buerstmayr et al. 2014). ‘Sumai3’, a Chinese spring wheat cultivar, is by far the best source of FHB resistance (Zhu et al. 2019). Sumai3 contains *Fhb1,* which confers the largest effect QTL for type II resistance (Bai and Shaner 1994; Buerstmayr et al. 2009). In addition, Sumai3 and its

derivatives have been shown to accumulate low DON levels in the kernels of infected spikes further implying the importance of this resistance source (Bai 2001). *Fhb1* is being used widely in spring-wheat breeding and is being used with increasing popularity in winter wheat (Bai et al. 2018; Shah et al. 2018). Six other QTL besides *Fhb1* have been formally assigned a gene name: *Fhb2, Fhb4,* and *Fhb5* derived from wheat and *Fhb3, Fhb6,* and *Fhb7* derived from alien species (Bai et al. 2018). Several QTL have been shown to have additive effects implying the feasibility of their pyramiding in locally adapted cultivars to achieve high levels of FHB resistance (Bai et al. 2018; Clark et al. 2016; Kolb et al. 2001; Rudd et al. 2001; Salameh et al. 2011). FHB resistance detected in locally adapted cultivars, on the other hand, seems to involve multiple genes with minor effects and largely unknown genetics thereby limiting their use in wheat breeding (Clark et al. 2016). Sources of FHB resistance that are most frequently used in current wheat breeding programs can be traced back to only a few parents, including Sumai3 and its derivatives (Bai and Shaner 2004; Buerstmayr et al. 2009; Chu et al. 2011). Deployment of only one or a few sources of resistance over large crop production areas poses vulnerability to resistance breakdown and severe disease epidemics. Therefore, in order to enhance and diversify FHB resistance in wheat, QTL analysis of more diverse resources to identify novel loci for FHB resistance is essential.

'Surpresa' is a Brazilian spring wheat cultivar characterized as having moderate resistance to FHB and DON accumulation and occurs in a collection of common wheat accessions in the National Small Grains Collection (NSGC) (Zhang et al. 2008). It was developed from a cross between cultivar Alfredo Chaves-6-21 and the cultivar Polyssu to withstand aluminum toxicity in acid soils of Brazil (Rajaram et al. 1988). Understanding the genetic basis of FHB resistance in Surpresa will be useful in wheat breeding programs

considering its unique pedigree and promising resistance to FHB and DON. Therefore, the objective of this study was to identify novel QTL for resistance to FHB in Surpresa.

Materials and Methods

Plant Materials

To detect and map QTL for resistance to FHB in Surpresa (PI 185843), a bi-parental mapping population containing 187 recombinant inbred lines (RILs) ($F_{2:7}$) was developed from the cross between Surpresa and the FHB-susceptible spring wheat cultivar Wheaton (PI 469271) using the single-seed descent inbreeding method.

Phenotypic Evaluation

The RILs, parents and checks were evaluated for reaction to FHB and related agronomic traits in three greenhouse and four field-experiments between 2016 and 2018. Alsen (PI 615543), known to have *Fhb1,* was used as resistant check in all experiments. Greenhouse evaluations were conducted in three growing seasons: fall of 2016 and 2017 (16GH and 17GH), and winter of 2018 (18GH). In each greenhouse experiment, the RILs and parents were grown in 6-inch clay pots filled with potting mix (Pro-mix biofungicide; Premier Tech Horticulture, Quakertown, PA) and supplemented with slow-release fertilizer (Osmocote Plus 15-9-12 N-P-K plus minors; Everris Inc., Dublin, OH) after planting. The pots were arranged on greenhouse benches in a completely randomized design (CRD) with three replications (pots) per line. The greenhouse was supplemented with artificial light for a 14 h photoperiod and temperature maintained between 20 and 22°C during early crop growth period (before anthesis). The inoculum was prepared using four pathogenic isolates collected from North Dakota (two isolates each producing 3ADON and 15ADON) and maintained at a concentration of 100,000 spores per mililitres (mL) (Puri and Zhong 2010). FHB inoculations were performed at or after 50% flowering, by injecting 10µl of

spore suspension into a floret in the central spikelet using a syringe (Stack et al. 2002). 8-10 spikes from each pot were inoculated. The inoculated spikes were then covered with a 13 cm transparent polyethylene bag with light misting for 48 h to provide high humidity. The inoculated plants were then maintained at 22-24°C in the greenhouse to ensure proper disease development.

All field evaluations were conducted in the FHB nursery located in Fargo, ND. The field experiments were conducted in the summer of 2016 (16FAR), 2017 (17FAR), and 2018 (18FAR) with arrangements for overhead misting to maintain optimum relative humidity. The RIL population and their parents were evaluated using a randomized complete block design (RCBD) with two replications in 16FAR and four replications in 18FAR. The number of heads inoculated per hill was low in 16FAR as the heads in each replication did not flower at the same time. To mitigate the effects of this problem in 17FAR, the RILs were planted in short rows of 6 feet with one replication allowing approximately 20-25 heads to be inoculated at the same time. We increased the number of replications in 18FAR to achieve the same goal as the space was not limited in 2018.

In the 2016 and 2018 field experiments, each line was planted with 10-15 seeds per hill. At 50% flowering stage, 8-10 spikes at similar developmental stage in a hill were inoculated into a floret in the central spikelet using a syringe as described above. For the field experiment in 2017, 30-40 seeds were planted in each short row. Approximately 20-25 heads from each row (each row is a line) were inoculated. The overhead misting was set up to for 5 min in 3 h intervals for 12 hours overnight (6:00 P.M. to 6:00 A.M.), until 14 days after inoculating the latest maturing lines.

In 2018, the RILs and parents were also assessed for type II FHB resistance using corn-spawn inoculation as described in Chu et al. (2011). For corn-spawn inoculation, autoclaved corn seeds infected with 15 fungal cultures: 5 producing nivalenol, 5 producing 3 acetyl-deoxynivalenol, and 5 producing 15 acetyl-deoxynivalenol and evenly broadcasted in the field three-weeks prior to initial flowering.

FHB severity was assessed 21 days post inoculation in all greenhouse and field experiments by visual assessment using a modified 1-9 Horsfall-Barrett disease rating scale with 9 categories of infection, to reflect 0, 7, 14, 21, 33, 50, 67, 80, and 100% of disease severity (Stack and McMullen 1998). The disease severity for each replication was calculated by averaging severities of all inoculated heads.

In addition, DON accumulation was assessed on one random 2.5g ground grain sample of each line from the 2018 greenhouse and field experiments. All inoculated heads of each line from point-inoculated experiments were harvested at maturity, all replications combined, threshed carefully to keep all the seeds and tombstones, ground into fine powder, and sent to USWBSI supported laboratory for DON analysis. Similarly, all heads from each replication of individual line inoculated by corn-spawn were harvested and processed for DON accumulation analysis.

Agronomic and phenological traits known to be associated with FHB reaction such as flowering date (days to flowering after planting) and plant height (PH) were also recorded to determine their relationship to FHB resistance. PH was measured from soil surface to the tip of spike (excluding awns) from 18FAR experiments only.

DNA Extraction and Two Enzyme Genotyping-by-Sequencing

Fresh leaf tissue of plants at the 2-3 leaf stage were collected in 96-deepwell plates, freeze dried using liquid nitrogen, and ground using a QIAGEN TissueLyser. The DNA extraction method of Tai and Tanksley (1990) was followed. The genomic DNA was then quantified with a Quant-iT PicoGreen assay kit (P7589; Thermo Fisher Scientific) and used for GBS-library preparation.

GBS library preparation followed the protocol described in Liu et al. (2019). In brief, 200 ng of genomic DNA was digested with *Pst*I and *Mse*I and ligated with a common and a unique barcoded adapter. Equal volumes of ligation product for each sample were pooled into a single tube, purified with a QIAquick PCR purification kit (28104; QIAGEN), and amplified by PCR. Each PCR reaction was a total volume of 200 µL with 2X Taq Master Mix (New England BioLabs Inc.), two primers (5 nmol each), and 50 ng/µl of genomic DNA for each sample. PCR amplification was performed with denaturation at 98°C for 10s followed by 18 cycles of annealing at 65°C for 30s, and finally 30s extension at 72°C. The PCR products were cleaned up again using a QIAquick PCR purification kit. The GBS library was then sequenced on an Illumina HiSeq 2500 to generate single-end, 100-bp reads at the Genomic Sequencing and Analysis Facility at the University of Texas Southwestern Medical Center in Dallas, Texas. GBS data were then analyzed for SNPs using the TASSEL-GBS pipeline with the *Triticum aestivum* IWGSC RefSeq v1.0 as the reference genome (IWGSC, 2018). SNP markers were filtered with an individual read depth greater than 1, *MAF* greater than 0.05, and missing data less than 30% to yield 5681 polymorphic SNP markers.

Statistical Analysis, Linkage Map Construction, and QTL Analysis

The distribution of phenotypic traits assessed in all experiments were tested for normality using the Shapiro-Wilk normality test and homogeneity of variances was verified using Levene's test ("car" package) in RStudio version 3.6.1 (Fox and Weisberg 2019; RStudio Team 2016). Correlation coefficients between disease severity and DON content were calculated using Spearman's correlation (a rank-order correlation) as it measures the relationship between two continuous, random variables without assuming normal distribution of variables. Broad-sense heritability, defined as $H^2 = V_G/V_{P,}$ for each trait was calculated with the restricted maximum likelihood (REML) method in RStudio using the Sommer package (Covarrubias-Pazaran 2018). Heritability coefficients were estimated from variance components with the equation $H^2 = V_G / (V_G + V_{GxY}/y + V_E/yr)$, where V_G is genotypic variance, V_{GxY} is the genotype-by-year interaction variance, V_E is the residual variance, y is the number of years, and r is the number of replications.

The SNP markers generated from two-enzyme genotyping-by-sequencing were evaluated for distorted segregation and missing values. SNPs with >30% missing values or distorted segregation were excluded from linkage mapping. A genetic linkage map with the remaining GBS-SNP markers was constructed using the Kosambi mapping function (Kosambi 1944) and 'regression' mapping algorithm in JoinMap® version 5.0 (Van Ooijen 2018). The minimum logarithm of odds (LOD) threshold of 3 was used to determine linkage groups. The long (L) and short (L) arms of each chromosome were estimated based on physical location of centromeres published in ChIP-seq data for CENH3 (Guo et al. 2016).

A total of 5370 SNP markers were used to detect genomic regions associated with FHB resistance and DON accumulation in the RILs. Seven phenotypic datasets for FHB severity and three for DON accumulation were analyzed individually for QTL mapping. In addition, QTL

analysis for PH and DH were also analyzed individually. A significantly associated QTL was determined using Composite Interval Mapping (CIM) (Jiang et al. 2007) in QGene v.4.4 (Joehanes and Nelson 2008). The LOD threshold for claiming significance of QTL at $P < 0.05$ was determined by performing 1000 permutation tests (Churchill and Doerge 1994).

Results

Variation in Disease Severity and DON Among RILs and Parents

The mean phenotypic values, range of values for RILs, values for parents, and broad-sense heritability for FHB resistance traits along with related agronomic traits are presented in Table 1. The distribution of disease severity (type II resistance) and DON accumulation (type III resistance) were found to be continuous in all experiments, indicating quantitative inheritance (Figures 1, 2, and 3). Transgressive segregation was observed at both the higher and lower levels of disease severity and DON accumulation (Figures 1, 2, and 3).

The resistant parent Surpresa showed moderate resistance to FHB in comparison with the susceptible parent Wheaton in all experiments. Alsen (PI 615543), known to possess *Fhb1* from a Sumai3 derivative, ND2710, showed a consistently higher level of FHB resistance in all the experiments when compared with Surpresa. Disease severities in greenhouse experiments overall were higher than in field experiments. FHB severity in the corn-spawn inoculated field experiment was relatively higher than in the other field experiments.

The mean DON accumulation in Surpresa and Wheaton varied in greenhouse and field experiments (Table 1). DON accumulation in Surpresa varied from 3.4 ppm in 18FAR-DON to 10.3 ppm in 18FAR_CORN-DON experiment. Wheaton showed higher DON accumulation ranging from 5.9 ppm in 18FAR-DON to 47.10 ppm in 18GH-DON. The mean DON accumulation in RILs varied from 0.33 to 202.40 ppm among the three experiments. Like the

disease severity, DON accumulation was found to be highest in greenhouse experiments with relatively higher accumulation in the corn-spawn inoculated field experiment. The normality test indicated deviation from a normal distribution for DON accumulation in RILs in each of the three experiments.

Table 1. Phenotypic data and broad-sense heritability for FHB severity and related agronomic traits in Wheaton/Surpresa RILs and parents.

Trait	Environments	Parents		RILs		
		Surpresa	Wheaton	Mean ± SD	Range	H^2
FHB Severity	16GH	na	0.86	0.73 ± 0.19	0.13 – 1.00	0.57
	17GH	0.40	0.89	0.60 ± 0.16	0.17 – 0.95	
	18GH	0.36	0.91	0.61 ± 0.15	0.22 – 0.97	
	16FAR	0.28	0.86	0.50 ± 0.17	0.14 – 0.97	0.15
	17FAR	0.59	0.85	0.37 ± 0.15	0.10 – 0.84	
	FAR18P	0.30	0.66	0.46 ± 0.10	0.19 – 0.71	
	FAR18C	0.35	0.76	0.55 ± 0.09	0.31 – 0.76	-
DON Content (ppm)	18GH	7.35	47.10	37.45 ± 30.81	0.33 – 202.4	
	FAR18P	3.40	5.90	11.42 ± 6.78	1.00 – 49.90	
	FAR18C	10.30	39.80	23.30 ± 12.48	6.80 – 72.10	
Days to Flower (DF)	16GH	na	69.00	54.06 ± 5.43	45.00 – 76.75	-
	17GH	81.00	73.00	54.99 ± 5.84	44.33 – 70.83	
	18GH	74.00	78.00	73.10 ± 4.26	65.67 – 88.33	
	16FAR	59.00	52.00	54.95 ± 5.23	48.00 – 70.00	-
	17FAR	55.00	56.00	55.48 ± 2.83	52.00 – 63.00	
	FAR18P	57.00	57.00	55.69 ± 2.60	49.50 – 62.25	
	FAR18C	59.00	58.00	57.12 ± 3.17	49.33 – 68.67	
Plant Height (PH)	FAR18P	37.25	27.36	31.84 ± 3.15	26.00 – 40.75	-
	FAR18C	35.50	26.95	31.07 ± 3.02	25.33 – 39.25	

H^2, broad-sense heritability; FHB severity, mean of the symptomatic proportions of infected spikes; FAR, field nursery at Fargo location; GH, greenhouse; P, point inoculation; C, corn-spawn inoculation.

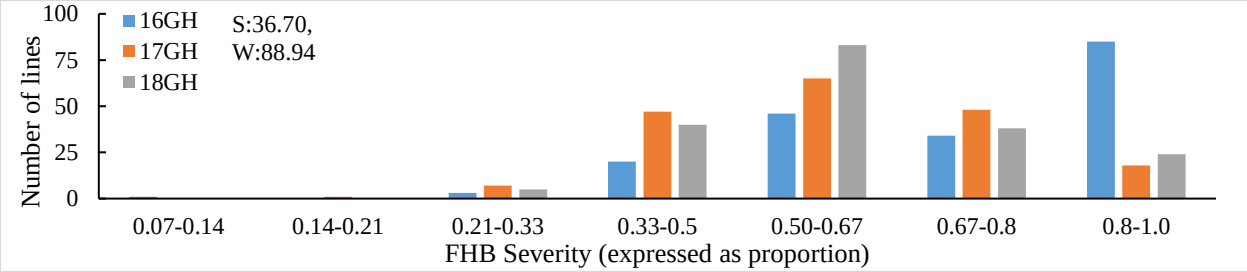

Figure 1. Frequency distribution of FHB severity in Wheaton/Surpresa RILs across greenhouse experiments.
GH, Greenhouse; S, Surpresa; W, Wheaton.

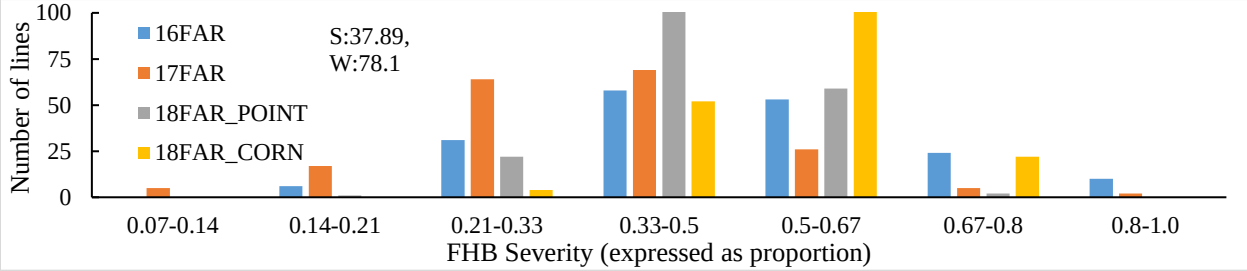

Figure 2. Frequency distributions of FHB severity in Wheaton/Surpresa RILs in field experiments.
FAR, field nursery at Fargo location; POINT, point inoculation; CORN, corn-spawn inocultion; S, Surpresa; W, Wheaton.

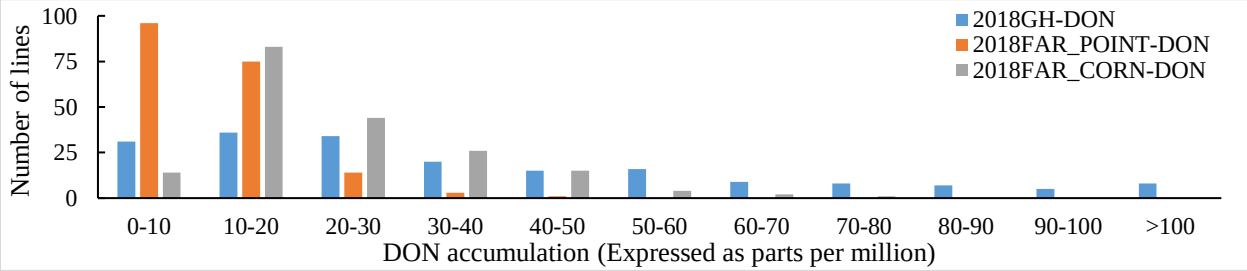

Figure 3. Frequency distributions of DON accumulation in Wheaton/Surpresa RILs.
DON, DON content on samples; FAR, field nursery at Fargo location; GH, greenhouse; POINT, point inoculation; CORN, corn-spawn inoculation; S, Surpresa; W, Wheaton.

Analyses of variance showed significant genotype and genotype-by-environment interactions for both disease severity and DON accumulation (Table 2). For disease severity in both field and greenhouse experiments, the variances explained by genotype and genotype-by-environment were significant ($P < 0.0001$) (Table 2).

The Spearman's correlation coefficient for disease severity ranged from 0.09 to 0.51 across all experiments (Table 3). No significant correlation was observed for disease severity in the 16FAR and 18FAR experiments, and between the 17FAR and FAR18C experiments. Associations ranging from negative to a very strong positive relationships between disease severity and DON content was observed across experiments. Strong positive correlation was observed between disease severity and DON accumulation in the greenhouse experiments; however, the association was weak in the field experiments. Heritability for disease severity was high, ranging from 0.76 to 0.81 for greenhouse experiments indicating that the assessment of FHB severity in greenhouse are reproducible.

Table 2. Analysis of variance results for FHB severity measured across greenhouse and field environments in 187 Wheaton/Surpresa RILs.

Source	Greenhouse			Field		
	df	MS	*F*-value	df	MS	*F*-value
Year (Y)	2	587.21	0.39^{ns}	2	1454.43	0.66^{ns}
Rep x Year	6	523.94	0.91^{ns}	4	22.73	1.00^{ns}
Genotype	186	9926.77	6.62***	186	4484.51	2.03^{***}
GenotypexYear	370	3194.35	2.13***	364	2895.00	1.31**

Year, Environment in which the analysis of variance is assessed; Rep, biological replication; MS, mean sum of squares; ***, $P<0.0001$; **, $P<0.001$; ns, $P > 0.05$.

Table 3. Spearman's correlation coefficient (**ρ**) between FHB disease severity (proportion of symptomatic spikelets) and deoxynivalenol (DON) levels calculated from individual experiments.

	16GH	17GH	18GH	16FAR	17FAR	FAR18P	FAR18C	DON18GH	DON18P	DON18C
16GH	…	…	…	…	…	…	…	…	…	…
17GH	0.36***	…	…	…	…	…	…	…	…	…
18GH	0.48***	0.48***	…	…	…	…	…	…	…	…
16FAR	0.29**	0.28***	0.25***	…	…	…	…	…	…	…
17FAR	0.17*	0.27***	0.28***	0.21**	…	…	…	…	…	…
FAR18P	0.27***	0.21**	0.35***	0.12ns	0.23**	…	…	…	…	…
FAR18C	0.19***	0.22**	0.34***	0.09ns	0.11ns	0.51***	…	…	…	…
DON18GH	0.33***	0.43***	0.72***	0.08ns	0.07ns	0.16*	0.17*	…	…	…
DON18P	-0.08ns	0.08ns	0.09ns	-0.06ns	0.16*	-0.0008ns	0.17*	0.06ns	…	…
DON18C	-0.003ns	0.18*	0.02ns	0.11ns	-0.14*	0.06ns	0.19*	0.16*	-0.06ns	…

***, $P<0.0001$; **, $P<0.001$; *, $P<0.05$; ns, non-significant.
GH, greenhouse; FAR, field experiment at Fargo location; P, point-inoculation; C, corn-spawn inoculation, DON, deoxynivalenol accumulation.

Linkage Map Construction

A total of 5681 polymorphic SNP markers with ≤ 30% missing data were identified by using two-enzyme genotyping by sequencing. Of the 5681 SNPs analyzed in the mapping population, 5370 (94.53%) were mapped to 21 linkage groups, equally assigned to each of the A, B, and D genomes, at minimum threshold LOD value of 3 (Figure 4). Most of the SNP markers were mapped to the B genome (50%), followed by genome A (43%) and genome D (7%). The genetic linkage map spanned 4249.14 cM covering all 21 chromosomes of wheat with an average distance of 1.07 cM between markers.

QTL for FHB Resistance and DON Accumulation

QTL analysis using CIM on individual phenotypic datasets detected 4 significant QTL for resistance to FHB mapped on chromosomes 2A (*Qfhb.ndwp-2AS, Qfhb.ndwp-2AL*), 3B (*Qfhb.ndwp-3BL*), and 4D (*Qfhb.ndwp-4D*) (Figure 5). The QTL, their positions, the environment in which the QTL were detected are reported in Table 4. All these QTL were derived from the resistant parent Surpresa.

Qfhb.ndwp-4D showed the largest effect explaining 15.8% of the phenotypic variation. This QTL was delineated to a 3.47 cM interval between SNPs S4D_68970439 and S4D_234703979. A QTL that affects plant height was co-localized with the QTL on chromosome 4D. *Qfhb.ndwp-2AS* and *Qfhb.ndwp-2AL* were detected in 16GH and 17GH and mapped approximately 28 cM apart from each other. The two loci explained 13.3-14.4% of the phenotypic variation, respectively. The first QTL on chromosome 2A, *Qfhb.ndwp-2AS*, was detected in the 17GH experiment and mapped to a 4.08 cM interval flanked by SNPs S2A_50055119 and S2A_51983004 with no polymorphic SNP markers between them. The second QTL, *Qfhb.ndwp-2AL,* was detected in the 16GH experiment and mapped within a 2.72 cM interval between flanking markers S2A_473904223 and S2A_498098498 with the peak at S2A_494977419.

The QTL on chromosome 3B, *Qfhb.ndwp-3BL,* was detected in the FAR18C experiment and mapped within a 6.28 cM interval between flanking markers S3B_792570263 and S3B_807079831. No SNPs were mapped within this QTL region. This QTL explained about 11.1% of the phenotypic variation.

Table 4. Summary of QTL detected for FHB severity (type II resistance) by composite interval mapping (CIM) in the RIL population derived from the cross between FHB-resistant Surpresa and FHB-susceptible Wheaton.

QTL	Chr	ENV	Flanking SNP markers	LOD	R^2	Add.	Assoc
Qfhb.ndwp-2AS	2A	17GH	S2A_50055119 – S2A_51983004	6.2*	0.14	-4.76	-
Qfhb.ndwp-2AL	2A	16GH	S2A_473904223 – S2A_498098498	5.8*	0.13	-6.33	-
Qfhb.ndwp-3BL	3B	FAR18C	S3B_792570263 – S3B_807079831	4.3*	0.10	-1.16	-
Qfhb.ndwp-4D	4D	17GH	S4D_68970439 – S4D_234703979	6.3*	0.15	4.90	*Rht-D*

QTL, quantitative trait loci; Chr, chromosome; ENV, experiment in which QTL was detected; LOD, logarithm of odds; R^2, proportion of phenotypic variance explained by each QTL; Add., additive effect denoting the contribution of resistant or susceptible allele; Assoc, association with other markers.

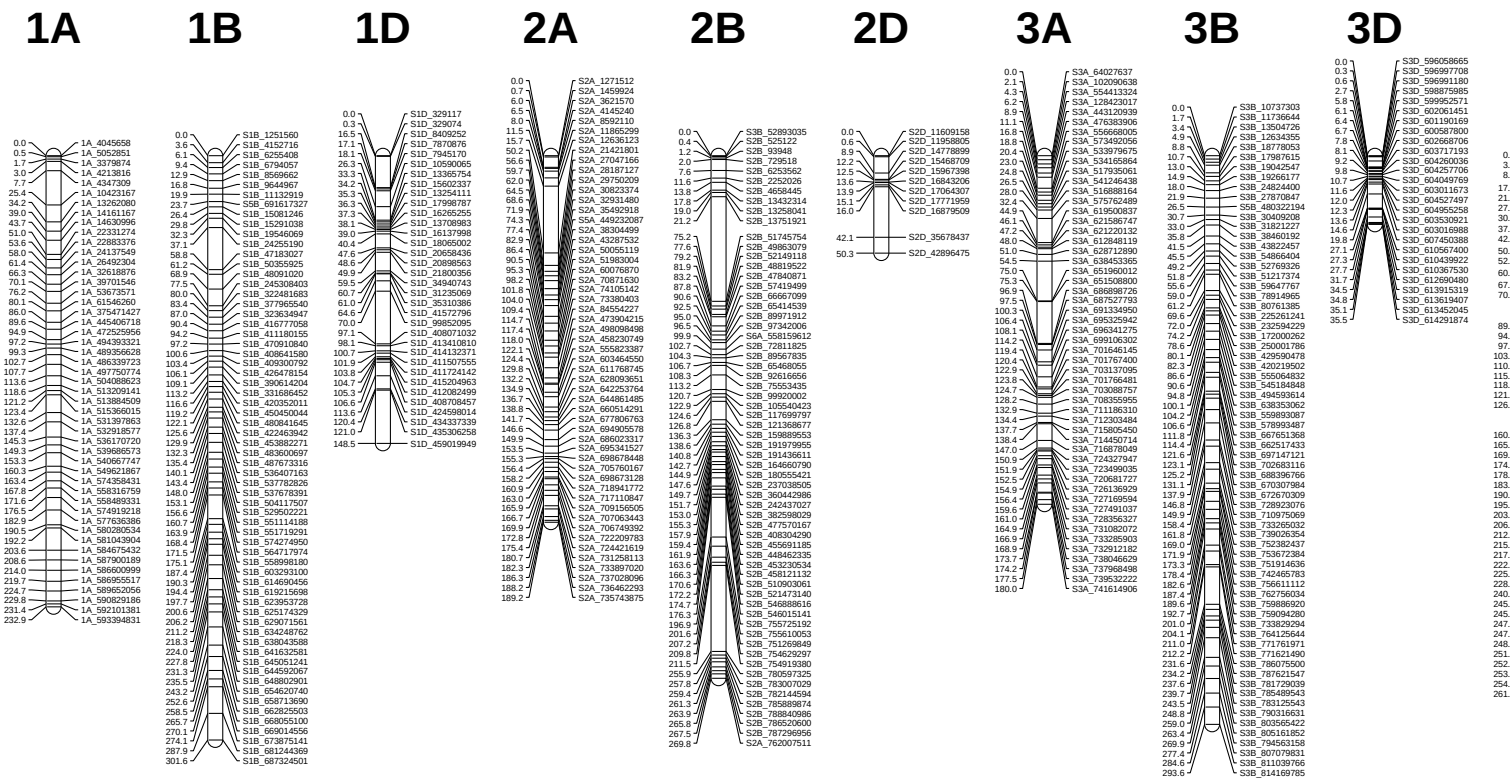

Figure 4. Genetic linkage maps constructed in the Wheaton/Surpresa derived RIL population.
The position of marker loci is shown to the right of the linkage groups and centiMorgan (cM) distances between loci are shown to the left.

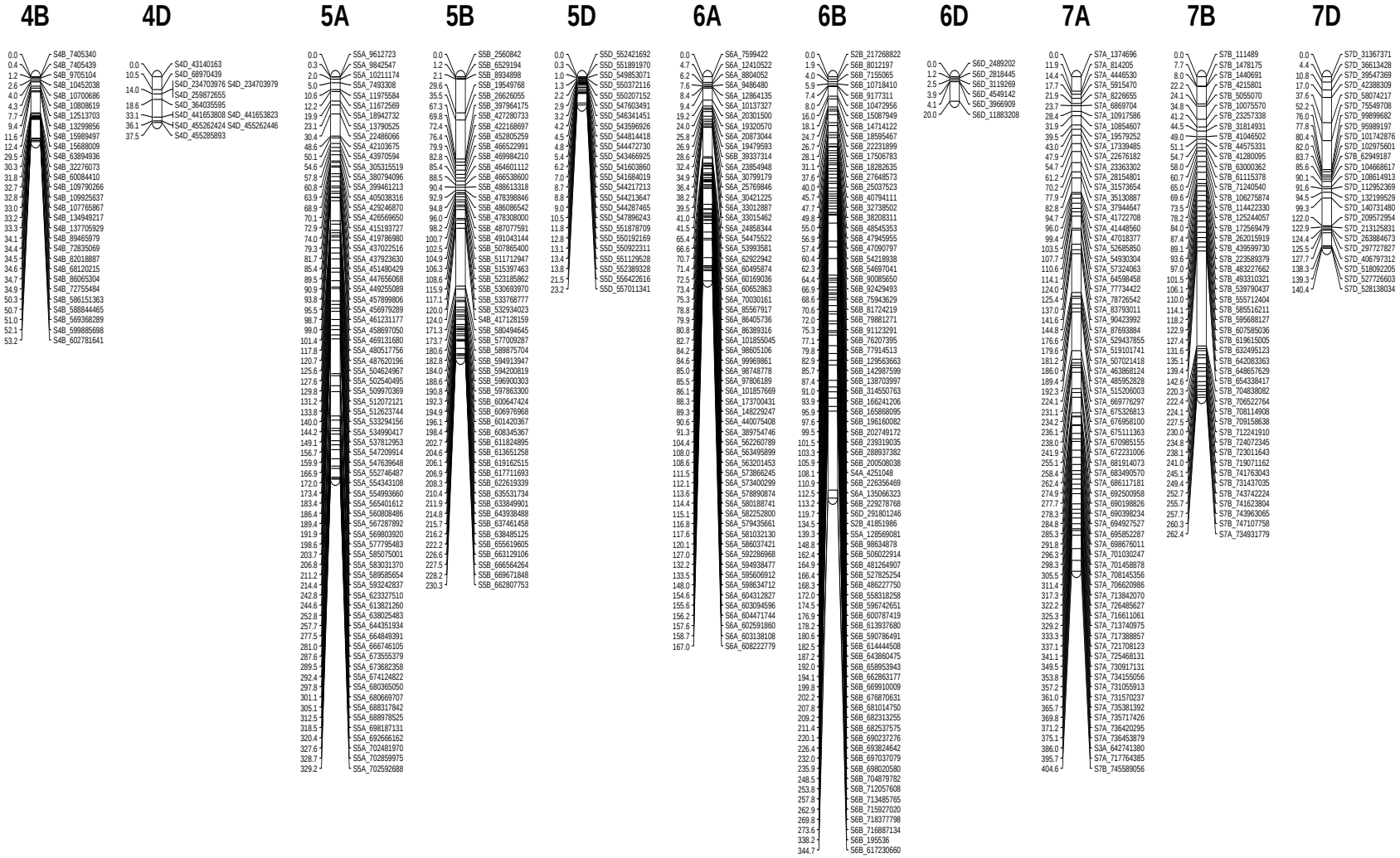

Figure 4. Genetic linkage maps constructed in the Wheaton/Surpresa derived RIL population (continued).

The position of marker loci is shown to the right of the linkage groups and centiMorgan (cM) distances between loci are shown to the left.

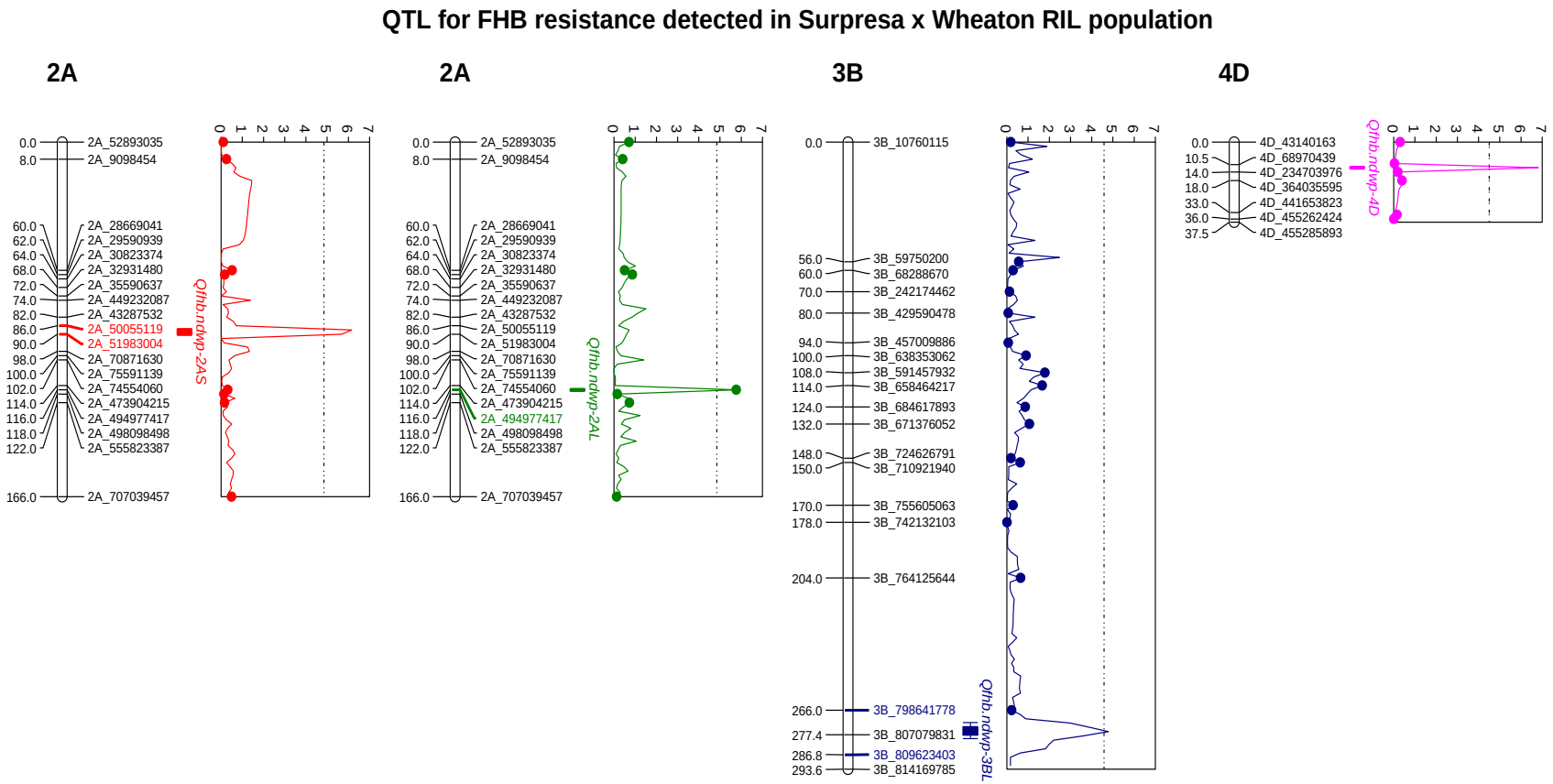

Figure 5. Linkage maps for chromosomes 2A, 3B, and 4D showing the respective QTL for type II resistance (disease severity) derived from the Wheaton/Surpresa - RIL population.

Discussion

Based on the phenotype and genotype data generated in this study, four QTL originating from Surpresa for type II FHB resistance were identified explaining 11.1-15.8% of the total phenotypic variation. The low proportion of phenotypic variance accounted for by these QTL could be the result of significant genotype-by-environment interaction. Frequency distributions of FHB severity and DON accumulation in Wheaton/Surpresa RILs across the different environments were continuous indicating quantitative inheritance of resistance. FHB severity was higher in the greenhouse than in the field. This may be due to the environmental conditions that were more conducive to disease development in the greenhouse, resulting in higher disease severities. ANOVA results (Table 2) showed significant genotype, and genotype-by-year interaction in all experiments. Broad-sense heritability for FHB severity in the greenhouse and field experiments were 0.57 and 0.15, respectively. This indicates that the assessments of FHB severity in controlled environment is more reproducible, however, a significant environment and genotype-by-environment interaction might limit reproducibility in field experiments.

The association of FHB severity and DON content has been studied extensively to understand the usefulness of disease severity for predicting DON levels in the harvested wheat grain. Considering FHB severity to be the first step in the DON accumulation process, a high correlation is expected between these two traits (He et al. 2019). However, the correlation between FHB disease severity and DON content has varied greatly with significantly high positive correlations, low significant correlations, negative correlations, or no correlation. In the current study, FHB severity showed significant positive correlation with DON accumulation under controlled greenhouse conditions while the association was weak under field conditions. In a meta-analysis conducted to understand the relationship between FHB disease severity and

DON accumulation, Paul et al. (2005) showed that several inherent factors including wheat genotype, pathogen population, weather conditions, as well as other random factors affect the overall correlation coefficients. Each RIL in the current study differ in the magnitude of resistance to FHB thereby producing a different amount of DON for a similar level of disease or vice-versa thereby producing weak correlations between disease severity and DON in the field.

It has frequently been reported that morphological and phenological traits, especially plant height and flowering date, are correlated with FHB resistance (Bai et al. 2018; Lu et al. 2013; Steiner et al. 2017). In this study, we also evaluated the RIL population for plant height and flowering date. We found a weak negative correlation between FHB severity and plant height as well as flowering date. This result agrees with previous reports of an association between plant height and FHB severity (He et al. 2016, Mesterhazy et al. 1995). In fact, the QTL on chromosome 4D, *Qfhb.ndwp-4D,* was found to occur in the same region as the *Rht-D1* locus. QTL for FHB resistance (type II) have previously been co-localized or closely linked with *Rht-D1* locus in European winter wheat cultivars Apache, History, Romanus, Spark, and Arina (Draeger et al. 2007; Holzapfel et al. 2008; Srinivasachary et al. 2008). In addition, QTL at the *Rht-D1* locus have also been reported to have the strongest effect on FHB (Draeger et al. 2007; Holzapfel et al. 2008; Srinivasachary et al. 2008). In this study, *Qfhb.ndwp-4D* conferred the strongest effect on type II resistance explaining 15.8% of the total phenotypic variation in disease severity. Disease escape or differences in micro-climate around the wheat heads have also been suggested as factors contributing to FHB resistance.

Overall, we identified four significant QTL for resistance to FHB spread, located on chromosomes 2A (*Qfhs.ndwp-2AS, Qfhb.ndwp-2AL*), 3B (*Qfhb.ndwp-3BL*), and 4D (*Qfhb.ndwp-4D*) (Figure 5). *Qfhb.ndwp-2AS* and *Qfhb.ndwp-2AL* were mapped approximately 28 cM (444

Mb) apart on chromosome 2A. *Qfhb.ndwp-2AS* was mapped to a 4.08cM interval between SNPs S2A_50055119 and S2A_51983004 in the 17GH experiment with no polymorphic SNP markers between them. Several studies have detected QTL on chromosome 2A following different inoculation approaches and markers closely linked to those QTL have also been identified (Angelica et al. 2016; Gervais et al. 2003; Häberle et al. 2009; Ma et al. 2006; Yi et al. 2018). In a mapping population (Arina//NK93604) evaluated by Semagn et al. (2007), a QTL was detected on chromosome 2AS that explained about 27% of DON accumulation after spray inoculation with *F. culmorum* isolates. The SSR locus *Xbarc124*.1 (physical location: 3784350-3784367), linked to this QTL, was located approximately 1 Mb proximal of SNP S2A_5005519 associated with QTL *Qfhb.ndwp-2AS.* Therefore, it is assumed that these QTL are at the same locus or closely linked. However, unlike the role of QTL detected by Semagn et al. (2007) in DON reduction, *Qfhb.ndwp-2AS* is involved in conferring type II resistance in the current study. Therefore, further study is required to determine whether the two QTL are different.

The second QTL on chromosome 2A, *Qfhb.ndwp-2AL*, was detected in the 16GH experiment explaining 13.3% of the phenotypic variation for type II resistance. It was mapped to a 2.72 cM interval between S2A_473904223 and S2A_498098498 with a peak at S2A_494977417. Holzapfrl et al. (2008) identified a QTL for type II resistance on long arm of chromosome 2A from a History/Rubens mapping population that explained 1.5-4.1% of the total phenotypic variation. The SSR locus *Xgwm425* (physical location: 19460982-473780340), associated with the QTL from History/Rubens mapping population, is located approximately 21 Mb away from SNP S2A_494977417 associated with *Qfhb.ndwp-2AL*. This suggests that *Qfhb.ndwp-2AL* is a novel QTL, different from the one detected by Holzapfrl et al. (2008).

The third QTL, *Qfhb.ndwp-3BL*, was mapped to a 6.28 cM interval on the long arm of chromosome 3B and explained 10.1% of the phenotypic variation. Several QTL have been identified on chromosome 3B but only two have been mapped onto the long arm (Bourdoncle and Ohm 2003; Cai et al. 2016). The QTL mapped on 3BL in Huapei 57-2 is closely linked to the SSR locus *Xgwm247* (physical location: 826 Mb), which is approximately 19 Mb apart from the marker linked to *Qfhb.ndwp-3BL*, implying that this is likely a new QTL.

In this study, the type II resistance conferred by Surpresa appears to be unstable across environments. Stability of resistance has been shown to depend on the level of resistance conferred by the resistant parent and in part to the genetic background of the susceptible parent used in the study (Draeger et al. 2007; Mesterhazy 1995). Evaluation of populations based on different susceptible backgrounds could possibly help to identify stable QTL for FHB resistance and elucidate the genetic basis of resistance in Surpresa. Furthermore, a year effect, as observed by Gervais et al. (2003), was also observed in the QTL reported in study as we observed the expression of QTL only in experiments when the disease pressure was high ($P<0.001$) (Figure B3). The QTL for FHB resistance on chromosome 4D was found to be associated with plant height in this study. Plant height was found to be negatively correlated with FHB resistance in this study and the results implicated the possible involvement of the *Rht-D1b* allele in increased susceptibility. Follow-up experiments with RILs with and without *Rht-D1b* would help clarify whether the QTL on chromosome 4D is true or a mere disease escape.

In summary, the genetic basis underlying FHB resistance appears to be complex and sveral QTL with small to moderate effect seem to govern the resistance to FHB. Clearly, such QTL will be difficult to select, pyramid, and verify in breeding programs in the absence of

markers with high allele-specificity, leaving uncertainty about its worth in wheat breeding programs.

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GENOME-WIDE ASSOCIATION MAPPING OF RESISTANCE TO FUSARIUM HEAD BLIGHT IN A GLOBAL PANEL OF SPRING WHEAT

Abstract

Resistance to Fusarium head blight is a complex trait controlled by multiple small to medium effect quantitative trait loci (QTL). Numerous genome-wide association studies (GWAS) have been performed on wheat germplasms from diverse regions to identify novel QTL for FHB resistance. A globally collected panel of 233 spring wheat accessions was evaluated for reaction to FHB in field and greenhouse experiments with the objectives of i) evaluating the phenotypic reaction to FHB and DON; and ii) identifying quantitative trait loci (QTL) associated with FHB resistance using GWAS. The population was genotyped with a 9K single nucleotide polymorphism (SNP) assembly at the USDA-ARS genotyping laboratory in Fargo, ND. The wheat accessions were significantly different ($p<0.05$) in their reaction to FHB with FHB severity ranging from 7 to 97% across environments. Spearman's correlation for FHB severity and DON content were high across environments. A total of 4390 SNP markers were used in the analysis. GWAS identified two significant QTL associated with type II and type III resistance. *Qfhb.ndwp*-3A conferred type II resistance and explained 10.03% of the total phenotypic variation while *Qfhb.ndwp*-2BL conferred type III resistance explaining 26.66% of the total phenotypic variation. *Qfhb.ndwp*-3A was co-localized with a QTL for plant height.

Introduction

Fusarium head blight (FHB) is a destructive disease in all wheat-growing regions of the world (Goswami et al. 2004). In North America, FHB in wheat is primarily caused by the ascomycete fungus *Fusarium graminearum*. The disease causes huge losses by reducing the yield and quality of the grains produced. Recurring epidemics of FHB almost every year in the

1990s led the disease to be regarded as the worst to hit the United States since the stem rust epidemics of the 1950s (Wood et al. 1999). The disease caused losses of over $3 billion in wheat and barley production during that period and caused many growers to switch to less risky crops (Windels et al. 2000). The disease still today maintains its notoriety for causing large amount of wheat crop losses in the United States and China (Savary et al. 2019).

Since the 1990's, breeding for resistance to FHB has been intensified by scientists around the globe (Zhu et al. 2019). Despite these efforts, no source of immunity to FHB has been discovered. Extensive evaluations of large genetic resources, including landraces, cultivars, breeding lines, and wild relatives of wheat, has led to the identification of numerous germplasm accessions with moderate to high FHB resistance (Buerstmayr et al. 2019). Resistance to FHB is genetically complex and controlled by numerous small to medium effect QTL. In addition, certain plant morphological and phenological traits such as plant height, flowering date, anther extrusion etc. are known to affect the reaction of plants to FHB (Bai et al. 2018; Lu et al. 2013; Steiner et al. 2017).

Marker-trait associations in plants are commonly determined using two approaches: bi-parental linkage mapping and genome-wide association mapping (Zhu et al. 2008). Linkage mapping is based on a population, segregating for the trait of interest, derived from a cross between two selected parents with contrasting phenotype, which allows identification of rare alleles with large effects (Nordborg and Weigel, 2008). Since the first published report of FHB resistance QTL in 1999 till date, close to 500 QTL conferring FHB resistance, on all 21 chromosomes, have been reported using bi-parental populations till date (Buerstmayr et al. 2019). However, only a handful of QTL, such as *Fhb1* (Cuthbert et al. 2006; Li et al. 2019; Liu et al. 2006; Rawat et al. 2016; Schweiger et al. 2016; Su et al. 2019), *Fhb2* (Cuthbert et al. 2007),

Fhb4, *Fhb5* (Jia et al. 2018), *Qfhs.ifa-5A* (Steiner et al. 2019), *Qfhs.ndsu-3AS* (Zhu et al. 2016), *Qfhb.nau-2B* (Li et al. 2019), and *Qfhb.mgb-2A* (Gadaleta et al. 2019) have been fine mapped. Of these, the most stable type II resistance QTL *Fhb1* reduces disease severity by 20-25% depending on the genetic background (Pumphrey et al. 2007) and has been extensively utilized in wheat breeding programs (Bai et al. 2018; Shah et al. 2018). For the remaining FHB resistance QTL, their utilization via marker-assisted selection (MAS) in breeding programs is still a challenge, owing to their minor effects, lack of diagnostic markers, and poor reproducibility across different genetic backgrounds (Buerstmayr et al. 2019; Li et al. 2016). Context dependency, caused by genotype-by-environment interaction or epistasis or both, further limits the utilization of QTL in MAS for improvement of complex traits (Podlich et al. 2004). The bi-parental mapping approach can only assess two alleles (from the parents) per locus, further limiting the utilization of QTL detected using this approach across different genetic backgrounds (Wang et al. 2012).

Genome-wide association study (GWAS) is an alternative method based on a diversity panel representing greater allelic diversity at a given locus and allows high-resolution mapping resulting from historical recombination events to identify significant marker-trait associations (MTA) (Zhu et al. 2008). GWAS offers the advantage of bypassing the need for constructing a mapping population and utilizes meiosis information that accumulated throughout the evolutionary history of a natural population (Wang et al. 2009; Zhu et al. 2008). In addition, this approach facilitates the evaluation of multigenic traits with multiple alleles and can overcome the context dependencies of QTL mapped in bi-parental populations (Pozniak et al. 2012). Miedaner et al. (2011) observed a significant positive epistatic effect between a *Fhb1*-linked SSR marker and the semi-dwarfing locus *Rht-B1b* that enhances resistance to FHB in a European soft winter

wheat population. This epistatic interaction could be exploited to achieve high-yielding wheat varieties with enhanced FHB resistance. However, it is important to note that kinship and population structure can result in false-positive marker-trait associations, which need to be considered when interpreting the QTL results of complex traits in GWAS (Buerstmayr et al. 2019).

Over the last decade, numerous GWAS of traits associated with FHB resistance in wheat have been performed in Europe, the United States, and China. Association studies on different European winter wheat collections identified small to medium effect QTL for FHB resistance (Kollers et al. 2013; Miedaner et al. 2011; Mirdita et al. 2015). Analysis of a soft winter wheat breeding panel from the mid-western and eastern regions of the United States indicated that the FHB resistance included a *Fhb1*-associated major resistance component (Arruda et al. 2016). On the contrary, evaluation of hard red spring wheat breeding lines from North Dakota State University showed that *Fhb1* had a low effect on type II FHB resistance in this spring wheat population although some other QTL for FHB resistance were found (Liu et al. 2019). No significant effect of the *Fhb1*-R allele, as compared to the *Fhb1*-S allele, was observed on FHB resistance in a panel of 256 soft red winter wheat lines (Tessmann et al. 2019). Spring wheat lines developed in the Pacific Northwest and CIMMYT showed resistance to FHB as a cumulative effect of multiple low to medium effect QTL, with no *Fhb1*-associated markers detected in the marker-trait association (Wang et al. 2017). GWAS of FHB resistance in a panel of 213 accessions in the elite wheat germplasm of China identified several small effect QTL, including *Fhb1*, conferring FHB resistance (Wu et al. 2019). These studies were performed in a population specific to a region and indicated a combined effect of numerous small to medium effect QTL conferring FHB resistance, with or without the major effect of *Fhb1*. Zhang et al.

(2008) screened 1,045 global spring wheat accessions that showed varying reactions to FHB. Numerous accessions showed low to moderate disease severity but the novelty and types of resistances in most of these accessions were not well understood. In this study, we evaluated a world-wide collection of 233 spring wheat accessions from the National Small Grains Collection (NSGC) including those screened by Zhang et al. (2008) for reaction to FHB over multiple years. The objective of this study was to identify novel QTL for resistance to FHB (type II and type III) using genome-wide association mapping based on Infinium 9K SNP markers.

Materials and Methods

Plant Materials

A panel of 233 spring wheat accessions from the National Small Grains Collection (NSGC) were evaluated for reaction to FHB over multiple years in this study. This panel comprised of two distinct sets (Set I and II) of spring wheat accessions evaluated in different years and locations. The first set (Set I) comprised of 73 spring wheat accessions previously reported to possess moderate to high levels of resistance to FHB (Zhang et al. 2008). The second set (Set II) comprised of 160 spring wheat accessions identified to have some levels of FHB resistances. Additional six spring wheat genotypes (ND 2710, Sumai3, PI 277012, Alsen, Steele ND, and Choteau) were included as checks. The two sets of wheat accessions are listed in Appendix Table A1 with their origin, number of accessions, crop improvement status, and pedigree.

Evaluation of Reaction to FHB and DON Accumulation

Wheat accessions from set I were evaluated for type II resistance (resistance to fungal spread) in two greenhouse and four field experiments between 2009 and 2010. Wheat accessions from Set II, on the other hand, were evaluated in three greenhouse and five field experiments

between 2016 and 2018. In the greenhouse, individual wheat accessions from set I were planted in rows in plastic buckets (30x20x35 cm^3), two rows per bucket and 8 to 10 seeds per row. Growing conditions were maintained at 23±2°C with 16h supplemental light, and fertilized with Multicote 4 (NPK::14:14:16 plus minor nutrient) (Heifa Biochemical, Israel). Plants were supplemented with liquid fertilizer (20:20:20, J.R. Peters, Inc. Allentown, PA) once a week. Individual wheat accessions from set II, on the other hand, were grown in 6-inch clay pots in a greenhouse, with three replicates (pots) per accession in a completely randomized design (CRD). Inoculum was prepared using four pathogenic isolates collected from North Dakota (two isolates producing 3ADON, and the other two isolates producing 15ADON) and maintained at a concentration of 100,000 spores per mL by mixing equal amounts of spores of each isolate. FHB inoculations were performed at 50% flowering using the single-spikelet inoculation technique as described by Stack et al. (2002). The plants were then moved to a room with a misting system (1 min of misting in every half hour) to facilitate disease development and returned to the greenhouse after 48 h of incubation.

Field evaluations of set I were conducted at two locations, Fargo and Prosper, in North Dakota while set II was evaluated only in the FHB nursery located in Fargo, ND. In 2009, 73 and 76 lines (including checks) from set I were evaluated at Fargo and Prosper, ND, respectively. In 2010, 75 lines (including checks) were tested at both the Fargo and Prosper locations. Individual lines were planted in hill plots in a randomized complete block design (RCBD) 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 apart.

For set II, all wheat accessions were evaluated in every field experiment. For field experiments in 2016 and 2018, the wheat accessions were evaluated for type II FHB resistance

using two inoculation techniques: i) single-spikelet inoculation (Stack et al. 2002), and ii) corn-inoculum inoculation (Puri et al. 2010). For 2017, wheat accessions were evaluated using only single-spikelet inoculation. For 2016 and 2018 field experiments, the wheat accessions were evaluated using randomized complete block design with three replications, while in 2017, the accessions were grown in short rows of 6 feet with one replication. These changes were made to accommodate enough heads to represent overall FHB reactions of individual wheat accessions studied in set II. Overhead misting for 5 min at 3 h intervals, for 12 hours daily (6:00 P.M. to 6:00 A.M.), was set up until 14 days after single-spikelet inoculation of the latest maturing accessions.

FHB disease severity (DS) was assessed 21 days post inoculation in all greenhouse and field experiments by visually assessing symptomatic spikes using a modified 1-9 Horsfall-Barrett disease rating scale with 9 categories of infection, to reflect 0, 7, 14, 21, 33, 50, 67, 80, and 100% of disease severity (Stack and McMullen 1998). The DS for each replication was calculated by averaging severities of all inoculated heads.

In addition, DON accumulation was assessed on one random 2.5 g ground grain sample from each accession in set II from 2018 greenhouse and field experiments. Inoculated heads from point-inoculated experiments were harvested at maturity, with all replications combined, threshed carefully to keep all the seeds including the tombstones, ground into fine powder, and sent to a USWBSI supported laboratory for DON analysis. For corn-spawn-inoculated experiments, all heads from each replication were harvested and processed for DON accumulation analysis. For set I, 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, Fargo, ND for mycotoxin analysis.

Genotypic Data

Genomic DNA samples from wheat accessions of set I were extracted from leaves of one-week old seedling plants using the method described by Riede and Anderson (1996). Each DNA sample was quantified using a NanoDrop (Wilmington, DE) and diluted to 50ng/μL for further use. Genotyping was conducted in the USDA-ARS Biosciences Research Lab, Fargo, ND, USA using the Infinum 9K chips.

For set II, the genotyping data were extracted from genotyping experiment NSGCwheat9K_spring available at The Triticeae Toolbox (T3)/wheat website (https://triticeaetoolbox.org/wheat/genotyping/display_genotype.php?trial_code=NSGCwheat9K_spring). NSGCwheat9K_spring genotyping was performed in an Infinum 9K platform by the National Small Grains Collection (NSGC) at USDA-ARS location in Fargo, ND in 2011. Single-nucleotide polymorphism (SNP) markers with minor allele frequency (MAF) less than 5% and missing data over 10% were removed from the analysis to avoid false marker-trait associations.

Phenotypic Data Analysis

Disease severity (DS) data from each set of the wheat accessions were tested separately for normal distribution using the Shapiro-Wilk test and homogeneity of variances were tested using Levene's test ("car" package) in RStudio version 3.6.1 (Fox and Weisberg 2019; RStudio Team 2016). Student's t-test was performed on each year's DS data of check varieties from set I and set II to test if true difference in means is not equal to 0 to be able to combine the datasets. Each dataset combined was considered as an environment (GH, YEAR1, YEAR2, YEAR3, DON1, DON2) in this study. Type III analysis of variance (ANOVA) for DS was calculated with

Satterthwaite's method for each environment using linear mixed effect model in lmerTest package (Kuznetsova et al. 2017) in RStudio v.3.6.1. Spearman's correlation coefficient was used to estimate correlation between DS and DON content. Broad-sense heritability, defined as $H^2 = V_G/V_P$, for each trait was calculated by using the restricted maximum likelihood (REML) method in RStudio in the Sommer package (Covarrubias-Pazaran 2018). Heritability coefficients were estimated from variance components with the equation

$$H^2 = V_G/(V_G + V_{GxY}/y + V_E/yr)$$

where V_G is genotypic variance, V_{GxY} is the genotype-by-year interaction variance, V_E is the residual variance, y is the number of years, and r is the number of replications.

Marker-Trait Association Analysis

Principal component analysis (PCA) was performed to determine the level of population structure of the panel. PCA reduces the complexity of high dimensional data by transforming them into fewer dimensions called principal components (PCs) while retaining their structure (Lever et al. 2017). A kinship matrix (K) was calculated based on the centered IBS method that is scaled to give a reasonable estimate of additive genetic variance. Linkage disequilibrium (LD) between SNP markers was calculated as squared allele frequency correlation (R^2) in TASSEL version 5.0. Genome-wide LD decay was estimated by plotting R^2 against the corresponding pairwise genetic distance (cM) (Wang et al. 2014).

The genome-wide association analysis (GWAS) was performed in Trait Analysis by aSSociation, Evolution and Linkage (TASSEL; Bradbury et al. 2007) version 5 that implements a general linear model and mixed linear model for controlling population and family structure. Individual sets, with their genotype and phenotype data, alongside a combined dataset was analyzed for GWAS. Four different statistical models were tested: i) Naïve model: GLM without any correction for population structure; ii) PCA-model: GLM with correction for population

structure; iii) K model: GLM with kinship matrix, and iv) PCA + K model: MLM with PCs and K-matrix as correction for population structure and familial relatedness. The significance of marker-trait associations was based on a false-discovery-rate (FDR) calculated from *p*-values using the R function *p*.adjust (method = fdr) Benjamini and Hochberg (1995). A significant marker-trait association is defined by FDR as the adjusted *p*-values of less than or equal to 0.1.

Results

Phenotypic Data Analysis

This genotype panel included landraces, cultivars, and breeding lines from 57 countries in five continents (Africa, n=26; Asia, n=79; Europe, n= 54; North America, n=16; and South America, n=62) (Appendix A1). Frequency distributions of FHB DS and DON content across environments showed deviation from normal distributions (Figure 6). FHB severity across environments (Table 5) ranged from 7 to 97% with mean FHB severity ranging from 35.93% to 48.96%. Mean DS was the highest in the GH environment as compared to the rest of environments. Among the 233 accessions, 25 accessions showed high resistance to FHB across field experiments, consistent with the results by Zhang et al. (2008) (Table 6). Analysis of variance showed significant differences among genotypes ($p<0.05$) for all traits evaluated in all environments (Table 7). In addition, significant genotype-by-environment interactions ($p<0.05$) were also observed for all traits. The Spearman's correlation coefficients for disease severity ranged from 0.50 to 0.68, all significant at $p<0.0001$ (Table 8). DON content was significant and positively correlated with FHB severity across all environments with the value of r ranging from 0.34 to 0.80 (Table 7). Broad-sense heritability estimated for DS was 0.86 in this experiment.

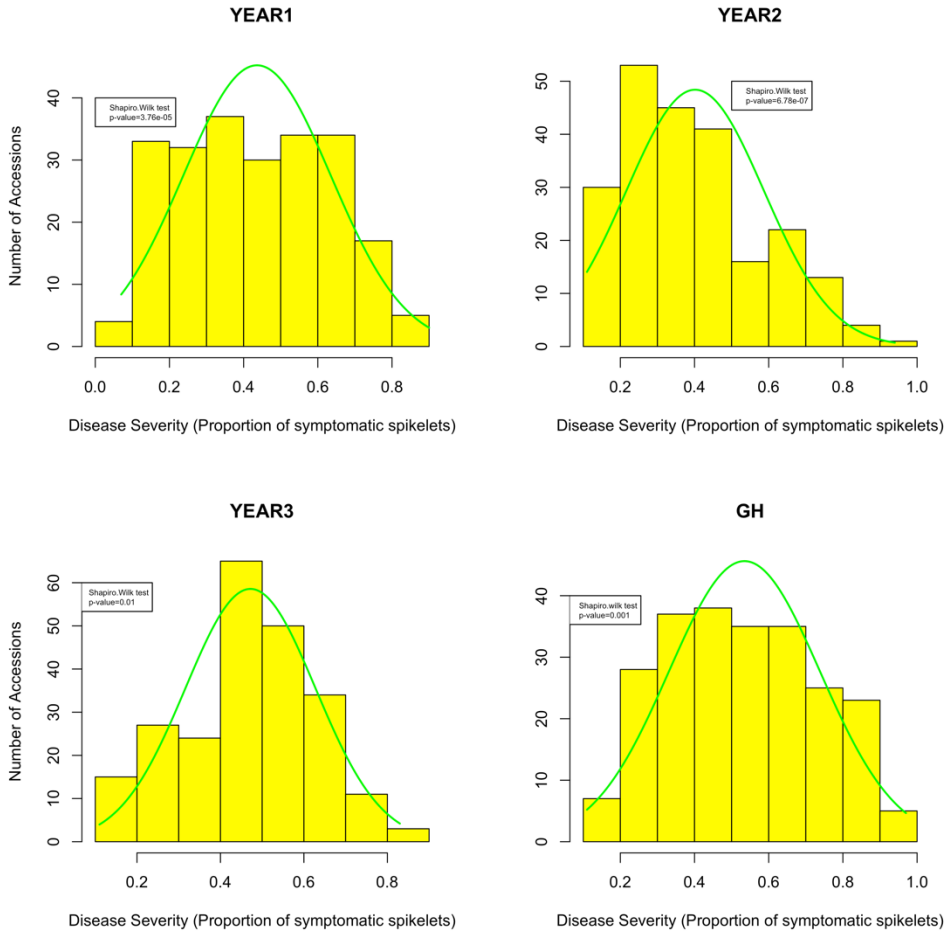

Figure 6. Histogram with a normal curve (green line) of Fusarium head blight disease severity in 233 spring wheat accessions.
YEAR1, dataset combining 2009 and 2016 field experiments conducted at Fargo; YEAR2, dataset combining the 2009 field experiment at Prosper and the 2017 field experiment conducted at Fargo; YEAR3, dataset combining the 2010 field experiment data from Fargo and Prosper and the 2018 field experiment from Fargo; GH, dataset combining greenhouse experiment data from 2009/10 and greenhouse experiments from 2016, 2017, and 2018; DON1, DON accumulation data obtained from combining DON data from the 2009 field experiment at Fargo, and DON accumulation dataset from the greenhouse experiment conducted in 2018; DON2, DON accumulation data obtained after combining datasets from the 2009 field experiment conducted at Prosper, and the DON accumulation dataset from the 2018 field experiment conducted at Fargo.

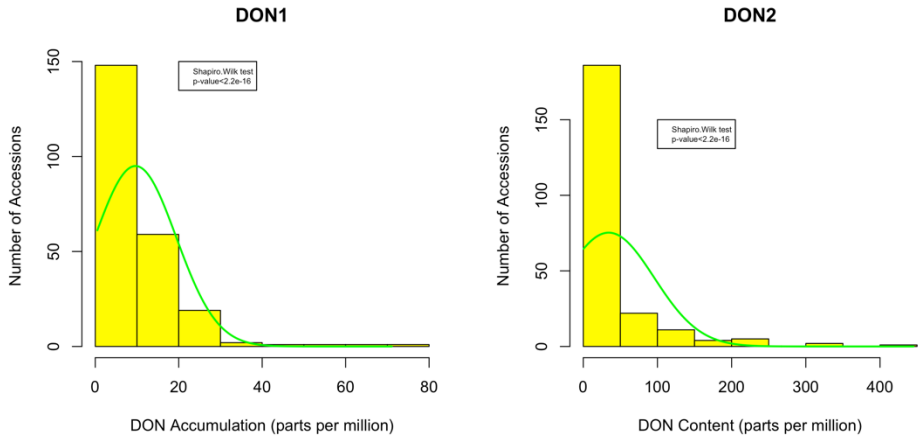

Figure 6. Histogram with a normal curve (green line) for Fusarium head blight disease severity in 233 spring wheat accessions (continued).

YEAR1, dataset combining the 2009 and the 2016 Fargo experiments; YEAR2, dataset combining the 2009 Prosper and the 2017 Fargo experiments; YEAR3, dataset combining the 2010 Fargo/Prosper and the 2018 Fargo experiments; GH, dataset combining the 2009, 2010, 2016, 2017, and 2018 greenhouse experiments; DON1, DON content data combining the 2009 Fargo experiment and the 2018 greenhouse experiment; DON2, DON accumulation data combining the 2009 Prosper experiment and the 2018 Fargo experiment.

Table 5. Summary of the phenotypic data and broad-sense heritability of 233 spring wheat accessions evaluated for their reaction to Fusarium head blight severity and DON accumulation across different environments.

Traits	Environments	Mean ± SD	Range	Normality	H^2
FHB Severity	GH	0.53 ± 0.20	0.11 – 0.97	*P*=0.001	0.86
	YEAR1	0.44 ± 0.20	0.07 – 0.90	*P*=3.76e-05	
	YEAR2	0.40 ± 0.19	0.11 – 0.94	*P*=6.78e-07	
	YEAR3	0.47 ± 0.16	0.11 – 0.83	*P*=0.01	
DON Content	DON1	9.64 ± 9.72	0.50 – 71.05	*P*<2.2e-16	-
	DON2	34.26 ± 61.07	0.50 – 445.1	*P*<2.2e-16	

FHB Severity, mean of the symptomatic proportions of infected spikes; DON content, deoxynivalenol accumulation.
GH, greenhouse; YEAR1, dataset combining 2009 and 2016 Fargo experiments; YEAR2, dataset combining 2009 Prosper and 2017 Fargo experiments; YEAR3, dataset combining 2010 Fargo/Prosper and 2018 Fargo experiments; DON1, deoxynivalenol content dataset from 2009 field nursery experiment in Fargo location and greenhouse experiment in 2018; DON2, deoxynivalenol content dataset from 2009 field nursery experiment in Prosper location and 2018 field nursery experiment in Fargo location.
SD, standard deviation; Normality, the Shapiro-Wilk test for normality; H^2, broad-sense heritability.

Table 6. List of 25 spring wheat accessions with consistent high FHB resistance along with their reactions to FHB measured by proportion of infected spikelets across environments and concentration of deoxynivalenol (DON).

Accession	Name	GH	YEAR1	YEAR2	YEAR3	DON1	DON2
PI 633976	ND 2710	0.11	0.1	0.12	0.11	0.5	0.5
PI 462151	Shu Chou Wheat No. 3	0.11	0.13	0.15	0.16	1.1	2.1
PI 277012	I 825	0.11	0.07	0.13	0.26	4.1	1.1
PI 182561	Sin Chunaga	0.28	0.17	0.14	0.19	1.5	1.3
PI 104131	Excelsior	0.31	0.12	0.21	0.19	7.6	1.5
PI 382140	Abura	0.28	0.22	0.12	0.22	1.7	0.5
PI 344467	Oncativo Inta	0.68	0.16	0.26	0.18	1.8	1.1
PI 182591	Norin 61	0.31	0.19	0.15	0.27	0.5	0.9
PI 351816	Froment du Japon	0.41	0.16	0.16	0.29	1.1	0.5
PI 182583	Chuko	0.17	0.2	0.13	0.29	0.5	0.5
PI 382167	16-52-9	0.25	0.12	0.18	0.32	2.5	1.1
PI 182586	Norin 43	0.36	0.21	0.17	0.24	2.1	1.2
PI 182568	Norin 34	0.33	0.21	0.2	0.22	1.8	0.5
PI 345731	Tezanos Pintos Precoz	0.56	0.22	0.23	0.18	1.2	1.0
PI 382153	Nobeoka Bozu	0.12	0.18	0.3	0.16	0.8	1.0
PI 351221	Newthatch Selection	0.4	na	0.24	0.2	20	0.5
PI 519790	274-1-118	0.32	0.22	0.31	0.15	1.7	0.7
PI 382154	Nyu Bai	0.26	0.18	0.28	0.23	1.2	0.5
PI 644122	Bahiense	0.31	0.24	0.28	0.18	2.5	3
PI 644137	1032	0.29	0.2	0.23	0.28	1.6	1.4
PI 344465	Laureano Alvarez Laah	0.24	0.19	0.37	0.17	1.8	1.8
PI 644119	…	0.43	0.26	0.31	0.16	1.3	3.4
PI 81791	Sapporo Haru Komugi jugo	0.33	0.24	0.22	0.29	3.5	1.1
PI 644135	220	0.37	0.1	0.16	0.49	2	0.9
PI 644132	Wabian	0.48	0.25	0.2	0.3	5.2	1.4

Accession, wheat genotype as listed in the GRIN database; Name, cultivar name in the country of origin.

GH, reaction of accessions to FHB in greenhouse; YEAR1, reaction of accessions to FHB in Fargo in 2009 and 2016; YEAR2, reaction of accessions to FHB at Prosper in 2009 and at Fargo in 2017;YEAR3, reaction of accessions to FHB at Fargo and Prosper combined from 2010 and at Fargo in 2018; DON1, concentration of deoxynivalenol in wheat accessions from 2009 Fargo experiment and 2018 greenhouse experiment; DON2, deoxynivalenol concentration in wheat accessions from 2009 Prosper experiment and 2018 Fargo experiment.

Table 7. Variance components of Fusarium head blight severity across environments for the panel of 233 spring wheat accessions.

Source	df	TSS	MSS	*F*-Value	Pr (>*F*)
Year (Y)	3	225738.94	725246.31	65.69	< 0.0001***
Rep x (Y)	12	235043.09	19586.92	17.10	<0.0001***
Genotype (G)	222	3945521.09	17772.62	15.52	<0.0001***
(Y) x (G)	648	2303961.40	3555.50	3.10	<0.0001***

Year, Environment in which the analysis of variance is assessed; Rep, biological replications; TSS, Total sum of squares; MSS, Mean sum of squares; ***, $P<0.0001$.

Table 8. Spearman correlation coefficients for Fusarium head blight severity and DON accumulation data in 233 spring wheat accessions.

Environments	GH	YEAR1	YEAR2	YEAR3	DON1
GH	…	…	…	…	…
YEAR1	0.55***	…	…	…	…
YEAR2	0.50***	0.68***	…	…	…
YEAR3	0.57***	0.66***	0.51***	…	…
DON1	0.53***	0.51***	0.34***	0.61***	…
DON2	0.80***	0.65***	0.51***	0.66***	0.68***

***, $P < 0.0001$.
GH, Greenhouse; YEAR1, dataset combining field nursery data of the 2009 and 2016 Fargo; YEAR2, dataset combining field nursery data of 2009 in Prosper and 2017 in Fargo; YEAR3, dataset combining field nursery data of 2010 in Fargo and Prosper and 2018 in Fargo; DON1, deoxynivalenol content dataset from the 2009 field nursery experiment in Fargo and greenhouse experiment in 2018; DON2, deoxynivalenol content dataset from the 2009 field nursery experiment in Prosper and the 2018 field nursery experiment in Fargo.

Marker Distribution, Population Structure, and LD

A total of 5,863 polymorphic SNP markers were identified in genotyping set I with the 9K-SNP array. Of the 5,863 SNPs analyzed, 5,411 (92.30%) were mapped to 21 linkage groups, 2,595 markers (47.96%) mapped to the A genome, 2,483 markers (45.89%) mapped to the B genome, and 333 markers (6.15%) mapped to the D genome.

For set II, the Infinium 9K SNP chip generated 5,303 SNP markers with minor allele frequency (MAF) less than 5% and missing data less than 10%. Of the 5,303 polymorphic SNP markers, 2,400 (45.26%), 2,149 (40.52%), and 581 (10.96%) markers were associated within the

A, B, and D genomes, respectively while the chromosomal location of 173 (3.26%) markers were unknown.

A total of 4,390 polymorphic SNP markers were found common to the two sets and were utilized in the GWAS analyses. The first six PCs explained 33.7, 6.4, 3.2, 2.8, 2.2, and 1.9% of the total variation, respectively. Hierarchical ward clustering implemented in rrBLUP indicated that the population structure of the panel had three major groups (groups 1, 2, and 3) (Figure 7). Group1 was further subdivided into three subgroups (groups 1A, 1B, and 1C) while group 2 was subdivided into two subgroups (groups 2A, and 2B). Group 3 on the other hand had no further subdivisions. Most of the wheat accessions in set I clustered together in groups 1 and 2 while wheat accessions in set II clustered into group 3. The clustering of the wheat accessions into three groups showed an admixture of wheat accessions of different crop improvement status but were not related due to the geographical origin of genotypes. The LD analysis was based on pairwise squared correlations (r^2) for all 4390 SNP markers. The mean LD decayed to 0.51 between markers, with physical distance < 1Mb and 0.45 with distance < 5 Mb (Figure 8).

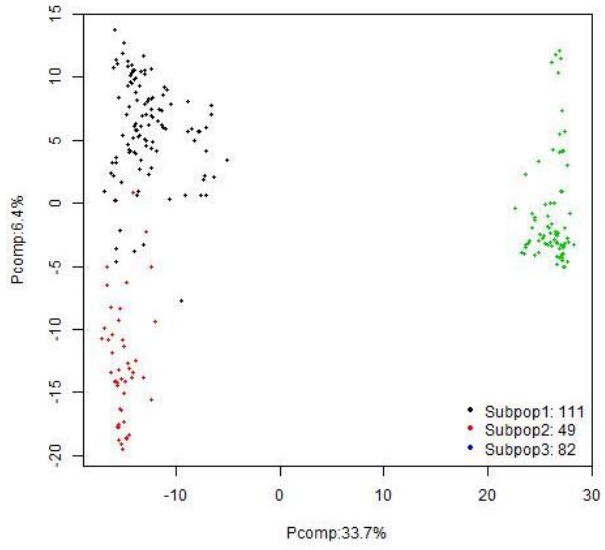

Figure 7. Population structure based on principal component (PC) analysis in 233 spring wheat accessions.
Three different colors represent clusters; black, group 1; red, group 2; green, group3.

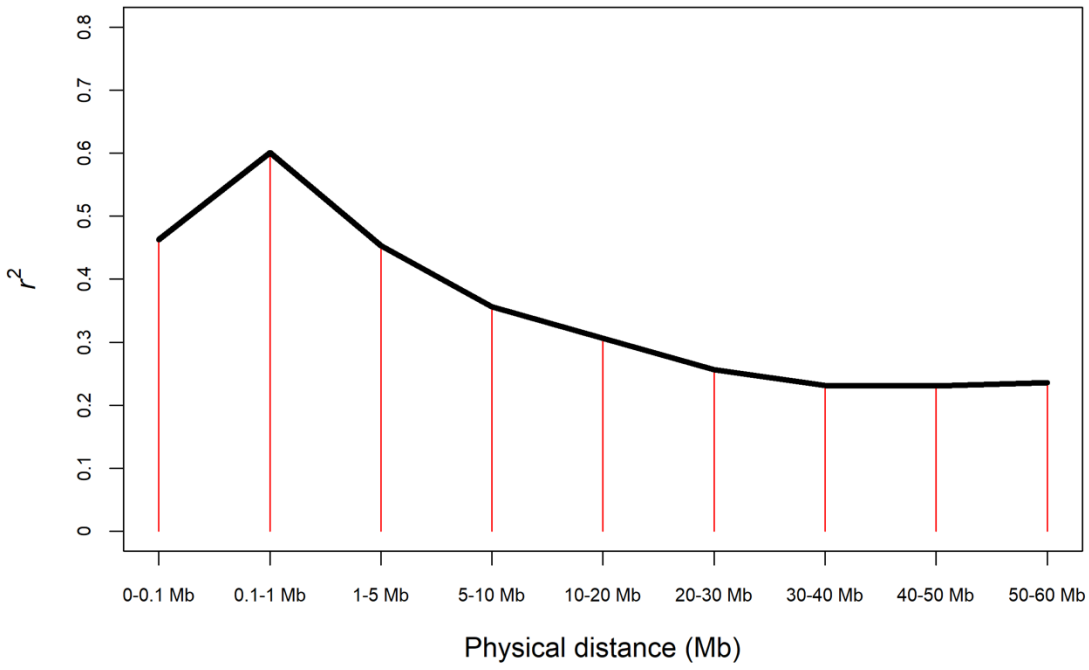

Figure 8. Genome-wide average linkage disequilibrium (LD) decay plot for 233 spring wheat accessions based on data for 4,390 single nucleotide polymorphisms (SNPs).
Line graph of LD r^2 between SNP pairs and incremental groups of physical distances (in megabases).

Marker-Trait Associations (MTAs)

The marker-trait association was analyzed for three different datasets; i) set I, ii) set II, and iii) combined datasets using all 233 wheat accessions to identify the loci conferring FHB resistance. Four statistical models were compared for their ability to detect marker-trait associations using 5,411, 5,303, and 4,390 SNPs, respectively. Models that did not account for population stratification, i.e. the "naïve" and the "K model", yielded the largest numbers of significant MTAs. The "naïve" model detected 7,035 and 22 significant MTAs for type II and type III resistances using the combined dataset, respectively at $P<0.01$ after permutation whereas the "K model" detected 6,955 and 12 significant MTAs for type II and III resistances, respectively at $P<0.01$ after 1,000 permutation tests. These associations were probably the result of not accounting for population structure as it is known that lack of appropriate correction for

population structure can lead to spurious associations. Based on the uniformity of p-values distribution between observed and expected p-values, the PCA+K model was selected for subsequent analysis of all the traits (Appendix B2).

The MTAs and magnitude of their effects are presented in Table 9; only MTAs with logarithm of odds (LOD) scores > 3 from the combined dataset are listed in the table. A mixed model accounting for population structure and familial relatedness detected 21 MTAs for type III FHB resistance in DON1 (significant after false discovery rate (fdr) correction at less than or equal to 0.1). The significant associations were located on chromosomes 1A, 3B, 4A, and 6A, respectively and explained between 35.56 to 38.31% of the total variation in DON accumulation. No significant association was detected for type II resistance. A mixed model applied to set II resulted in just one significant MTA associated with type III resistance in DON2 at fdr ≤ 0.1. In total, two significant MTAs were detected in this study, one for type II resistance and the other for type III resistance (Figure 9). *Qfhb.ndwp*-3A (tagged SNP: IWA7564) was significantly associated with type II resistance at $P<0.1$ after fdr correction and was mapped on the long arm of chromosome 3A. *Qfhb.ndwp*-3A explained 10.03% of the total phenotypic variation.

Qfhb.ndwp-2BL (tagged SNP: IWA4900) was significantly associated with type III resistance at $P<0.0001$ after fdr/Bonferroni correction and was mapped on the long arm of chromosome 2B. It explained 26.66% of the total phenotypic variation in DON accumulation.

The frequency distribution of favorable alleles for FHB resistance in six subgroups and five continents was also analyzed (Appendix A3). Favorable allele frequency for reduced disease severity was found to be highest in subgroup 1B followed by subgroups 3, and 1A while lower in subgroups 2B, 1C, and 1D, respectively. Based on the geographic origin of wheat accessions, South American wheat accessions possessed the highest frequency of favorable alleles for

reduced DS followed by European, Asian, and North American wheat accessions. Wheat accessions of African origin possessed the lowest frequency of favorable alleles for reduced DS.

On the other hand, all wheat accessions in subgroup 3 possessed the favorable allele for reduced DON content. On the contrary, none of the other subgroups possessed any of the favorable alleles for reduced DON content.

Table 9. Genome-wide association study (GWAS) of 233 spring wheat accessions. Only single-nucleotide polymorphisms (SNPs) with logarithm of odds (LOD) score > 3 (p<0.001) are shown. The effect of each SNP is expressed as a percentage of the mean of each trait.

Trait	ENV	Marker	Chr	Pos	*P* value	FDR	R^2
FHB Severity	YEAR1	IWA8460	3A	36230840	1.19E-04	0.39	0.07
	YEAR1	IWA4275	2B	605001517	4.98E-04	0.75	0.08
	YEAR1	IWA4618	4B	670440340	9.37E-04	1	0.05
	YEAR2	IWA7564	3A	559532786	6.13E-06	0.08	0.10
	YEAR2	IWA1604	3A	558884759	2.43E-05	0.16	0.09
	YEAR2	IWA3772	3A	559531071	2.43E-05	0.16	0.09
	YEAR2	IWA2925	3A	558887570	5.50E-05	0.23	0.08
	YEAR2	IWA6907	3A	558886118	6.02E-05	0.23	0.09
	YEAR2	IWA3600	UNK	200	6.02E-05	0.23	0.09
	YEAR2	IWA3771	3A	559530532	1.54E-04	0.45	0.10
	YEAR2	IWA7970	3A	560681188	2.18E-04	0.52	0.11
	YEAR2	IWA7891	3A	560681461	2.18E-04	0.52	0.11
	YEAR3	IWA6396	3A	699858331	3.32E-04	0.60	0.09
	YEAR3	IWA5677	3B	241391873	7.89E-04	1	0.08
	YEAR3	IWA1707	2B	690029335	8.13E-04	1	0.07
DON content	DON1	IWA6127	UNK	0	3.08E-04	0.60	0.09
	DON1	IWA3304	3B	586512142	3.19E-04	0.60	0.10
	DON1	IWA2550	3B	292024034	3.42E-04	0.60	0.09
	DON1	IWA6938	6A	545832250	3.74E-04	0.62	0.08
	DON2	IWA4900	2B	696356261	9.30E-11	2.45E-06	0.27
	DON2	IWA5304	1A	333617499	5.14E-04	0.75	0.06
	DON2	IWA2924	2B	640341216	7.50E-04	1	0.05
	DON2	IWA6259	1B	341931621	9.61E-04	1	0.05
	DON1	IWA6127	UNK	0	3.08E-04	0.600552	0.09

FHB Severity, mean of the symptomatic proportions of infected spikes; DON content, deoxynivalenol accumulation.
GH, greenhouse; YEAR1, dataset combining the 2009 and 2016 Fargo experiments; YEAR2, dataset combining 2009 Prosper and 2017 Fargo experiments; YEAR3, dataset combining 2010 Fargo/Prosper experiment and 2018 Fargo; DON1, deoxynivalenol content dataset from 2009 field nursery in Fargo and the greenhouse experiment in 2018; DON2, deoxynivalenol content dataset from the 2009 field nursery in Prosper and 2018 field nursery in Fargo.
Chr, Chromosome; UNK, unknown chromosomal location; Pos, Physical position of the SNP marker on wheat reference genome;
R^2, phenotypic variation explained by the marker.

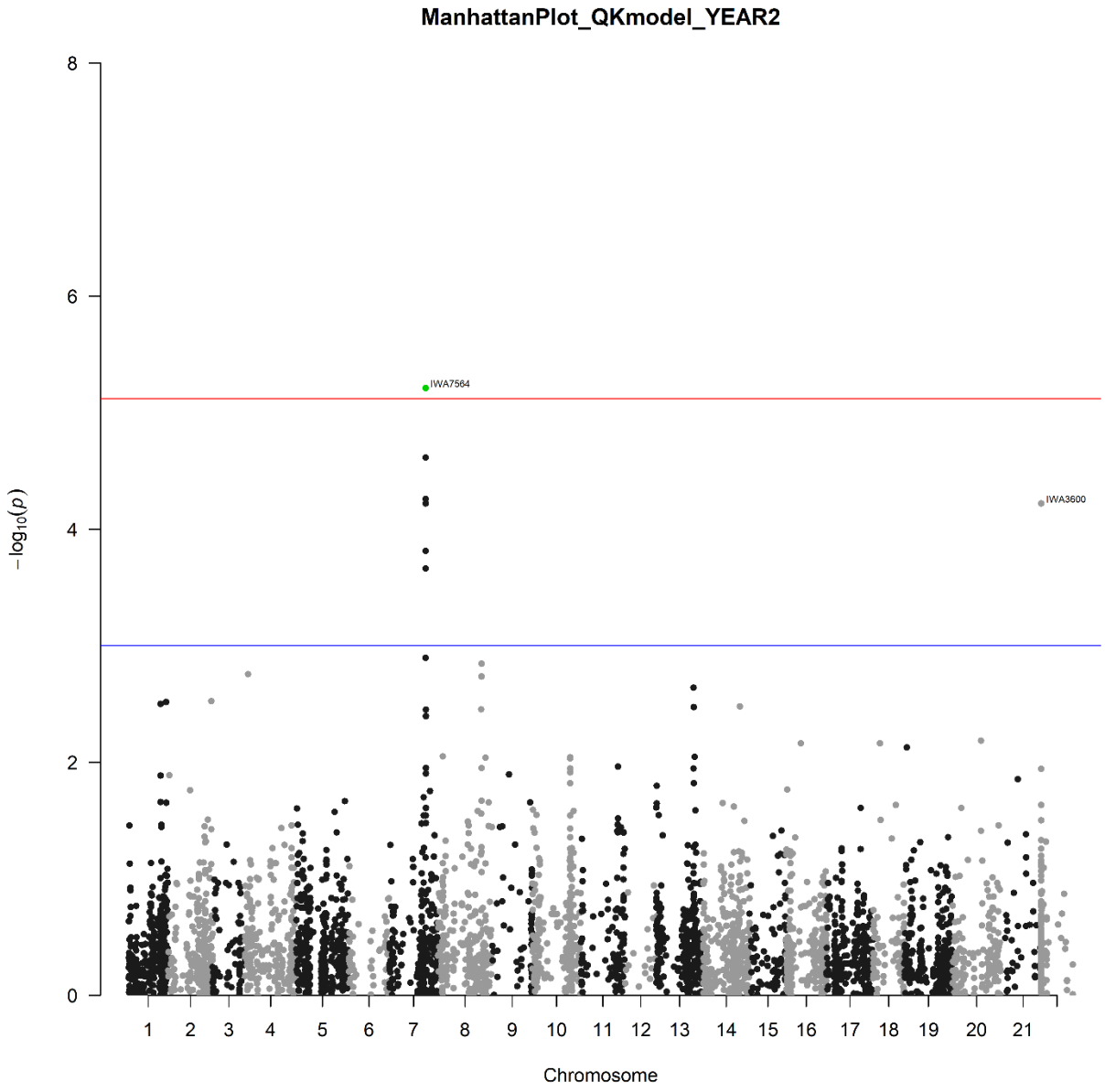

Figure 9. Manhattan plot showing the genome-wide scan of markers associated with resistance to DS and DON content in 233 spring wheat accessions evaluated across YEAR2 and DON2 respectively.
YEAR2, dataset combining field nursery data of 2009 in Prosper location and 2017 in Fargo location; DON2, deoxynivalenol content dataset from 2009 field nursery experiment in Prosper location and 2018 field nursery experiment in Fargo location.
Horizontal blue line indicates significance at $P < 0.05$ (LOD > 3) while horizontal red line indicates significance at fdr < 0.1.

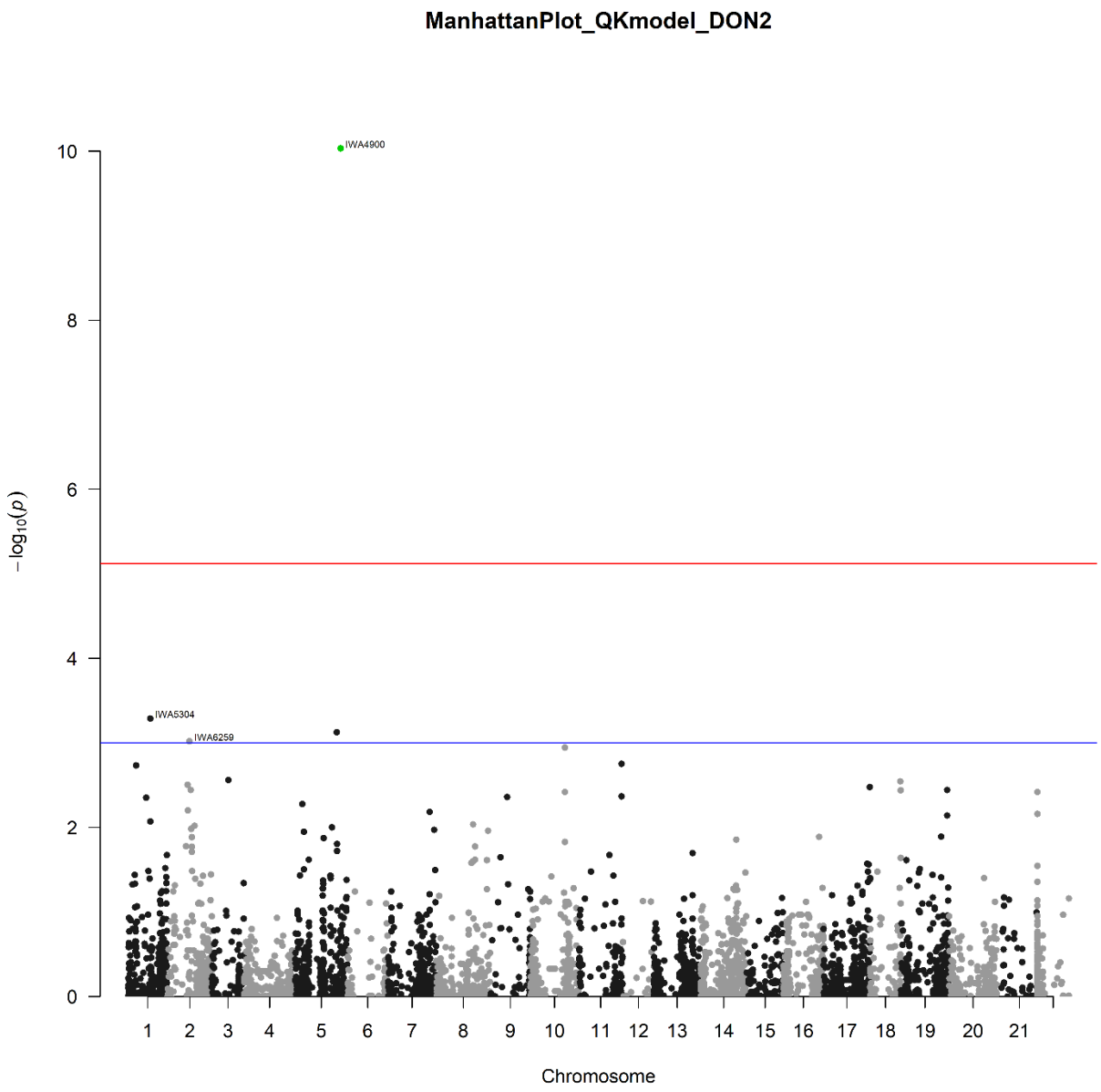

Figure 9. Manhattan plot showing the genome-wide scan of markers associated with resistance to DS and DON content in 233 spring wheat accessions evaluated across YEAR2 and DON2 respectively (continued).

YEAR2, dataset combining field nursery data of 2009 in Prosper location and 2017 in Fargo location; DON2, deoxynivalenol content dataset from 2009 field nursery experiment in Prosper location and 2018 field nursery experiment in Fargo location.
Horizontal blue line indicates significance at $P < 0.05$ while horizontal red line indicates significance at fdr < 0.1.

Discussion

Genome-wide association studies (GWAS) is a powerful tool that offers high-resolution mapping of genetic loci contributing to complex quantitative traits. It has been successfully applied in wheat to map QTL for traits such as yield, quality, and disease resistance. In addition to providing high resolution mapping, GWAS bypasses the need to construct a mapping population and utilizes more alleles than bi-parental linkage mapping. Numerous GWAS of FHB resistance traits have been conducted worldwide (Arruda et al. 2016; Kollers et al. 2013; Liu et al. 2019; Miedaner et al. 2011; Tessmann et al. 2019; Wang et al. 2017; Wu et al. 2019). In this study, we assessed FHB resistance of a spring wheat panel of global origin in seven trials over three years and identified several QTL associated with FHB resistance.

GWAS analysis was performed on three datasets: set I; set II, and all wheat accessions combined. The results from all three GWAS analyses were compared to realize the advantages and disadvantages of GWAS. A mixed linear model using 71 wheat accessions evaluated in set I identified 21 significant MTAs for DON accumulation on chromosomes 1A, 3B, 4A, 5A, and 6A at fdr≤0.1. Of the 21 MTAs, 14 were located on chromosome 4A, and three on chromosome 6A were co-segregating SNPs. All the significant marker-trait associations were identified in one environment, DON1, indicating their unstability across environments. GWAS is a powerful tool that connects variation in trait with its causative underlying genetics. A successful GWAS analysis relies on several factors including the use of many individuals to increase genetic variance among them (Korte and Farlow, 2013). If the model assumptions are not met, GWAS might identify false positive causative SNPs, especially, when a pattern is detected among loci and factors causing variation in the trait analyzed (Platt et al. 2010). GWAS can both identify rare variants that explain a large proportion of the variation in phenotype or common variants

with minor effects on the phenotype. When a trait is polygenic, one approach to improve the power of detecting meaningful associations is to maximize the genetic variance within the population by increasing sample size. In addition to increasing sample size for maximizing genetic variance, using geographically distant accessions can also maximize the genetic variance within the sample, however, it has the potential of increasing genetic heterogeneity, leading to the loss of power to detect a variant (Korte and Farlow, 2013). In addition, genetic heterogeneity can lead to a non-causative marker to be significant explaining a larger portion of phenotypic variation (Platt et al. 2010). The mixed linear model serves the purpose to control spurious associations arising from complex correlation structures due to the genetic heterogeneity and relatedness. The first set, set I, is comprised of 71 individuals from geographically distant locations and therefore, to improve the power of detecting true association, increasing the number of individuals in the analysis with more individuals from similar geographic regions would be ideal (Kote and Farlow, 2013). A mixed linear model applied to set II comprised of 164 global spring wheat accessions, detected one MTA, significant after fdr correction at ≤0.1. The significant association was detected for type III resistance on chromosome 2B and explained 28.80% of the total phenotypic variation. Although the number of accessions evaluated in set II is enough, adding more genotypes could increase chances of identifying true associations.

Within the combined dataset, a mixed linear model identified two significant MTAs at fdr ≤ 0.1, one for type II and the other for type III FHB resistance. The MTA identified in the combined dataset includes the SNP significantly associated with type III FHB resistance in set II. In addition to the SNP identified in set II, the combined dataset detected a new MTA that was not detected in either of the sets. However, none of the MTAs detected in this study were significant across multiple environments. This could be the result of differences in environmental

conditions among experiments affecting the reaction of wheat accessions to FHB. FHB resistance is known to be a complex quantitative trait strongly affected by environments (Steiner et al. 2017) and significant genotype-by-environment interaction was observed in this study.

The first QTL, *Qfhb.ndwp*-3A, identified in this study contributed to type II resistance and explained 10% of the total phenotypic variation. *Qfhb.ndwp*-3A had a positive effect of 16.4%, indicating that it increased the DS. The SNP tagged to *Qfhb.ndwp*-3A, IWA7564, has been associated with canopy temperature depression (grain fill), harvest index, heading date, test weight, and grain yield in wheat (https://wheat.pw.usda.gov/jb/?data=%2Fggds%2Fwhe-iwgsc2018&loc=chr3A%3A559532713..559532837&tracks=DNA%2Ct3_GWAS&highlight=). FHB resistance has been frequently associated with plant height and the extent of anther retention after flowering (Lu et al. 2013; Steiner et al. 2017). The co-localization of *Qfhb.ndwp*-3A with a plant height QTL on chromosome 3A in this study could indicate the contribution of plant height in escaping the disease. However, a study conducted by He et al. (2016) suggested a direct effect of FHB resistance QTL colocalized with plant height QTL on chromosome 2DL in reducing FHB DS, independent of the role in disease escape. The contribution of *Qfhb.ndwp*-3A in this study could also be a direct effect of resistance QTL rather than disease escape. Therefore, further validation might be needed to more accurately understand the association between *Qfhb.ndwp*-3A and the associated plant height QTL.

Several QTL for FHB resistance have been mapped on chromosome 3A utilizing a range of markers from RFLP (Restriction Fragment Length Polymorphism) and SSR (Simple Sequence Repeat) to SNPs (Anderson et al. 2001; Bourdoncle and Ohm 2003; Giancaspro et al. 2016; Mardi et al. 2006; Paillard et al. 2004; Steiner et al. 2004; Zhang et al. 2012). Based on the meta-analysis of the QTLome of FHB resistance in hexaploid wheat by Venske et al. (2019), at least

20 QTL have been identified on chromosome 3A. QTL derived from a Chinese cultivar 'Huapei 57-2' explained 8.1% of the total phenotypic variation for type II resistance (Bourdoncle and Ohm et al. 2003). The marker locus linked to this QTL (*Xgwm5*; physical location: 552 Mb) is 7 Mb distal to IWA7564 identified in this study. Therefore, based on the physical location, *Qfhb.ndwp*-3A identified in the current study might be the same or at the same locus as the QTL identified from 'Huapei 57-2'.

The second QTL, *Qfhb.ndwp*-2BL (tagged SNP: IWA4900) detected in this study is for type III FHB resistance and was mapped on the long arm of chromosome 2B and explained 26.66% of the total phenotypic variation. A previous study conducted by Wang et al. (2017) reported a QTL, *Qfhb-2B-3*, significantly associated with type III resistance in CIMMYT germplasm. *Qfhb-2B-3* (physical location: 749 to 760 Mb) is located 53 Mb away from the QTL *Qfhb.ndwp*-2BL detected in this study and suggests that it should be considered novel.

The wheat accessions used in this study clustered unequally into two major groups and seven subgroups. The population was structured while showed an admixture of wheat accessions at different crop improvement status and geographical origin. The exchange of genetic materials within and across geographical boundaries between different breeding programs might be factored in to explain the complex population structure observed in this study.

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GENOME-WIDE ASSOCIATION STUDY OF VIRULENCE, DON PRODUCTION, FUNGICIDE SENSITIVITY, AND SPORE PRODUCTIVITY IN A *FUSARIUM GRAMINEARUM* POPULATION COLLECTED FROM NORTH DAKOTA

Abstract

Fusarium head blight (FHB) is a destructive disease of small grains. The disease is predominantly caused by a haploid ascomycete fungus *Fusarium graminearum* in North America. To understand the genetics of quantitative traits in this fungal pathogen, we conducted a genome-wide association study (GWAS) of virulence, mycotoxin production, spore productivity, and fungicide sensitivity to two demethylation inhibition (DMI) class fungicides, tebuconazole and prothioconazole, using 183 *F. graminearum* isolates collected between 1981 and 2013 from North Dakota. The isolates were phenotyped for virulence on two spring wheat cultivars, Alsen and Wheaton, in two greenhouse seasons (2017 and 2018), while mycotoxin production was evaluated on Alsen only in a 2018 greenhouse experiment. The isolates were also evaluated for spore productivity on mung-bean agar media while the effective concentration of two triazole fungicides, tebuconazole and prothioconazole, that inhibits isolate growth in-vitro by 50% was calculated. Two-enzyme genotyping-by-sequencing (GBS) was used to gentotype the 183 isolates and 11,965 single-nucleotide polymorphism markers were used in GWAS. The results showed that two MTAs were significantly associated with deoxynivalenol (DON) production, and two with fungicide sensitivity after fdr correction. No SNPs were significantly associated with traits of virulence and spore productivity. The genes around the SNPs associated with DON production have not been previously reported for their roles in mycotoxin production. Further characterization of these genes involved in metabolic pathways of *F. graminearum* could help to develop new strategies for management of the disease.

Introduction

Fusarium head blight (FHB) is a devastating disease of wheat and barley world-wide. The disease causes significant yield and quality reduction in wheat and barley with an estimated $3 billion loss during the 1990s in the United States (Windels 2000). At least 16 phylogenetically related species of *Fusarium,* referred to as the Fusarium graminearum species complex (FGSC), are the causal agents of this disease (Sarver et al. 2011). In the United States, *F. graminearum* is the main causal agent for FHB. The *F. graminearum* population in North America has long been considered very diverse and dominated by a single panmictic population (O'Donnell et al. 2000; Zeller et al. 2003). Large genetic and phenotypic differences in virulence (severity of fungal infection, used interchangeably with aggressiveness), chemotype, and mycotoxin production were observed among isolates collected from different regions of the United States (Gale et al. 2007; Puri and Zhong 2010; Ward et al. 2008). The upsurge in virulence and mycotoxin production was associated with isolates of *F. graminearum* typically producing 3-acetyl-deoxynivalenol (3ADON) instead of the typical 15-acetyl-deoxynivalenol producing isolates (Kelly et al. 2018; Puri and Zhong 2010). Virulence, often perhaps inappropriately referred to as aggressiveness, is an important trait of the fungus along with DON production to determine the potential of an isolate to cause yield loss. Understanding the genetics of virulence and DON production could help minimize losses due to this disease (Talas et al. 2012). A candidate gene-based mapping approach was utilized to identify significant marker-trait associations in virulence and mycotoxin production in *F. graminearum* in Germany (Talas et al. 2012). However, a candidate-gene based mapping approach will detect the effects of SNPs within previously identified genes; it cannot identify novel genes affecting a pathogen trait (Talas et al. 2016).

Thus, genome-wide association mapping of a natural pathogen population is a better approach to identify novel genes associated with virulence and mycotoxin production.

Along with planting resistant wheat cultivars, applying fungicides in a timely manner is a recommended measure to manage FHB (Cowger et al. 2020). Demethylation inhibitor (DMI) fungicides are effective on reducing FHB and DON when applied at early anthesis (Willyerd et al. 2012). The DMI fungicides inhibit sterol biosynthesis in fungal membrane by targeting C14-demethylase, thereby degrading membrane integrity and limiting cell survival of the fungal pathogens (Liu et al. 2011). The efficacy of DMI fungicides, however, is weather dependent and can reduce FHB and DON by 70% when used with a moderately resistant cultivar (Paul et al. 2019). In addition, the high variability in the efficacy of the DMI fungicides has been hypothesized to depend on the variation in sensitivity to the fungicides within and among fungal populations (Paul et al. 2008). The sensitivity of *F. graminearum* to DMI fungicides has been found to be variable depending on the presence of one of the three *CYP51* paralogs (Liu et al. 2011). Yin et al. (2009) identified a tebuconazole-resistant isolate of *F. graminearum* from infected wheat heads in China, however, no difference was observed in the expression levels or DNA sequences of *CYP51A* and *CYP51B* between resistant and susceptible isolates. Besides the role of *CYP51* paralogs, point mutation in the target gene *CYP51,* over-expression or energy-dependent drug efflux mechanisms are also known to affect sensitivity or resistance in different fungal species (Délye et al. 1998; Ma et al. 2006; Reimann and Deising 2005). In the United States, the use of tebuconazole for managing FHB began with the crisis exemption during 1997 in the state of North Dakota, however, its use in several other foliar disease of cereals began much earlier (McMullen et al. 2012). Paul et al. (2008) compared the efficacy of DMI fungicides through a multivariate meta-analysis of over 100 uniform field trials and reported the

combination of prothioconazole and tebuconazole to be the most effective in suppressing FHB. Consistent use of DMI fungicides to manage plant diseases have resulted in DMI-resistance in many economically important fungal pathogens including *Blumeria graminis* f. sp. *tritici* (Godet et al. 1998), *Colletotrichum cereal* (Wong et al. 2007), *Erysiphe graminis* (Delye et al. 1998), *Mycosphaerella graminicola* (Mavroeidi et al. 2005), and *Venturia inequalis* (Köller et al. 1997) indicating a need to incorporate effective measures. By 2014, a tebuconazole-resistant field isolate of *F. graminearum* was identified from New York (Spolti et al. 2014). The tebuconazole resistant isolate was shown to produce larger amounts of DON as compared to the tebuconazole sensitive isolates indicating pathogenic fitness associated with fungicide resistance. No such study of variation in fungicide sensitivity exists for the *F. graminearum* population from North Dakota. With emerging fungicide resistance reports in the United States and associated pathogenic fitness, there is a need to evaluate the *F. graminearum* population from North Dakota for DMI sensitivity and associated pathogen fitness.

Genome wide association study (GWAS) is a powerful tool that emerged with the advancement of sequencing technologies to identify genomic regions in an organism that govern quantitative traits taking advantage of recombination events accumulated over generations (Bartoli and Roux 2017). It facilitates identifying genomic regions that are associated with a phenotype of interest to a fine level if corrected for false positives arising from population structure and familial relatedness between the individuals under study. GWAS of quantitative traits such as DON production, fungicide sensitivity and aggressiveness in *Fusarium sp* causing FHB has been conducted in Europe in recent years (Castiblanco et al. 2018; Castiblanco et al. 2017; Talas et al. 2016; Talas et al. 2012). Talas et al. (2016) identified 26 candidate genes for aggressiveness, 17 for DON production and 51 for propiconazole sensitivity with a false

discovery rate (fdr) correction at 0.05 from a diverse collection of *F. graminearum* isolates from Germany. However, no similar studies have been reported in North America. The objectives of this study were to: (i) assess phenotypic variation among the *F. graminearum* isolates collected from North Dakota between 1980-2000 (R isolates), 2008, 2010 and 2013; (ii) conduct GWAS of aggressiveness, mycotoxin production, spore productivity and fungicide sensitivity.

Materials and Methods

Fungal Isolates and Identification

A total of 165 *F. graminearum* isolates used in a previous study (Puri and Zhong 2010) along with 18 other isolates collected from the state of North Dakota between 1980 and 2013 were used in this study. Of the 183 isolates, 105 isolates were collected in 2013, 23 in 2010, 34 in 2008 and 21 from 1980-2000 (Appendix A2). All the isolates were identified as *Fusarium graminearum* based on morphology and PCR amplifications of portions of the reductase and histone H3 genes.

DNA Extraction and Two Enzyme Genotyping-by-Sequencing (GBS)

DNA was extracted from mycelia grown for 4-5 days on potato dextrose agar (PDA) media using the DNeasy Plant Mini Kits (QIAGEN, Cat No./ID:69104). DNA concentration of each isolate was quantified using a Qubit 2.0 Fluorometer (ThermoFisher Scientific). DNA concentrations were adjusted to ~50 ng/µL and stored at -20°C until submitted for sequencing.

Genomic data for 183 *F. graminearum* isolates were obtained using the two-enzyme genotyping-by-sequencing (GBS) approach (Poland et al. 2012). Briefly, the genomic DNA of each isolate was digested with enzymes *PstI* and *MspI*. The resulting fragments were size selected for 300 bp followed by ligating adapters. The adapters ligated to an individual DNA fragment comprised of a unique barcode adapter and a common adapter. Individual fragments

were then pooled, purified, amplified, and sequenced using 150-bp single-end reads on an Illumina HiSeq2000 at the USDA-ARS Genotyping Laboratory, Fargo, ND. The quality of resulting reads was checked using FASTQC v.0.11.7. Based on per base sequence quality report in fastqc, all sequences were trimmed to achieve the least quality of 32 using FASTX toolkit v.0.0.14 fastx_trimmer v0.0.6. Only sequences longer than or equal to 120 bp were retained. All alignments were performed using Bowtie2 v.2.3.0. Samtools 1.5 was used to sort and index the sequences based on their barcodes. Reference isolate *F. graminearum* PH-1 (Rresv4.0, King et al .2015) was used to call SNPs using Samtools 1.5. Filtering conditions were set as minimum sequence quality =20. BCFtools 1.1 was used to discard any SNPs that did not meet these criteria.

Phenotyping

Virulence

Virulence (based on disease severity on a host genotype) of 183 isolates was evaluated in a greenhouse over two years (2017GH and 2018GH) on two spring wheat cultivars Alsen and Wheaton. The isolates were evaluated for their ability to produce DON based on DON accumulation in Alsen cultivar in the 2018 greenhouse experiment. Alsen, developed at NDSU, was derived from the cross ND674//ND2710/ND688 and exhibits moderate resistance to FHB due to it having *Fhb1* (Frohberg et al. 2006) while Wheaton is highly susceptible to FHB (Wilcoxson et al. 1992). The experiment was laid out as a completely randomized design (CRD) with three replications for each isolate and all isolates were randomized within each replication. For each wheat genotype, 3 seeds per replication were planted in 6” clay pots (S-6 6” Standard pot – Red Clay/Basalt, Diameter-6”, Height-5.25”, model:GSP-MC-M-01) filled with PRO-MIX Biofungicide growing medium (Sphagnum peat moss (75-85%), perlite, vermiculite, limestone

and biofungicide). The experiment was conducted at 18 ± 2^0C with 16 h of supplemental light (600-watt high pressure sodium lamps by P.L. Light Systems, Inc., Beamsville, Ontario, Canada) until anthesis. Plants were irrigated daily and fertilized with Peters General Purpose 20-20-20 fertilizer (JR Peters, Inc., Allentown, Pennsylvania).

To prepare spore suspensions, the *F. graminearum* isolates were inoculated in-vitro onto 6 cm Petri plates (60 x 15 mm style, Corning™Falcon™) containing mung-bean agar media by smearing a 4 mm mycelial plug with a policeman loop. The Petri dishes were incubated at room temperature for 7 days (12 hours light/dark cycle) under fluorescent and near UV light. Spores were harvested from plates by adding distilled water and scraping the agar surface with a rubber policeman loop, filtered through a layer of mira cloth and quantified in a hemocytometer (Spencer Improved Neubauer 1/10 mm deep US Pat. No. 2.660.091). The final inoculum suspension was adjusted to 10^5 spores/mL in water. When the plants were at 50% anthesis, 10 µL of freshly prepared spore suspension was inoculated into the central spikelet of a spike (Stack et al. 2002) using a syringe (10 mL BD syringe, Becton Dickinson & CO., NJ) fitted with a needle (26$G^{1/2}$ Precision Glide® Needle, Becton Dickinson & CO., NJ). Approximately 6-8 spikes were inoculated per pot. Inoculated wheat spikes in each pot were then bagged with a clear polythene bag sprayed with water to maintain high humidity for 48 hours in order to facilitate disease development. After 48 hours of incubation, bags were removed. The plants were maintained in the same greenhouse room at 23 ± 2^0C until maturity. Disease severities were recorded 21 days post inoculation. For each inoculated spike, percentage of infected spikelet (PIS) was estimated based on a 0 (no infection) to 100% (each spikelet on each head infected) disease severity scale described by Stack et al. (2002) and mean DS caused by each isolate was calculated by averaging PISs of all spikes inoculated.

DON production in planta

The inoculated heads of Alsen for each replication of isolates were hand-harvested and threshed. Grains from replications for the same isolate were then combined and ground to fine powder. DON content was analyzed following the method of Mirocha et al. (1998). A standard curve of 0 to 160 ppm of DON was used to generate a linear equation which was used to calculate the DON values of each sample.

Fungicide sensitivity assay

Sensitivity to eight concentrations (0, 0.5, 1,5, 10, 25, 50, and 100 micrograms per mililitres) of demethylation inhibitor fungicides (DMI), tebuconazole and prothioconazole, was determined in-vitro for 183 isolates using a microplate assay described by Talas and McDonald (2015) except that the plates were incubated in a shaker at 100 rpm at 26°C for 4 days. Spore suspensions for each isolate were produced in-vitro following the procedure described by Puri and Zhong (2010) and adjusted to 10^5 spores/mL. The fungicides were dissolved in acetone to make a stock solution of 10 mg/mL and working stocks were prepared by serial dilutions in acetone. The acetone concentration was kept constant in all dilutions (2.6% v/v) to get final concentrations of 0, 0.5, 1, 5, 10, 25, 50 and 100 µg/mL in potato dextrose broth (PDB) media. The fungicide amended PDB of each working stock was mixed with 0.33 µg streptomycin sulfate per mL to prevent any bacterial contamination. One hundred and fifty microlitres of this product were added to each well of sterile flat bottom micro titer plates (Greiner Bio-One). Fifty microlitres of spore suspension of 10^5 spores per mL were added to each well. The plates were sealed with parafilm, covered with tinfoil and placed in a shaker at 100 rpm at 26°C for 4 days. For each isolate, the experiment was repeated three times with three replications in each run. The optical density (OD) value of each well was measured on an ELISA plate reader (EPOCH

microplate spectrophotometer, BIOTEK) at 620 nm wavelength as recommended by Fungicide Resistance Action Committee (FRAC) for monitoring DMI resistance in *Fusarium graminearum* (https://www.frac.info/docs/default-source/monitoring-methods/approved-methods/gibbze-microtiter-monitoring-method-bcs-2006-v1.pdf?sfvrsn=659a419a_4).

The mean optical density of fungal growth in each fungicide concentration was converted into a percentage inhibition of fungal growth relative to the untreated control. These data were regressed against the logarithm of the fungicide concentration, and the concentration that effectively inhibited the fungal growth by 50% relative to the untreated control (EC50) was determined by interpolation of the 50% intercept. The analysis was performed using the general linear model procedure in Statistical Analysis System v9.4 (SAS Institute, Cary, NC).

Spore productivity assay

Spore productivity of 183 isolates was evaluated in three replications and the experiment was conducted three times. The initial spore suspension for each isolate was produced by placing an agar-plug on mung-bean agar plates and incubating them for 5 days under fluorescent and near UV light conditions with a 12/12 h of light/dark cycle. The spores were washed off the plates with distilled water and final spore concentration was maintained at 5000 spores per mL. One hundred microlitres of spore suspension was used as inoculum for estimating spore productivity of each isolate. MBA plates smeared with the inoculum were incubated for 7-9 days at 26 °C temperature under light conditions as described above. After 7-9 days of incubation, 5mL of distilled water was used to wash off spores. Spore concentration was measured in spores/mL using a hemocytometer (Spencer bright-line, Neubauer-type, Cambridge Instruments, Inc., MA).

Statistical Analysis

Frequency distribution of all traits evaluated in this study were tested for normality using the Shapiro-Wilk test and homogeneity of variances was verified using Levene's test ("car" package) in RStudio version 3.6.1 (Fox and Weisberg 2019; RStudio Team 2016). Spearman's rank-correlation coefficients between virulence, DON production, spore productivity, and fungicide sensitivity to two triazole fungicides were calculated. Type III analysis of variance (ANOVA) for each trait was calculated with Satterthwaite's method for each environment using linear mixed effect model in the lmerTest package (Kuznetsova et al. 2017) in RStudio v.3.6.1.

GWAS Analysis

A total of 11,965 SNPs was used to detect significant marker-trait associations in TASSEL v5.2.51. Principal component analysis (PCA) was performed to detect the level of population stratification in the population while the familial relatedness was estimated through identity-by-state (K matrix).

Four statistical models, Naïve, PCA, K, and PCA+K, were tested in TASSEL v5.1 and the best model was selected based on the quantile-quantile (QQ) plot. The significance of the marker-trait association was determined based on false discovery rate (FDR) with threshold set to a cutoff of ≤ 0.1.

Results

Variation in Phenotypic Traits

Spring wheat varieties Alsen and Wheaton showed a wide range of reactions to the *F. graminearum* isolates evaluated in this study. Virulence scored as the percentage of infected spikelets (PIS) displaying symptoms of Fusarium head blight (FHB) ranged between 6.14 to 100% across experiments on Alsen while it ranged between 7 to 100% on Wheaton (Table 10).

Frequency distribution of isolates for virulence on both wheat varieties deviated from a normal distribution (Figure 10). Average virulence of isolates was significantly higher on Wheaton as compared to Alsen at $P<0.0001$ across all the environments (data not shown). Furthermore, the mean virulence of isolates grouped by the year of collection was also analyzed but no significant difference was observed between isolates collected in different years for their virulence on either of the wheat varieties. Analysis of variance showed significant differences among isolates, trial, and isolates-by-trial ($p<0.01$) for virulence on both Alsen and Wheaton (Table 11).

DON production by each isolate on spring wheat cultivar Alsen was assessed in the 2018 greenhouse trial only. All the isolates showed significant difference in their ability to produce DON on Alsen and the DON accumulation values ranged between 0.7 to 52.61 parts per million (ppm) with an average DON production of 7.50 ppm. The frequency distribution of isolates for DON production revealed a deviation from a normal distribution ($P<0.0001$).

Isolates showed significant variation at $P < 0.0001$ for spore productivity on mung-bean agar media assessed at 7-9 days after inoculation (Table 12). Levene's test for homogeneity of variances held true between trials and therefore spore productivity data of each isolate across trials were combined. The frequency distribution of spore productivity revealed deviation from a normal distribution ($P<0.0001$) (Figure 10). Spore productivity for isolates across trials ranged between 0.3 to 140 million spores per plate with mean spore productivity of 15.97 million spores/plate.

Fungicide sensitivity of the *F. graminearum* isolates to two triazole fungicides, tebuconazole and prothioconazole, did not follow a normal distribution (Figure 10) with mean effective concentration inhibiting 50% of the fungal growth (EC50) values of 1.4 mgL^{-1} and 3.28 mgL^{-1}, respectively. EC50 values ranged from 0.02 to 9.97 mg/L for tebuconazole while the

range was 0.02 to 25.12 mg/L for prothioconazole. For reference, the sequenced strain of *F. graminearum*, PH-1, was also evaluated for fungicide sensitivity to tebuconazole and prothioconazole. EC50 values of the PH-1 strain for tebuconazole ranged from 0.02 to 1.60 mg/L, while for prothioconazole, the values ranged from 0.02 to 3.21 mg/L. Four isolates (Fg13_44, R1214, R1171, R1261, R1246) showed more than 100-fold higher EC50 values for tebuconazole as compared to the most-sensitive isolate (Fg10_135_2). On the other hand, nine isolates (Fg13_65, R1246, R1214, Fg08_12, Fg13_78, Fg08_7, Fg08_4, Fg13_66, and R1707) showed over 100-fold higher EC50 values for prothioconazole as compared to the most-sensitive isolate (Fg10_121_03). Analysis of variance showed significant differences among isolates and isolates-by-trial for fungicide sensitivity to both tebuconazole and prothioconazole ($p<0.0001$) (Table 13). No significant difference was observed between trials as can be expected when the experiments were conducted under controlled laboratory conditions. Broad-sense heritability for fungicide sensitivity were 0.91 and 0.89, for tebuconazole and prothioconazole, respectively.

Table 10. Summary of trait phenotypes of 183 *Fusarium graminearum* isolates collected from the state of North Dakota.

Traits	Cultivar/ Fungicide	Environments	Mean ± SD	Range	H^2
Virulence	Alsen	17GH	0.27 ± 0.14	0.06 – 0.78	0.46
		18GH	0.54 ± 0.16	0.13 – 1.00	
	Wheaton	17GH	0.87 ± 0.22	0.07 – 1.00	0.73
		18GH	0.92 ± 0.12	0.20 – 1.00	
DON production	Alsen	18GH	7.50 ± 6.72	0.70 – 52.61	…
Spore productivity (million spores/plate)		Trial 1	16.25 ± 23.07	0.20 – 255	0.94
		Trial 2	15.59 ± 18.65	0.25 – 128.75	
		Trial 3	16.02 ± 21.14	0.20 – 165.00	
Fungicide Sensitivity (EC50)	Tebuconazole	Trial 1	1.03 ± 0.76	0.02 – 7.22	0.91
		Trial 2	1.06 ± 0.81	0.02 – 9.97	
		Trial 3	1.02 ± 0.74	0.02 – 7.82	
	Prothioconazole	Trial 1	3.33 ± 4.15	0.02 – 24.75	0.89
		Trial 2	3.26 ± 3.87	0.02 – 25.12	
		Trial 3	3.25 ± 3.86	0.02 – 24.51	

Virulence, severity of fungal infection based on mean symptomatic proportions of infected spikes; DON production, deoxynivalenol produced by 183 *Fusarium graminearum* isolates on Alsen in 2018 greenhouse; Spore productivity, Spores produced 183 isolates on mung-bean agar media plates; Fungicide sensitivity, EC50 value of 183 isolates evaluated for triazole fungicides tebuconazole and prothioconazole.
Cultivar, spring wheat cultivar from which the data was obtained; Tebuconazole, Prothioconazole, triazole fungicides.
GH, greenhouse; Trial, trials performed under laboratory conditions; SD, standard deviation; H^2, broad-sense heritability.

Table 11. Analysis of variance to differentiate components contributing to phenotypic variation in virulence of 183 isolates on spring wheat cultivar Alsen.

Sources of Variation	df	TSS	MSS	F-Value	Pr (>F)
Trial	1	4265.74	4265.74	2.82	0.09^{ns}
Rep (Trial)	4	59.41	14.85	0.01	1.00^{ns}
Isolates	181	1215287.18	6714.29	4.45	<0.0001***
Trial: Isolates	173	551909.86	3190.23	2.11	<0.0001***
Error	711	0.04365	0.00006		

***, $P<0.001$; **, $P<0.01$; ns, $P>0.05$; df, degrees of freedom; TSS, total sum of squares; MSS, mean sums of squares.

Table 12. Analysis of variance (type III) with Satterthwaite's method for spore productivity of 222 *Fusarium graminearum* isolates.

Sources of Variation	df	TSS	MSS	*F*-Value	Pr (>F)
Trial	2	29.80	14.90	0.02	0.98^{ns}
Rep (Trial)	6	69.15	11.52	0.02	1.00^{ns}
Isolates	221	7186921.13	32520.00	47.30	<0.0001***
Trial (Isolates)	442	172804.92	390.96	0.57	1.00^{ns}

***, *P*<0.0001; df, degrees of freedom; TSS, total sum of squares; MSS, mean sum of squares.

Table 13. Analysis of variance to separate the different variance components contributing to phenotypic variation in fungicide sensitivity of isolates.

Sources of Variation	df	Tebuconazole			df	Prothioconazole		
		MSS	F-Value	Pr(>F)		MSS	F-Value	Pr(>F)
Trial	2	0.14	0.66	1.00	2	5.81	0.95	0.51
Isolates	181	4.67	21.38	<0.0001***	184	111.98	18.24	<0.0001***
Trial: Isolates	362	0.25	1.15	0.041*	363	8.91	1.45	1.71e-06***
Error	1628				1595	0.004		

***, *P*<0.05; ***, *P*<0.0001; df, degrees of freedom; MSS, mean sum of squares.

Correlation Analysis

Spearman's correlation analysis revealed a moderate but significant positive correlation between virulence and DON production in all experiments (*P*<0.01) (Table 13). Correlation coefficients between virulence and DON production in Alsen ranged between 0.36 to 0.44. However, no significant correlation was observed between virulence and spore productivity of *F. graminearum* isolates in any experiment. The correlation coefficients for sensitivity to triazole fungicides and virulence of *F. graminearum* isolates on Alsen were mostly negative ranging between -0.1 to -0.16. A moderate but significant negative correlation was observed between DON production and sensitivity to tebuconazole fungicide while the correlation was non-significant between DON production and sensitivity to prothioconazole fungicide.

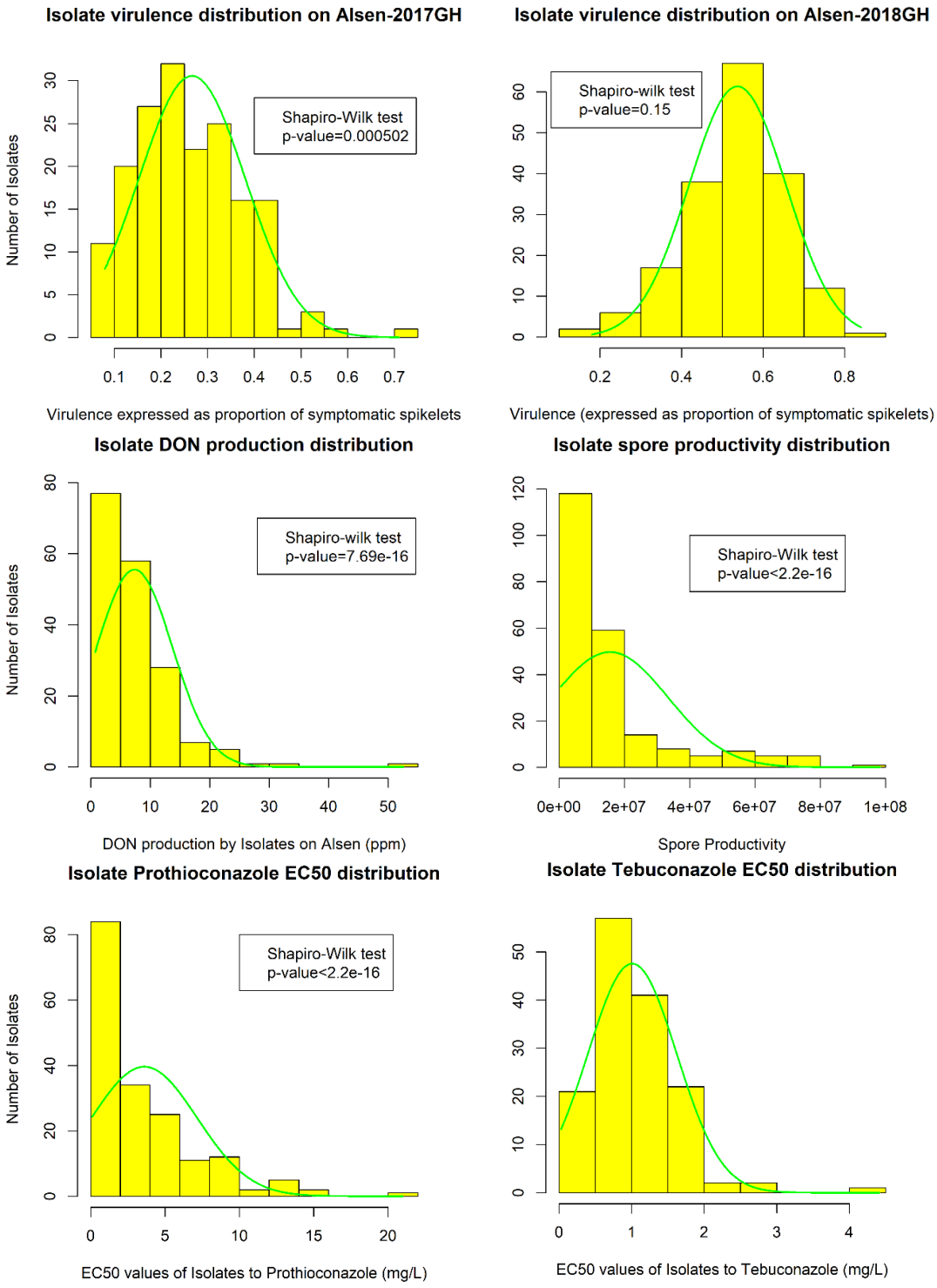

Figure 10. Histogram plots with normal curve (green line) for traits of virulence evaluated in 183 *Fusarium graminearum* isolates collected from North Dakota.
Shapiro-Wilk test, test for normality; ppm, parts per million; EC50, effective concentration of the fungicide that inhibits fungal growth by 50%.

Table 14. Spearman's correlation coefficients among traits of virulence, spore productivity, DON production, and fungicide sensitivity to tebuconazole and prothioconazole fungicides of 183 *Fusarium graminearum* isolates collected from North Dakota.

	Vir18GH	Vir17GH	Spore	DON	TEB	PRO
Vir18GH	…	0.34***	-0.05^{ns}	0.44***	-0.16^{ns}	-0.1^{ns}
Vir17GH	…	…	0.09^{ns}	0.36***	-0.15^{ns}	0.00^{ns}
Spore	…	…	…	0.21**	0.00^{ns}	-0.03^{ns}
DON	…	…	…	…	-0.30***	-0.09^{ns}
TEB	…	…	…	…	…	0.10^{ns}
PRO	…	…	…	…	…	…

***, $P<0.0001$; **, $P<0.01$; ns, $P>0.05$; Vir, virulence data from 2017 or 2018 greenhouse evaluation; Spore, spore productivity experiment; DON, DON production on Alsen in 2018 greenhouse experiment; TEB, fungicide sensitivity to tebuconazole fungicide; PRO, fungicide sensitivity to prothioconazole fungicide.

Marker Distribution, Population Structure, and Linkage Disequilibrium

The two-enzyme Genotyping-by-Sequencing (GBS) generated a total of 104770 polymorphic SNP markers distributed evenly across four chromosomes of the *F. graminearum* genome. Of the 104,770 SNPs analyzed, 30.13% were mapped on chromosome 1, 26.89% on chromosome 2, 20.91% on chromosome 3, and 22.06% on chromosome 4.

The SNPs were filtered for a minimum allele frequency of 5% along with further filtering to allow a maximum of 10% missing SNPs data. This filtering resulted in a total of 11,965 SNPs to be used for genome-wide association analysis. The hierarchical ward clustering analysis revealed two major clusters in the GWAS panel (groups 1, and 2) and group 2 was further sub-divided into three subgroups (2A, 2B, and 2C) (Figure 11). These subdivisions were further supported by the principal component analysis (Figure 12). The first five principle components explained 10.5, 4.9, 3.3, 2.8, and 2% of the total genotypic variation, respectively. The first four PCs clustered the GWAS panel into four groups (Figure 12). The composition of groups and subgroups based on year of isolate collection and chemotype of isolates is presented in Table 14.

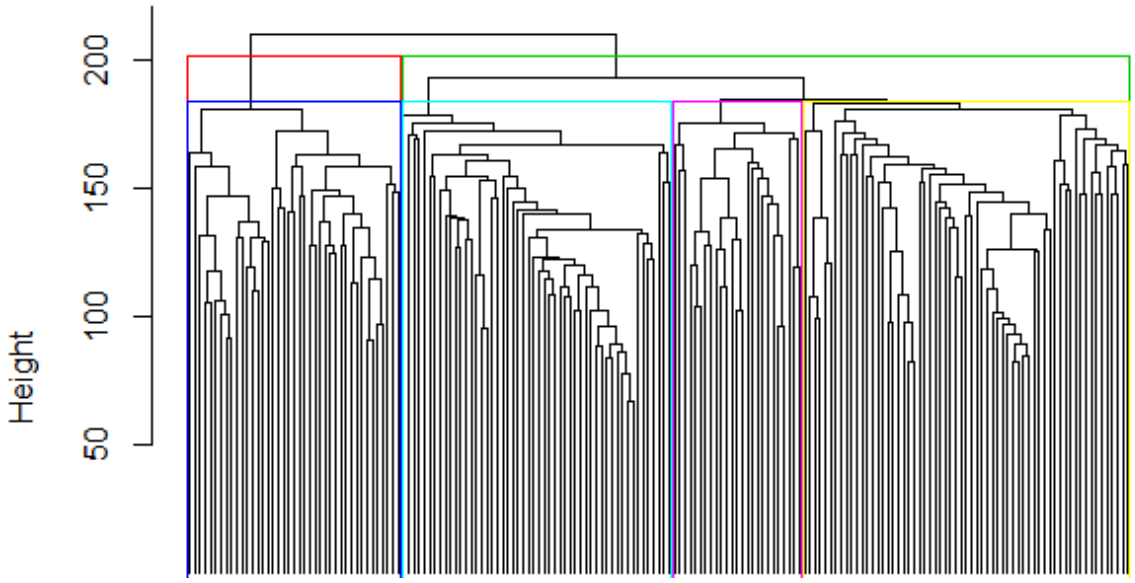

Figure 11. Population structure analysis of 183 *Fusarium graminearum* isolates by hierarchical Ward clustering.
Vertical red and green lines separate the two major groups (group 1 and group 2) while vertical purple, blue, pink, and yellow lines separates groups and subgroups (group1, subgroup 2A, 2B, and 2C).

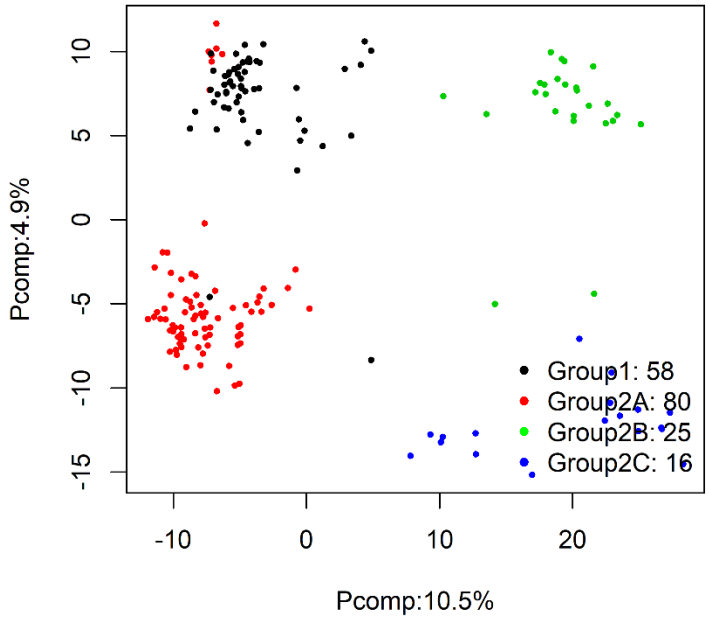

Figure 12. Population Structure based on principal component analysis in 183 *Fusarium graminearum* isolates.
Four different colors represent clusters: black = Group 1; red = Group 2A, green = Group 2B; and blue = Group 2C.

Table 15. Proportion of isolates clustered into groups and subgroups based on year of collection and chemotype.

Group	Subgroup	# of Isolates	Proportion						
			R	2008	2010	2013	3ADON	15ADON	UNK
1	1	39	0.14	0.11	0.13	0.62	0.67	0.31	0.03
2	2A	52	0.35	0.31	0.25	0.09	0.10	0.41	0.49
	2B	26	0.04	0.00	0.08	0.88	0.12	0.77	0.12
	2C	64	0.02	0.00	0.06	0.92	0.14	0.79	0.06

Group and subgroups, based on ward clustering; #, number; R isolates, isolates collected before 2008; 3ADON, 15ADON, UNK, isolates of 3ADON, 15ADON and undetermined chemotype.

The LD analysis based on pairwise squared correlations (r^2) for all 11,965 polymorphic SNP markers exhibited a rapid decay in LD within a physical distance of 5 Kb. The mean LD decayed to 0.36 between markers, with physical distance of < 1 Kb and 0.19 at <5 Kb (Figure 13).

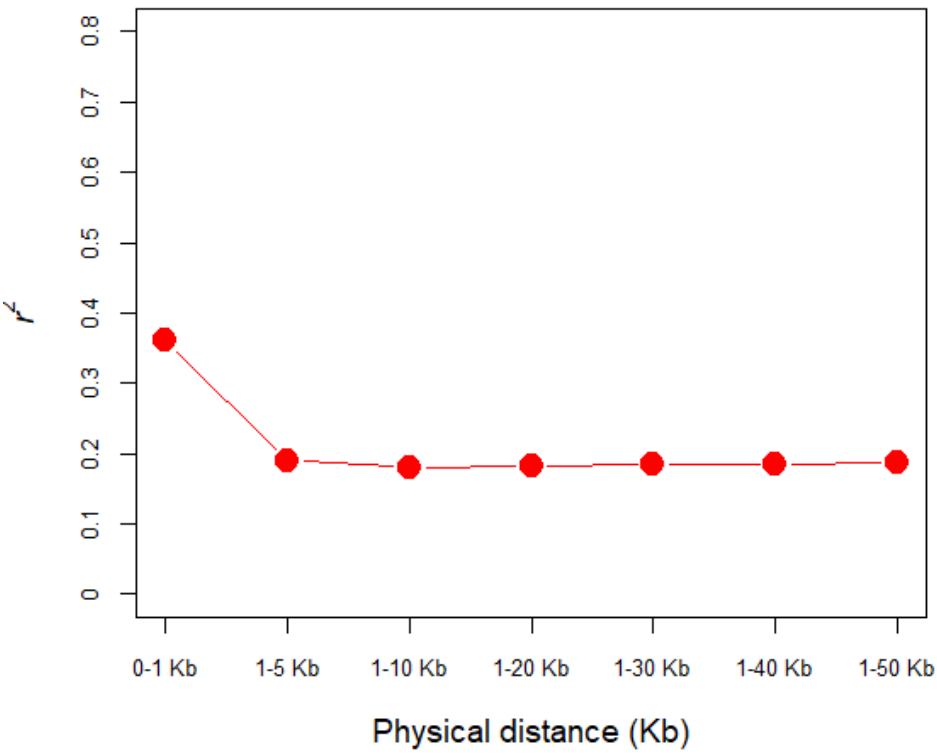

Figure 13. Genome-wide distribution of the pairwise LD measure r^2 based on the physical distance between 11,965 SNPs.

Marker-Trait Associations (MTAs)

Based on the least deviation observed in the QQ plot, mixed linear model (MLM) which accounts for familial relatedness (K-model), was the best fit (Figure 14). The model detected a total of 504 MTAs for DON production, 504 MTAs for virulence, 521 MTAs for spore productivity, 520 for tebuconazole sensitivity, and 548 for prothioconazole sensitivity ($P<0.05$). The significance threshold adjusted to an FDR cutoff of ≤ 0.1 resulted into two significant MTAs for DON production, one for tebuconazole sensitivity, and one for prothioconazole sensitivity (Figure 15).

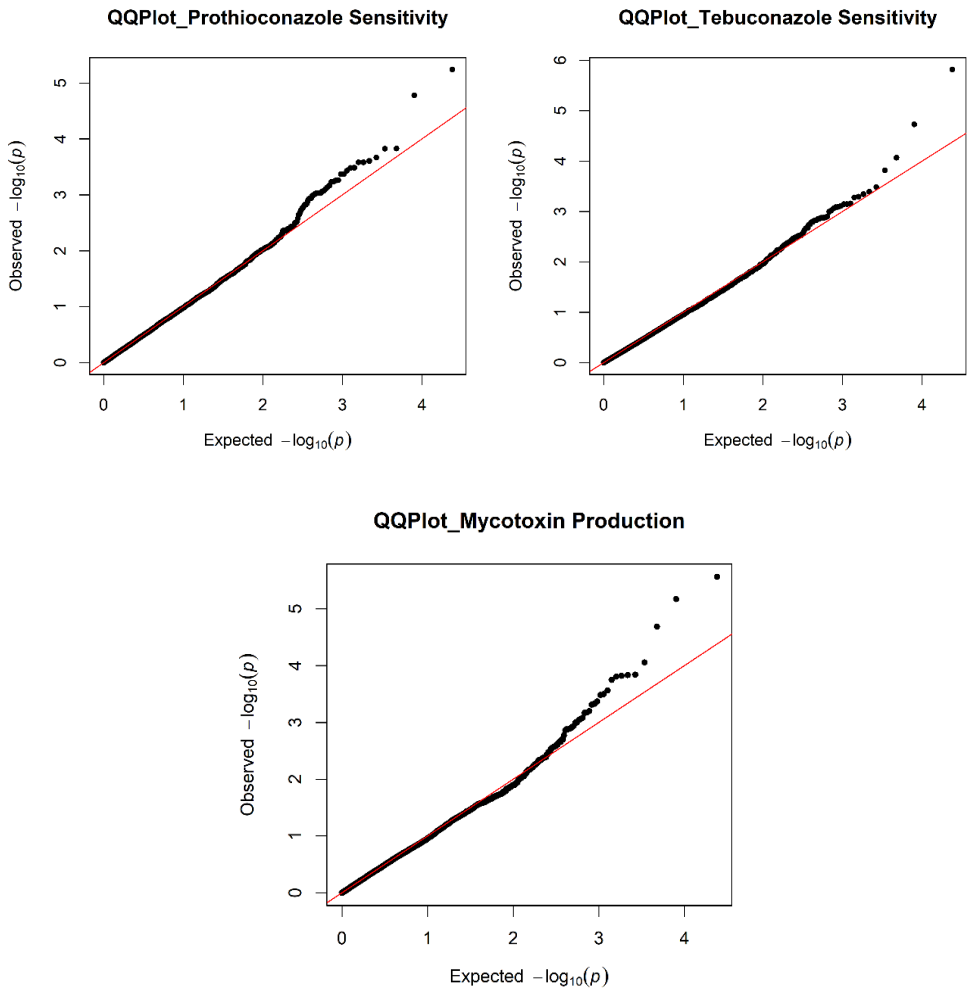

Figure 14. Quantile-quantile plots between expected and observed *P* values (-log10) for the traits of virulence, DON production on Alsen, spore productivity, and fungicide sensitivity.

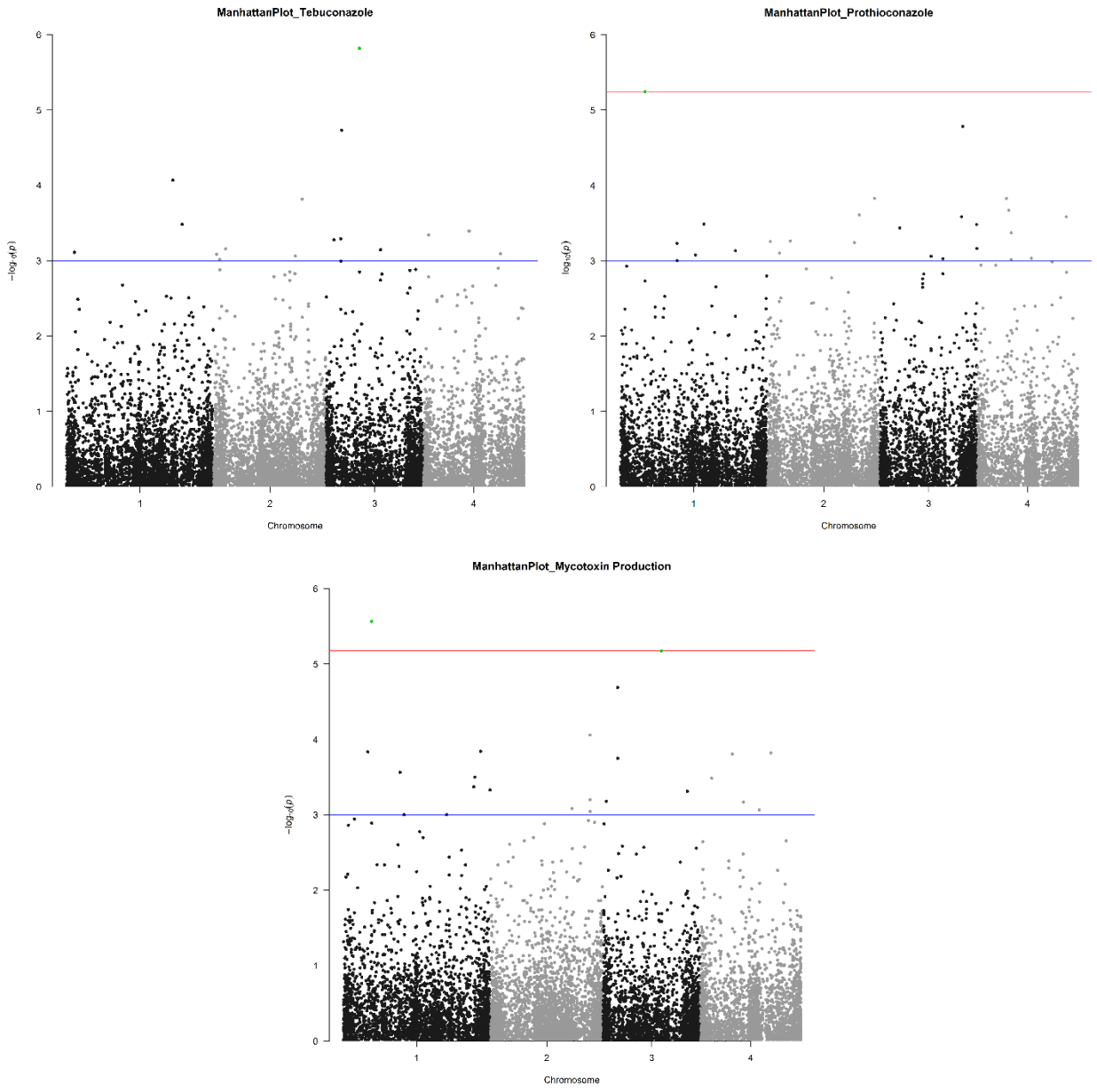

Figure 15. Manhattan plot showing marker-trait association across 183 *Fusarium graminearum* isolates for traits of virulence, DON production, spore productivity, and fungicide sensitivity. Horizontal blue line indicates a logarithm of odds (LOD) at 3.0 while red line indicates a LOD at fdr ≤0.1.

Based on the physical locations of SNPs in the reference genome of *Fusarium graminearum* strain PH-1, genes and their predicted functions were derived from annotations based on Rothamsted research. DON production of isolates was associated with two significant SNPs. The most strongly associated MTA for DON production on Alsen was located 1.6 Kb

upstream of the FGRAMPH1_01G01773 gene on the forward strand in chromosome 1, while 0.5Kb upstream of FGRAMPH1_01G01771 gene on the reverse strand, and explained 14.13% of total phenotypic variation in DON production (Table 15). The gene FGRAMPH1_01G01773 codes for a protein FGRAMPH1_01T01773 with no known function while FGRAMPH1_01G01771 codes for a glycosyltransferase with a nucleotide-diphospho-sugar transferases domain.

The second MTA for DON production was in a protein encoding gene FGRAMPH1_01G19767 on chromosome 3 and explained 12.95% of the total phenotypic variation. The gene has three coding exons and encodes for a GTP-binding protein SAS1 that belongs to the Ras family.

The MTA for tebuconazole sensitivity was located on a protein coding gene with two coding exons on chromosome 3 and explained 17.37% of the total phenotypic variation. The gene FGRAMPH1_01G18195 encodes a 4-aminobutyrate aminotransferase protein that is involved in the gamma-aminobutyric acid metabolism. The MTA for prothioconazole sensitivity was located on FGRAMPH1_01G01577, a gene encoding peroxisomal membrane protein 4, on chromosome 1. It explained about 14.75% of the total phenotypic variation in sensitivity to prothioconazole sensitivity in *F. graminearum* isolates.

Table 16. Significant single nucleotide polymorphisms (SNPs) associated with the traits of DON production and fungicide sensitivity in *Fusarium graminearum.*

Trait	Marker	Chr	Pos	*P*-value	FDR	R^2	Effect
DON	SFG1_2292465	1	2292465	2.71e-06	0.08	14.14	-1.07
	SFG3_4671721	3	4671721	6.70e-06	0.10	12.95	1.50
TEB	SFG3_2669008	3	2669008	1.51e-06	0.08	17.37	0.77
PRO	SFG1_1997745	1	1997745	5.70e-06	0.10	14.75	4.96

DON, deoxynivalenol production; TEB, sensitivity to tebuconazole fungicide; PRO, sensitivity to prothioconazole; Marker, single nucleotide polymorphism marker; Chr, chromosome; Pos, position in the chromosome; R^2, percentage of phenotypic variation explained by SNP; Effect, Effect of the reference allele on phenotype.

Discussion

In this study, we first evaluated 183 *Fusarium graminearum* isolates collected from the state of North Dakota over several years for variation in the traits of virulence, DON production, spore productivity, and fungicide sensitivity to two triazole fungicides. The same isolates were also genotyped by two-enzyme genotyping-by-sequencing using 11,965 polymorphic SNP markers. A significant range of variation was observed between isolates in terms of virulence on Alsen and Wheaton (P<0.01). Alsen is a hard-red spring wheat variety released by North Dakota State University known to possess *Fhb1* gene (Frohberg et al. 2006) derived from a Sumai3 derivative ND2710 and is resistant to FHB while Wheaton is a susceptible variety (Wilcoxson et al. 1992). As expected, the average virulence of isolates on Alsen was significantly lower as compared to that on the FHB-susceptible cultivar Wheaton at P<0.0001. We further analyzed the virulence of isolates on Alsen by the year of collection to see if the isolates were gradually able to overcome resistance conferred by Alsen. However, no significant difference was observed for virulence of isolates when grouped by year of collection in both years. In addition, no significant difference was observed for virulence when isolates were grouped by chemotype. Nevertheless, three isolates collected in 2013 and one isolate collected in 2008 (Fg13_44, Fg13_41, Fg13_56,

Fg08_5) consistently showed higher virulence on Alsen (>50% disease severity) and produced DON at higher levels compared to other isolates indicating a possible risk of resistance breakdown. More isolates should be collected in the regions where the isolates with high virulence on Alsen were sampled and evaluated for virulence on Alsen. This information is important to ensure the effectiveness of continually using *Fhb1* in wheat cultivars to reduce impact of FHB on wheat production.

Fungicide sensitivity of 183 isolates to two triazole fungicides, tebuconazole and prothioconazole, evaluated in this study across three trials showed significant differences in the EC50 values ($P<0.0001$). Sensitivity of *F. graminearum* isolates to tebuconazole fungicides varied between 0.02 to 9.97 mg/L for tebuconazole while the range was 0.02 to 25.12 mg/L for prothioconazole. The EC50 value of isolates observed in this study is greater than the EC50 value of isolate Gz448NY11 reported by Spolti et al. (2014). The EC50 values of PH-1 strain for tebuconazole ranged from 0.02 to 1.60 mg/L, while for prothioconazole, the values ranged from 0.02 to 3.21 mg/L. Based on the most-sensitive isolate identified for each fungicide, isolates with over 100-fold higher EC50 values are considered resistant (TEB-R/PRO-R), isolates with 10-100 fold EC50 values were considered as reduced sensitive (TEB-RS/PRO-RS), and isolates with less than 10-fold EC50 values were considered as sensitive (TEB-S/PRO-S). Five isolates were found to be resitant to tebuconazole, 154 isolates had reduced sensitivity, and 12 isolates were sensitive. For prothioconazole fungicide, 9 isolates were found to be resistant (PRO-R), 129 isolates had reduced sensitivity (PRO-RS), while 37 isolates were sensitive (PRO-S). 74% and 75% of the isolates overall with reduced sensitivity to tebuconazole and prothioconazole fungicides, respectively, were of 15ADON chemotype (data not shown). This observation is

consistent with the observation made by Spolti et al. (2014) that isolates with greater resistance factor for tebuconazole and metconazole fungicides were of 15ADON chemotype.

GWAS analysis identified two marker trait associations significantly associated with DON production and for sensitivity to each of the fungicides, respectively. The highly significant MTA explaining 14.13% of the measured variation in DON production was detected 1.6 Kb upstream of the gene FGRAMPH1_01G01773 in chromosome 1. The gene FGRAMPH1_01G01773 codes for an uncharacterized hypothetical protein FGRAMPH1_01T01773 with no known function in *F. graminearum*. On the reverse strand, the SNP was located 0.5 Kb upstream of a FGRAMPH1_01G01771 gene that encodes protein with nucleotide-diphospho-sugar transferases domain (IPR029044). The nucleotide-diphospho-sugar transferases domain is usually found in a diverse family of glycosyl transferases that catalyzes the transfer of sugar moiety from UDP-glucose to a range of substrates forming glycosidic bonds (Campbell et al. 1997). UDP-glycosyl transferases (UGTs) in wheat, barley, and *Arabidopsis thaliana,* are shown to convert DON to an inactive or less toxic DON-3-O glucoside as a mechanism of resistance to FHB. However, the question of why the pathogen would produce such enzymes to reduce DON production remains unclear .

A second MTA explaining 12.95% of the variance in DON production was located in chromosome 3 on a protein coding gene FGRAMPH1_01G19767. The gene encodes a GTP-binding protein SAS1 that belongs to the Ras family. Disruption of *RAS2,* a gene encoding Ras-GTPases, impaired the virulence of *F. graminearum* on wheat in a study conducted by Bluhm et al. (2007). *Erf2,* another gene associated with RAS protein, has also been shown to be involved in virulence (Talas et al. 2016; Talas et al. 2012). Zhang et al (2013) associated FgRho4, a RAS GTPase, with DON production in *F. graminearum*. The SAS1 protein, through its role in DON

production, could possibly have resulted in increased virulence since DONs have long been considered virulence factors in *F. graminearum.*

Two MTAs were significantly associated with triazole sensitivity in *F. graminearum* in this study. The MTA for tebuconazole sensitivity in FGRAMPH1_01G18195 in chromosome 3 explained 17.37% of the total phenotypic variation. This gene encodes 4-aminobutyrate aminotransferase, a protein that is involved in the gamma-aminobutyric acid (GABA) metabolism. The gene encoding GABA along with various cell wall degrading enzymes (CWDE) were found to be upregulated when *F. graminearum* was grown on minimal media containing hop cell wall indicating a possible role in the induction of CWDE (Carapito et al. 2008). In other filamentous fungi, GABA is known to be an intracellular metabolite. In light of the sensitivity to tebuconazole, GABA may play a role in the induction of CYP450 enzymes to reduce the effect of fungicide on ergosterol biosynthesis, thereby conferring resistance to azole fungicides.

The MTA for prothioconazole sensitivity was located on FGRAMPH1_01G01577, a gene encoding peroxisomal membrane protein 4. Peroxisomal membrane protein 3, PEX3, was found to be regulating traits of vegetative growth, virulence, and sexual and asexual production in *F. graminearum* (Kong et al. 2019). However, the gene FGRAMPH1_01G01577 has not previously been identified for its role in reduced fungicide sensitivity to DMI fungicides in *F. graminearum.*

Studies to manage the disease have focused on increasing host resistance, however, researches focusing on understanding the pathogen biology related with virulence are limited. Knowledge on factors associated with virulence in the pathogen could potentially lead to development of wheat cultivars with improved resistance to FHB.

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APPENDIX A. SUPPLEMENTAL TABLES

Table A1. Origin, GRIN ID, pedigree, and crop improvement status of 223 global spring wheat accessions collected at the National Small Grains Collection in Aberdeen, Idaho used in the genome-wide association analysis.

SET	GRIN	PEDIGREE	ORIGIN	CROP STATUS
1	GHIRKA		Belarus	Cultivar
1	CI7605		Russia	Landrace
1	CI7652		Russia	Landrace
1	AUSTRALIAN_WHITE		Mexico	Uncertain Improvement Status
1	5339-3B-13T-2B	Egypt 101/Timstein//Frontana/4/Timstein/Kenya 58//Frontana/3/Yaqui/Timstein	Colombia	Breeding material
1	II-9430-6B-3T-1B-2T	Frocor/Yaqui//Kentana/3/Kenya 350AD9C2/Gabo/4/Frocor/3/McMurachy//Kentana/Yaqui	Colombia	Breeding material
1	II-9982-8T-3B-7T-1B-1T	Frocor/3/McMurachy/Kentana//Yaqui/4/Frocor/Cinco//Mayo 54/5/Frocor	Colombia	Breeding material
1	II-11380-1B-7T-1B	Kentana/Thatcher//Mentana/3/Frocor	Colombia	Breeding material
1	II-11380-3B-1T-1B	Kentana//Thatcher/Mentana/3/Frocor	Colombia	Breeding material
1	H648		Argentina	Breeding material
1	LEBARATA		Venezuela	Landrace
1	CITR 9045		Iraq	Landrace
1	J25		China	Landrace
1	CAGAYAN		Philippines	Landrace
1	3		Armenia	Landrace
1	57		Afghanistan	Landrace
1	BENVENUTO_INCA	Mentana/Lin Calel M.A.	Argentina	Cultivar
1	FLORENCE193		Portugal	Uncertain Improvement Status
1	RITANA1267		Brazil	Uncertain Improvement Status
1	II-116-2C-5C-(1-3C)-12C	Newthatch/Marroqui	Mexico	Breeding material
1	JO3|PI190450	selection from landrace	Norway	Cultivar
1	H32D13395		Portugal	Uncertain Improvement Status
1	GASPINO		Portugal	Uncertain Improvement Status
1	MAHON73|PI191734	selection from Mahon	Tunisia	Cultivar
1	LUIZIA_STRAMPELLI		Italy	Cultivar
1	BXRA8_10142		Argentina	Uncertain Improvement Status
1	AMERICANO44D	selection from landrace	Uruguay	Cultivar
1	ACORES		Portugal	Uncertain Improvement Status
1	GRANADERO_FS		Mozambique	Cultivar
1	ROEMER|PI192071		Mozambique	Uncertain Improvement Status
1	3511		Mozambique	Uncertain Improvement Status
1	FLORENCE		Mozambique	Uncertain Improvement Status
1	3618		Mozambique	Uncertain Improvement Status
1	KADOLZER_III	selection from Slovakian landrace	Czechoslovakia	Cultivar
1	3721	Svalov Kolben II/Schlanstedte	Sweden	Breeding material
1	NORDMER		Norway	Cultivar

Table A1. Origin, GRIN ID, pedigree, and crop improvement status of 223 global spring wheat accessions collected at the National Small Grains Collection in Aberdeen, Idaho used in the genome-wide association analysis (continued).

SET	GRIN	PEDIGREE	ORIGIN	CROP STATUS
1	EXTRA_KOLBEN_II	selection from Extra Kolben I	Sweden	Cultivar
1	RUMANIEN|PI192397		Romania	Uncertain Improvement Status
1	TREMESINO		Spain	Landrace
1	CINCO_A		Colombia	Breeding material
1	FRONTANA3671		Colombia	Cultivar
1	17		Japan	Breeding material
1	PI 202672		Finland	Breeding material
1	FROMBINA		Paraguay	Uncertain Improvement Status
1	D.I.V.6723		Argentina	Breeding material
1	190/52		South Africa	Breeding material
1	TAICHUNG_NO.23|PI241596	Saitama 27/Florence	Taiwan	Cultivar
1	TAICHUNG_NO.29|PI241597	selection from landrace Chingtao Shantung	Taiwan	Cultivar
1	TAICHUNG_NO.31|PI241598	selection from landrace Chingtao Shantung	Taiwan	Cultivar
1	CRIOLLO_SANTA_IRENE		Guatemala	Landrace
1	ANDES55	Kentana 48/Frontana//Mayo 48	Colombia	Cultivar
1	CDF33-1	Cadet/Florence 386	Israel	Breeding material
1	NFT6-2	Newthatch/Florence 386	Israel	Breeding material
1	PI254029		Europe	Uncertain Improvement Status
1	505.M.I.D.7	318AJ/Regent	Kenya	Breeding material
1	148/57		Croatia	Breeding material
1	SARATOVSKAJA210	Lutescens 2074/Sarroza	Russia	Cultivar
1	DIRK		Pakistan	Uncertain Improvement Status
1	CRETE15		Greece	Landrace
1	ALEPPO21		Syria	Landrace
1	TUNIS23		Tunisia	Landrace
1	TAICHUNG_NO.2|PI278740	Shoawase/Saitama 27	Taiwan	Cultivar
1	TAICHUNG_NO.31|PI278743	selection from landrace Chingtao Shantung	Taiwan	Cultivar
1	OSTKA_SUSKA	selection from Polish landrace	Poland	Cultivar
1	OSTKA_WIERZBIENSKA		Poland	Cultivar
1	KARNOBATSKA_RANASREIKA	N159/Gluretty	Bulgaria	Cultivar
1	UBILEINA_III		Bulgaria	Uncertain Improvement Status
1	CAILLOUX	Florence/Aurore	Tunisia	Cultivar
1	DANBATTA		Nigeria	Uncertain Improvement Status
1	WHITE_SPITZKOP		South Africa	Cultivar
1	SUGAMUXI68	Frontana/Kenya 58//Newthatch/3/2*Bonza	Colombia	Cultivar
1	SAMACA68	Bonza/Kentana 54//2*Africa Mayo	Colombia	Cultivar
1	MIRAMAR63	composite variety of 10 isolines of Frocor	Colombia	Cultivar
1	CIANO67	Pitic 62/Chris sib//Sonora 64 = II19957	Mexico	Cultivar
1	FLORENCE_AURORE|PI342641	Florence/Aurore	Lebanon	Cultivar

Table A1. Origin, GRIN ID, pedigree, and crop improvement status of 223 global spring wheat accessions collected at the National Small Grains Collection in Aberdeen, Idaho used in the genome-wide association analysis (continued).

SET	GRIN	PEDIGREE	ORIGIN	CROP STATUS
1	GIRUA	Willet/Colonias	Brazil	Cultivar
1	IJUI	Frontana*2/Kenya 58	Brazil	Cultivar
1	PEL-A683-64		Brazil	Breeding material
1	135D		Switzerland	Breeding material
1	M20-38		Switzerland	Breeding material
1	ROTER_LOWE	Koga II/Nos Nordgau	Mozambique	Cultivar
1	Z.88.116		Switzerland	Breeding material
1	62IT53		Switzerland	Breeding material
1	NO.9		Iran	Landrace
1	HIYOKU_KOMUGI	Shikoku 87/Saikai 95	Japan	Cultivar
1	2/1/2002		Mali	Uncertain Improvement Status
1	1/1/1994		Mali	Uncertain Improvement Status
1	C38-1		Philippines	Uncertain Improvement Status
1	15		Iran	Landrace
1	MIRIAM	Chapingo 53//Norin 10/Brevor 26/3/Yaqui 54/4/Merav	Israel	Cultivar
1	34-4		Ethiopia	Landrace
1	5114-111	Kanqueen/Florence Aurore//Miriam 1	Israel	Breeding material
1	GOGATSU_KOMUGI	Gokuwase 2/Norin 61	Japan	Cultivar
1	SAKIGAKE_KOMUGI	Danchikomugi/Chugoku 81	Japan	Cultivar
1	PI429321		Yemen	Landrace
1	R.R.21		Yemen	Breeding material
1	NW46A		Nepal	Landrace
1	G-40	T. durum/T. dicoccoides G-25//T. aestivum	Israel	Breeding material
1	PAVLODARSKAJA_I	Veselopodolyanskaja 610/Saratovskaja 29	Kazakhstan	Cultivar
1	FAO51.886		Nepal	Landrace
1	SIMLE		Nepal	Landrace
1	FAO51.904		Nepal	Landrace
1	FAO51.826		Nepal	Landrace
1	BOHR_GAMH		Yemen	Uncertain Improvement Status
1	YANG_CHOU_WHEAT_NO.3		China	Uncertain Improvement Status
1	MG27041		Greece	Landrace
1	MG17968		Algeria	Landrace
1	TRIGO|PI477878		Peru	Landrace
1	TRIGO|PI477899		Peru	Landrace
1	MG31396		Ethiopia	Landrace
1	MG31613		Ethiopia	Landrace
1	IQ54		Iraq	Landrace
1	PI481922		Sudan	Landrace
1	SY270		Jordan	Landrace
1	ALHAMA|PI490399		Mali	Uncertain Improvement Status
1	ALKAMU_BERI|PI490401		Mali	Uncertain Improvement Status

Table A1. Origin, GRIN ID, pedigree, and crop improvement status of 223 global spring wheat accessions collected at the National Small Grains Collection in Aberdeen, Idaho used in the genome-wide association analysis (continued).

SET	GRIN	PEDIGREE	ORIGIN	CROP STATUS
1	ZFA3629		Zambia	Uncertain Improvement Status
1	V764-1-J3-B2-J-3	Bluebird Tordo//Lachish/3/Nursi/T. dicoccoides (strain G-25)	Israel	Breeding material
1	V879-B2-B12-J2	Nursi/T. dicoccoides (strain G-25)//708-26/3/VC293	Israel	Breeding material
1	V882-F22-F2-F3-F2-J2	Nursi/T. dicoccoides (strain G-25)//Merav/3/V378-751	Israel	Breeding material
1	1117/83	Nursi/T. dicoccoides (strain G-25)//708-26/3/Cajeme-71	Israel	Breeding material
1	II-13831-3J-3J-2J-3L-L-J-1CZ	Yaqui 53/3/Frocor/Frontana/Yaqui	Peru	Breeding material
1	L1360-3838	Giza 139/Gabo	Egypt	Breeding material
1	ND71-12-111	ND 373*3/Giza Hegazy Ahmer	United States	Breeding material
1	8363-1B-1T-3B	Frontana/3/Mida//Kenya117A/Santa Catalina/4/Kenya	Colombia	Breeding material
1	FROHBERG12-107	ND 480//Polk/Wisconsin 261	United States	Breeding material
1	LE2070	Preludio/L 10//Sonora 64/Knott #2	Uruguay	Breeding material
1	SE381-4S-1S-6S-OS	Kavkaz/4/Chile/Inia/3/Ciano//El Goucho/Sonora	Syria	Breeding material
1	JACUI	S 8/Toropi	Brazil	Cultivar
1	GHATI|PI542666		Algeria	Landrace
1	LERMA		Bolivia	Landrace
1	TRIGO|PI565240		Bolivia	Landrace
1	PERLA		Bolivia	Landrace
1	AW7200/90		Georgia	Landrace
1	P8921-Q4C5	HY358/M82-2102//2*HY320/3/W8600	Canada	Breeding material
1	IWA8604316		Iran	Landrace
1	IWA8606150		Iran	Landrace
1	IWA8607570		Iran	Landrace
1	IWA8609285		Iran	Landrace
1	IWA8609318		Iran	Landrace
1	IWA8609365		Iran	Landrace
1	IWA8609415		Iran	Landrace
1	IWA8610797		Iran	Landrace
1	IWA8611737		Iran	Landrace
1	IWA8611858		Iran	Landrace
1	IWA8612097		Iran	Landrace
1	IWA8612122		Iran	Landrace
1	IWA8612591		Iran	Landrace
1	IWA8612778		Iran	Landrace
1	IWA8613053		Iran	Landrace
1	IWA8613332		Iran	Landrace
1	IWA8613626		Iran	Landrace
1	IWA8613963		Iran	Landrace
1	IWA8614310		Iran	Landrace

Table A1. Origin, GRIN ID, pedigree, and crop improvement status of 223 global spring wheat accessions collected at the National Small Grains Collection in Aberdeen, Idaho used in the genome-wide association analysis (continued).

SET	GRIN	PEDIGREE	ORIGIN	CROP STATUS
1	IWA8614520		Iran	Landrace
1	93-11-14-2-2	Ning 7840/Wangshuibai (Wong Su)	Canada	Breeding material
1	93-11-2-3-2	Ning 7840/Wangshuibai (Wong Su)	Canada	Breeding material
1	KAREE-DN2	Karee*4/PI 262660.	South Africa	Breeding material
1	SSL10-11	selection from Funo, CI14349	United States	Breeding material
1	SSL19-24	selection from Ning 7840, PI531188	United States	Breeding material
1	SSL67-68	selection from Sumai 3, PI481542	United States	Breeding material
1	SSL74-77	selection from Tilchifun 2, CI15166	United States	Breeding material
2	CENTENARIO	Artigas/Larranaga	Uruguay	Cultivar
2	SURPRESA	Polyssu/Alfredo Chaves 6-21	Brazil	Cultivar
2	PI163439		Argentina	Uncertain Improvement Status
2	IV C 390 1/2 10132		Argentina	Uncertain Improvement Status
2	533B		Switzerland	Landrace
2	ENCRUZILHADA	Fortaleza/Kenya Farmer	Brazil	Cultivar
2	Z.88.54		Switzerland	Breeding material
2	MENTANA	Rieti/Wilhelmina//Akagomughi	Italy	Cultivar
2	KOOPERATORKA	selection from Krymka	Ukraine	Cultivar
2	CLUJ 49-926		Romania	Cultivar
2	NYU BAI		Japan	Landrace
2	RENACIMIENTO	Americano 25C open pollinated	Uruguay	Cultivar
2	CHUDOSKAJA		Poland	Uncertain Improvement Status
2	VAULION		Switzerland	Cultivar
2	ACADEMIA 48	selection from Romanian landvariety	Romania	Cultivar
2	FUNO	Duecentodieci/Damiano	Italy	Cultivar
2	NOBEOKA BOZU		Japan	Landrace
2	ODESSKAJA 13	Erythrospermum 7623-1/Lutescens 62	Ukraine	Cultivar
2	KLEIN TRIUNFO	Americano 25C/Pelon 33C1	Argentina	Cultivar
2	STEPNJACHKA	selection from Banatka Khersonskaya	Ukraine	Cultivar
2	PANTANEIRO	Sonora 63*2/Lagoa Vermelha	Brazil	Cultivar
2	PI163429		Argentina	Uncertain Improvement Status
2	NEW THATCH SELECTION		Switzerland	Uncertain Improvement Status
2	TRINTECINCO	Alfredo Chaves 3-21/Alfredo Chaves 4-21	Brazil	Cultivar
2	ARTEMOWSKA		Bulgaria	Uncertain Improvement Status
2	SIN CHUNAGA		Japan	Uncertain Improvement Status
2	SHU CHOU WHEAT NO.3		China	Uncertain Improvement Status
2	FUJIMI KOMUGI	Norin 67/2*Norin 26	Japan	Cultivar
2	111A		Greece	Landrace
2	274-1-118	Bage/Tehuacan/3/Frontana/Kenya 58/Newthatch/RL 4151	Uruguay	Breeding material
2	LONTOI		Hungary	Cultivar
2	NORIN 34	Shinchunaga/Eshimashinriki	Japan	Cultivar

Table A1. Origin, GRIN ID, pedigree, and crop improvement status of 223 global spring wheat accessions collected at the National Small Grains Collection in Aberdeen, Idaho used in the genome-wide association analysis (continued).

SET	GRIN	PEDIGREE	ORIGIN	CROP STATUS
2	1032		Italy	Landrace
2	JAPON 2		Japan	Uncertain Improvement Status
2	LII/14-B		Yugoslavia	Landrace
2	H 51	Americano 25e/Favorito//Universal	Argentina	Breeding material
2	WHESTPHALEN	CNT 10/Burgas 2//Jacui	Brazil	Cultivar
2	JASI 10T		Romania	Cultivar
2	ABURA		Brazil	Cultivar
2	NORIN 61	Fukuoka 18/Shinchunaga	Japan	Cultivar
2	RIO NEGRO	Surpresa/Centenario	Brazil	Cultivar
2	PRODIGIO ITALIANO		Italy	Uncertain Improvement Status
2	BELGRADE 4		Yugoslavia	Uncertain Improvement Status
2	MANCHURIAN		China	Landrace
2	BAHIENSE	Klein Sinmarq/Eureka F.C.S.	Argentina	Cultivar
2	PRODIGIO ITALIANO		Italy	Uncertain Improvement Status
2	WABIAN		Paraguay	Uncertain Improvement Status
2	NORIN 43	Shiromansaku/Akakomugi 3//Shichunaga	Japan	Cultivar
2	CITR 7175		China	Landrace
2	220		Greece	Landrace
2	KLEIN CONDOR	Standard F.C.S./Sud Oeste F.C.S	Argentina	Cultivar
2	CHUKO		Japan	Landrace
2	FROMENT DU JAPON		Switzerland	Uncertain Improvement Status
2	FRONTANA	Fronteira/Mentana	Brazil	Cultivar
2	LAUREANO ALVAREZ LAAH	Benvenuto Inca/Klein 157	Argentina	Cultivar
2	HATVANI	selection from landrace from Potisi	Hungary	Cultivar
2	SAPPORO HARU KOMUGI JUGO		Japan	Cultivar
2	EXCELSIOR	Arminda/Virtue	Argentina	Cultivar
2	16-52-2		Brazil	Breeding material
2	MAGYAROVAR 81		Hungary	Cultivar
2	BUCK AUSTRAL	Sola 50//Quivira/Guatrache/3/Massaux No. 1/Buck Quequen 2-2-11	Argentina	Cultivar
2	TEZANOS PINTOS PRECOZ	Frontana//Thatcher/Sinvalocho	Argentina	Cultivar
2	274		Argentina	Landrace
2	COLOTANA 266/51	Colonista/Frontana	Brazil	Breeding material
2	3084		Argentina	Uncertain Improvement Status
2	ONCATIVO INTA	Thatcher/Sinvalocho M.A.//Beckman 1971	Argentina	Cultivar
2	TOKAI 66		Brazil	Cultivar
2	I 826	Extremo Sur/Argelino//T.timopheevii	Spain	Breeding material
2	16-52-9	Red Hart/Ponta Grossa 1	Brazil	Breeding material
2	GOGATSU KOMUGI	Gokuwase 2/Norin 61	Japan	Cultivar
2	ND 2710	Sumai3/Wheaton//Grandin.	United States	Uncertain Improvement Status
2	ESTANZUELA YOUNG	Bage/4/Thatcher/3/Frontana//Kenya 58/Newthatch	Uruguay	Cultivar
2	ALSEN		United States	Uncertain Improvement Status

Table A1. Origin, GRIN ID, pedigree, and crop improvement status of 223 global spring wheat accessions collected at the National Small Grains Collection in Aberdeen, Idaho used in the genome-wide association analysis (continued).

Set, the set in which each wheat accession was evaluated for traits of Fusarium head blight resistance; GRIN, Germplasm Resource Information Network ID; Pedigree, the recorded ancestry for a particular wheat accession; Origin, Country where the wheat accession was developed or collected from; Crop Improvement Status, the state of a particular accession in terms of plant breeding.

Table A2. Frequency distribution of favorable alleles for FHB resistance QTL in 233 global spring wheat accessions based on hierarchical clustering into subgroups and geographical origin of the accessions.

QTL	Tag-SNP	Allele	RAF	Subgroups					
				1A	1B	1C	2A	2B	3
Qfhb.ndsu-3A	IWA7564	C/T	0.46	0.40	0.93	0.25	0.15	0.29	0.6
				Geographical Origin					
				EU	AF	SA	AS	NA	
				0.46	0.17	0.55	0.42	0.44	
Qfhb.ndsu-2BL	IWA4900	A/C/T/G	0.52	1A	1B	1C	2A	2B	3
				0.45	0	0.46	0.17	0.12	1
				EU	AF	SA	AS	NA	
				0.45	0	0.46	0.17	0.12	

QTL, Quantitative trait locus; Tag-SNP, single nucleotide polymorphisms (SNPs) associated with the trait at fdr ≤ 0.1; Allele, alleles present at the particular SNP locus; RAF, resistance allele frequencies of the tagged SNP; Subgroups, Frequency of favorable alleles based on subgroups based on relatedness; Geographical Origin, Frequency of favorable alleles in wheat accessions collected from particular geographical origin; EU, Europe; AF, Africa; SA, South America; AS, Asia; NA, North America.

Table A3. Name, collection date, origin, PCR based chemotype, and identification of 223 *Fusarium graminearum* isolates collected from North Dakota.

Isolate	Year	County	Chemotype	Remarks
Fg13_2	7/16/2013	Grant	15ADON	*F. graminearum*
Fg13_3	7/16/2013	Morton	15ADON	*F. graminearum*
Fg13_4	7/16/2013	Morton	15ADON	*F. graminearum*
Fg13_5	7/16/2013	Oliver	15ADON	*F. graminearum*
Fg13_6	7/16/2013	Oliver	15ADON	*F. graminearum*
Fg13_7	7/16/2013	Oliver	15ADON	*F. graminearum*
Fg13_8	7/16/2013	Oliver	15ADON	*F. graminearum*
Fg13_9	7/16/2013	Mercer	15ADON	*F. graminearum*
Fg13_10	7/16/2013	Mercer	15ADON	*F. graminearum*
Fg13_11	7/16/2013	Mercer	15ADON	*F. graminearum*
Fg13_12	7/16/2013	Mercer	15ADON	*F. graminearum*
Fg13_13	7/16/2013	Dunn	15ADON	*F. graminearum*
Fg13_14	7/16/2013	Dunn	15ADON	*F. graminearum*
Fg13_16	7/16/2013	Dunn	15ADON	*F. graminearum*
Fg13_17	7/16/2013	Billings	15ADON	*F. graminearum*
Fg13_23	7/16/2013	Stark	15ADON	*F. graminearum*
Fg13_24	7/16/2013	Stark	15ADON	*F. graminearum*
Fg13_26	7/16/2013	Hettinger	15ADON	*F. graminearum*
Fg13_28	7/16/2013	Slope	15ADON	*F. graminearum*
Fg13_33	7/16/2013	Bowman	15ADON	*F. graminearum*
Fg13_34	7/16/2013	Bowman	15ADON	*F. graminearum*
Fg13_35	7/16/2013	Adams	15ADON	*F. graminearum*
Fg13_38	7/16/2013	Adams	15ADON	*F. graminearum*
Fg13_39	7/16/2013	Hettinger	15ADON	*F. graminearum*
Fg13_40	7/16/2013	Hettinger	15ADON	*F. graminearum*
Fg13_41	7/16/2013	Grant	15ADON	*F. graminearum*
Fg13_42	7/16/2013	Grant	15ADON	*F. graminearum*
Fg13_44	7/16/2013	Grant	15ADON	*F. graminearum*
Fg13_45	7/16/2013	Morton	3ADON	*F. graminearum*
Fg13_46	7/16/2013	Sioux	15ADON	*F. graminearum*
Fg13_47	7/16/2013	Sioux	15ADON	*F. graminearum*
Fg13_48	7/16/2013	Sioux	15ADON	*F. graminearum*
Fg13_49	7/16/2013	Burleigh	15ADON	*F. graminearum*
Fg13_50	7/16/2013	Emmons	15ADON	*F. graminearum*
Fg13_51	7/16/2013	Emmons	15ADON	*F. graminearum*
Fg13_52	7/16/2013	Emmons	15ADON	*F. graminearum*
Fg13_53	7/16/2013	Emmons	15ADON	*F. graminearum*
Fg13_54	7/16/2013	McIntosh	15ADON	*F. graminearum*
Fg13_55	7/16/2013	McIntosh	15ADON	*F. graminearum*
Fg13_56	7/16/2013	McIntosh		*F. graminearum*
Fg13_57	7/16/2013	McIntosh	15ADON	*F. graminearum*
Fg13_58	7/16/2013	Logan	15ADON	*F. graminearum*
Fg13_60	7/16/2013	Logan	15ADON	*F. graminearum*
Fg13_62	7/24/2013	Stutsman	15ADON	*F. graminearum*
Fg13_63	7/24/2013	La Moure	15ADON	*F. graminearum*
Fg13_64	7/24/2013	La Moure	15ADON	*F. graminearum*
Fg13_65	7/24/2013	La Moure	15ADON	*F. graminearum*
Fg13_66	7/24/2013	Dickey	15ADON	*F. graminearum*
Fg13_67	7/24/2013	Dickey	15ADON	*F. graminearum*
Fg13_68	7/24/2013	Dickey	15ADON	*F. graminearum*

Table A3. Name, collection date, origin, PCR based chemotype, and identification of 223 *Fusarium graminearum* isolates collected from North Dakota based on morphological and molecular features (continued).

Isolate	Year	County	Chemotype	Remarks
Fg13_69	7/24/2013	Dickey	15ADON	*F. graminearum*
Fg13_71	7/24/2013	Sargent	15ADON	*F. graminearum*
Fg13_72	7/24/2013	Ransom	15ADON	*F. graminearum*
Fg13_73	7/24/2013	Ransom	15ADON	*F. graminearum*
Fg13_74	7/24/2013	Sargent	15ADON	*F. graminearum*
Fg13_78	7/24/2013	Barnes	15ADON	*F. graminearum*
Fg13_79	7/24/2013	Barnes	3ADON	*F. graminearum*
Fg13_80	8/13/2013	Cavalier	15ADON	*F. graminearum*
Fg13_82	8/13/2013	Cavalier	15ADON	*F. graminearum*
Fg13_83	8/13/2013	Towner	3ADON	*F. graminearum*
Fg13_84	8/13/2013	Towner	15ADON	*F. graminearum*
Fg13_85	8/13/2013	Towner	15ADON	*F. graminearum*
Fg13_87	8/13/2013	Rolette	15ADON	*F. graminearum*
Fg13_90	8/13/2013	Pierce		*F. graminearum*
Fg13_91	8/13/2013	Pierce	15ADON	*F. graminearum*
Fg13_92	8/13/2013	Benson	3ADON	*F. graminearum*
Fg13_93	8/13/2013	Benson	3ADON	*F. graminearum*
Fg13_101	7/17/2013	Pierce	3ADON	*F. graminearum*
Fg13_102	7/17/2013	Nelson	3ADON	*F. graminearum*
Fg13_103	7/17/2013	Walsh	15ADON	*F. graminearum*
Fg13_105	7/17/2013	Cavalier	15ADON	*F. graminearum*
Fg13_109	7/17/2013	Traill	15ADON	*F. graminearum*
Fg13_111	7/17/2013	Nelson	15ADON	*F. graminearum*
Fg13_113	7/17/2013	Pierce	15ADON	*F. graminearum*
Fg13_114	7/17/2013	Rolette	3ADON	*F. graminearum*
Fg13_115	7/17/2013	Ward	3ADON	*F. graminearum*
Fg13_118	7/17/2013	Walsh	3ADON	*F. graminearum*
Fg13_127	7/17/2013	Traill	15ADON	*F. graminearum*
Fg13_128	7/17/2013	Barnes	3ADON	*F. graminearum*
Fg13_131	7/17/2013	Towner	3ADON	*F. graminearum*
Fg13_138	7/17/2013	Ward	15ADON	*F. graminearum*
Fg13_142	7/17/2013	Divide	15ADON	*F. graminearum*
Fg13_143	7/17/2013	Divide	15ADON	*F. graminearum*
Fg13_146	7/17/2013	Rolette	15ADON	*F. graminearum*
Fg13_147	7/17/2013	Stutsman		*F. graminearum*
Fg13_148	7/17/2013	Traill	15ADON	*F. graminearum*
Fg13_152	7/17/2013	McLean	3ADON	*F. graminearum*
Fg13_157	7/17/2013	Wells	15ADON	*F. graminearum*
Fg13_159	7/17/2013	Wells	15ADON	*F. graminearum*
Fg13_160	7/17/2013	Ward	15ADON	*F. graminearum*
Fg13_161	7/17/2013	Wells	15ADON	*F. graminearum*
Fg13_163	7/17/2013	Bottineau	15ADON	*F. graminearum*
Fg13_174	7/17/2013	Wells	15ADON	*F. graminearum*
Fg13_175	7/17/2013	Burke	15ADON	*F. graminearum*
Fg13_177	7/17/2013	Ward	15ADON	*F. graminearum*
Fg13_179	7/17/2013	Barnes	15ADON	*F. graminearum*
Fg13_180	7/17/2013	Cass	15ADON	*F. graminearum*
Fg13_181	7/17/2013	Bottineau	15ADON	*F. graminearum*
Fg13_185	7/17/2013	Renville	3ADON	*F. graminearum*
Fg13_187	7/17/2013	McLean	3ADON	*F. graminearum*

Table A3. Name, collection date, origin, PCR based chemotype, and identification of 223 *Fusarium graminearum* isolates collected from North Dakota based on morphological and molecular features (continued).

Isolate	Year	County	Chemotype	Remarks
Fg13_188	7/17/2013	McLean	15ADON	*F. graminearum*
Fg13_189	7/17/2013	McLean	3ADON	*F. graminearum*
Fg13_190	7/17/2013	McLean	15ADON	*F. graminearum*
Fg13_191	7/17/2013	Wells	15ADON	*F. graminearum*
Fg13_192	7/17/2013	McLean	15ADON	*F. graminearum*
Fg13_154	7/17/2013	Burke	3ADON	*F. graminearum*
R366	8/20/1981		15ADON	*F. graminearum*
R370	8/20/1981			*F. graminearum*
R372	8/20/1981			*F. graminearum*
R397	9/29/1981			*F. graminearum*
R1171	8/8/1991	Cavalier	15ADON	*F. graminearum*
R1213	10/1/1993			*F. graminearum*
R1217	10/1/1993			*F. graminearum*
R1226	10/1/1993			*F. graminearum*
R1231	10/1/1993			*F. graminearum*
R1236	10/1/1993			*F. graminearum*
R1237	10/1/1993			*F. graminearum*
R1238	10/1/1993			*F. graminearum*
R1240	10/1/1993			*F. graminearum*
R1247	10/1/1993		15ADON	*F. graminearum*
R1250	10/1/1993			*F. graminearum*
R1261	1993	Cavalier		*F. graminearum*
R1262	1993	Cavalier		*F. graminearum*
R1305	10/5/1995			*F. graminearum*
R1316	10/5/1995	Cass		*F. graminearum*
R1694	6/9/2004	Cass		*F. graminearum*
R1698	6/9/2004	Cass		*F. graminearum*
Fg08_2	2008	Barnes	3ADON	*F. graminearum*
Fg08_3	2008	Griggs	3ADON	*F. graminearum*
Fg08_4	2008	Barnes	3ADON	*F. graminearum*
Fg08_5	2008	Foster	3ADON	*F. graminearum*
Fg08_6	2008	Griggs	3ADON	*F. graminearum*
Fg08_7	2008		15ADON	*F. graminearum*
Fg08_9	2008		3ADON	*F. graminearum*
Fg08_10	2008	Barnes	3ADON	*F. graminearum*
Fg08_11	2008	Foster	3ADON	*F. graminearum*
Fg08_12	2008	Barnes	3ADON	*F. graminearum*
Fg08_13	2008	Steele	15ADON	*F. graminearum*
Fg08_18	2008	Griggs	15ADON	*F. graminearum*
Fg08_19	2008	Foster	3ADON	*F. graminearum*
Fg08_20	2008	Barnes	3ADON	*F. graminearum*
Fg08_21	2008	Barnes	3ADON	*F. graminearum*
Fg08_25	2008		3ADON	*F. graminearum*
Fg08_26	2008		15ADON	*F. graminearum*
Fg08_27	2008			*F. graminearum*
Fg08_28	2008			*F. graminearum*
Fg08_29	2008		3ADON	*F. graminearum*
Fg08_30	2008		15ADON	*F. graminearum*
Fg08_31	2008			*F. graminearum*
Fg08_32	2008			*F. graminearum*

Table A3. Name, collection date, origin, PCR based chemotype, and identification of 223 *Fusarium graminearum* isolates collected from North Dakota based on morphological and molecular features (continued).

Isolate	Year	County	Chemotype	Remarks
Fg08_33	2008			*F. graminearum*
Fg08_34	2008		15ADON	*F. graminearum*
Fg08_35	2008			*F. graminearum*
Fg08_36	2008		15ADON	*F. graminearum*
Fg08_37	2008		15ADON	*F. graminearum*
Fg08_38	2008			*F. graminearum*
Fg08_39	2008			*F. graminearum*
Fg08_40	2008			*F. graminearum*
Fg08_44	2008			*F. graminearum*
Fg08_47	2008			*F. graminearum*
Fg08_59	2008			*F. graminearum*
Fg10_137_5	2010	Burke	15ADON	*F. graminearum*
Fg10_121_3	2010	Williams	15ADON	*F. graminearum*
Fg10_120_2	2010	Williams	3ADON	*F. graminearum*
Fg10_125_01	2010	McLeans	3ADON	*F. graminearum*
Fg10_168H2_1	2010	Towner	3ADON	*F. graminearum*
Fg10_541_1	2010			*F. graminearum*
Fg10_79_1	2010	Trail	3ADON	*F. graminearum*
Fg10_140H1_3	2010	Mountrail	15ADON	*F. graminearum*
Fg10_135_2	2010	Burke	3ADON	*F. graminearum*
Fg10_4H2_1	2010	Cass	15ADON	*F. graminearum*
Fg10_61_1	2010	Cavalier	3ADON	*F. graminearum*
Fg10_136_4	2010	Burke	3ADON	*F. graminearum*
Fg10_148H1_3	2010	Divide	15ADON	*F. graminearum*
Fg10_23H1_2	2010	Dicky	15ADON	*F. graminearum*
Fg10_55_1	2010	Pembina	3ADON	*F. graminearum*
Fg10_160H2_2	2010	Rolette	3ADON	*F. graminearum*
Fg10_156H1_1	2010	Pierce	15ADON	*F. graminearum*
Fg10_141H2_2	2010	Mountrail	3ADON	*F. graminearum*
Fg10_124_1	2010	Mcleans	15ADON	*F. graminearum*
Fg10_127_3	2010	McLeans	3ADON	*F. graminearum*
Fg10_141H1_4	2010	Mountrail	15ADON	*F. graminearum*
Fg10_6H1_2	2010	Sargent	15ADON	*F. graminearum*
Fg10_166H1_3	2010	Towner	3ADON	*F. graminearum*

Isolate, Name of the fungal isolate; Year, Date or year on which the particular isolate was collected; County, North Dakota county from where the isolate was collected; Chemotype, Identification of isolate based on the type of mycotoxin they produce; Remarks, Result from morphological and molecular identification of individual isoaltes.

APPENDIX B. SUPPLEMENTAL FIGURES

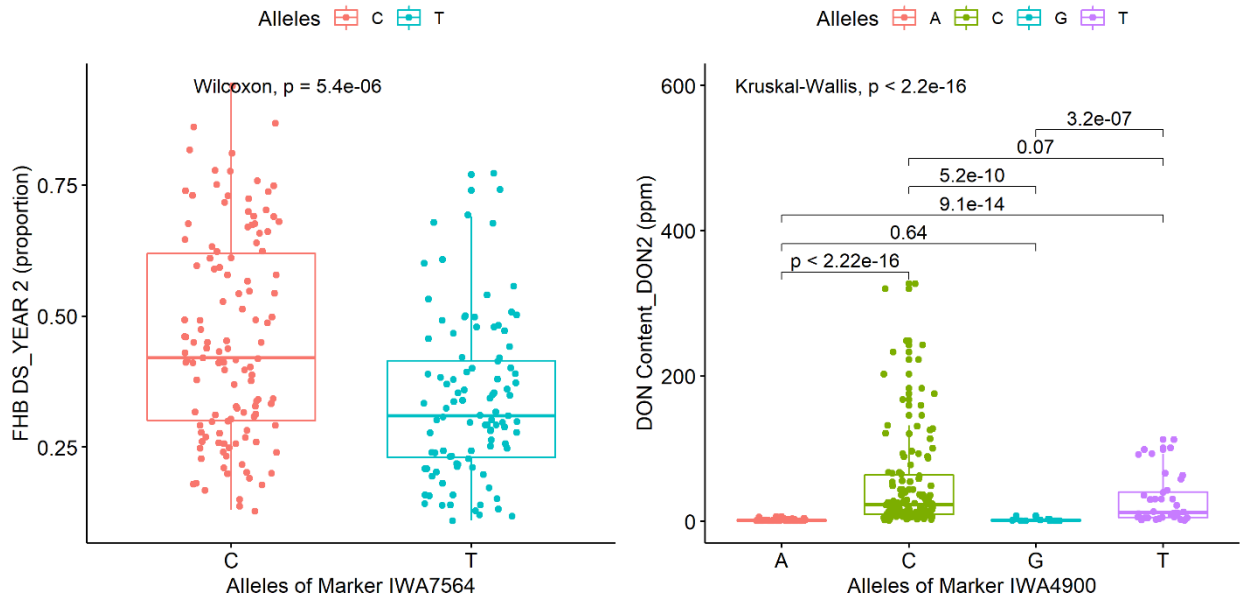

Figure B1. Effect of favorable allele on reducing FHB DS and DON content in 233 spring wheat accessions based on unpaired mean comparison.

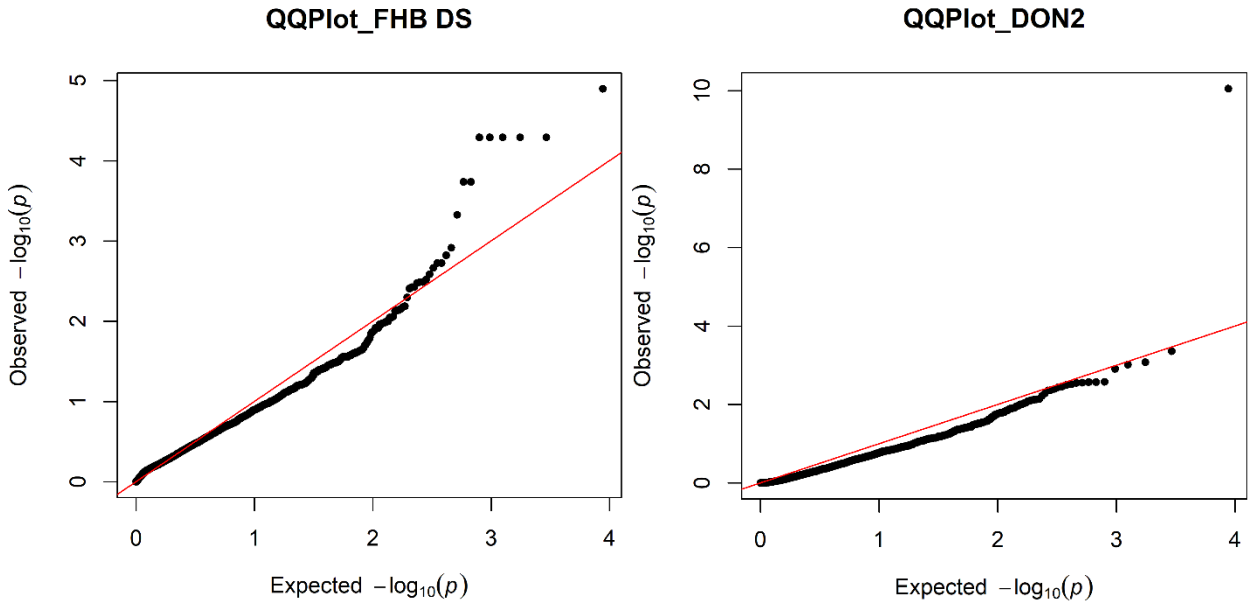

Figure B2. Quantile-quantile plots based on mixed model that accounts for population structure and relationship matrix between expected and observed *P* values (-log10) for traits of FHB disease severity and DON content in 233 spring wheat accessions.

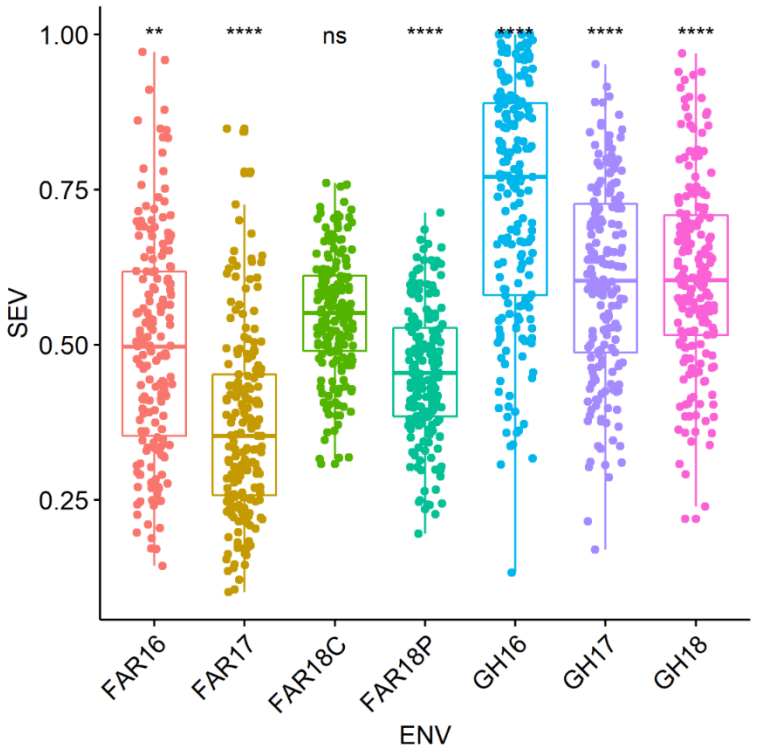

Figure B3. Pairwise-mean comparisons of FHB disease severity on WPDS lines across years of evaluation.