

Genomics-assisted assessment of quantitative disease resistance: An evaluation of genetic resistance of two-row barley (*Hordeum vulgare* L.) to fusarium head blight (*Fusarium graminearum*) and deoxynivalenol accumulation.

by

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ABSTRACT

James R. Tucker, Ph.D., University of Manitoba, February 2022. Genomics-assisted assessment of quantitative disease resistance: An evaluation of genetic resistance of two-row barley (*Hordeum vulgare* L.) to fusarium head blight (*Fusarium graminearum*) and deoxynivalenol accumulation. Ph.D. Co-Supervisors: Dr. W. G. Dilantha Fernando and Dr. Ana Badea.

Fusarium head blight (FHB) incited by *Fusarium graminearum*, is currently the most devastating disease of barley (*Hordeum vulgare* L.) in western Canada. Type B trichothecene mycotoxins e.g. deoxynivalenol (DON) associated with FHB are closely monitored by both malting and livestock feed industries. Breeding disease-resistant varieties is a sustainable disease management practice, however, this is complicated by quantitative-based resistance. To date, breeding efforts have largely relied on labour-intensive screening nurseries. Two studies focus on the evaluation of a single nucleotide polymorphic (SNP) marker array (Barley 50K SNP) in i) genome-wide association study (GWAS); ii) genomic estimated breeding values (GEBV) whole-genome marker prediction. While GWAS identified numerous genomic associations with FHB and DON, they generally were small effects explaining a low proportion of the phenotypic variance. Genomic estimated breeding values (GEBVs) were calculated by classical statistical and machine learning approaches, where both were able to demonstrate moderate prediction is possible for FHB and DON content. Genomic selection is thus recommended as an efficient breeding approach vs. considerations of individual SNP markers. The *F. graminearum* population of western Canada has shifted towards 3-acetyl-deoxynivalenol (3ADON)-producers, which tend to accumulate higher levels of DON. *In vitro* selection (IVS) has been successfully used to identify resistant somaclonal variants. Another study involves poly-A RNA-sequencing

contrast of *IVS* derived variety Norman vs. its parent, CDC Kendall. Different transcriptional alternations were detected in response to infection by 3ADON, 15ADON, and nivalenol (NIV) – producing strains of *F. graminearum*. In the final experiment, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to evaluate mycotoxin composition in rachis and grains for a group of field-inoculated varieties with differential resistance levels. The conjugate form deoxynivalenol-3-glucoside (DON3G) was found to occur at a significant level (26%) of total toxins. While varieties differed in DON content, the ratio of DON3G/DON appeared stable across genotypes. In summary, the dissertation contributed to novel research through the identification of feasible genomics-based breeding strategies for developing FHB resistance and lowering DON, contributed to an understanding of *IVS* resistance of Norman, identified gene targets of differentially expressed genes between the varieties, and has identified toxin-conjugation as a resistance factor.

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FOREWORD

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CONTRIBUTIONS OF AUTHORS

Chapter 2 Literature Review

Fernando, W.G.D., Oghenekaro, A.O., Tucker, J.R., Badea, A. 2021. Building on a foundation: advances in epidemiology, resistance breeding, and forecasting research for reducing the impact of fusarium head blight in wheat and barley. Can J Plant Pathol. doi: 10.1080/07060661.2020.1861102 (IF=2.442)

This was published as a review article in honour of W.G.D.F.'s capacity as the immediate past president of the Canadian Phytopathological Society. A review of literature pertaining to barley was conducted by J.R.T., while the wheat review was written by A.O.O. Only materials originally written by J.R.T. are included in the dissertation. Revisions were done by W.G.D.F and A.B.

Chapter 3 Genetic structure and genome-wide association study of a genomic panel of two-row, spring barleys (*Hordeum vulgare* L.) with differential reaction to Fusarium head blight (*Fusarium graminearum* Schwabe) and deoxynivalenol production.

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Chapter 4 GPTransformer: A transformer-based deep learning method for predicting Fusarium-related traits in barley.

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Author Contributions: JT, AB, and WF conceived and designed the experiments. JT and WL conducted field analysis. JT grew plant and fungus, conducted inoculations, performed the RNA isolation, and wrote the manuscript. JT, SM, WX, and ZY analyzed the data. CH conducted a microarray analysis. SS conducted mycotoxin assays. AB and WF held supervisory roles. JT, AB, WF, and WX revised the manuscript. All authors read and approved the final manuscript.

Chapter 6 Deoxynivalenol-3-glucoside content is highly associated with deoxynivalenol levels in two-row barley genotypes of importance to Canadian barley breeding programs.

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CHAPTER 1

GENERAL INTRODUCTION

Barley (*Hordeum vulgare* L.) is a member of the grass family Poaceae, of monocotyledonous plants which also includes other grains of major economic importance: *Zea mays* L. (maize), *Triticum aestivum* L. (wheat), *Avena sativa* L. (oat), and *Secale cereale* L. (rye). Barley is a diploid organism, with an extremely large size (5 Gb) genome composed of seven chromosomes (labelled 1H, 2H, 3H, 4H, 5H, 6H, 7H) that vary in size. Approximately 10,000 years ago, this ancient crop was domesticated in the Fertile Crescent from a near, wild-relative *Hordeum vulgare subsp. spontaneum* (Badr et al. 2000). Barley is an extremely versatile crop that can be used in multiple ways. Two-thirds of the crop is primarily used as animal fodder, which can be cut following seed development and used to produce silage, or harvested at maturity as grain. Traditionally it is used in the production of malt, where grains are germinated through soaking and laid in germination beds where they undergo an enzymatic modification of starches followed by kilning (heat dried). Malt is used in the manufacture of fermented beverages such as beer or distilled products such as whiskey, or as a nutrient additive. Barley grains are also considered a healthy food (Health Canada, 2012), and are consumed directly as pearled or hulless (without husk) food barley. Barley is rich in certain B vitamins along with uniquely-high vitamin E and is high in soluble fiber. It is considered to be a robust cereal crop, which tolerates poorer quality soils and low temperatures. It is an efficient crop with minimal input requirements and contributes to sustainable production services through carbon sequestration in soils. Barley is an important crop for Canada, with over 10 million tonnes of annual production (FAOSTAT, 2019).

In barley, floret is the basic unit of a grass flower are arranged in triplets within spikelets and run in alternative-manner up the rachis (central stalk). Two common morphologies are found in cultivated barley: two-row spike (*Vrs1*, functional allele) which has fertile central florets, but sterile lateral florets; six-row spike (*vrs1*, loss-of-function allele) where fertility is restored to lateral florets. As a result, two-row barley grain tends to be symmetrical and more uniform in size than six-row types which produce thinner, lateral kernels. While a significant American export market had existed for six-row barley in the past, barley production in western Canada is currently dominated by two-row barley varieties. Uniform, plump kernels are desired by

both livestock producers and the malting/ brewing industries. In general, two-row barley has lower protein content (typically 11-13%), a thinner husk, and more starch than six-row barley. Recent advances in two-row yield genetics, have closed the gap on traditionally higher-yielding six-row barleys. It is an inexpensive crop to grow with minimal inputs, which contributes to agro-environmentally sustainability services, through contribution to organic carbon sequestration. Barley is considered an easy crop to grow and is well adapted to a short growing season due to its early maturation.

Barley grown in the western Prairies may be infected by several diseases which can cause economic loss under epidemic conditions: surface borne smuts (*Ustilago nigra* Tapke; *Ustilago hordei* [Pers.] Lagerh), net blotch *Pyrenophora teres* Drechs. (anamorph: *Drechslera teres* [Sacc.] Shoem.), net-form net blotch (*Pyrenophora teres* f. sp. *teres*), spot-form net blotch (*Pyrenophora teres* f. sp. *maculata*), stem rust (*Puccinia graminis* Pers.:Pers. f. sp. *tritici* Erikss. & E. Henn.), spot blotch (*Cochliobolus sativus* Ito & Kuribayashi) Drechs. ex Dastur (anamorph: *Bipolaris sorokiniana* (Sacc.) Shoemaker), scald (*Rhynchosporium commune* Zaffarano, McDonald, and Linde). Pathology research conducted in western Canada over many years has contributed to incorporating resistance genes to these economically-important pathogens. However, one of the most devastating of all diseases is Fusarium head blight (FHB) caused by *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schweinitz) Petch). While yield losses can occur under higher infections, concerns about this disease are principally associated with mycotoxin (deoxynivalenol, DON) production which can reduce grain quality. Likewise, ingestion of toxins from infected grains may contribute to health concerns for humans and animals, such that maximum allowable standards are set for DON in food and feed. Breeding for FHB resistance and lowering DON content is a very high priority for western Canadian barley breeders. Due to its quantitative nature, FHB resistance has been difficult to incorporate into elite germplasm, while maintaining quality. While progress has been slow, several contemporary varieties have been released with moderately resistant (MR) reactions.

Huge advances in barley genomics science have occurred in the last decade. The development of Next-Generation sequencing technologies and the drop in sequencing costs have allowed for an exponential level of data generation. Barley not only has one of the largest diploid genomes

but is also largely composed of repetitive transposable elements (Rimpau et al. 1980). As a result, lowered sequencing costs have fostered the development of numerous high-quality reference genomes (Mascher et al. 2017 [Morex V1]; Dai et al. 2018; Monat et al. 2019 [Morex V2]; Jayakodi et al. 2020; Zeng et al. 2020; Xu et al. 2021; Mascher et al. 2021 [Morex V3]). Improved genome assemblies have also facilitated the development of genome-wide SNP marker micro-array platforms, which allow for convenient, high-throughput genotyping (Bayer et al. 2017). The strong foundation of genomic science recently advanced within barley, will permit the use of this information to further investigate resistance to pathogens occurring in complex host-pathogen interactions. This dissertation presents the groundwork for the application of several new biotechnologies to enhance resistance to the destructive pathogen *F. graminearum*.

Objective 1

Study genetic variance and identify genomic regions of interest in two-row spring through genome-wide association mapping (GWAS) in a two-row barley panel chosen for differential reaction to Fusarium head blight (FHB) and deoxynivalenol (DON) accumulation.

Objective 2

Evaluate the application of genome-wide, single nucleotide polymorphic (SNP) molecular markers in genomic prediction of Genomic Estimated Breeding Values (GEBV) using classical statistically based and machine learning approaches.

Objective 3

Identification of transcriptional alterations in an *in vitro* selected, moderately-resistant variety in contrast with its parent-donor, challenged by three different chemotype strains of *Fusarium graminearum* (3ADON, 3-acetyl-deoxynivalenol; 15ADON, 15-acetyl-deoxynivalenol; NIV, nivalenol).

Objective 4

Investigation of the ratio of conjugated a form of deoxynivalenol (DON3G, deoxynivalenol-3-glucoside) to DON in a set of barley varieties with different resistance to DON accumulation.

CHAPTER 2

LITERATURE REVIEW

Part of this Literature Review has now been published: Fernando, W.G.D., Oghenekaro, A.O., Tucker, J.R., Badea, A. 2021. Building on a foundation: advances in epidemiology, resistance breeding, and forecasting research for reducing the impact of fusarium head blight in wheat and barley. Can J Plant Pathol. doi: 10.1080/07060661.2020.1861102

2.1 The pathogen of barley - *Fusarium graminearum*

Fusarium head blight (FHB) is a global, cereal disease attributable to several causal fungi, which is generally associated with warm and moist growing conditions (Parry et al. 1995). In North America, FHB of barley is caused by the *Fusarium* species complex including *Fusarium graminearum* Schwabe (teleomorph = *Gibberella zeae* (Schwein.) Petch, *F. poae* (Peck) Wollenweb., *F. sporotrichioides* Sherb, and *F. avenaceum* Ellis and Everhart. (teleomorph: *Gibberella acuminata* Wollenw.) (Salas et al. 1999). *Fusarium graminearum* is a pathogen of heightened concern for barley industries due to its high pathogenicity and ability to cause severe disease (Xue et al. 2006). While alternate pathogens are prevalent in some specific countries, *F. graminearum* is the most frequently encountered FHB pathogen worldwide. *F. graminearum* was the dominant *Fusarium* species on barley in Quebec, followed by *F. poae* and *F. avenaceum* (Bourdages et al. 2006). While *F. graminearum* and *F. poae* were found as equally dominant pathogens under a recent survey conducted in Ontario, *F. graminearum* was isolated at much-elevated levels (210%) during years of epidemic conditions (Xue et al. 2019). DON produced by *F. graminearum*, is the most frequently detected trichothecene of barley grains and consequently is the primary focus of monitoring efforts conducted by industry.

F. graminearum is considered a pathogen at greater risk of evolving means to overcome host resistance genes due to its mixed reproduction system (McDonald and Linde, 2002). It possesses both sexual and asexual reproductive states and allows for genetic recombination and propagation of clones. *F. graminearum* is homothallic and thus does not require a compatible strain to produce perithecia (i.e. are sexual, self-fertile) (Cavinder et al. 2012). The genome of *F. graminearum* shows low levels of repetitive elements, due to targeting mechanisms

associated with a repeat-induced point mutation (RIP) (Cuomo et al. 2007). Repeat induced point mutation (RIP) is a fungal-specific mutagenesis process confirmed in *F. graminearum*, which may influence the evolution of its genome, promoting rapid adaptation to selection pressures (Hane et al. 2015). Fusarium genomics has demonstrated the existence of specialized, pathogenicity chromosomes with evidence of horizontal acquisition (Ma et al. 2010). Pan-genome analysis of North American isolates of *F. graminearum* identified abundant signatures of selection within genomic regions of dispensable accessory genes associated with pathogen specialization (Kelly and Ward, 2018). Practical analysis via vegetative compatibility groupings (VCG) using nitrate-non utilizing mutants, demonstrated extremely high genetic diversity in isolates collected from barley spikes from southern Manitoba (McCallum et al. 2001). High VCG diversity was found at regional (0.95), local (0.94), and within-spike (0.90) levels. The extreme level of diversity observed within this pathogen on barley should be taken into consideration within breeding strategies.

Advancements in Fusarium genomics have elucidated the genes that the fungus uses for the invasion which may be elicitors of host defense response. Cuomo et al. (2007) discovered high degrees of polymorphism in telomeric regions, which contained an abundance of pathogenicity genes, commonly expressed under host-pathogen interaction. The secretome of *F. graminearum* is now well documented (Brown et al. 2012; King et al. 2015), with over 600 proteins included. Secreted proteins which function as cell wall degrading enzymes (CWDEs) provide the fungus with an arsenal of virulence effectors to enter and colonize the host. Biochemical characterization of the enzyme cocktail produced by *F. graminearum* in response to growth on media with sole carbon source from glucose or hop cell wall material demonstrated a significant increase in activities of the latter (5 vs. 17 CWDE activities) and enhanced ability to convert plant cell wall substrates into a variety of sugars (Phalip et al. 2009). Extracellular peptidase gene expression produced by *F. graminearum* during the infection of wheat and barley were highly similar ($r^2 = 0.87$), whereas the contrast of *in planta* vs. *in vitro* conditions demonstrated weak association ($r^2 = 0.22$) indicating host-induced pathways (Lowe et al. 2015). Serine peptidase (eg. FGSG_1086) was upregulated in barley vs. wheat contrast, possibly in response to the presence of barley-specific Bowman-Birk type (trypsin-like enzyme) peptidase inhibitors (Pekkarinen, 2003). *Fusarium graminearum* strains of varying aggressiveness express the same genetic program and show similar effector protein

profile during infection of wheat, however, more aggressive strains accumulate these substances in higher abundance (Fabre et al. 2019). Aggressiveness thus was not determined by specific effectors, but rather by their general rate of production. The pathogen carries numerous pathogenicity factors that can be employed under various circumstances. The alternative ways this pathogen can utilize to invade a host places limitations on control strategies via genetic resistance.

Four kinds of trichothecenes are produced by fungi (A, B, C, D), where *Fusarium* spp. can produce types A or B. *F. graminearum* produces various types of secondary metabolites including B-type trichothecene mycotoxins [Deoxynivalenol (DON) and its acetylated forms (3-acetyl-4-deoxynivalenol = 3ADON and 15-acetyl-4-deoxynivalenol = 15ADON), nivalenol (NIV) and its acetylated form 4-acetylnivalenol]. In North America, *F. graminearum* strains are primarily DON producers. This population is further sub-divided into those which preferentially produce 3-acetyl-4-deoxynivalenol (3-ADON) versus 15-acetyl-4-deoxynivalenol (15-ADON) trichothecenes. The genetic basis of chemotype i.e. 3-ADON vs. 15-ADON is controlled by the trichothecene biosynthetic gene *TRI8* (Alexander et al. 2011) in the *TRI* gene cluster which encodes an esterase which differentially removes the acetyl group from C-3 or C-15 position (McCormick and Alexander, 2002). While 3-ADON or 15-ADON may possess different toxicities, these acetylated forms are commonly found at much lower levels than DON itself. As a sub-population characteristic, chemotype information is useful information for investigating population dynamics.

Following significant changes in the cropping environment arising from alterations in agronomic practices such as conservation tillage or climatic changes, pathogens and their relationships with their hosts are expected to evolve. *Fusarium* spp. demonstrate variability in their toxigenic potential of isolates from different environments (Miller et al. 1991), indicating that aggressiveness may evolve. Historically, the origin of the *F. graminearum* population in Canada was the 15-ADON chemotype (Miller et al. 1983). Ward et al. (2008) documented an adaptive shift of 3-ADON replacing the 15-ADON population in Canada with a decline in 3ADON proportion observed from the eastern coast to the Prairie provinces. However, in Ontario the native 15ADON chemotype remains dominant (Crippin et al. 2020). Population structure of chemotypes isolated from wheat originating across eight Canadian provinces

showed that genetic structure was strongly associated with chemotype (Kelly et al. 2015). The endemic population (NA1) was dominated by 15-ADON chemotypes, whereas the alternate population (NA2) included mostly 3-ADON chemotype. In a similar study in the upper Midwestern USA of isolations from barley, Liang et al. (2014) found more divergence and less admixture between chemotype sub-populations, suggesting less recombination between NA1 and NA2 in barley than wheat.

Of concern to cereal production are findings that 3-ADON chemotypes produces higher levels of toxin (Ward et al. 2008; Von der Ohe, 2010; Puri and Zhong, 2010; Foroud et al. 2012). As toxins may act as a virulence factor, the level of disease is also expected to rise under infection by pathogens with elevated toxigenic potential. Along with increased toxicity, other pathogenic characteristics are also associated with the 3-ADON chemotype (fecundity, growth rate) implicating a general increase of aggressiveness (Ward et al. 2008). 3-ADON forms are also more resilient, showing higher DON and faster mycelial growth following exposures to extreme heat and cold stress (Vujanovic et al. 2012). Implications on the existence of more aggressive forms of pathogens, dictate requirements to identify and use strongly pathogenic isolates when screening barley breeding lines for resistance.

The *F. graminearum* population of North America is dynamic, displaying changes and/ or introduction of distinctive chemotypes. Nivalenol (NIV) producers have been documented within the USA and show an ability to dominate in select environments e.g. southeast USA (Gale et al. 2011). Isolations from southern Canada and the northern USA were found to produce a novel type A trichothecene (NX-2) through an altered *TR1* gene (Kelly et al. 2016). While they were observed under low frequency (1.8%), the unique occurrence of type A chemotypes in *F. graminearum* demonstrates evolvability of the species, which could involve events of horizontal gene transfer. Marks of selection pressure exist for genomic divergence at the trichothecene toxin gene cluster, where variations in toxigenesis may contribute to increased virulence (Kelly and Ward, 2018). The recent changes in North America parallel the global situation, which is also characterized by recent population shifts in FHB-causing species including replacement of *F. graminearum* by *F. poae* in Italy; replacement of *F. culmorum* by *F. graminearum* in Europe; more aggressive 3-ADON strains of *F. asiaticum* population replacing NIV producers in China (Valverde-Bogantes, 2019). As toxins associated with FHB

demonstrate proclivity to evolve, they are a central element in forecasting alterations in host-pathogen interaction.

2.2 Epidemiology of *Fusarium* head blight of barley

Fusarium graminearum belongs to the *Ascomycota*, a phylum characterized by the formation of an ascus or sac where sexual spores develop. Infective bodies are primarily derived from infected residues of preceding crops (Dill-Macky and Jones, 2000) and/ or non-crop grass species (Turkington et al. 2011). Infection is initiated by the release of sexual spores (ascospores) from perithecial bodies developing on crop residues and carried to host spikes by air (Figure 2.1). Ascospores are released 2-4 days after major rainfall events in a diurnal fashion, starting between 16:00-18:00 with a peak achieved by midnight (Paulitz, 1996). Airborne ascospores are the primary inoculum source, and the disease is generally characterized as monocyclic in the Canadian environment (Fernando et al. 1997). To a lesser extent, the infection can occur as the result of splash dispersion of asexually produced spores (macroconidia) directly from crop residues or via secondary infections arising from primary infection sites. While *F. graminearum* can also infect non-spike tissues and is one of several *Fusaria* commonly associated with crown rot, the fungus is not capable of systemic growth from crown to spike (Xi et al. 2008; Moretti et al. 2014). Following contact with the host spike, ascospores and/or macroconidia germinate where germ tubes differentiate to hyphae. Spores were observed to germinate very quickly within 6-12 h on wheat (Pritsch et al. 2000), but took a longer period of 24 h on barley (Boddu et al. 2006). In contrast to wheat, *F. graminearum* also displays a longer period from colonization to the onset of pathogenicity-related gene expression in barley (Lysøe et al. 2011) and relatively lower gene expression overall for this host (Harris et al. 2016). Concerning other *Fusarium* species, *F. graminearum* displays an elevated propensity to form an expansive hyphal network and particularly under warm, humid conditions (Bushnell et al. 2003). Hyphal networks are established to explore the host-surface, in response to stimuli associated with accumulating nutrients. Hyphal growth on exterior surfaces is considered an important opportunistic step of the infection process, which allows the pathogen to discover stomata or other entry points.

Anthesis is a period of high susceptibility of the host to the fungus, particularly for wheat (*Triticum aestivum*). Optimal conditions for host infection at anthesis may differ between

wheat and barley, where the latter shows more moderate temperatures (15°C) are favourable to severity (Schöneberg et al. 2018) vs. warmer temperatures for wheat (> 20°C) (Osborne and Stein, 2007). Flowering in wheat is associated with the swelling of lodicules which open the floret, making the host vulnerable to infection. While florets are temporarily open, airborne spores may directly land inside the floret or hyphae may grow into the inner cavity. This vulnerability is prolonged further by dehiscent anthers that are caught between closed lemma and palea allowing extended access to hyphae. Senescing anthers are also a stimulant for fungal colonization (Strange and Smith, 1971; Strange et al. 1974). While open-flowered barley exists, closed-flowering varieties are commonly seen in Canada. Pollination of barley commonly also occurs while the spike is in the boot, thereby providing a physical barrier of protection during this developmental stage. Once spikes emerge, the lignified thick-walled epidermal cells of the abaxial surfaces of the lemma and palea offer defence to direct penetration. However, the pathogen can gain access to the floret interior through the floret mouth or via the overlapping region of lemma and palea (Lewandowski et al. 2006).

Once inside the floral cavity, the fungus has a high probability of completing the infection process. The adaxial surface of the lemma and palea has a thin epidermis, which is easily penetrated by infection pegs. Likewise, as revealed by the use of a strain transformed by a green fluorescent protein gene (GFP), brush hairs (ovary epithelial hairs) of barley were a target for colonization (Skadsen and Hohn, 2004). *F. graminearum* has an arsenal of cell wall degrading enzymes at its disposal to facilitate this process (detailed later). The fungal hyphae differentiate into specialized infection structures such as foot structures or compound appressoria e.g. lobate appressoria or infection cushions (Jansen et al. 2005; Boenisch and Schäfer, 2011; Qiu et al. 2019). The fungus lives for a short biotrophic period within the apoplast for a couple of days, during which it does not appear to cause harm to the host.

Following the brief initial biotrophic period, the pathogen alters its approach to that of necrotrophic nature. At approximately 72 h post-infection, a dramatic increase in DON is observed in barley (Evans et al. 2000; Boddu et al. 2006). The increase in mycotoxins at this stage is associated with the increased proliferation of fungus and intracellular growth. It is well documented that DON is a virulence factor for wheat and is required for the fungus to bypass the barrier imposed by the rachis and spread throughout the spike via the vascular tissues

(Jansen et al. 2005). Barley differs from wheat in this regard, demonstrating effective type II resistance (defined Mesterhazy, 1995) even in susceptible varieties. Beyond the path through the rachis, under conditions of high humidity, hyphae may grow outside of the spike surface to reach and infect distal florets. Stomates, or injury points, may also provide the fungus with alternative, stochastic entry routes. Silica cells associated with trichomes of the lemma and palea can be directly penetrated and may also be a vulnerability for later infection in barley (Imboden et al. 2018). *Fusarium* infection in barley can occur over two weeks following heading, where this extended period can increase the chance of exposure to events of favourable infection conditions (McCallum and Tekauz, 2002). While open-flowering barley is susceptible during anthesis, as in wheat, closed flowering barley demonstrates increased susceptibility at a later stage (Yoshida et al. 2007; Yoshida et al. 2008).

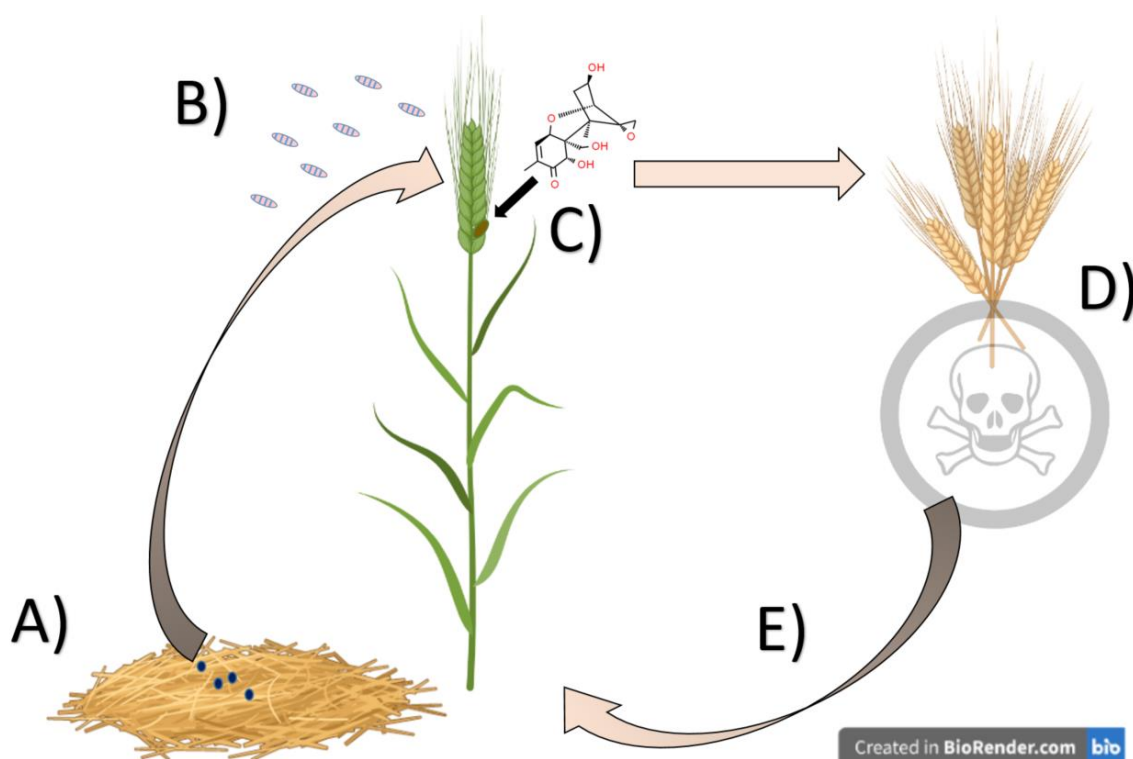


Figure 2.1 Life cycle of *Fusarium graminearum*: A) Perithecia form on crop residues; B) airborne ascospores ejected from the perithecia landing on spikes; C) Fusarium head blight symptoms distinguished by brown-discolouration; D) Ripe grains containing deoxynivalenol and other associated mycotoxins; E) Fungus overwintering on crop residues.

2.3 Host plant defense of barley to *Fusarium graminearum*

2.3.1 Physical plant defense

Plants may escape fungal invasion through multiple morphological features and/ or temporal displacement of developmental stages (reviewed by Steffenson et al. 2003). Plant characteristics often associated with lower infection include plants of tall stature (Zhu et al. 1999; de la Pena et al. 1999; Ma et al. 2000; Buerstmayr et al. 2004; Choo et al. 2004; Horsley et al. 2006; He et al. 2015); hulless grain (Clear et al. 1997; Legzdina and Buerstmayr, 2004); grain pigmentation (Choo et al. 2004; Choo et al. 2015); lax inflorescence (Zhu et al. 1999; Ma et al. 2000;); two-row vs. six-row spike type (*vrs1* locus) (Mesfin et al. 2003; Choo et al. 2004; He et al. 2015); lodging resistance (Choo et al. 2004); extreme heading date (early or late) (Mesfin et al. 2003; Khatibi et al. 2012). Cuticular wax may prevent spore adhesion and/or prevent water retention. Structural defense barriers may also prohibit fungal invasion through constitutive properties of strengthened plant cellular components such as cutin, suberin, lignin, cellulose, hemicellulose, and pectin. While many physical characteristics have been correlated with FHB resistance and/or low DON accumulation, determining whether such character associations are the result of genetic linkage or pleiotropy has largely been elusive in past genetic studies due to inadequate genetic resolution.

2.3.2 Molecular plant defense

Beyond the physical barriers that contribute to the escape or slowing of the infection process, barley displays an intrinsic interaction with the pathogen in ways that resemble the typical relationship of the host-pathogen interface. As in other plants, barley combats invasion of the pathogen via the engagement of an advanced innate immune system. The first line of defence for barley is triggered by the detection of conserved molecular signatures termed microbe (or pathogen) associated molecular patterns (MAMPs or PAMPs). This basal level of immune response is termed Pattern-triggered Immunity (PTI). Pathogens circumvent this basal level of defence through active suppression of host defence via the production of effector molecules, termed effector-triggered susceptibility (ETS). In turn, hosts which recognize effector proteins via intracellular resistance gene products, commonly nucleotide-binding site leucine-rich repeat proteins (NBS-LRR), thereby protecting themselves through a stronger type resistance named effector-triggered immunity (ETI). ETI is characterized by localized programmed cell

death and hypersensitive response which act to contain the pathogen from spreading into adjacent tissues. These processes are also accompanied by the transfer of defensive signals to distal tissues, where systemic induction of resistance occurs. The systemic acquired resistance (SAR) is considered to provide longer-lasting host protection and is associated with the induction of pathogenesis-related (PR) proteins (Ali et al. 2018). This action-reaction scenario describes the four-phased ‘Zig-Zag’ model which is the foundation of the plant immune system model as originally proposed by Jones and Dangl (2006). The immune reaction is yet to be seen in barley, where quantitative resistance is the common case vs. qualitative gene-for-gene resistance patterns. Through the extension of this model, host-*Fusarium* interaction is further characterized by the pathogen's ability to metabolize small toxic molecules which may also be considered effectors, delivering suppression of host plant resistance. Again, host plants may induce the third level of defence where mycotoxins are detoxified through molecular modification via conjugation employing enzymatic action and/or transportation out of cells, binding within cell walls, or storage in vacuoles.

Phytohormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are intrinsically involved in response to pathogen attack, where SA is commonly associated with biotrophic relationships whereas JA/ ET are common to necrotrophic plant defence (Glazebrook, 2005). Phytohormones are produced during PTI and ETI where plants trigger coordinated immune responses such as reactive oxygenated species (ROS) generation, cellular Ca^{2+} spikes, and MAP kinase activation resulting in transcriptional reprogramming (Tsuda and Somssich, 2015). Induced defence response may involve the induction and expression of a suite of genes involved in cell wall fortification features (lignification), hydrolytic enzymes, protease inhibitors, phytoalexins, and transcription factors that regulate the expression of such genes. SA is a plant hormone that is well defined for its commanding role in the disease resistance of many plants (Vlot et al. 2009). The importance of SA in FHB resistance is evident through experiments demonstrating increased resistance in wheat transformed with the *Arabidopsis* NPR1 gene (*AtNPR1*), a hyper-responsive regulator of SA-signalling (Makandar et al. 2006; Makander et al. 2012). Salicylic acid (SA) may be produced through two biosynthetic routes of the shikimic acid pathway, involving differential regulators i.e. isochorismate synthase (ICS) or phenylalanine ammonia-lyase (PAL) (Dempsey et al. 2011). Through transgenic methods, Hao et al. (2018) demonstrated that suppression of the ICS

pathway compromised barley resistance, whereas suppressing the PAL pathway did not. They concluded that ICS plays a critical role in basal resistance of barley through modulating ROS accumulation.

Alternatively, the oxylipin pathway is involved as the main component of plant defence, which may be generally emphasized in regulation in monocots such as cereals. Oxylipins are formed through the oxidation of free polyunsaturated fatty acids via enzymatic activity of lipoxygenases (LOX) with the main forms being 9-LOXs or 13-LOXs (oxidation of 9 or 13 carbon positions), where the 13-LOXs lead to the formation of JA and JA-compounds. Nalam et al. (2015) demonstrated in *Arabidopsis* that loss of function in *LOX1* or *LOX5* (9-LOX genes) resulted in increased disease resistance. Likewise, in wheat, silencing 9-LOX homologue *TaLpx-1* enhanced resistance, such that they concluded that 9-LOXs are susceptibility factors that *F. graminearum* uses to attenuate SA expression. While accelerated SA articulation may underlie the basal resistance to *F. graminearum* of preliminary infection and JA-signalling may constrain this effect, this may be in contradiction to later stages of infection where JA contributes to the promotion of resistance (Makander et al. 2012). Li and Yen (2008) investigated transcription patterns of the resistant wheat cultivar ‘Sumai 3’ through gene chip array and concluded that JA and ET signalling were the dominant regulatory hormones, while SA did not significantly contribute to the process. The importance of JA and ET signalling was also supported by Gottwald et al. (2012) who investigated the resistance of wheat cultivar ‘Dream’ and observed enrichment of genes in the 13-LOX pathway. Ding et al. (2011) demonstrated the complexities of hormone signalling through analysis of resistant wheat cultivar ‘Wangshuibai’ and a susceptible mutant, where they observed in the resistant genotype a SA peak 3 hours post-infection with a quick decline thereafter and then a stronger peak in JA at 12 hours. They characterized effective resistance as a coordinated and ordered expression of signalling pathways. Attenuation of ET through the interference of *Ethylene Insensitive 2 (EIN2)* in wheat via RNAi, reduced both FHB and DON content (Chen et al. 2009). The authors of this study identified *EIN2* as a susceptibility target that *F. graminearum* uses to exploit ET signalling to cause DON-induced cell death. However, ET signalling can also lead to enhanced defence response, where the response demonstrates an interaction with the condition of host resistance (Foroud et al. 2019a). Along with main defence related hormone responses of SA and JA/ ET, several other hormones have been highlighted with

importance in FHB resistance including abscisic acid (ABA) and gibberellic acid (GA) (Buhrow et al. 2016; Qi et al. 2016); auxin (Brauer et al. 2020; and brassinosteroids (BRs) (Ali et al. 2014). Wang et al. (2018a) provide a comprehensive study that integrates transcriptomic data and quantification of all major plant hormones, describing the complexities of integration in *Fusarium*-wheat interaction and rate of expression in pathways. The role of hormonal signals in FHB resistance appears to be complex and time-dependant, likely the result of defence mechanisms tracking a pathogen that shifts its trophism over the host-pathogen interaction. While the integration of hormone signalling is convoluted, hormones do appear to have a centralized and important role in the dynamics of controlling *Fusarium*-induced disease.

2.3.3 Pathogen-associated molecular pattern (PAMP)-Triggered Immunity (PTI)

In higher plants, Pathogen-associated molecular pattern (PAMP)-Triggered Immunity (PTI) (PTI) is the first stage of defence on potentially pathogenic microorganisms achieved through Ca^{2+} signalling and MAPK cascades which lead to alterations in transcription patterns that contribute to protection. Initial contact of *F. graminearum* spores with the barley host may differ from that of wheat, where an extended initial period (excess of 12-24 hours) is observed before spore germination and development of specialized infection structures (Boddu et al. 2006). Morphological or differential chemical signatures may be at the foundation of this prolonged period, which could extend barley a longer period to respond to and induce PTI defence via pathogen recognition receptors (PRRs). A receptor-like kinase (MLOC_12033.1) termed *HvLRRK-6H* was demonstrated to be important to barley resistance to infection via a leaf assay with *F. culmorum* (Thapa et al. 2018). The *HvLRRK-6H* gene was originally highlighted in FHB resistant ‘uzu’ (semi-dwarf) barley lines carrying a gene encoding the LRR receptor kinase, brassinosteroid insensitive1 (*BRI1*) (Ali et al. 2014). Chitin elicitor receptor kinase gene, *HvCERK1* has been implicated in the resistance response of barley genotype CI 9831 (Karre et al. 2017). The chitin elicitor gene *HvCERK1* induced path resulted in upregulation of transcription factor *WRKY23* which promotes the production of metabolites belonging to flavonoid and hydroxycinnamic acid amides (HCAA) pathways (Karre et al. 2019). Sarowar et al. (2019) demonstrate that resistance in *Arabidopsis* and wheat can be enhanced by flg22, a bacterial microbe-associated molecular pattern (MAMP) accompanied by

upregulation of *WRKY29*. PTI is the first line of defence, where a response that can undergo rapid induction could be a highly useful means in circumventing FHB.

Patterns of disease resistance in barley do not follow classical gene-for-gene interaction typical of ETI response. The majority of R-genes observed in the plant kingdom contain a nucleotide-binding site (NB) and a leucine-rich repeat domain (LRR). While classic NB-LRR proteins do appear to be induced in barley upon initial infection by *Fusarium*, this resistance does not appear to be powerful enough to neutralize pathogen infection (Kazan et al. 2012). Such proteins often associated with hypersensitive responses and programmed cell death may ultimately contribute to a contradictory response once the pathogen switches from biotrophic to necrotrophic lifestyle. While *F. graminearum* is recognized to initially demonstrate biotrophic behaviour, it is more strongly characterized by the display of necrotrophy that follows where cell death aids the fungus in this regard. Development of PTI resistance may offer a means to hasten identification of *Fusarium* threat and induce resistance to prevent infection.

2.3.4 Protein expression during infection

The proteome plays an important part in biotic stress response, where proteins represent the physical embodiment of processes that contribute to lessening biotic stress on the plant. Multiple studies have been conducted in barley to evaluate *Fusarium* resistance response, typically through contrast protein profiles of genotypes infected/ non-infected treatment. Proteins that are induced or upregulated in a short period following an attack of a pathogen are termed pathogenesis-related proteins (PR proteins). While diversified in their biochemical function, these proteins are characterized by low molecular weight, pH stability, and are generally resistant to proteolytic enzymes, and thus can withstand environmental changes induced by pathogen invasion (reviewed by Ferreira et al. 2007). Geddes et al. (2008) evaluated protein response to *F. graminearum* 3 days post-infection for a set of barley lines of contrasting resistance levels and observed increases in pathogenesis-related proteins PR-3 (chitinase) and PR-5 (thaumatin-like) in resistant genotypes vs. decreases observed in the susceptible genotype. Increases in proteins associated with oxidative-burst/ stress-response (malate dehydrogenase and peroxidase) were associated with susceptible genotypes which were hypothesized to be indicators of elevated oxidative stress which would prepare tissues for promotion of saprophytic growth. Yang et al. (2010a) evaluated the *Fusarium* response of

barley cultivar ‘Scarlett’, where they also observed up-regulated pathogenesis-related proteins (PR-1,2,3,5,9,15) and proteins involved in energy metabolism (elevated cellular respiration and glycolysis). The contrast of resistant ‘Chevron’ to a susceptible cultivar infected by *F. culmorum* at a later stage (10 days post-infection, mid-dough stage) demonstrated up-regulation of carbohydrate metabolism (amylase inhibitor) and defense response (chitinase, xylanase inhibitor, serpins, universal stress proteins) (Klára et al. 2017). Several studies have also been conducted to evaluate differential infection response on mature barley grains, where differences in protein profiles demonstrated persistence through the ripening process (Yang et al. 2010b; Zantinge et al. 2010; Eggert and Pawelzik, 2011). Elevated levels of serpin proteins (serine protease inhibitors) and amylase inhibitors were commonly upregulated in infected, matured grains.

2.3.5 Resistance to deoxynivalenol

Resistance mechanisms specific to the lower accumulation of toxins are termed Type V and are divided into: a) chemical transformation leading to degradation and/or detoxification eg. glycosylation; b) inhibition of trichothecene biosynthesis through host-manufactured compounds (Boutigny et al. 2008). While DON is produced at low concentrations in an earlier phase of the infection process, by 72 hours post-infection DON content quickly rises in barley spikes and is associated with the lifestyle switch of the pathogen from biotrophy to necrotrophy (Boddu, 2006). DON has been identified as a virulence factor in barley, where wild-type cultures produced higher disease severity in spikes when contrasted with *TRI5* gene loss-of-function mutants (Boddu et al. 2007). DON inhibits protein synthesis (Desjardins, 2006) through binding to the 60S subunit of the eukaryotic ribosome inside the peptidyl transferase center (Garreau de Loubresse et al. 2014), thereby interfering with the translation of messenger RNA. Given the essential life-barring activities of the ribosome, its components are highly conserved, thus making it easily targeted by pathogens (Foroud, 2019). Through disruption of the translation process, the pathogen sequentially interferes with potential protein-driven host defence mechanisms. Programmed cell death (PCD) is an induced mechanism used to halt pathogen spread. In a cell culture system study of the model plant species *Arabidopsis thaliana*, DON showed an ability to inhibit and disarm apoptosis-like PCD typical of hypersensitive response induced defence (Diamond et al. 2013). While DON is not required for

F. graminearum to infect its host tissues, specialized infection structures are associated with the production of DON, albeit at low levels (Boenisch and Schäfer, 2011). Early production of DON during the biotrophic phase may be associated with suppression of plant defence mechanisms, representing the first phase of DON-host interaction.

Deoxynivalenol is a secondary metabolite produced by *F. graminearum*, acting as a multifaceted stress responder when encountering conditions in the environment. Conditions met by the fungus which promote DON in nature can be abiotic or biological. Stress response may be adapted for both interaction with antagonistic microorganisms during the saprophytic phase or with the host during host infection (Audenaert et al. 2013). Deoxynivalenol production is induced in *F. graminearum* by several stimuli including low pH (Gardiner et al. 2009a; Merhej et al. 2010), sugars (Jiao et al. 2008), and arginine-polyamine pathway products (Gardiner et al. 2009b; Gairdner et al. 2010). Deoxynivalenol (DON) is also prompted in response to conditions of oxidative stress associated with hydrogen peroxide (H_2O_2), which is commonly produced in early plant defence response (Ponts et al. 2006; Ponts et al. 2007; Ponts, 2015). Induction of H_2O_2 in wheat by application of prothioconazole fungicide increases DON content (Audenaert et al. 2010). In *F. graminearum*, ROS-equilibrium is tightly controlled, where the loss of virulence is observed in mitogen-activated protein kinase gene *FgOS-2* deficient mutants through downstream impact on ATF/CREB transcription factor *Atf1* (Nguyen et al. 2012; Nguyen et al. 2013). Using a sensitive fluorescent indicator protein (HyPer-2), Mentges and Bormann (2015) demonstrated via confocal laser scanning microscopy, elevations of intracellular H_2O_2 occurring in the specialized infection cushions of *F. graminearum*, implicating its role in early host-pathogen interaction. The fungus stimulates a host stress response and then uses this as an opportunity to trigger further DON production under a positive feedback cycle. Responsive increases in DON further promote plant defense mechanisms and increase oxidative state, resulting in damages to its cellular components. In this sense, DON acts as an effector, where it elicits plant host defences which eventually lead to cell death (Desmond et al. 2008). Elevations in DON prepare plant cells for the next phase of necrotrophic infection following movement from the apoplast to intracellular space, where it derives its nutrients from dead host tissues.

2.3.6 Deoxynivalenol conjugation

Common resistance is observed in barley (Type II), where the infection is generally restricted from spreading through the rachis from initial infection to distal kernels (Bai and Shaner, 2004). Comparison of single-floret point inoculations of wild type vs. *TRI5* deficient mutant near-isogenic strains of *F. graminearum* demonstrated no significant difference in the spread, indicating that for barley, DON is not a virulence factor on Type II resistance (Langevin et al. 2004; Maier et al. 2006). This situation is in contrast with wheat, where DON is an important virulence factor involved in facilitating fungal spread through the rachis (Proctor et al. 1995; Bai et al. 2002). Histological examination using fluorescent *F. graminearum* strains demonstrated that barley is restricted from distribution beyond the rachis node, where even highly susceptible barleys possess an adequate level of resistance (Jansen et al. 2005).

Molecular mechanisms of Type II resistance have been well characterized in barley. Previous gene expression studies involving +/- *TRI5* *F. graminearum* challenge (Boddu et al. 2007) or topical DON application (Gardiner et al. 2010) have documented strong up-regulation of DON-specific resistance in barley. A predominant defense is achieved through the conjugation of DON with a glucose molecule to form deoxynivalenol-3-glucoside (D3G). This is achieved through resistance response enzymes, uridine diphosphate (UDP)-glucosyltransferases (UGTs). Four barley UGT genes were functionally characterized through heterologous expression in yeast, where only *HvUGT13248* (*MLOC_65675*; *HORVU5Hr1G047150*), demonstrated DON resistance (Schweiger et al. 2010). The function of *HvUGT13248* in DON resistance has been further evaluated through the transformation of *A. thaliana* (Shin et al. 2012) and wheat (Li et al. 2015). Using transgenic wheat, the function of this gene has been further characterized to confer resistance and convert nivalenol (NIV) to less toxic nivalenol-3-glucoside (N3G) with even greater efficiency than DON (Li et al. 2017).

2.3.7 Restriction of deoxynivalenol biosynthesis

A series of metabolic profiling studies have been conducted with cereals to identify *F. graminearum* or *F. culmorum* resistance-related metabolites through the application of mass spectrometry (as reviewed by Gauthier et al. 2015). Several studies of barley have repeatedly detected significant fold-changes in specific metabolite groups in contrasts involving resistant genotypes to susceptible control(s) (Bollina et al. 2010; Eggert et al. 2010; Bollina et al. 2011; Kumaraswamy et al. 2011a & 2011b, Kumaraswamy et al. 2012; Cajka et al. 2014; Chamarthi

et al. 2014). Chemical groups represented as resistance-related metabolites detected were: flavonoid phenylpropanoids, non-flavonoid phenylpropanoids, fatty acids (jasmonic acid, linolenic acid), terpenoids, hydroxycinnamic acid amides, and alkaloids. The most commonly induced metabolite group is flavonoids (Gauthier et al. 2015). Flavonoids have strong antioxidative properties, resulting in production reduction and quenching of ROS through several mechanisms (Mierziak et al. 2014). Reductions in oxidative stress are hypothesized to result in negative feedback on trichothecene production (Ponts et al. 2006; Ponts et al. 2007; Ponts, 2015). As trichothecene metabolism is dependant on the presence of molecular oxygen to complete several reaction steps, the presence of anti-oxidative compounds may also inhibit their synthesis. Boutigny et al. (2008) provide a review on the role of phenolics as anti-oxidant compounds on inhibition of mycotoxin formation.

Studies identifying associated compounds have led researchers to investigate these further. Properties of resistance-related metabolites have been examined through assays directed to evaluate the activities regarding the inhibition of mycelial growth or trichothecene production. Bollina and Kushalappa (2011) evaluated 10 selected molecules from phenolic, flavonoid, and fatty acid chemical groups and demonstrated complete trichothecene inhibition by lauric, p-coumaric, sinapic and ferulic acids, naringenin, quercetin, and methyl jasmonate. Ponts et al. (2011) studied five phenolic acids (ferulic, coumaric, caffeic, syringic, and p-hydroxybenzoic acids) and found general inhibition of fungal growth and a decrease in toxin production for p-hydroxybenzoic acid, but increased toxin for ferulic and coumaric acids. Schöneberg et al. (2018) demonstrated that ferulic acid inhibited fungal growth on three *Fusarium* species, but p-hydroxybenzoic acid, vanillic acid, quercetin, and rutin stimulated growth. Identification of metabolites that effectively reduce DON production could be pursued through the development of convenient assays for plant breeders to use as bio-markers. Manifestation of compounds in specific tissues may be of importance, for example through the use of mutants Skadhauge et al. (1997) demonstrated the importance of proanthocyanidins in the testa layer which prevented the further penetration of hyphae of *F. culmorum* in barley seed. Resistance strategies that rely on limiting the initial production of toxins vs. detoxification such as glycosylation, may be preferred as conjugated toxins are readily converted back to their more toxic form in the mammalian gut.

2.4 Breeding *Fusarium* head blight resistance in barley

2.4.1 Identification of resistance from exotic resistance sources

Identification of FHB resistant barley has been a long-term process, which has involved the screening of tens of thousands of gene bank accessions through various methods by numerous research programs across the world. In the United States of America, screening efforts were conducted in the late-1920s in response to epidemics of that time, where several moderately resistant accessions were identified through the effort. Chevron, a Swiss landrace accession, was identified to both carry the *Rpg1* stem rust resistance gene (*Puccinia graminis* f. sp. *tritici*) and moderate FHB resistance (Shands, 1939). This line has proven to be one of the most resistant, six-row accessions ever identified and has been a foundational source for breeding FHB resistance. MNBrite, an American six-row variety with Chevron (Ciho 1111, PI 38061, Swiss landrace) in its ancestry, carries moderate FHB resistance developed through indirect selection for kernel brightness. Kernel staining is associated with a complex of pathogens, including *Fusarium* species (Rasmusson et al. 1999). Resistance of MNBrite was further combined with crosses involving Chinese resistant accession, Zhedar 2 (two-row) to develop Quest (PI 663183) a moderately-resistant, six-row variety with malting quality (Smith et al. 2013).

Epidemics varying in severity have been common to eastern Asian countries (Choo, 2009), where significant efforts in evaluating germplasm to identify resistance sources of barley occurred in the previous century. In Japan, several studies were carried out to identify resistant germplasm (Ikeda et al. 1955; Heta and Hiura, 1963; Gocho and Hirai, 1987; Takeda, and Heta, 1989; Takeda and Wu, 1996). Likewise, significant screening efforts were performed in China (Chen et al. 1982; Chen et al. 1991; Zhou et al. 1991). Following epidemics in the mid-1990s, revitalized screening efforts were conducted in North America, including in USA (Prom et al. 1997; Scholz et al. 1999; Steffenson and Scholz, 2001; Skoglund and Menert, 2002), Canada (McCallum et al. 2004; Tucker et al. 2009) and Mexico (Gilchrist, 2001; Gilchrist et al. 2001). Evaluation of germplasm has also been conducted in Europe (Buerstmayr et al. 2004; Vančo et al. 2007). Many of the accessions identified earlier in eastern Asia have been validated in the North American growing environment, but poor malting quality (Urrea et al. 2005) and unadapted nature due to photoperiod sensitivity have posed problems in their utility for breeding

(Franckowiak, 2001). Huang et al. (2013) studied a panel of 78 moderately resistant accessions using 1,727 Diversity Array Technology (DArT) markers, where population structure analysis indicated clustering within spike type and growth habit and broad diversity within groups. Haplotype analysis of genomic positions associated with FHB QTLs (2H bins 8, 10, 13, and 6H bin 7) has also been conducted, with a few accessions with distinct haplotypes being identified. From the immense collective efforts by researchers globally over the past decades (collectively > 30, 000 accessions), little more than 100 lines have been identified with moderate resistance, where notably immune response has not been identified (Steffenson et al. 2003; Steffenson et al. 2016).

In the 1980s more frequent isolations of *F. graminearum* and its associated mycotoxins were first observed from wheat samples originating in the Red River Valley region of western Canada (Clear and Abramson. 1986; Abramson et al. 1987; Clear and Patrick, 1990). While not initially regarded as a problem for other cereals, *F. graminearum* was soon to adapt to the barley crops in the region (Tekauz et al. 2000). In only a couple of years, observations of infected barley went from a sporadic incidence to detection in nearly all fields surveyed within Manitoba (Clear et al. 1996; Clear et al. 2000; McCallum et al. 2000). Since it was originally observed in eastern Manitoba, FHB has spread progressively to occupy barley growing regions further west (Turkington et al. 2002), such that the situation in Canada is currently dire. Crosses were initiated in 1996 in the two-row malting barley breeding program at Agriculture and Agri-Food Canada, Brandon Research and Development Centre (AAFC-BRDC), where it remains a high priority breeding objective to date. Over 50 resistance sources have been used within this program. However, very few resistant breeding lines were advanced to cooperative registration testing in the early 2000s (Legge et al. 2004). Some of the mentionable resistance exotic sources that have resulted in successful crosses include Harbin (origin China) and Svanhals (origin Sweden). Crosses made with elite breeding line TR04282 (Harbin/TR253//TR253) have resulted in the release of two moderately resistant malting barley cultivars that incorporate resistance to other diseases of economic importance combined with malting quality: AAC Connect (Legge et al. 2017) and AAC Goldman (Legge et al. 2018). Fusarium head blight breeding efforts at the Field Crop Development Centre, Olds College has focussed on the development of multi-disease resistance using wide-crosses through a partnership with ICARDA/ CIMMYT, Mexico (International Center for Agricultural Research in the Dry Areas/ International Maize and Wheat

Improvement Center) using resistance sources like Shri and Gobernadora (He et al. 2015; Osman et al. 2019). Breeding for FHB resistance in eastern Canada has focussed on Chevron and Quest resistance within the six-row breeding program (R. Khanal, personal communication) and has used Mimai 114 (origin China) to develop 2-row, hulless cultivar AC Alberte (Choo et al. 2001). Breeding with exotic materials has generally been difficult, requiring multiple breeding cycles with back-crossing to elite germplasm to achieve progeny with improved agronomics, and in particular, suitable malting quality.

2.4.2 Resistance from the native gene pool

While breeding efforts using exotic barley germplasm may provide resistant alleles uncommon to the Canadian gene pools, identification of resistance within adapted materials has also provided heritable variation for developing resistant cultivars. While resistance may not be considered as strong, this may represent an alternative for breeding resistant cultivars, especially for a crop such as barley where adequate resistance sources are lacking. Two-row barley is a generally more resistant type than six-row barley (Choo et al. 2004), and founder genetics of Canadian germplasm such as Harrington (TR441 = Klages///Gazelle/Betzes//Centennial) and AC Oxbow (TR226 = TR233/WPG8020//WPG823/TR222) have contributed to general resistance levels of modern cultivars (Rudd et al. 2001; Tekauz et al. 2000). Betzes (C.I. 6398), a heritage two-row variety, originating from Germany, but introduced to North America through Poland (Wiebe and Reid, 1961), is a reoccurring parent common to both lineages. Known resistance sources such as Svanhals and Chevron (via Chevron-selection Peatland) also exist as distant parents in pedigrees of Canadian founder lines, which may have conferred resistance alleles amongst others inherently carried within European-based breeding pools. Transgressive segregation for resistance occurs in barley (Zhu et al. 1999) and appears to be common in crosses for FHB resistance, where progeny lines may display higher resistance levels than their parents. As resistance levels may supercede either parent, combining ability is demonstrated to be an important factor and should be considered in breeding. Fusarium Head Blight (FHB) resistance breeding was initiated much sooner in eastern Canada where Island (TBR635-25 /'Symko'), a moderately-resistant, general-purpose cultivar with a European resistance foundation was developed (Choo et al. 2006). Conlon (ND13299 =

Bowman*2/Brigitta//ND10232) an American two-row cultivar that was not bred specifically for FHB resistance, demonstrates moderate-resistance. Complementary crosses between elite Canadian lines have resulted in progeny with superior resistance than their parents and the development of moderately-resistant cultivars adapted to western Canada, such as CDC Mindon (TR04378 = TR339/TR251, Rossnagel, et al. 2008) and Lowe (TR13609 = H92017203Z/'Ponoka' where H92017203Z = 'AC Oxbow'/'Leo', Juskiw, et al. 2019). While CDC Mindon exhibits an intermediate reaction to FHB infection, it displays a significant reduction in DON accumulation, thereby demonstrating the complexities of host resistance. It is possible that through the creation of fitting crosses, further improvements in resistance may be achieved through matching additive effects and/ or possible epistatic contributions.

2.4.3 Resistance from *in vitro* selection (IVS)

Breeding malting barley cultivars, which must meet the minimum standard for numerous characteristics, has been a challenge for breeding FHB resistance. The use of exotics, which tend to carry very poor quality, requires multiple breeding cycles to reduce the linkage drag of non-adapted alleles. A more immediate solution to FHB resistance development is to use *in vitro* selection (IVS) techniques to expose variation and apply selection on callus in growth media. Chawla and Wenzel (1987) performed *in vitro* selection to develop resistance in barley through the application of fusaric acid. This breeding method is particularly efficient when paired with a doubled haploid system, which fixes alleles by removing heterozygous conditions under a single generation. An anther co-culture assay for the regeneration of doubled haploids under media with trichothecenes has been used to develop FHB resistant wheat lines (Eudes et al. 2008). A similar approach was taken at AAFC-BRDC for IVS application using mycotoxins in an anther culture system (growth media containing 1.71 mg kg⁻¹ DON). Norman (TR05915 = CDC KendallDT002-4, Legge et al. 2011) a doubled-haploid, two-row malting cultivar, demonstrates a 25-30% decrease in DON from parent cultivar CDC Kendall (TR133 = Manley/SM85221, where SM85221 = WM7619-4147/Harrington). 'Taylor', a doubled haploid, hulless malting variety was selected from a segregating cross (HB705 = BM0058DT-5-1 = CDC Freedom/Rivers) (Legge et al. 2013) using anther culture and growth media with multiple mycotoxins (1.43 mg kg⁻¹ DON; 1.43 mg kg⁻¹ 3ADON; 2.86 mg kg⁻¹ T2). 'Taylor' displays reduced DON content within the hulless class, which is the least-toxic type of barley attributable

to the physical separation of the hull which retains half of all kernel mycotoxins (Clear et al. 1997). A microspore technique was also developed at AAFC-BRDC and applied to the development of breeding populations through *IVS* (Banik et al. 2005). This success of *IVS* was genotype-specific, with the resistant lines identified under sporadic occurrence rather than results of generalized effect (Legge et al. 2004). While the loss of resistance in *IVS* cultivars can occur, the resistance in these *IVS* cultivars such as Norman and Taylor has shown stability over time.

2.5 Molecular Breeding

Marker-assisted selection (MAS) is a breeding process where a marker (morphological, biochemical, or genetic) is used to indirectly select a desirable trait through linkage to the marker. With routine development and application of molecular markers in plant breeding during the late 1990s, a large number of bi-parental mapping studies were conducted in an attempt to identify quantitative trait loci (QTL) associated with FHB resistance and low DON accumulation (reviewed by Massman et al. 2011; Foroud et al. 2019b). While resistance sources are more commonly two-row, studies have primarily focussed on the identification of resistant QTLs within the six-row background. Multiple resistance sources have been investigated including Aza-Fran (CMB 643), two-row (Zhu et al. 1999); Chevron, six-row (de la Pena et al. 1999; Ma et al. 2000; Canci et al. 2004); CI 4196, two-row (Horsley et al. 2006); Frederickson, two-row (Mesfin et al. 2003; Smith et al. 2004); Gobernadora (Shenmai 1 or Zhenmai 1), two-row (Zhu et al. 1999); Harbin, two-row (Hori et al. 2006; Sato et al. 2008); Russia 6, two-row (Hori et al. 2005); Zhedar 2, two-row (Dahleen et al. 2003); Zhenongda 7, two-row (Yu et al. 2010). These studies have identified significant QTLs of all seven chromosomes of barley and some in multiple chromosomal regions. Quantitative trait loci (QTLs) associated with FHB have often been coincident with those for DON, but this is not universal, demonstrating the difficulties of breeding for this disease where resistance for both characters is desirable. Quantitative trait loci (QTLs) have tended to show minor effects, subject to genotype x environment interactions (i.e. environmentally specific interactions), and display large confidence intervals (> 20 cM), limiting their breeding applications.

While several QTLs have been identified for FHB severity and/ or DON content, many have coincident occurrences with QTLs for adverse agronomic characters, and thus have not been useful to breeding programs (Huang et al. 2018). Chromosome 2H has been a source of major

QTLs, occurring in bins 8 and 10 (defined as Qrgz-2H-8 and Qrgz-2H-10 respectively). However, these QTLs are associated with heading date and/or plant height (de la Pena et al. 1999; Ma et al. 2000; Dahleen et al. 2003; Mesfin et al. 2003; Horsley et al. 2006; Lamb et al. 2009; Huang et al. 2018) and the *Vrs1* locus which controls two-row spike type. The short arm of chromosome 2H possesses multiple major genes controlling photoperiod response (*Ppd-H1*, Laurie et al. 1994) and early maturity (*Eam6*, Franckowiak and Konishi, 2002). The lack of adequate genetic resolution in many experiments has made it difficult to conclusively define whether an association between resistance and two-row morphology is due to genetic linkage, or pleiotropy. However, fine-mapping analysis of Qrgz-2H-8 has implied that tight linkage is the possible mechanism of morphological association (Nduulu et al. 2007). Furthermore, resistance derived from accession CI 4196 was incorporated in a six-row background in a germplasm line, 6NDRFG-1 (PI 615583), with a level of resistance comparable with its parent line (Urrea et al. 2002). The QTL associated with *vrs1* was also identified in a six-row association mapping population, providing evidence that genetic resistance in that region may be independent of morphology (Massman et al. 2011). However, Hori et al. (2006) and Sato et al. (2008) did not identify an FHB resistance QTL on 2H in the peri-centromeric region using two-row x two-row crosses. Resistance alleles contributed by Harbin were found in proximity to the cleistogamy locus (*cly1/Cly2*) that determines open/closed flowering type, implicating its involvement in the resistance. Yu et al. (2010) reported an FHB resistance QTL on the 2H chromosome in bin 14 (defined as Qrgz-2H-14) which was derived from an adapted North American parent. This QTL, which described 14% of the phenotypic variation in FHB resistance, was not associated with heading date or plant height.

Of the many QTLs reported for FHB resistance (78) and DON accumulation (42), only a couple demonstrate independence from other characters (Steffenson et al., 2016). Along with major QTLs observed on 2H, genetic studies involving Swiss landrace, Chevron have identified QTLs for FHB severity and DON content on chromosomes 1H (de la Pena et al. 1999; Ma et al. 2000) and 7H (Ma et al. 2000), but these are also associated with late heading and tall stature. Horsley et al. (2006) identified a QTL on chromosome 4H (bin 2) derived from Chinese accession CI 4196 which explained 9-14% of the variation for DON accumulation, which was subsequently designated QDON-4H-2. A QTL for FHB severity and DON content on 4H was also detected in a study by Zhu et al. (1999), which was associated with plant height

but not heading date. A QTL derived from Chinese accession Zhedar 2 for FHB severity and DON content that was identified on chromosome 6H (Dahleen et al. 2003), is also associated with the late heading. Another QTL associated with kernel staining on 6H (Canci et al. 2004) is accompanied by elevated protein content (Canci et al. 2003), a character considered to be highly undesirable for malting barley. Huang et al. (2018) used a 93 entry RIL population with partial FHB resistance derived from elite cultivar Rasmusson, to investigate QTLs for FHB resistance and DON accumulation in the context of associated agronomic traits. They found QTLs and agronomic character associations similar to those contributed by Chevron, however, a native, minor QTL on 3H (bin 4) donated by Rasmusson was found to lower DON content and was independent of plant height. Correlated response of negative characters (via pleiotropy or linkage disequilibrium) has highly limited marker-assisted breeding for low FHB and DON.

2.6 Modern biotechnologies and how they facilitate Fusarium head blight research

Technological improvements in genomics science have made it possible to contend with difficulties common to cereal crop species of the Triticeae tribe, which are considered challenging to work with given their extremely large size and numerous repetitive elements throughout. Chromosome capture sequencing (Hi-C) methodologies, optical mapping, and positional information from dense genetic maps through collaborative efforts of the International Barley Genome Sequencing Consortium (IBSC) have aided in overcoming such challenges where significant-resolution improvements have been made on previous undertakings of the past decade (The International Barley Genome Sequencing Consortium, 2012) and have resulted in the production of the first high-quality reference assembly for the 5 Gb barley genome (Beier et al. 2017; Mascher et al. 2017). With the creation of this biotechnological tool, new possibilities exist for plant breeders to exploit this information to develop improved and adapted barley cultivars for use in sustainable production systems (Smith et al. 2018). Past decades of research devoted to developing FHB resistant cultivars have proven to be exceptionally challenging with very slow progress. However, genomics advancements provide new hope and revitalized abilities to contend with this devastating disease and build on past successes to improve barley resistance.

Barley breeders now have convenient access to high throughput application and evaluation of breeding populations, with the development of genotyping-array barley platforms including

diversity arrays technologies (DArT, Wenzl, et al. 2004) or more-recently SNP (single nucleotide polymorphism) assays as Illumina GoldenGate (Close et al. 2009) and Illumina Custom Genotyping BeadChip assays of 9k (Comadran et al. 2012) or 50k (Bayer et al. 2017) markers respectively. However, reductions in sequencing costs and the advent of methodologies utilizing restriction enzymes i.e. Genotyping-by-sequencing (GBS), which helps reduce complexity via reduced representation of the genome, have made it possible to effectively cover large cereal genomes such as barley. Genotyping-by-sequencing (GBS) has proven effective for barley using a single enzyme protocol (Elshire et al. 2011) or two-enzyme systems with common and random cutting properties (Poland et al. 2012). Using improvements in computational efficiencies and improved statistical methodologies to impute missing data, GBS has become an attractive possibility for application in genomic-assisted breeding, which demonstrates more independence of ascertainment bias of fixed genotype arrays (Rasheed et al. 2017). Techniques allowing thorough coverage of the immense barley genome, coupled with a high-quality reference genome that defines positions of highly repetitive DNA elements, have set the stage for novel advancements in the understanding of barley genetics.

Development of genome-wide molecular marker systems and common access to such technologies has facilitated genome-wide association study (GWAS) in plant science, providing advancements in approach and new opportunities to identify QTLs and/ or genes for complex characters of interest (Gupta et al. 2005; Burke et al. 2007; Zhu et al. 2008; Varshney et al. 2009; Rafalski, 2010). Genome-wide association study (GWAS) makes use of diversity panels of unrelated germplasm which demonstrate a phenotypic range in characters, to examine character-marker associations occurring as a result of linkage disequilibrium between markers and functional polymorphisms of alleles for genes. This method holds advantages over bi-parental QTL mapping due to the potential of greater recombination events over an extended period. Furthermore, alleles that are identified through GWAS may represent those common to breeding gene pools vs. those introgressed from exotic sources and may carry fewer problems associated with linkage drag. With the increased availability of genome-wide markers which have expanded depth of coverage, practical resolution of genetic associations and tests of marker positions is now commonly available at unprecedented levels. Specifically, the GWAS approach has contributed to genetic improvement in barley over the past decade

through the identification of multiple favourable alleles eg. agronomic components, grain quality, and biotic/abiotic stress tolerance, which can be employed in crop breeding (recently reviewed by Alqudah et al. 2019).

Due to their large influence on the phenotype and ease of breeding, qualitative disease resistance genes (effector-triggered-resistance) associated with major R-genes have been more commonly exploited by plant breeders. Quantitative resistance has generally been a more durable form of resistance, as defined by the persistence of resistance in cultivars grown over large areas under conditions conducive to disease (Cowger and Brown, 2019). Genome-wide association study (GWAS) has been a beneficial tool for the identification of QTLs associated with quantitative disease genetic resistance for multiple pathogens of barley. Recent applications of GWAS procedures have been used to identify resistance-related QTLs for stem rust (causal agent *Puccinia graminis* f. sp. *Tritici*) using populations of *H. vulgare* (Turuspekov et al. 2016) or *H. vulgare* subsp. *spontaneum* accessions (Sallam et al. 2017); barley stripe rust (causal agent *Puccinia striiformis* f. sp. *hordei*) (Belcher et al. 2018; Visionsi et al. 2018); leaf rust (causal agent *Puccinia hordei*) (Singh et al. 2018); net-form net blotch (*Pyrenophora teres* f. sp. *teres*) (Richards et al. 2017; Amezrou et al. 2018; Daba et al. 2019; Rozanova et al. 2019); spot-form net blotch (*Pyrenophora teres* f. sp. *malculata*) (Burlakoti et al. 2016; Daba et al. 2019); scald (causal agent *Rhynchosporium commune*) (Daba et al. 2019); spot blotch (causal agent *Cochliobolus sativus*) (Roy et al. 2010; Bykova et al. 2017; Wang et al. 2017; Gyawali et al. 2018; Novakazi et al. 2020). The many QTLs identified in recent years reinforce the utility of GWAS as a powerful tool for the discovery of quantitative resistance, where many of such QTLs may also be useful for further investigation of FHB resistance, albeit in the context of synergistic or antagonistic effects.

Massman et al. (2011) utilized a GWAS panel, consisting of 768 lines from advanced breeding populations from four USA breeding programs in the Upper Midwest, to analyze associations of FHB and DON with Illumina GoldenGate bead-array SNP assay (BOPA1; Close et al. 2009). Several minor QTLs explaining 1-3% of the phenotypic variation for FHB (4) or DON (8) were identified, however, genomic regions were less coincident with heading date and/or plant height. This study identified strong clustering of breeding programs and row-type indicating little crossing between groups. Very few FHB resistance QTLs were detected in the

two-row vs. six-row sub-population, owing to less phenotypic variation overall within the two-row class and/or gene expression within different genetic backgrounds. Mamo et al. (2015) evaluated a diverse set of 298 Ethiopian and Eritrean barley landraces with Illumina iSelect Infinium 9K SNP array (BOPA1 and BOPA2; Comadran et al., 2012). These accessions were phenotyped for multiple agronomic characters as well as FHB and DON content by inoculation with a mixture of 19 isolates of *F. graminearum*. While this study identified QTLs for FHB and DON seen in the previous study, negative associations with agronomic characters were not present indicating that this germplasm could be useful in breeding programs without the confounding effects from heading date or plant height. Bedawy et al. (2018) conducted leaf and spike FHB disease assays on a diversity panel comprised of 140 genotypes in combination with DArT and iSelect SNP marker assays, identifying QTLs for each character on chromosomes 1H and 5H, respectively. They also identified positive epistatic interactions, contributing to FHB disease reduction. As GWAS of FHB resistance in barley has generally identified many genes of minor effect, a more comprehensive approach to breeding such as genomic selection may be more productive (Massman et al. 2011).

Lande and Thompson (1990) introduced the concept of using genome-wide molecular markers to improve quantitative traits. Limits on molecular marker methodologies of the time could not support high throughput application, but as they stated, “Although the scale of this endeavor may exceed the current capability of most molecular genetic laboratories, we anticipate that improved technology soon will make these procedures feasible.” Recent advancements in genomics have lowered the costs of genotyping, such that molecular marker coverage is no longer a limitation for breeding practice. Genomic selection, as conceived by Meuwissen et al. (2001), is a breeding approach founded on the principle that with dense enough marker coverage, each allele contributing to a character will be in linkage disequilibrium with a polymorphic molecular marker e.g. SNP marker. A comprehensive summation of marker effects across entire genomes may be used to predict the merit of characters for individuals and to perform the selection of candidates. Many complex models have been pursued to improve the accuracy of the method (Desta and Ortiz, 2014; Wang et al. 2018b), however, simplistic models may often be adequate (de los Campos, 2013). Basic principles involve synchronized phenotyping of a ‘training’ population which differentiates for a character of interest and genotyping via genome-wide molecular markers. Character-marker associations are used to

calculate a genomic estimated breeding value (GEBV) for individuals in a population for which the breeder wishes to perform selection based on genomic information alone. As predictions are based on associations formed in the ‘training set’, the accuracy of initial phenotyping along with a close genetic relationship to that for which selection is applied are important determinants of prediction accuracy (Habier et al. 2013). While this approach was more readily adopted by animal breeders, it has recently gained favour in the plant breeding community and is expected to revolutionize the breeding of complex characters of crops (Bhat et al. 2016; Crossa et al. 2017; Robertsen et al. 2019).

Due to the global nutritional and economic importance of wheat, significant efforts have been devoted to the genetic improvement of the FHB resistance of this crop. Literature review (Buerstmayr et al. 2009; Buerstmayr et al. 2020) and meta-analysis (Venske et al. 2019) have identified numerous QTLs from studies conducted over the past two decades. While molecular markers are now commonly used to breed for incorporation of several major QTLs (Fhb1, Fhb2, Qfhs.ifa - 5A), it is recognized that breeding values of QTLs may have been over-estimated and QTLs may interact in complex ways under field situations. Genomic selection has been used as an alternative or complementary method to MAS for breeding FHB resistant wheat (reviewed by Steiner et al. 2017; Buerstmayr et al. 2019). Genomic prediction of FHB and/or DON has shown great potential, where several studies have demonstrated moderate prediction accuracies in hexaploid (Rutkoski et al. 2012; Arruda et al. 2015; Jiang et al. 2015; Mirdita et al. 2015; Hoffstetter et al. 2016; Dong et al. 2018) and durum wheat (Steiner et al. 2019; Moreno-Amores et al. 2020). Genomic selection models may show an advantage over traditional MAS through the benefit of higher prediction accuracy (Arruda et al. 2016). Overall, the implementation of a genomic-assisted breeding strategy within wheat to improve FHB resistance in wheat has been very promising, in consideration of polyploidy and immensity of genomic size ~17 Gb (Shi and Ling, 2018).

Emerging barley genomics tools offer prospects for sustaining barley improvement in the face of difficult breeding objectives such as FHB resistance (Smith et al. 2018). Before implementation, multiple studies have performed *in silico* analysis for pre-evaluation of genomic prediction in six-row barley. Real data of 1,325 SNP markers (BOPA1, Close et al. 2009) identified in 863 barley breeding lines was used to study 100 hypothetical QTLs for a

character of interest, where moderate prediction accuracies endorsed genomic selection in barley breeding programs (Iwata and Jannink, 2011). The simulation was further implemented through the application of phenotypic information of FHB and DON in six-row barley lines, concluding that due to excessive linkage disequilibrium marker numbers could be reduced to as little as 384 within a population of 200 with minimal effect on genomic prediction (Lorenz et al. 2012). Increasing population size by the addition of neighbouring barley breeding programs, where little genetic information is exchanged showed no advantage when incorporated into the training population (Lorenz et al. 2012; Lorenz et al. 2015). Positive preliminary results of simulated studies have encouraged further research into the application of genomic prediction in breeding methods to reduce FHB in cereals.

Sallam and Smith (2016) evaluated multiple genomic prediction models of FHB and DON for a dynamic six-row barley population comprised of 647 breeding lines. They concluded that prediction accuracy was not improved in more complex models. Abed et al. (2018) also demonstrated successful genomic selection for six-row barley, where models which included both additive and epistatic components proved to demonstrate better predictability. These studies have demonstrated moderate genomic predictions for FHB and DON in barley, such that significant resources could be spared by genomic selection application. Both studies also demonstrated significant reductions in the set of genome-wide markers which could be implemented without significant loss of prediction accuracy. Genomic selection for increasing FHB resistance and lowering DON content in six-row barley could make use of more conservative genomic panels with reduced targets.

Beyond the use of genome-wide markers to impose genomic selection within segregating populations, a more ambitious goal is in their application on informative parental selection. Modern methods (Mohammadi et al. 2015) which utilize phenotype-genotype relationships and informative segregating markers have improved the prediction of genetic variance (σ^2_G). Such a method ‘PopVar’, has demonstrated reasonably accurate prediction of σ^2_G for FHB severity within 40 historic, six-row bi-parental breeding populations (Tiede et al. 2015). Abed and Belzile (2019) used ‘PopVar’ to predict yield and DON in silico for the simulated progeny of 245 pairwise crosses of six-row barley parents in a training population and then validated these crosses using data of registration trials in Eastern Canada. Neyhart and Smith (2019) used a

training population of two-row barley lines, to predict superior parental combinations, which were validated through phenotyping 27 bi-parental populations. These studies have been able to predict progeny means well, but the genetic variance to a lesser degree (Abed and Belzile, 2019). Overall, such studies support the feasibility for plant breeders to use genome-wide markers to determine better choices for crossbreeding.

2.7 Use of modern genomic tools (transgenics and RNA interference) to combat Fusarium head blight and deoxynivalenol accumulation

While cereals exhibit a defense response to *F. graminearum* invasion i.e. expression of pathogenicity-related proteins, these generally do not occur quick enough, with enough abundance, or in required tissues. Wheat and barley have been transformed with several targets conferring anti-fungal protein products (reviewed by Dahleen et al. 2001). Most targets were on fungal-specific cell wall components such as chitinase (PR-3) and β -1,3-glucanases (PR-2), thaumatin-like proteins (PR-5), thionin (PR-13), and ribosome-inactivating proteins (RIPs) which target/inhibit protein synthesis in fungal cells. Abebe et al. (2006) demonstrated the application of the Lem2 gene promoter of barley for tissue-specific expression of transgenics, which could restrict trans-gene expression within organs important to Fusarium-defense (lemma/palea and coleoptiles), thereby conserving energy in the defense response.

Genetic engineering of cereal crops has also focussed on the detoxification of DON. The function of trichothecene genes (TRI genes) has been well characterized. *F. graminearum* uses the TRI101 gene to protect itself from the toxicity of DON through acetylation of the C-3 hydroxyl group. It was suggested that host plants expressing this gene would also lead to reduced toxicity (Kimura et al. 1998). While TRI101 trans-genes were able to reduce FHB and DON in wheat (Okubara et al. 2002) and barley (Manoharan et al. 2006), this mechanism did not confer field-based resistance possibly due to reversion to DON in planta. Transgenic wheat plants with barley UGT gene *HvUGT13248* display higher levels of resistance to FHB (Li et al. 2015) and crown root rot (Mandalà et al. 2019). As the Ribosomal Protein L3 (RPL3) is so essential to biological function it is generally conserved, however, it is also the target of DON binding which interferes with protein synthesis. Investigations have been conducted on altered RPL3 from rice (Harris and Gleddie, 2001) or tomato (Mitterbauer et al. 2004) with variable success. The epoxide on C12/C13 is the primary toxicity determinant of DON

(Eriksen et al. 2004). Several bacterial species produce enzymes that can de-epoxidize DON to a non-toxic form and are used as amendments in industrial processing to detoxify grains. Genes coding for enzymes that confer de-epoxidation of DON would represent excellent targets for the transformation of cereals.

Technological advancements in gene-editing platforms such as CRISPR-Cas9 offer new potential to easily remove susceptibility targets from host genomes. CRISPR-mediated genome editing was applied to a wheat ortholog (*TaNFXL1*) of Arabidopsis (*Arabidopsis thaliana*) trichothecene-responsive defense gene NFXL1. *TaNFXL1* is a transcription factor that represses *F. graminearum* resistance, and thus represents a potential breeding target to reduce FHB. CRISPR editing of additional susceptibility (TaABCC6) and resistance (TansLTP9.4) targets was demonstrated (Cui et al. 2019). Su et al. (2019) showed that deletion of susceptibility gene TaHRC (putative histidine-rich calcium-binding protein) via CRISPR-Cas9 increased resistance. Lipxygenases are associated with JA-mediated defense which attenuates the SA response, thus representing targets for gene editing (Borisjuk et al. 2019). Wang et al. (2018c) demonstrated CRISPR editing is possible for *TaLpx-1* gene (9-lipoxygenase). The CRISPR-Cas9 system has been successfully applied to edit barley (Lawrenson et al. 2015; Gasparis et al. 2018). FHB resistance-related susceptibility targets such as putative 2-oxoglutarate Fe (II)-dependent oxygenase (2OGO); ethylene insensitive 2 (EIN2) are being evaluated (Low et al. 2019).

RNA interference (RNAi) is a new genomic tool for silencing genes that may promote disease in plants, which may provide new promise in the battle to control FHB in cereals (Machado et al. 2018). RNA molecules are used to inhibit gene expression or translation by targeting mRNA through the introduction of sequence-specific double - stranded RNAs (dsRNA). Koch et al. (2013) demonstrated reduced growth in *F. graminearum* through host - induced gene silencing (HIGS) of cytochrome P450 lanosterol C14 alpha - demethylase - encoding (CYP51) genes which are essential for ergosterol biosynthesis. Targeting chitin synthase via HIGS demonstrated reduced infection in wheat (Cheng et al. 2015), while a small *F. graminearum* RNA (Fg-sRNA1) silenced the wheat resistance chitin elicitor gene (TaCEBiP) in *Nicotiana benthamiana* to enhance *F. graminearum* infection (Jian and Liang, 2019). Chen et al. (2016) investigated HIGS of β -1, 3-glucan synthase gene FcGls1 of *F. culmorum* which

induced cell wall abnormalities expressed as swollen hyphae. Baldwin et al. (2018) demonstrated that RNAi vectors could also target a positive-regulatory gene (TRI6) in the trichothecene cluster of *F. graminearum* which reduced the expression of TRI5 i.e. the first gene in the DON production pathway. Overall, RNAi using the HIGS approach appears to be a promising technology that could complement FHB resistance breeding efforts.

RNAi can also be applied in a non-transgenic approach through external application via spray - induced gene silencing (SIGS). Exogenous formulations of long dsRNA or siRNAs (short interfering RNA) can be applied and subsequently taken up by cells (host or pathogen target). Koch et al. (2016) demonstrated in *Arabidopsis* and barley spraying long dsRNAs targeting CYP51 genes facilitated disease control. This method is publicly appealing due to its non-transgenic nature, high specificity, the potential for application against multiple pathogens, and the ephemeral nature of applied RNA molecules in the environment.

Preamble: Chapter 3

Barley is a major crop well suited for growth in the western Canadian environment. Grains were brought to Canada by European colonists and were grown and used to supply local breweries as early as 1668 (Metcalf, 1995). Since colonization, there has been a long history of testing barley for adaptation to the western Canadian environment, followed by improvements through selective breeding. While many varieties exist for general-purpose production including livestock feed, the historical focus of two-row barley breeding has largely been on the development of malting barley. The quality of Canadian barley has been systematically improved over recent decades, through an evaluation system based on analytical methods (Edney et al. 2014). Through historical events, foundational genetics and selection actions applied within the barley breeding of western Canada have given rise to modern varieties under current production. Breeding of two-row barley has pursued the development of an ideotype (a hypothetical plant representing a balance of yield, quality, and disease resistance). The current study provides an analysis of the genetic structure of modern, elite two-row barley germplasm adapted for production in western Canada.

Following several FHB epidemics in the mid-90s which resulted in massive economic loss, significant investments in breeding have been made to minimize the impact of this disease through the development of genetically resistant varieties. The next chapter explores the structure of genetic variance of a germplasm collection of two-row spring barleys selected for differential reaction to *Fusarium* head blight (FHB) and deoxynivalenol (DON) production. While these traits have been extensively studied in six-row barley, two-row barley has been evaluated to a much lesser degree. Barley is sold by class, where two-row barley is the dominant form of barley grown in the major barley-producing areas of western Canada. Two-row barley generally displays a higher level of FHB resistance and accumulates less DON in contrast to six-row barley. Phenotypic variance in FHB resistance and DON accumulation is narrow in two-row barley, thus response to selection may be limited by lack of genetic variance.

The objectives of this chapter were to describe the existing genetic variance structure of two-row barley adapted for production in western Canada and identify genomic regions associated with FHB resistance and lowered DON accumulation. The research was conducted on an

assembly of elite barley lines and registered cultivars collected from the western cooperative registration trials or advanced breeding material bred for FHB resistance acquired directly from the breeding programs. A new technology (Bead-based microarray technology, Illumina Inc.) was applied to evaluate single nucleotide polymorphism (SNP) molecular markers across the whole-genome of barley and applied to the panel. Through genome-wide association mapping (GWAS), genetic resistance of FHB and DON content was explored in two-row barley in the context of materials adapted to the western Canadian agri-environment.

CHAPTER 3

Genetic structure and genome-wide association study of a genomic panel of two-row, spring barley (*Hordeum vulgare* L.) with differential reaction to *Fusarium* head blight (*Fusarium graminearum* Schwabe) and deoxynivalenol production.

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3.1 Abstract

Fusarium head blight (FHB) primarily incited by *Fusarium graminearum* Schwabe, is the most devastating disease of barley (*Hordeum vulgare* L.) in Canada. Contaminated grains are unsuitable for use as livestock feed or malting industries primarily due to contamination of mycotoxins, such as deoxynivalenol (DON). Reducing DON content has been a long-term goal of barley breeders, however, complex genetics of resistance and laborious testing have made it difficult to develop resistant cultivars. An Illumina 50K SNP beadchip was used to genotype a diverse collection (n=400) of two-row barley genotypes, selected primarily from North American breeding programs. This genomic panel was phenotyped in three environments in Manitoba, Canada, over two growing seasons. Genotypes were evaluated for heading date, height, FHB score, and DON content. A genome-wide association study (GWAS) was carried out using a mixed linear model (*MLM*: $Q + K$) accounting for population structure (Q) and kinship (K) as covariates. This study has characterized the population structure of two-row barley germplasm important to North American breeding programs. Significant marker-trait associations were identified on all chromosomes for FHB and all chromosomes for DON content except 1H and 6H. Individual marker effects were small, explaining only up to 5% of the phenotypic variation. Genes within genomic regions that were associated with FHB traits displayed multiple functions linked with disease resistance. While numerous minor, marker-

trait associations were identified, phenotypic evaluations are still the best predictors of these traits even though they are time-consuming and dependent on environmental conditions.

Keywords: barley, fusarium head blight, deoxynivalenol, linkage mapping, GWAS, quantitative disease resistance

3.2 Introduction

Fusarium head blight (FHB) is a devastating disease of barley (*Hordeum vulgare* L.) and other cereals, caused primarily by *Fusarium graminearum* Schwabe (teleomorph: *Gibberella zeae* (Schwein.) Petch), which can significantly reduce yield and quality of grain. Fungal infections are characterized by stained and/or shriveled kernels, which are sometimes covered with pink mycelium. Of particular importance are B-type trichothecene mycotoxins, such as deoxynivalenol (DON), which are commonly associated with this disease (Laitila, 2015). Contaminated barley grains are not acceptable for use as livestock feed or malt production. Deoxynivalenol is thermostable and it and its conjugated form (deoxynivalenol-3-glucoside, D3G) can be transmitted to beer made from infected barley (Lancova et al., 2008). Due to food safety issues, strict maximum limits have been set for DON by the malting industry for commercial use ($< 0.5 \text{ mg kg}^{-1}$) (He et al. 2015). Since the mid-1990s (Clear et al. 1996; Tekauz et al. 2009) FHB has become the most damaging threat to barley production in western Canada.

The development of genetically resistant cultivars remains the most sustainable approach to FHB management. However, resistance sources are limited in barley (Bai and Shaner, 2004) and provide only partial resistance. Fusarium head blight resistance is complex and under polygenic control. Numerous bi-parental QTL (quantitative trait loci) mapping studies have been conducted with numerous FHB resistance sources (de la Peña et al. 1999; Zhu et al. 1999; Ma et al. 2000; Dahleen et al. 2003; Mesfin et al., 2003; Hori et al. 2005 & 2006; Horsley et al. 2006; Yu et al. 2010). Quantitative trait loci for FHB were commonly associated with plant height and heading date and were often environmentally specific. Near-isogenic lines (NILs) and transcriptomics have been used to study functions of QTLs in six-row barley located on chromosome 2H Bins 8 & 10; 3H Bin 6; 6H Bin 7 (Jia et al. 2010; Huang et al. 2016). Linkmeyer et al. (2013) applied marker-assisted selection (MAS) to incorporate a resistant allele for *Qrgz-2H-8*, but concluded that the introgressions did not attain resistance as high as

for the Swiss-landrace ‘Chevron’, which is one of the best sources of partial resistance. Therefore, MAS has had limited application as a breeding method for developing FHB resistance in barley.

Modern technologies based on single nucleotide polymorphism (SNP) markers have emerged to facilitate the application of whole-genome scans in barley (Waugh et al. 2009). The Illumina 9K iSelect SNP chip (7,842 markers) consists of 2,832 original Illumina GoldenGate BOPA SNPs (Close et al. 2009) plus over an additional 5K of Next-Generation sequence-based SNPs (Comadran et al. 2012) were applied in QTL-mapping of a RIL (recombinant inbred lines) population to identify genomic regions involved in FHB resistance. Huang et al. (2018) mapped FHB and DON QTLs in a RIL population involving ‘Rasmusson’, a six-row barley cultivar with ‘Chevron’ (Moderately-resistant - MR) in ancestry. Several QTLs were identified on multiple chromosomes for FHB (2H, 5H, 6H, and 7H) and DON content (2H, 3H, and 7H). Ogrodowicz et al. (2020) also used the 9K SNP chip for QTL mapping in a two-row RIL population and identified regions associated with FHB index on several chromosomes (2H, 3H, 5H, and 7H), but found no association for DON content. However, several of the resistance QTLs in these studies were also negatively associated with important agronomic traits.

Genome-wide association study (GWAS) is a powerful tool that uses whole-genome markers and offers an alternative approach to QTL mapping which is restricted to bi-parental populations. Genome-wide association study has the benefits of higher resolution by relying on more-extensive, historical recombination events (Bush and Moore, 2012), however, its power is dependent on sample size and the number of molecular markers used. Using the Illumina GoldenGate or the iSelect 9K SNP chip, GWAS has identified numerous QTLs for FHB and DON within six-row contemporary breeding germplasm from American barley breeding programs (Massman et al. 2011; Sallam et al. 2015). Mamo and Steffenson (2015) applied GWAS within a mixed panel of 2-row/6-row landraces from Ethiopia and Eritrea, where they identified associations in the 2-row sub-group for FHB (1H, 4H, and 7H) and DON content (2H, 4H, and 5H). Bedawy et al. (2018) used a combination of 9K SNP markers and diversity array (DArT) markers to identify QTLs associated with a *F. graminearum* leaf assay and spike inoculations in a greenhouse (1H and 5H).

The majority of past research contributions in genetics have been focused on six-row resistance development, while two-row barley is by far the dominant type produced in Canada. Over the past two decades, FHB resistance has been enhanced through an accumulation of minor alleles in lines tested in large screening nurseries (Legge et al. 2004). Progress has led to the release of moderately-resistant cultivars with lowered DON content, developed through exotic introgressions e.g. ‘AAC Connect’, ‘AAC Goldman’, (Legge et al. 2017 & 2018), or identification of transgressive-segregants, e.g. ‘CDC Mindon’ (Rosnagel et al. 2008) and ‘Lowe’ (Juskiw et al. 2019). In this study, a large genomic panel of contemporary two-row barley (n=400) along with the more recent, larger barley 50K SNP microarray chip (containing 44,040 molecular marker assays) (Bayer et al. 2017), was used to assess the population structure of the North American assembled germplasm collection and perform GWAS to identify genomic regions associated with quantitative resistance to FHB and low DON accumulation under western Canadian field conditions. This study provides valuable information that could support the barley breeders in their quest to develop resistance to this destructive disease.

3.3 Materials and methods

The plant materials used in this study consisted of 400 two-row, hulled barley genotypes with spring-habit, primarily adapted to production within North America. Germplasm was selected based on the previous reaction to FHB and DON content, as tested primarily in a FHB nursery at Brandon, MB. Genotypes originating from elite by elite breeding crosses and/or exotic introgressions were incorporated. Pedigrees were taken into consideration to increase diversity and exclude half-sibs.

The majority of the panel (> 75%) consisted of cultivars and advanced breeding lines from the three western Canadian barley breeding institutes: Brandon Research and Development Centre, Agriculture and Agri-Food Canada, Brandon, MB (AAFC-BRDC); Crop Development Centre, University of Saskatchewan, Saskatoon, SK (CDC); Field Crop Development Centre, Olds College, Lacombe, AB (FCDC) (Figure 3.1). In addition to these breeding institutions, genotypes were selected from other breeding programs organizations such as Busch Agricultural Resources Inc. (BARI); WestBred/ Highland Specialty Grains (WB/ HSG); North Dakota State University (NDSU), Ottawa Research and Development Centre, Agriculture and

Agri-Food Canada (AAFC-ORDC), Lethbridge Research and Development Centre, Agriculture and Agri-Food Canada (AAFC-LeRDC), Molson Coors Brewing Co (COORS) and Agricore United (AU). Additionally, a small portion (< 5%) of the panel included international cultivars or landrace accessions. Furthermore, the panel consisted of genotypes that could be classified as malting (n = 171) and general-purpose (n = 229, feed/forage) barley. For the majority of the barley genotypes, all of the seed used in the study was received directly from the breeders except for the landraces, which were received from a gene bank (Plant Gene Resources of Canada).

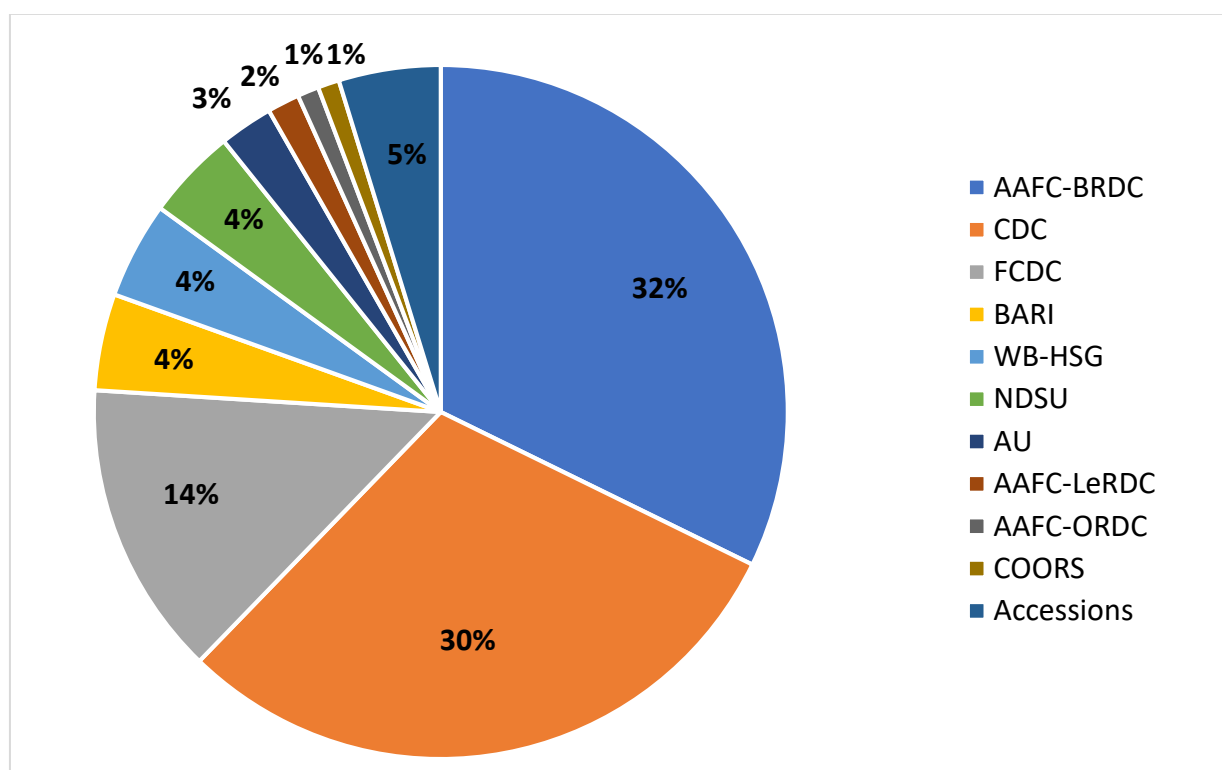


Figure 3.1. Pie chart of genetic panel composition by the breeding institutions (Agriculture and Agri-Food Canada, Brandon Research and Development Centre [AAFC-BRDC]; Crop Development Centre [CDC]; Field Crop Development Centre [FCDC]; D = Busch Agricultural Resources, Inc.[BARI]; WestBred LLC/ Highland Specialty Grains [WB/HSG]; North Dakota State University [NDSU]; Agriculture and Agri-Food Canada, Lethbridge Research and Development Centre [AAFC-LeRDC]; Agriculture and Agri-Food Canada, Ottawa Research and Development Centre [AAFC-ORDC]; Molson Coors Beverage Company [COORS]; Agricore United [AU]; Accessions are landrace or exotic cultivars).

3.3.1 Phenotype: A field experiment

In 2014 and 2015, the experiments were conducted in field FHB nurseries at three locations in Manitoba: Brandon (49°51'56.1"N 99°58'57.9"W), Carman (49°30'05.1"N 98°01'43.5"W) and Carberry (49°52'24.8"N 99°24'30.8"W). Standard agronomic practices were applied within locations, with the following seeding dates: Brandon (May 28, 2014; May 20, 2015), Carberry (May 19, 2014; May 20, 2015), Carman (May 29, 2014; June 1, 2015). All 400 genotypes of the panel were grown together in a randomized complete block design (RCBD), at a seeding rate of 30-40 seeds per meter. All sites were irrigated with various methods: Brandon (PVC risers with NaanDanJain 502-H sprinkler head); Carberry (center-pivot overhead irrigation); Carman (mist irrigation). Inoculation methods differed by site, however all used mixtures of two isolates each of 3ADON and 15ADON chemotypes of *F. graminearum*. Before inoculation, isolates were mixed in equal proportions by volume. Grain spawn (maize kernels colonized by *F. graminearum*) was spread on the soil surface (5 g m⁻²) at Brandon and Carberry, whereas Carman plots were sprayed three times, first at 75% spike emergence with a macroconidial suspension (5 x 10⁴ spores ml⁻¹) then at three-day intervals thereafter. Following inoculation at Carman, mist irrigation was applied at ten-minute intervals for ten hours.

Plots were rated for FHB score 3.5 weeks following 50% heading, during the soft dough growth stage (Zadoks - Z85, Zadoks et al. 1974). A visual 0-5 scale (A. Tekauz, personal communication) was used to evaluate a composite measure of incidence and severity: 0 = no infection; 1 = incidence low, up to 5% of spikes; severity low, 1 or 2 kernels per spike affected (up to 7% of spike); 2 = incidence low to moderate, 5 to 15% of spikes infected; severity low to moderate, 1 to 4 kernels (up to 15% of spike); 3 = incidence moderate, 15 to 30% of spikes; severity moderate, 2 to 8 kernels (up to 25% of spike); 4 = incidence moderate to high, 30 to 50% of spikes infected; severity moderate to high, 4 to 12 kernels (up to 40% of spike) and 5 = incidence high, 50% or more spikes affected; severity high, 5 to 15+ kernels (up to 50%+ of head diseased). Heading (50% spike emergence) (HD50) was recorded only at Brandon and Carberry and used in the calculation of days to heading by subtracting the seeding date. Height (HT) was measured as the distance from the soil surface to the top of the spike excluding awns and was recorded as an average of three measurements at all three locations.

Grains were harvested at maturity with stationary research combined with a low wind speed setting to retain thin kernels. Grains were cleaned using an SLN3 sample cleaner (Pfeuffer GmbH, Kitzingen, Germany). A 20 g subsample that was free of debris and/or chaff was then ground using a Perten 3610 lab mill with a fine particle disc set (Perten Elmer Inc. Waltham, MA, USA). Deoxynivalenol content was analyzed on a 2-gram sub-sample by enzyme-linked immunosorbent assay (ELISA) technique using Veratox ® 5/5 (Neogen Corporation, Lansing, MI, USA) as per the manufacturer's protocol. Sample extracts were analyzed in pairs, where analysis was repeated for deviations above 10%.

The least-square (LS) means of all traits were calculated for genotypes using SAS JMP Genomics 9.1 software (SAS Institute Inc., 1989-2019) in a model overall years with variables as fixed effects. Least-square means were used for descriptive statistics and inputs for modelling genome-wide association study (GWAS). Pearson correlation coefficients were calculated in R statistic environment (R core team 2021) using the 'cor' function and heat map graphics were produced with the 'ggcorrplot' package.

3.3.2 DNA Extraction

Two barley seeds were surface-sterilized in 6% vol/vol sodium hypochlorite and rinsed in distilled water. Seeds were germinated on moist cotton balls and grown under natural light until the two-leaf stage. Leaves were randomly cut from a single plant and flash frozen in liquid-N, then freeze-dried in a lyophilizer (Labconco Corporation, Kansas City, MO, USA). Total genomic DNA was extracted from 100 mg of lyophilized tissue per genotype using Qiagen, DNeasy 96 Plant Kit (Qiagen, Canada). The quality and concentration of DNA were evaluated by agarose gels and a Nanodrop™ 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). Samples were normalized to 50 ng μl^{-1} (20 μl) of DNA using micro-pure DNase free water.

3.3.3 High Throughput Genotyping

DNA samples were assayed on an Illumina iScan (Illumina, San Diego, CA, USA) using a custom iSelect - 50K SNP microarray chip consisting of 44,040 molecular markers (Bayer et al. 2017) at AAFC, Morden Research and Development Centre, Morden, MB. A custom cluster file (Trait Genetics, GmbH, Germany) was used to call SNP alleles using Illumina

Genome Studio v2.0.5 software. Allelic data in Plink format were imported into TASSEL v5.2.58 then filtered for minor allele frequency (MAF) > 5% and for missing data <10% (total of 25,068 SNPs). Missing values were numerically imputed by the mean value method using five nearest neighbours. Data were imported into SAS JMP Genomics 9.1 (SAS Institute Inc., 1989-2019) and converted into numerical values (0 = major allele; 1 = heterozygous; 2 = minor allele).

3.3.4 Linkage disequilibrium and population structure analysis

Resolution of trait mapping is affected by linkage disequilibrium (LD). LD decay was analyzed separately for each chromosome. Squared allele frequency correlations (r^2) were calculated pairwise for all filtered molecular markers using SAS JMP Genomics version 9.1 (SAS Institute Inc. 1989-2019). Intra-chromosomal graphs were produced by plotting r^2 values of marker pairs against physical genomic map position (base pair) over each chromosome with a smoothing line function ($\alpha=0.005$).

STRUCTURE v2.3.4 software (Pritchard et al. 2000) was used to perform a Bayesian clustering analysis of population structure. A sub-set of > 2,000 polymorphic SNP markers of equidistant spacing overall seven chromosomes was used with a burn-in of 100,000 iterations and 100,000 Markov Chain Monte Carlo (MCMC) iterations. Population sub-populations from K1 (no structure) to K5 were tested using an admixture model. Results were analyzed using Structure Harvester v0.6.94 (Earl and vonHoldt, 2012) where most likely sub-populations were identified via the Evanno method (Evanno et al., 2005). A ΔK index was calculated and plotted against the K value to identify the peak value and determine the most likely number of sub-populations. A Q matrix (sub-population coefficients) was extracted from the population structure analysis based on two sub-populations. Visual analysis of the Q matrix output by STRUCTURE was conducted using Structure Plot v2.0 (Ramasamy et al. 2014). Alternatively, population structure was evaluated by TASSEL v5.2.58 through principal component analysis (PCA) along with the production of a neighbour-joining tree visualized by Archaeopteryx v0.9909 to evaluate patterns of relatedness. A matrix of pairwise kinship values (K matrix) was also calculated within SAS JMP Genomics version 9.1 (SAS Institute Inc. 1989-2019) via an identity-by-descent method. The K-matrix was used in subsequent GWAS analysis as a random factor.

Association mapping was conducted using all 400 genotypes for traits via various methods (with Q-matrix based on PCA or STRUCTURE results; general linear model (*GLM* + *Q*) and mixed linear model (*MLM* + *Q* + *K*) were evaluated by quantile-quantile (Q-Q) plots for best fit (Appendix 1.1). For simplicity, results herein are attributed to the *Q* (STRUCTURE) + *K* mixed model. Association analysis was conducted on overall means and within a location. SNP markers $-\text{Log}_{10} [\text{P-value}] \geq 3$ were considered statistically significant, a commonly accepted limit for the GWAS study (Chan et al. 2010). Manhattan plots were generated in SAS JMP Genomics 9.1 (SAS Institute Inc. 1989-2019) to visually identify peaks of trait associations by physical chromosome position.

3.3.5 Candidate gene prediction

Genes were determined by the physical position of the SNP allele on the IBSC_v2 (International Barley Sequencing Consortium, Morex v1) release of the barley reference genome (Mascher et al. 2017), or if unavailable, using the median value of start and end of markers. Associated genes of interest were identified through evaluation of SNP effect models (Bayer et al. 2017) and/or inspection of genes in nearest proximity to genomic position (± 50 Kbp) using Ensembl Plants genomic web browser within (URL plants.ensembl.org accessed Oct. 27, 2021). High confidence gene annotations were determined through BARLEX genome explorer (URL www.ipk-gatersleben.de/en/bioinformatics-tools accessed Oct 27, 2021). Gene functions were further evaluated by associated gene ontology (GO) terms (Ashburner et al. 2000, <http://geneontology.org/>); Pfam domains (Mistry et al. 2020, <http://pfam.xfam.org/> accessed Oct. 27, 2021); InterPro terms (Blum et al. 2000, URL www.ebi.ac.uk/interpro/ accessed Oct. 27, 2021).

3.4 Results

3.4.1 Population Structure Analysis

STRUCTURE software was applied to evaluate sub-structures within the panel of 400 genotypes. The optimal number of subpopulations (*K*) determined by STRUCTURE analysis, identified *K*=2 sub-populations (Appendix 1.2). Individuals from Cluster 1 (> 90% subpopulation membership) included 104 lines primarily contributed by the AAFC-BRDC breeding program, or other programs where an AAFC-BRDC parent was involved in a cross.

Representative individuals of Cluster 1 included cultivars and elite breeding materials such as: ‘AAC Connect’, ‘AAC Goldman’, ‘AAC Synergy’, ‘AC Oxbow’, ‘CDC Fraser’, ‘Cerveza’, ‘CDC Kindersley’, ‘Ellice’, ‘Major’ ‘Newdale’. Cluster 2, comprised of all remaining entries, included cultivars and lines from various programs and accessions (Figure 3.2). Genotypes in extreme opposition to Cluster 1, included feed cultivars such as ‘Brahma’, ‘McLeod’, ‘Ursa’, and ‘Xena’. This structure was also reflected in the phylogenetic tree constructed by the neighbor-joining method (Figure 3.3).

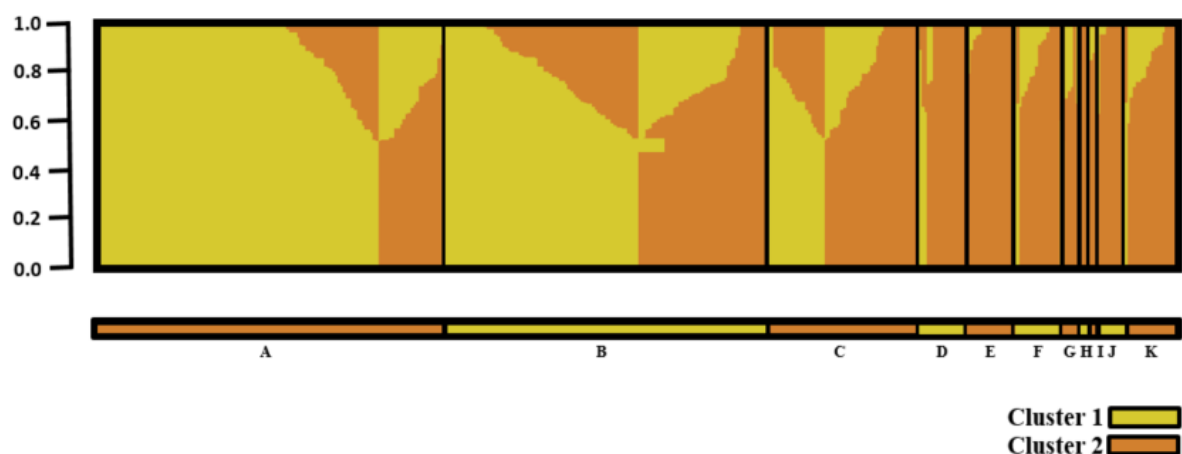


Figure 3.2. STRUCTURE Sub-population membership for 400 two-row barley grouped by breeding institution (A = Agriculture and Agri-Food Canada, Brandon Research and Development Centre [AAFC-BRDC]; B = Crop Development Centre [CDC]; C = Field Crop Development Centre [FCDC]; D = Busch Agricultural Resources, Inc. [BARI]; E = WestBred LLC/ Highland Specialty Grains [WB/HSG]; F = North Dakota State University [NDSU]; G = Agriculture and Agri-Food Canada, Lethbridge Research and Development Centre [AAFC-LeRDC]; H = Agriculture and Agri-Food Canada, Ottawa Research and Development Centre [AAFC-ORDC]; I = Molson Coors Beverage Company [Coors]; J = Agricores United [AU]; K = Accession. Yellow = Cluster 1 and Orange = Cluster 2). The Y axis represents the proportion of cluster (K=2) membership.

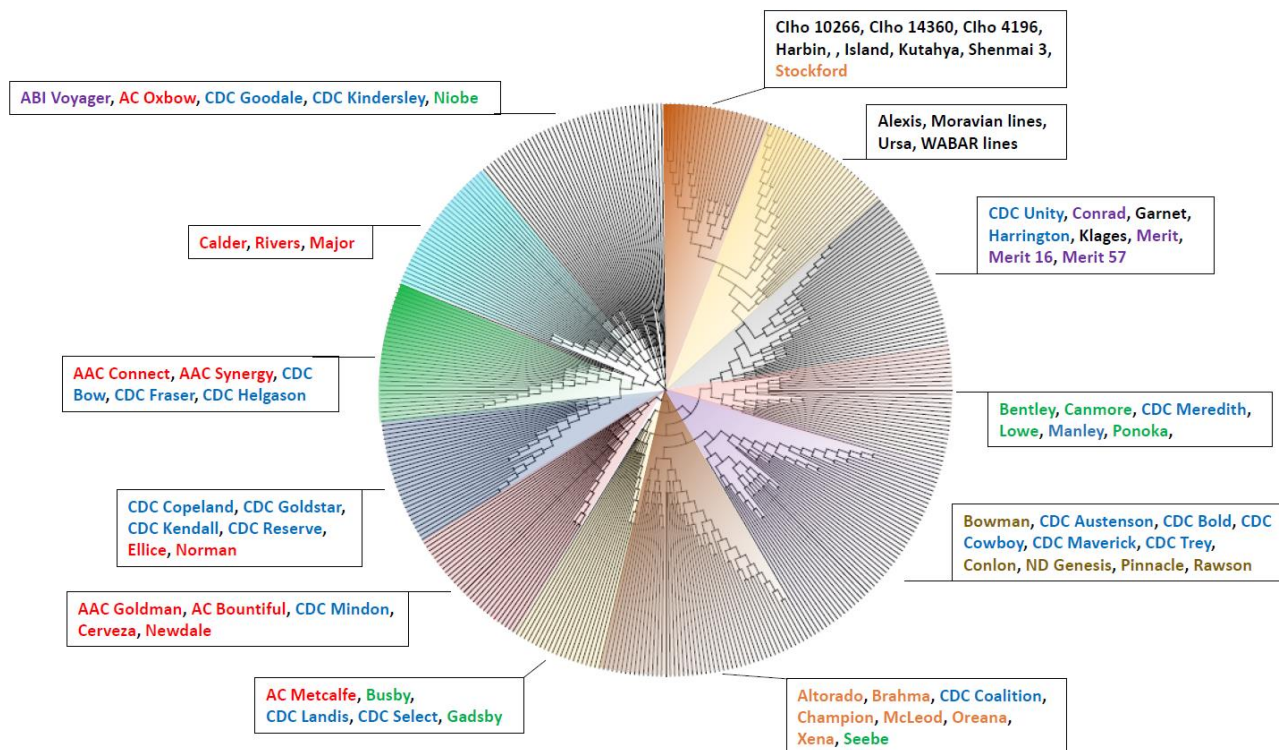


Figure 3.3. Phylogenetic tree constructed by neighbor-joining (NJ) method based on single-nucleotide polymorphism (SNP) genetic markers (25K) displaying selected cultivars and germplasm from the various institutions (Agriculture and Agri-Food Canada, Brandon Research and Development Centre [red]; Crop Development Centre [blue]; Field Crop Development Centre [green]; D = Busch Agricultural Resources, Inc. [purple]; WestBred LLC/ Highland Specialty Grains [orange]; North Dakota State University [brown]).

Principal component analysis (PCA) was also conducted to analyze the panel structure. Analysis of principal components demonstrated clustering within barley type (malt or feed) and separation on the primary principal component axis. Results of the PCA are presented by type (Figure 3.4A) and by breeding program origin (Figure 3.4B). Patterns of genetic relatedness from various analyses were confirmed by pedigree information (breeders' personal communication).

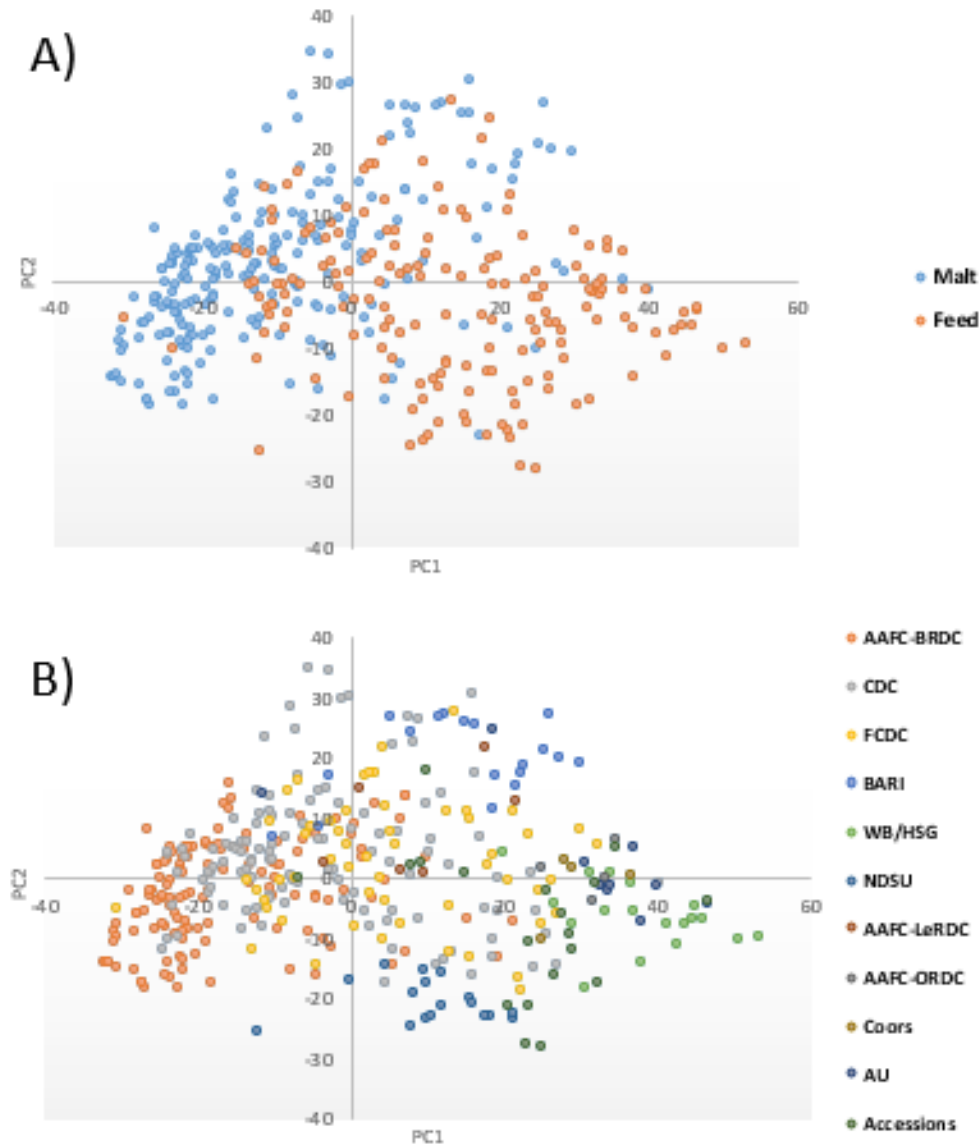


Figure 3.4. Plots of the first two principal components of genotype information (single-nucleotide polymorphism genetic markers) of the genomic panel with colouration highlighting A) malting or feed barley types; B) breeding institution origin. (Agriculture and Agri-Food Canada, Brandon Research and Development Centre [AAFC-BRDC]; Crop Development Centre [CDC]; Field Crop Development Centre [FCDC]; D = Busch Agricultural Resources, Inc. [BARI]; WestBred LLC/ Highland Specialty Grains [WB/HSG]; North Dakota State University [NDSU]; Agriculture and Agri-Food Canada, Lethbridge Research and Development Centre [AAFC-LeRDC]; Agriculture and Agri-Food Canada, Ottawa Research

and Development Centre [AAFC-ORDC]; Molson Coors Beverage Company [COORS]; Agricore United [AU]; Accessions are landrace or exotic cultivars).

3.4.2 Linkage Disequilibrium and polymorphic SNP coverage

The genomic panel was primarily composed of adapted breeding materials, where it is expected to show some level of LD decay as a result of selective breeding. The degree of population structuring is also related to the level of LD decay. The plot of squared allele frequency correlations (r^2) vs. genomic position (Mbp) demonstrated a decay of LD values with the genetic distance in the genomic panel (Appendix 1.3). The mean distance at which r^2 fell below 0.2 was approximately 5 Mbp. Of the 44K SNP markers evaluated, 57% (> 25K) were found to be polymorphic and useful for GWAS. The SNP markers displayed adequate genomic coverage, however, some gaps were observed over certain chromosomes (1H, 2H, and 4H), particularly in centromere regions (Appendix 1.4).

3.4.3 Phenotype

Overall means for all traits measured are presented by year for each location in Table 3.1. Fusarium head blight occurred in all locations in all years. Deoxynivalenol was detected in all of the harvested grains and displayed a continuous distribution (Appendix 1.5). Disease symptoms were similar for Brandon and Carman, yet considerably lower at Carberry. Deoxynivalenol content varied by location and year. While the lowest FHB infection level was observed at Carberry and particularly in 2014, this location produced the highest DON content for that year (Table 3.1). Carman displayed the highest consistency in overall DON content.

Table 3.1. Means and standard errors of height (HT, cm), fusarium head blight (FHB, 0-5), days to fifty percent heading (HD50, days), and deoxynivalenol (DON, mg kg⁻¹) content over years and locations.

Location	Year	HD50 ¹	Min-Max	HT ²	Min-Max	FHB ³	Min-Max	DON ⁴	Min-Max
Brandon	2014	57.4±0.1	49.0-62.5	83.5±0.4	55.5-121.5	2.0±0.1	0.0-5.0	7.1±0.2	0.7-30.3
	2015	53.6±0.1	39.0-64.0	82.2±0.4	53.0-113.0	2.4±0.1	0.0-5.0	19.7±0.5	4.6-90.0
Carberry	2014	71.6±0.1	57.0-76.0	75.9±0.4	53.0-110.0	0.4±0.0	0.0-5.0	27.2±0.5	2.7-69.8
	2015	61.4±0.2	43.0-67.5	67.6±0.3	45.0-89.5	0.7±0.0	0.0-4.5	6.0±0.1	0.8-19.4
Carman	2014	-	-	89.3±0.4	60.0-126.5	2.6±0.1	0.0-5.0	12.2±0.3	1.7-57.0
	2015	-	-	81.4±0.4	55.5-103.0	2.4±0.1	0.5-5.0	11.7±0.4	2.3-32.6

¹HD50 = days to 50% of plot headed; ²HT = height from ground to top of spike excluding awns; ³FHB = fusarium head blight (0-5); DON = deoxynivalenol content (mg kg⁻¹). Min=minimum, Max=maximum. Heading notes were not recorded at Carman.

Relationships between traits over locations were analysed by comparison of Pearson correlation coefficients (Appendix 1.6). Fusarium head blight displayed moderate positive association with DON content ($r^2 = 0.15$, P-value < 0.05). Height displayed a negative relationship with FHB ($r^2 = 0.36$, P-value < 0.0002), but showed no association with DON ($r^2 = 0.04$, P-value = 0.35).

3.4.4 FHB reaction and DON content

Overall, there was a wide range of mean FHB reactions among genotypes, where some genotypes displayed minimal infection and others had near-maximum scores (FHB, 0.3 - 4.8). Likewise, the DON content among genotypes ranged from 4.9 to 36.9 mg kg⁻¹. Genotypes previously identified as resistant at a single location (Brandon), were confirmed in this study by testing conducted in additional environments and using alternative evaluation methods (conidial spray). A list of genotypes with consistently low FHB and/or DON content across locations is presented in Table 3.2. The group of landrace exotics generally held both the lowest FHB score and DON content. In this group, germplasm such as CIho 4196 (Horsley et al. 2006) and Harbin (Hori et al. 2006) have been used extensively as resistance sources for breeding. However, several breeding lines with adaptive characters for production in North

America demonstrated resistance to DON accumulation near those exotic accessions including genotypes such as NDSU cultivars ‘Bowman’ and ‘Conlon’ and Canadian breeding lines incorporating resistance from: ‘Svansota’, ‘MNBrite’, ‘Shyri’ and ‘Gobernadora’. In some cases, high FHB scores did not result in high DON content as exemplified by HDE84194-622-1, which produced a high FHB score, but low DON content values at all locations.

Table 3.2. Sample of germplasm with low Fusarium head blight (FHB) score or deoxynivalenol (DON) content. Numbers represent grand mean over site years, with standard errors in brackets.

Name	Type	Mean FHB (0-5)	Mean DON (mg kg ⁻¹)
CN 5317 (396)	exotic	0.5 (0.2)	5.7 (0.9)
CIho 14360	exotic	0.3 (0.1)	6.0 (1.2)
Kutahya	exotic cultivar	0.3 (0.1)	6.6 (1.4)
CIho 4196	exotic	0.4 (0.1)	8.0 (1.7)
CI 2208	exotic	0.4 (0.1)	9.3 (2.1)
Harbin	exotic cultivar	0.3 (0.1)	9.7 (1.2)
HDE84194-622-1	exotic	1.1 (0.3)	6.5 (1.3)
Bowman	NDSU ¹ cultivar	4.7 (0.2)	5.2 (1.3)
Conlon	NDSU cultivar	1.4 (0.3)	6.9 (1.4)
2ND27421	NDSU line	3.0 (0.5)	4.9 (0.9)
2ND24263	NDSU line	1.9 (0.5)	7.1 (1.5)
TR06676	FCDC ² line	2.1 (0.5)	7.0 (1.5)
TR10692	FCDC line	1.0 (0.3)	5.8 (1.4)
SB071676	CDC ³ line	1.7 (0.3)	6.7 (1.5)
BM0332D-280	AAFC ⁴ line	0.5 (0.2)	9.5 (1.9)
BM0124-347-1	AAFC line	0.5 (0.2)	10.3 (2.1)
BM9637-53	AAFC line	0.4 (0.1)	11.1 (2.5)
CDC Bold [†]	CDC cultivar	3.9 (0.4)	26.3 (5.6)
BM9925-192 [†]	AAFC line	2.6 (0.4)	36.9 (5.5)

¹NDSU = North Dakota State University; ²FCDC = Field Crop Development Centre;

³CDC = Crop Development Centre; ⁴AAFC = Agriculture and Agri-Food Canada; [†]susceptible lines included for contrast.

3.4.5 Association mapping

Genome-wide association study (GWAS) identified a total of 128 SNP markers (Figure 3.5) associated with HD50, where approximately half (52%) of these markers were located on chromosome 2H (Appendix 1.7), with several peak regions (65.1, 184.8, 407.3, 570.1, 613.6, 626.3, 730.0 Mbp). Chromosome 3H held the second-highest number of significant markers with peaks at 40.6 and 670.2 Mbp. A total of 14 marker associations were observed on chromosome 7H with a peak at 55.0 Mbp. Significant marker associations were observed on all other chromosomes including peaks on chromosomes 4H: 590.4 Mbp and 5H: 661.2 Mbp.

A total of 60 SNP markers associated with HT were identified, and located across all chromosomes (Figure 3.5). As seen for HD50, the largest number of markers (40%) were located on chromosome 2H (Appendix 1.7), with association peaks (28.6, 46.1, 432.9, 483.3 Mbp). Chromosome 5H held the second-highest number of significant markers with peaks at 12.4, 564.9, and 665.6 Mbp.

Fusarium head blight (FHB) was associated with a total of 75 SNP markers (Figure 3.5), located across all chromosomes (Table 3.3). Chromosome 5H held the highest number of markers (23%), where peaks were observed at 28.6 Mbp (SCRI_RS_7368) and 564.9 Mbp (JHI-Hv50k-2016-321425), and the latter coincided with height-associated regions. A region near the end of chromosome 4HS was associated with FHB, with a peak at 2.3 Mbp (JHI-Hv50k-2016-227104). Likewise, three peak regions were identified on chromosome 7H including peaks at 28.1 Mbp (SCRI_RS_219709), 502.0 Mbp (JHI-Hv50k-2016-488582), and 611.1 Mbp (JHI-Hv50k-2016-498181). A region for FHB on chromosome 2H at peak 613.6 Mbp (JHI-Hv50k-2016-101173), coincided with a region associated with days to heading. Other regions associated with FHB were identified on chromosomes 1H: 11.4 Mbp (JHI-Hv50k-2016-10994), 3H: 614.2 Mbp (JHI-Hv50k-2016-201074), and 6H: 52.1 Mbp (JHI-Hv50k-2016-386764).

Deoxynivalenol content (DON) was associated with a total of 42 SNP markers (Figure 3.5; Table 3.4), which mostly occurred on chromosome 5H (40%). Associations were observed over multiple regions of chromosome 5H with peaks occurring at 5.6 Mbp (SCRI_RS_114102), 38.6 Mbp (JHI-Hv50k-2016-288841), 54.4 Mbp (JHI-Hv50k-2016-290535), 67.5 Mbp (JHI-Hv50k-2016-291313), and 602.7 Mbp (JHI-Hv50k-2016-337389).

Likewise, peaks were observed on chromosome 4H at 7.6 Mbp (JHI-Hv50k-2016-228552) and 603.4 Mbp (JHI-Hv50k-2016-261905). Peaks were also observed on chromosome 3H at 444.7 Mbp (JHI-Hv50k-2016-180623) and 512.9 Mbp (JHI-Hv50k-2016-186010). Multiple regions were also identified on chromosomes 2H (12.2 and 19.9-21.2 Mbp) and 7H (2.4 and 642.2 Mbp), while no significant markers were identified in association with DON on chromosomes 1H nor 6H.

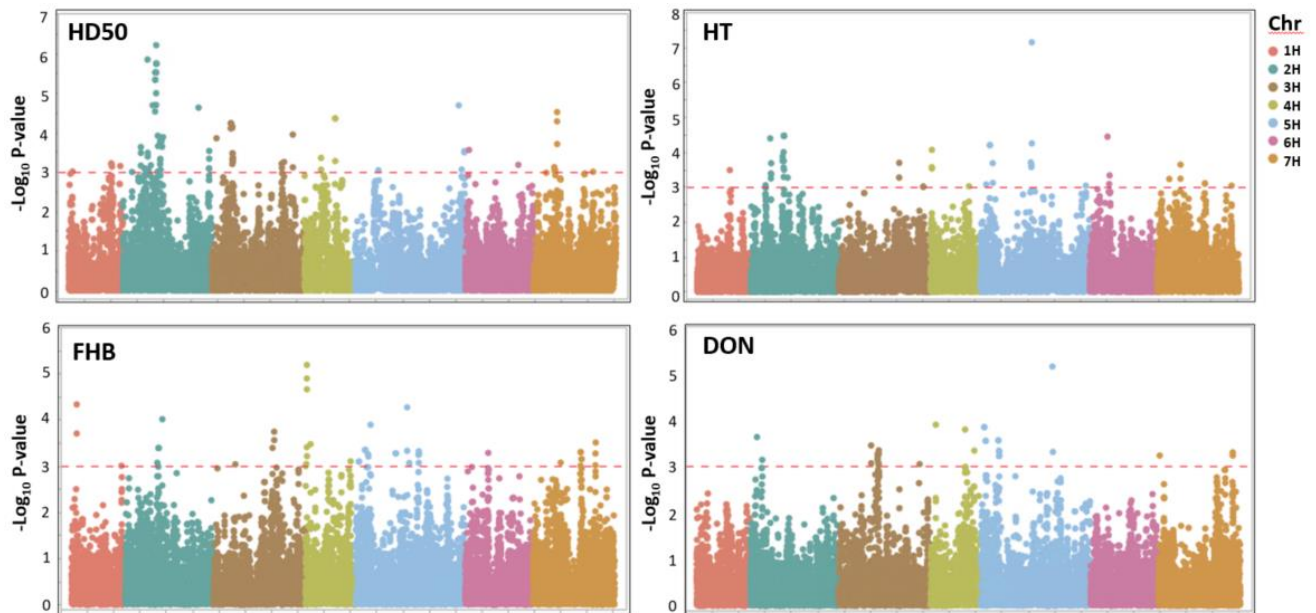


Figure 3.5. Manhattan plots of all site years of 400 genotypes for days to fifty-percent heading (HD50), height (HT), FHB (Fusarium head blight score), and deoxynivalenol content (DON) over the chromosomal position. The horizontal red dashed line represents a genome-wide significance threshold ($-\text{Log}_{10} \text{P-value} \geq 3.0$). SNP markers are displayed cumulatively by chromosome (Chr) which are represented by colour.

3.4.6 Candidate genes

3.4.6.1 I) Days to heading (HD50) and height (HT)

Marker-trait associations were often coincidental for HD50 and HT. Plant height (HT) was associated on chromosome 2H (28.6 Mbp) and borderline ($-\text{Log}_{10} \text{P-value} = 2.9$) for HD50. Photoperiod response regulator gene (HORVU2Hr1G013400, *Ppd-H1*), which determines the

sensitivity to long days is located at this position (Turner et al. 2005). Similar association patterns over locations corroborate the influence of chromosome 2H for these traits.

Plant height (HT) was associated with chromosome 1H at 515.7 Mbp in proximity to HORVU1Hr1G076420 (514.1 Mbp), RNA ligase/cyclic nucleotide phosphodiesterase family protein, *HvFT3*, *Ppd-H2*, and HORVU1Hr1G076730 (515.5 Mbp) gibberellin 2-oxidase (*HvGA2ox4*). Days to heading (HD50) showed a marker-trait-association on chromosome 2H at 614.2 and 730.0 Mbp which are respectively associated with HORVU2Hr1G084540 (*HvFTL5*, Flowering locus T) and HORVU2Hr1G113880 (*HvAP2*, ethylene-responsive transcription factor). Both HD50 and HT showed a marker-trait-association in 2H: 403-407 Mbp region, which are respectively associated with HORVU2Hr1G060390 (early-responsive to dehydration stress protein, *HvERD4*) and HORVU2Hr1G060890 (light dependant short hypocotyls 6 protein). Days to heading (HD50) was associated with positions on chromosome 3H: 34.8 and 40.9 Mbp. Cytochrome P450 superfamily proteins (HORVU3Hr1G014930, HORVU3Hr1G014990) are located at 34.8 Mbp, whereas *Six-rowed spike4* [*Vrs4*, syn. *Intermedium-E*], HORVU3Hr1G016690 (LOB domain-containing protein 25) is located at 40.9 Mbp. *Vrs4* is a major morphometric gene that controls the two-row spike morphology through *Vrs1* (HORVU2Hr1G092290) and its suppression of lateral spikelet fertility (Koppolu et al. 2013).

Plant height (HT) also showed association with several nearby regions on chromosome 5H, including 563.6 Mbp HORVU5Hr1G08121 (Cytochrome P450 superfamily protein) and 564.9 Mbp HORVU5Hr1G081520 (Hexosyltransferase). Height was also associated with the region of 565.2 Mbp is HORVU5Hr1G081620 (Two-component response regulator-like *PRR95*). Days to heading (HD50) and HT showed marker-trait-association on chromosome 5H at 661.2 Mbp in the position of HORVU5Hr1G12207 (glycosyl group transferase) and close to (665.3 Mbp) gibberellin 20 oxidase 2 (*HvGA20ox1*). Both traits also demonstrated association on chromosome 5H at 666.1 Mbp in the proximity of HORVU5Hr1G124360 (Jasmonate O-methyltransferase) and HORVU5Hr1G124390 (2-oxoglutarate [2OG] and Fe[II]-dependent oxygenase superfamily protein). Days to heading (HD50) and HT were associated on chromosome 7H at 550.0 Mbp, in proximity to HORVU7Hr1G029420 (sugar-phosphate exchanger 2).

3.4.6.2 II) Fusarium head blight

Genome-wide association study (GWAS) of FHB score demonstrated association on chromosome 1H at 14.6 and 551.8 Mbp in proximity to regions containing HORVU1Hr1G005290 (ENTH/ANTH/VHS superfamily protein) and HORVU1Hr1G092950 (Transducin family protein / WD-40 repeat family protein), respectively. Multiple locations on chromosome 2H were associated with multiple protein kinase proteins: HORVU2Hr1G038950 (185.3 Mbp); HORVU2Hr1G063910 (432.9 Mbp) and HORVU2Hr1G084730 (613.6 Mbp).

Another association of FHB score was observed on chromosome 2H at 483.3 Mbp in proximity to HORVU2Hr1G068660 (*MLO*-like protein 1). On chromosome 3H, FHB at 614.2 Mbp was associated with HORVU3Hr1G085770 (Pentatricopeptide repeat-containing protein).

On chromosome 4H, FHB was associated at 1.1 Mbp close to HORVU4Hr1G000610 (cellulose synthase-like A3), HORVU4Hr1G000620 (ATP binding cassette subfamily B1), and HORVU4Hr1G000030 (Leucine-rich receptor-like protein kinase). Fusarium head blight association was also seen with the adjacent region 2.3 Mbp in the position of genes HORVU4Hr1G001420 (GDSL esterase/lipase) and HORVU4Hr1G001450 (Fatty acyl-CoA reductase 3).

On chromosome 5H, FHB at 28.6 Mbp was in proximity of HORVU5Hr1G011070 (Chaperone protein DnaJ). Also associated with FHB were regions: 534.0 Mbp, HORVU5Hr1G071940 (UDP-Glycosyltransferase superfamily protein), and HORVU5Hr1G072070 (caffeoyl-CoA 3-O-methyltransferase); 564.0 Mbp, HORVU5Hr1G081520 (Hexosyltransferase) and 573.9 Mbp HORVU5Hr1G085070 (Protein kinase superfamily protein). Other detected FHB associations with genes were chromosome 6H 52.1 Mbp, HORVU6Hr1G019320 (pentatricopeptide repeat 336), and chromosome 7H, 501.8 Mbp HORVU7Hr1G083220 (Ribosomal RNA small subunit methyltransferase), 611.1 Mbp, HORVU7Hr1G102030 (Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein).

Table 3.3. Significant molecular markers ($-\text{Log}_{10} [\text{P value}] \geq 3$) associated with Fusarium head blight (FHB) averaged over all site years (n=6).

SNP marker ID ^A	Chr ^B	Allele ^C	Position ^D	Estimate	F value	$-\text{Log}_{10}$ P value ^E	R ² (%)
JHI-Hv50k-2016-10994	1H	A/G	11457366	0.3	16.9	4.3	4.1
JHI-Hv50k-2016-55687	1H	A/G	551839452	-0.1	11.0	3.0	2.7
JHI-Hv50k-2016-90430	2H	A/C	185336025	0.3	11.3	3.1	2.8
JHI-Hv50k-2016-92837	2H	A/G	403313659	0.3	10.9	3.0	2.7
JHI-Hv50k-2016-93148	2H	A/G	432905090	0.3	12.7	3.4	3.1
JHI-Hv50k-2016-94189	2H	A/G	472751660	0.3	12.7	3.4	3.1
JHI-Hv50k-2016-94503	2H	A/C	483278937	0.3	12.7	3.4	3.1
JHI-Hv50k-2016-101173	2H	G/A	613633521	0.3	15.5	4.0	3.8
JHI-Hv50k-2016-163997	3H	G/A	43840448	-0.3	11.1	3.0	2.7
JHI-Hv50k-2016-200089	3H	A/G	607677401	-0.2	12.7	3.4	3.1
JHI-Hv50k-2016-201074	3H	G/A	614159072	0.2	13.5	3.6	3.3
JHI-Hv50k-2016-201914	3H	A/G	619780034	0.2	10.8	3.0	2.7
JHI-Hv50k-2016-225695	4H	A/G	54127	0.3	11.0	3.0	2.7
JHI-Hv50k-2016-226348	4H	G/A	1092143	0.3	11.2	3.0	2.7
JHI-Hv50k-2016-226367	4H	A/G	1101951	0.3	11.2	3.0	2.7
JHI-Hv50k-2016-227048	4H	A/G	2027527	0.2	12.8	3.4	3.1
JHI-Hv50k-2016-227095	4H	G/C	2259274	0.3	19.5	4.9	4.7
JHI-Hv50k-2016-227104	4H	G/A	2259971	0.3	20.9	5.2	5.0
JHI-Hv50k-2016-227292	4H	C/G	2790475	-0.3	11.9	3.2	2.9
JHI-Hv50k-2016-228556	4H	G/A	7627786	0.3	13.1	3.5	3.2
JHI-Hv50k-2016-271609	4H	C/G	636432470	-0.2	11.4	3.1	2.8
JHI-Hv50k-2016-278585	5H	T/A	4733455	0.3	11.4	3.1	2.8
JHI-Hv50k-2016-278627	5H	C/G	4930992	0.3	11.4	3.1	2.8
JHI-Hv50k-2016-282091	5H	G/C	12380255	0.3	12.5	3.3	3.1
JHI-Hv50k-2016-282179	5H	G/A	12496840	0.3	12.5	3.3	3.1
JHI-Hv50k-2016-283470	5H	C/G	17076804	0.2	10.9	3.0	2.7
JHI-Hv50k-2016-284034	5H	C/A	19372939	0.2	12.1	3.3	3.0
JHI-Hv50k-2016-284439	5H	A/T	20346518	0.3	11.9	3.2	2.9
JHI-Hv50k-2016-286464	5H	G/A	28306179	0.2	15.0	3.9	3.6
SCRI_RS_7368	5H	A/G	28556331	0.3	21.0	5.2	5.0
SCRI_RS_196566	5H	G/A	533836295	0.3	12.2	3.3	3.0
JHI-Hv50k-2016-312973	5H	G/A	534003424	0.3	12.2	3.3	3.0
BOPA1_6833-658	5H	C/A	534311158	0.3	11.0	3.0	2.7
JHI-Hv50k-2016-313362	5H	C/A	534766150	0.3	12.2	3.3	3.0
JHI-Hv50k-2016-321425	5H	A/G	564904419	0.4	16.7	4.3	4.0
JHI-Hv50k-2016-321503	5H	A/G	565158282	0.3	12.4	3.3	3.0
JHI-Hv50k-2016-324079	5H	G/C	573947415	0.3	11.3	3.1	2.8
JHI-Hv50k-2016-332519	5H	A/G	591222237	0.2	12.4	3.3	3.0
JHI-Hv50k-2016-372853	6H	G/A	9325976	-0.2	10.9	3.0	2.7
JHI-Hv50k-2016-386716	6H	A/G	51116796	0.3	10.8	3.0	2.6
JHI-Hv50k-2016-386764	6H	G/A	52052535	0.3	12.2	3.3	3.0
SCRI_RS_219709	7H	G/A	28112055	0.1	11.7	3.2	2.9
JHI-Hv50k-2016-468635	7H	G/A	72973960	0.3	11.3	3.1	2.8
BOPA1_3232-201	7H	G/C	506517938	0.2	10.8	3.0	2.6
JHI-Hv50k-2016-488566	7H	G/C	501167934	0.2	11.0	3.0	2.7
JHI-Hv50k-2016-488570	7H	A/G	501750617	0.2	12.3	3.3	3.0
JHI-Hv50k-2016-488582	7H	T/A	501985533	0.2	12.3	3.3	3.0
JHI-Hv50k-2016-488814	7H	G/A	507360707	0.2	11.6	3.1	2.8

JHI-Hv50k-2016-488919	7H	G/A	509444751	0.2	11.6	3.1	2.8
JHI-Hv50k-2016-498095	7H	G/A	610791597	0.3	11.1	3.0	2.7
JHI-Hv50k-2016-498181	7H	A/G	611106760	0.3	12.2	3.3	3.0

^A Molecular marker of 50K SNP array (Bayer et al. 2017); ^B Chr = Chromosome; ^C Allele (Major/Minor); ^D Genomic position in base pairs (bp), reference genome IBSC v2 'Morex v1' gene models (Mascher et al., 2017). ^ENegative Log₁₀ Cochran-Armitage trend test probability.

3.4.6.3 III) Deoxynivalenol (DON) content

Multiple DON associations were observed on chromosome 2H 12.2 Mbp, HORVU2Hr1G005650 (Cullin-associated NEDD8-dissociated protein 1), and 21.2 Mbp, HORVU2Hr1G010490 (Leucine-rich receptor-like protein kinase family protein) and HORVU2Hr1G010800 (expansin 11). On chromosome 3H associations occurred at 444.7 HORVU3Hr1G058830 (TRICHOME BIREFRINGENCE-LIKE 19), 507.8 Mbp HORVU3Hr1G066840 (IQ-domain 6, a calmodulin-binding protein) and 512.9 Mbp HORVU3Hr1G067510 (WRKY DNA-binding protein 50), and 670.7 Mbp HORVU3Hr1G106260 (GRAS family transcription factor).

DON was associated with several genomic regions of chromosome 5H, 4.7-6.5 Mbp with notable genes: HORVU5Hr1G001670 (Early-responsive to dehydration stress protein [ERD4]), HORVU5Hr1G001920 (Leucine-rich receptor-like protein kinase family protein) and HORVU5Hr1G002000 (Neutral ceramidase). A CBS domain-containing protein HORVU5Hr1G012770 was identified at 38.6 Mbp. Several associations with DON content were seen between 48.7-67.5 Mbp in the region of HORVU5Hr1G014200 (serine/protein kinase); HORVU5Hr1G 014710 (WRKY DNA-binding protein 74); HORVU5Hr1G016350 (*DA1*, a ubiquitin-activated limiter of seed size); HORVU5Hr1G016840 (Protein kinase superfamily protein); HORVU5Hr1G017410 (Two-component response regulator ORR22) - Myb-like DNA-binding. Mapping to 602.7 Mbp, a SNP (JHI-Hv50k-2016-337389) explained 4.9% of the phenotypic variance for DON content (-Log₁₀ P-value = 5.1), associated with HORVU5Hr1G096940 (Cytochrome P450 superfamily protein). This location also maps to the vernalisation gene HORVU5Hr1G095630 [*VRN-H1*] (599.1 Mbp).

Table 3.4. Significant molecular markers ($-\text{Log}_{10} [\text{P value}] \geq 3$) associated with deoxynivalenol (DON) content averaged overall site years (n=6).

SNP marker ID ^A	Chr ^B	Allele ^C	Position ^D	Estimate	F value	$-\text{Log}_{10}$ P value ^E	R ² (%)
JHI-Hv50k-2016-65236	2H	G/A	12238955	1.3	13.7	3.6	3.3
JHI-Hv50k-2016-69936	2H	A/G	19959481	1.3	10.8	3.0	2.6
JHI-Hv50k-2016-70485	2H	C/A	21198556	1.4	11.6	3.1	2.8
JHI-Hv50k-2016-180623	3H	G/C	444738369	-1.8	12.9	3.4	3.2
JHI-Hv50k-2016-185349	3H	A/G	507760269	1.5	11.8	3.2	2.9
JHI-Hv50k-2016-185889	3H	A/G	511670303	1.5	11.3	3.1	2.8
JHI-Hv50k-2016-186010	3H	A/G	512945194	1.6	12.5	3.3	3.0
JHI-Hv50k-2016-216594	3H	G/A	670706415	1.5	11.2	3.0	2.7
JHI-Hv50k-2016-228552	4H	C/G	7604046	1.7	14.9	3.9	3.6
JHI-Hv50k-2016-261905	4H	A/G	603360860	1.4	14.5	3.8	3.5
JHI-Hv50k-2016-262043	4H	A/C	604057659	1.3	10.9	3.0	2.7
JHI-Hv50k-2016-270157	4H	C/G	633359109	-1.4	12.4	3.3	3.0
SCRI_RS_179365	5H	G/A	4870357	1.5	11.8	3.2	2.9
JHI-Hv50k-2016-278585	5H	T/A	4733455	1.8	14.7	3.8	3.6
JHI-Hv50k-2016-278627	5H	C/G	4930992	1.8	14.7	3.8	3.6
SCRI_RS_114102	5H	C/A	5560124	1.7	15.0	3.9	3.6
SCRI_RS_155555	5H	G/A	5590683	1.7	13.1	3.5	3.2
JHI-Hv50k-2016-279292	5H	C/A	6500198	1.8	13.4	3.5	3.3
JHI-Hv50k-2016-288841	5H	G/A	38558864	2.3	13.4	3.5	3.3
JHI-Hv50k-2016-288993	5H	G/A	39814934	2.3	13.4	3.5	3.3
JHI-Hv50k-2016-290535	5H	G/C	54424436	1.6	12.4	3.3	3.0
JHI-Hv50k-2016-290781	5H	A/G	61095947	2.0	11.9	3.2	2.9
JHI-Hv50k-2016-290990	5H	C/A	63230992	2.0	11.9	3.2	2.9
JHI-Hv50k-2016-291313	5H	A/G	67531377	2.1	12.3	3.3	3.0
JHI-Hv50k-2016-337389	5H	A/T	602676669	1.7	20.6	5.1	4.9
JHI-Hv50k-2016-337660	5H	G/A	603536995	1.6	12.3	3.3	3.0
JHI-Hv50k-2016-437412	7H	G/A	2423123	-1.5	12.0	3.2	2.9
JHI-Hv50k-2016-512584	7H	G/A	642107444	0.9	12.3	3.3	3.0
BOPA1_8758-564	7H	A/G	642239467	0.9	12.3	3.3	3.0

^A Molecular marker of 50K SNP array (Bayer et al. 2017); ^B Chr = Chromosome; ^C Allele (Major/Minor); ^D Genomic position in base pairs (bp), reference genome IBSC v2 'Morex v1' gene models (Mascher et al., 2017); ^E Negative Log₁₀ Cochran-Armitage trend test probability.

3.5 Discussion

We assembled genotypes primarily of adapted barley breeding lines and varieties that displayed a wide range of FHB infection in the current study, which resulted in varying levels of DON accumulation. Deoxynivalenol production was positively associated with *Fusarium* infection, and as seen in other studies the relationship is only moderate (Buerstmayr et al. 2004; Choo et al. 2004; He et al. 2015; Tucker et al. 2019). Late infections, which do not

produce high FHB levels, may result in very high mycotoxin accumulation. For example, FHB at Carberry in 2014 was the lowest level of infection but produced some of the highest DON levels in the study. This demonstrates the complexities in the relationship between FHB and DON, both of which are highly subjective to environmental conditions. This also illustrates the essential need to conduct DON testing in barley.

Patterns of relatedness were analyzed for germplasm of the genomic panel, where clustering of materials occurred mainly by barley end-use i.e. malting or livestock feed. The genetic variance was narrower within the malting class of barley. This is to be expected given the restrictions posed by malting quality standards. As a result, the use of elite crosses is required to maintain Canadian malting quality. In addition to type, the genotypes also demonstrated clustering by the institution. However, a substantial level of genetic transfer was apparent through the documented admixture within breeding pools of the Canadian breeding programs. The STRUCTURE analysis identified two main, genetically distinct sub-populations. Modern malting barley cultivars such as ‘AAC Connect’, ‘AAC Synergy’, and related breeding lines from the AAFC-BRDC program, clustered distinctly from the remaining entries in the genomic panel (Figure 3.3).

Fusarium head blight-marker association was observed on chromosome 1H at 11.5 Mbp. A QTL (*Qlds.1H*) has been previously detected by Bedawy et al. (2018) on chromosome 1H 18.9 Mbp and associated with SCRI_RS_239784 through greenhouse spike inoculation and *F. graminearum* leaf assay. Likewise, on chromosome 1H, an association was observed at 551.8 Mbp, which coincided with a previously identified QTL for FHB (BOPA1_2572-986, 547.3 Mbp) in two-row barley (Mamo and Steffenson, 2015). An association with FHB was observed at 185.3 Mbp close to protein kinase, HORVU2Hr1G038950. The previous association was reported between this region and a QTL, *FHB2H.50-56* (bin 6-7), (Massman et al. 2011) and SNP marker SCRI_RS_130072 for DON content (Mamo and Steffenson, 2015). Interestingly, FHB symptoms were not associated with the *Ppd-H1* region in this study, as previously reported in bi-parental QTL studies (Huang et al. 2018; Ogrodowicz et al. 2020, QTL termed *QFHB.IPG-2H_1*).

Genome-wide association study (GWAS) showed several regions of chromosome 2H between 403.3-432.9 Mbp associated with FHB, where several markers were previously associated with

DON content (SRCI_RS_230984, 403.9 Mbp; BOPA1_1062-478, 406.9 Mbp; SCRI_RS_197997, 408.0 Mbp) (Mamo and Steffenson, 2015). In the current study, this region (403-407 Mbp) was also associated with HD50 and HT. Mildew Locus O (*MLO*) proteins like HORVU2Hr1G068660 (483.3 Mbp) are susceptibility factors. Mutant forms of these genes (*mlo*) confer resistance to powdery mildew fungus but enhance susceptibility to hemibiotrophic organisms. It is possible the FHB association a 613.6 Mbp may coincide with major QTL *Qrgz-2H-8* on 2H (bin 8, 73 cM ~ 621 Mbp) previously documented in many studies (Canci et al. 2004; Dahleen et al. 2003, de la Penna et al. 1999; Horsley et al. 2006; Ma et al. 2000, Mesfin et al. 2003, and Zhu et al. 1999), which was also linked to heading date in these studies. However, no association was seen with *Qrgz-2H-10* on 2H (bin 10) in proximity to *vrs1* (row morphology) also commonly identified in these studies. Several associations were found for chromosome 3H with a peak at JHI-Hv50k-2016-201074 (614.2 Mbp), which is located with HORVU3Hr1G085520 (laccase 7) at 612.9 Mbp. On chromosome 4H, FHB was associated with multiple SNPs between 0.1-7.6 Mbp. A QTL *DON-QTL-4H-12_11485* associated with DON resistance was previously identified in proximity at 6.0 Mbp (Mamo and Steffenson, 2015) and associated with QTL *DON4H.03* (bin 1) (Massman et al. 2011). Several genes with potential resistance contributions in this region include HORVU4Hr1G000030 (LRR protein), HORVU4Hr1G000610 (cellulose synthase-like); HORVU4Hr1G000620 (ATP binding cassette subfamily B1); HORVU4Hr1G001450 (Fatty acyl-CoA reductase 3). A GDSL esterase/lipase gene, HORVU4Hr1G001420 associated with JHI-Hv50k-2016-227104 was highly significant ($-\text{Log}_{10}$ P-value = 5.2). While no single marker explained a majority of variation observed for DON content, markers identified were linked to genes previously documented in functions of resistance to FHB and DON production.

Resistance factors can be induced or possess a constitutive basis (Kushalappa et al. 2016). Lipids are important regulators of plant defense, where the well-known defense hormone, jasmonic acid (JA) is a derivative of lipid metabolism. Genes contributing to lipid catabolism such as GDSL esterase/lipases and non-specific lipid-transfer proteins (nsLTP, HORVU7Hr1G102030), have been implicated with FHB resistance in transcriptional studies (Foroud et al. 20212; Gottwald et al. 2012; Huang et al. 2016; Tucker et al. 2021). McLaughlin et al. (2021) demonstrated the importance of nsLTP genes, through the use of transgenics *AtLTP4.4* from *Arabidopsis* in wheat, which suppressed DON production in the field, where

they hypothesized it reduced DON-induced ROS. Fatty acyl-CoA reductase 3 (HORVU4Hr1G001450) which is involved in cuticular wax production, may be an important barrier for barley resistance to *F. graminearum* (Kumar et al. 2016). Caffeoyl-CoA 3-O-methyltransferase (HORVU5Hr1G072070), is involved in production of feruloyl-CoA lignin. Laccase (HORVU3Hr1G085520) catalyzes the synthesis of lignin through oxidative reaction which is deposited in the fortification of the cell wall. Structural-related proteins were identified which can aid fungal penetration such as expansin (HORVU2Hr1G010800) which causes cell loosening or cellulose synthase (HORVU4Hr1G000610) and UDP-glucose 6-dehydrogenase (HORVU4Hr1G008270) leading to hemicellulose and pectin.

On chromosome 5H, FHB association was seen at 28.6 Mbp (SCRI_RS_7368, $-\text{Log}_{10}$ P-value = 5.2). Genes in this region included HORVU5Hr1G010440 (WD-40 repeat family protein) at 26.2 Mbp. Fusarium head blight association was seen over the range 533.8-534.8 Mbp. A major QTL (*Qsds.5Hc*) explaining 39% of genetic variance was identified in a greenhouse study in association with SNP marker SCRI_RS_174710 located on 5H at 532.8 Mbp (Bedawy et al. 2018). This association was in proximity to HORVU5Hr1G071940 (UDP-Glycosyltransferase superfamily protein) and HORVU5Hr1G072070 (caffeoyl-CoA 3-O-methyltransferase). Also associated was JHI-Hv50k-2016-321425 ($-\text{Log}_{10}$ P-value = 4.3) at 564.9 Mbp beside HORVU5Hr1G081520 (Hexosyltransferase) and HORVU5Hr1G081620 (Two-component response regulator-like PRR95) and 573.9 Mbp beside HORVU5Hr1G085070 (Protein kinase superfamily protein). FHB association was seen on chromosome 6H at 51.1-52.1 Mbp in the same region of QTLs for FHB, *FHB6H.42-61*, and DON content *DON6H.42-67* (bins 5-7, Massman et al. 2011) and corresponding genes HORVU6Hr1G019320 (pentatricopeptide repeat 336) and MLOC_10242 (Disease resistant protein, 49.2 cM). While some loci associated with FHB did overlap with height and heading date, the majority did not. The biological function describing such genes identified in FHB-marker associated regions supports their contributions to resistance levels of barley albeit within limitations. Given that regions associated with FHB resistance may contain plausible genes with resistance function, linkage vs. pleiotropy may be responsible for correlations with negative agronomics where selective breeding could remove linkages.

Regions of association with DON did not always coincide with those of FHB and showed associations on chromosomes 2H- 5H and 7H. Deoxynivalenol associations on 3H occurred at numerous regions independently of FHB, which corresponded to genes of interest: 444.7 Mbp (52.0 cM) - HORVU3Hr1G058830 (TRICHOME BIREFRINGENCE-LIKE 19); 507.8 Mbp (57.1 cM) HORVU3Hr1G066840 (IQ-domain 6, a calmodulin-binding protein) and 512.9 Mbp (57.4 cM) - HORVU3Hr1G067510 (WRKY DNA-binding protein 50) a region with QTL *DON3H.52-65* identified with DON content (Massman et al. 2011). A DON association was also observed at 670.7 Mbp which was coincident with the association for FHB and the number of days to heading. Located nearby is HORVU3Hr1G106260, belonging to the GRAS transcription factor family (Scarecrow-like protein 3, *SCL3*), which may influence growth patterns through regulation of gibberellin acid (*GA*) under *GA-GID1-DELLA* mediated signaling by the trans-activation activity of DELLAs (Yoshida and Ueguchi-Tanaka, 2014). Deoxynivalenol association for overall analysis was identified in the tip of chromosome 4HS at 7.6 Mbp which corresponds with the FHB association at the region (0.1-7.6 Mbp) and aforementioned DON QTLs. Deoxynivalenol (DON) also showed an association on chromosome 4H at 603.3-604.1 Mbp in the region of HORVU4Hr1G077360 (Ethylene-responsive transcription factor 1, *ERF1*). On chromosome 5H, multiple regions were associated with DON content. Several associations were seen between 4.7-6.5 Mbp, with a peak at 5.6 Mbp ($-\text{Log}_{10}$ P-value = 3.9). These regions overlapped those of FHB association and include several receptor genes. A large association was observed at 602.7 Mbp ($-\text{Log}_{10}$ P-value = 5.1) in the position of HORVU5Hr1G096940 (Cytochrome P450 superfamily protein). A QTL for FHB severity was previously reported at this location, *QFHB.IPG-5H* close to marker SCRI_RS_184066 (601.1 Mbp) (Ogrodowicz et al. 2020). Loci identified in the current study demonstrated some overlap with previous genetic studies, particularly those following GWAS methodology.

Several leucine-rich receptor-like protein kinase (LRR-RLK) family proteins were identified in association with FHB or DON (HORVU2Hr1G010490, HORVU2Hr1G063910, HORVU4Hr1G000030, HORVU5Hr1G001920). LRR-RLK proteins are broad-spectrum, pattern-recognition receptor (PRR) proteins localized to cell surfaces and are an important component of the surveillance system for the detection of microbial signatures playing an important part in the initial stage of plant defense (Tang et al. 2017). These play a role in

pattern-triggered immunity (PTI) signaling by recognition of pathogen-associated molecular patterns (PAMPs). Following PAMP recognition, PTI response activates MAP kinases and reactive oxidative species (ROS) production. Sucrose nonfermenting-1 (*SNF1*)-related protein kinases (*SnRKs*) like HORVU5Hr1G014200 (serine/threonine-protein kinase 1) also play an important role in regulating stress-related metabolism (Chen et al. 2021). In wheat, *TaSnRK1α* interacts with a *F. graminearum* virulence factor *OSP24* and DON-induced orphan protein *TaFROG* to increase resistance (Perochon et al. 2019; Jiang et al. 2020).

Transcription factors are important factors in modulating transcriptional programming in immune response (Tsuda and Somssich, 2015). Genes of interest included those carrying the myeloblastosis (MYB) domain (HORVU5Hr1G017410), which are key regulators of phenylpropanoid metabolism (Liu et al. 2015). Also identified by this study as a gene of interest was ethylene-responsive transcription factor 1 (*ERF1*, HORVU4Hr1G077360) where ethylene signaling is known to be highly involved in FHB outcomes albeit complex (Foroud et al. 2019). Barley cultivars have demonstrated differential responses in WRKY transcription factors (Huang et al. 2016; Tucker et al. 2021), with some contributing to resistance while others to susceptibility to *F. culmorum* crown rot. (Uluhan et al. 2019). Karre et al. (2019) demonstrated *HvWRKY23* as a major component of FHB through regulation of flavonoid production. HORVU3Hr1G067510 (WRKY DNA-binding protein 50) and HORVU5Hr1G014710 (WRKY DNA-binding protein 74) are both genes of interest identified in the study. *Arabidopsis* gene *AtWRKY50* is associated with salicylic acid (SA) defense response through binding to the *PR1* (Pathogenesis-related 1) promoter region and activating its expression (Hussain et al. 2018).

While barley genotypes differed in their response to FHB and DON accumulation, none showed immunity, or the ability to prevent DON production entirely. Defense strategies may involve limiting DON creation, but also involve chemical modification toxins, which may reduce their toxicity, but also may increase mobility for storage in vacuoles or cell walls (Boutigny et al. 2008). Several gene classes have been commonly connected to DON-specific resistance and/ or detoxification (Gunupuru et al. 2017) including those in the current study: Cytochrome P450 (HORVU5Hr1G096940), ABC transporters (HORVU4Hr1G000620) and UGTs (HORVU5Hr1G071940).

Numerous marker-trait associations for both FHB (51 SNPs over 22 regions) and DON (29 SNPs over 13 regions) were identified in the current study, however, no regions could be defined as a major QTL. This study has identified several loci which have been previously documented in other studies, while a larger portion of marker-trait associations were previously undocumented. Low correlations for marker-trait associations are typical of GWAS. The current study had employed the 50K SNP chip which provided sufficient marker coverage at > 25K polymorphic markers. This finding, along with the observed continuous distribution of FHB-related traits, implies that a large number of minor genes likely contribute to the overall phenotype of two-row barley supporting the polygenic nature of FHB in barley. Several regions also coincided with HD50 or HT, implying that correlations between traits may be the result of pleiotropy, however, gene functions may imply tight linkages. Due to small additive effects and/or percentage of total variation explained by associated regions, genomic regions of interest may have limited breeding value in the marker-assisted selection but could be monitored. Thus a more holistic approach should be employed when breeding FHB resistant two-row barley, such as genomic selection (Meuwissen et al. 2001) which considers the joint merit of all molecular markers to predict genomic estimated breeding values (GEBVs) of breeding lines. Molecular markers identified in this study could provide additional information for developing prediction models, in GWAS-directed genomic selection modeling (using GWAS identified regions as covariates under fixed effects). However, singular SNP molecular markers could not adequately predict FHB and DON, where phenotypic evaluations are still the best predictors of these traits even though they are time-consuming and dependent on environmental conditions.

Preamble: Chapter 4

In the preceding chapter, it is shown that genomic regions conferring genetic resistance to Fusarium head blight (FHB) and deoxynivalenol (DON) content in barley are extremely limited in two-row barley in their magnitude. While genomic regions are associated with FHB and DON, molecular markers only explain a small percentage of the phenotypic variation observed which is largely due to the environmental component. While many of the markers were found in regions associated with genes that carry functional roles in resistance, comprehensive resistance is complex and the summation of numerous genes with small effects. Hence, the development of individual molecular markers for a marker-assisted selection (MAS) program is not feasible, as a plant breeder would need to coordinate dozens to 100's of regions to produce progeny lines with an adequate level of resistance.

The lower cost of genomic analysis brought on by advancements in sequencing technologies has increased the accessibility of genome-wide marker data. With increased coverage, it becomes possible to saturate the genome such that all genes contributing to resistance may be in linkage disequilibrium with a molecular marker. Theory and genetic models have been available for some time, allowing the use of all markers simultaneously in the genomic prediction of breeder values for individuals (Meuwissen et al. 2001). Selection placed on these genomic estimated breeding values (GEBVs) could be used to increase genetic gains over time, as it separates the selection process from requirements to measure phenotypes of progeny lines. The current chapter makes such predictions and contrasts approaches, based on the foundation of classical statistical modelling and machine learning. It is demonstrated that genomic prediction is possible for FHB and DON content of barley and that a whole-genome approach to genetic prediction is a useful method for developing resistance in segregating breeding populations.

CHAPTER 4

GPTransformer: A transformer-based deep learning method for predicting Fusarium related traits in barley

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4.1 Abstract

Fusarium head blight (FHB) incited by *Fusarium graminearum* Schwabe is a devastating disease of barley and other cereal crops worldwide. Fusarium head blight is associated with trichothecene mycotoxins such as deoxynivalenol (DON), which contaminates grains making them unfit for malting or animal feed industries. While genetically resistant cultivars offer the best economic and environmentally responsible means to mitigate disease, parental lines with adequate resistance are limited in barley. Resistance breeding based upon quantitative genetic gains has been slow to date, due to the intensive labour requirements of disease nurseries. The production of a high-throughput genome-wide molecular marker assembly for barley permits use in the development of genomic prediction models for traits of economic importance to this crop. A diverse panel consisting of 400 two-row spring barley lines was assembled to focus on Canadian barley breeding programs. The panel was evaluated for FHB and DON content in

three environments and over two years. Moreover, it was genotyped using an Illumina Infinium High-Throughput Screening (HTS) iSelect custom beadchip array of single nucleotide polymorphic molecular markers (50K SNP), where over 23K molecular markers were polymorphic. Genomic prediction has been demonstrated to successfully reduce FHB and DON content in cereals using various statistical models. Herein, we have studied an alternative method based on machine learning and compared it with a statistical approach. The bi-allelic SNPs represented pairs of alleles and were encoded in two ways: as categorical (-1, 0, 1) or using Hardy-Weinberg probability frequencies. This was followed by selecting essential genomic markers for phenotype prediction. Subsequently, we applied a transformer-based deep learning algorithm to predict FHB and DON. Apart from the transformer method, we also implemented a Residual Fully Connected Neural Network (RFCNN). Pearson correlation coefficients were calculated to compare true vs. predicted outputs. Models which included all markers generally showed marginal improvement in prediction. Hardy-Weinberg encoding generally improved the correlation for transformer for FHB (6.9%) and DON (9.6%). This study demonstrates the potential of the transformer-based method for genomic prediction of complex traits such as FHB or DON, having performed better or equally compared with existing machine learning and statistical method.

4.2 Introduction

Barley (*Hordeum vulgare* L.) is one of the most ancient grains and is currently the fourth-most produced cereal globally, measured both in area harvested and yield (FAOSTAT, 2019). Barley is primarily grown as animal fodder, or used by the malting and brewing industries. As a cash crop, malting barley necessitates maximized yield performance and requires strict management of numerous grain-quality characteristics with specific parameter ranges (Izydorczyk and Edney, 2017). Barley that meets these superior standards can be sold into the lucrative malting barley market, where it returns a significant premium to the barley producer. Fusarium head blight (FHB), caused by *Fusarium graminearum* Schwabe [teleomorph: *Gibberella zeae* (Schwein.) Petch], is a devastating disease of barley. Primary concern of the disease is due to associated trichothecene mycotoxins such as deoxynivalenol (DON), which are potent inhibitors of protein synthesis (Pestka, 2010). Due to potential adverse toxic effects,

DON and its alternative forms, are highly regulated with maximum concentration limits set for humans and livestock (EFSA CONTAM Panel, 2017).

Breeding FHB resistant varieties is a sustainable disease management solution, which has been achieved mainly through large disease nurseries. Studies have generally demonstrated a positive association between visual symptoms of FHB infection and DON content in matured grains. However, this correlation is often moderate, at best (Buerstmayr et al., 2004; Choo et al., 2004; Buerstmayr et al., 2004; He et al., 2015; Huang et al., 2018; Tucker et al., 2019). Mycotoxin quantification is highly technical, where sampling protocols, quality controls, and choice of analytical technologies are all important factors (Tittlemier et al., 2021). Analytical chemistries are expensive and labour requirements for harvest and processing grains are substantial.

FHB and DON content resistances are both under quantitative genetic control in barley (affected by many genes, each with a small effect). Significant undertakings have been made in genetic studies of biparental populations to identify quantitative trait loci (QTL) for breeding resistant barley cultivars (Fernando et al., 2021). While QTLs have been identified for FHB and DON, they are limited by the minimal level of genetic variance they explain, environmental specificity, and common association with negative agronomic traits such as extreme heading date (very early or late) and tall stature. Incorporating major QTLs such as *Qrgz-2H-8* from moderately resistant source ‘Chevron’ into elite backgrounds did not result in sufficient resistance levels (Linkmeyer et al., 2013). Some commercial success has been achieved in developing moderately resistant cultivars such as six-row, malting barley ‘Quest’, through pyramiding of multiple resistances (Smith et al., 2013). Association mapping was able to identify QTLs independent of negative agronomic traits, however, these only explained 1-3% of the observed variance (Massman et al., 2011).

Cereal crops (Poaceae family, Triticeae tribe) are characterized by their large genomes, with frequent repetitive elements (Mascher et al., 2017). With plummeting cost of genomic tools and the availability of highly improved reference genomes, modern breeding approaches are now possible, which take advantage of genome-wide marker capabilities for use in predicting complex traits (Jannink et al., 2010). Genomic prediction of FHB has been possible using statistically-based methodologies in hexaploid (bread) wheat (Rutkoski et al., 2012; Arruda et

al., 2015; Jiang et al., 2015; Mirdita et al., 2015; Hoffstetter et al., 2016; Dong et al., 2018); durum (pasta) wheat (Steiner et al., 2019; Moreno-Amores et al., 2020) and six-row barley (Sallam and Smith, 2016; Abed et al., 2018). While cereal genomes are complex, initial results of genomic prediction for FHB and DON content are very promising-and demand further investigation.

Traditional statistical algorithms such as Best Linear Unbiased Prediction (BLUP) and variants (Burgueno et al., 2012; Cuevas et al., 2017; Ferrao et al., 2017; Cuevas et al., 2019; Howard et al., 2019) have been applied in many genomic prediction problems. These models are mostly linear and perform well for additive traits. Machine learning methods have been applied in genomic prediction with moderate success (Heslot et al., 2012; Ogutu et al., 2011; Poland et al., 2012; Gonzalez-Camacho et al., 2018). The machine learning methods are acclaimed to capture non-additive effects better than the statistical methods (Heslot et al., 2012). Deep learning is a subset of machine learning that is gaining popularity for genomic prediction (Rachmatia et al., 2017; Ma et al., 2018; Jubair and Domaratzki, 2019; Khaki and Wang, 2019). Deep learning differs from traditional machine learning by applying artificial neural networks that often imitate how the human brain learns and identifies patterns based on the learned representations. Under the training phase of prediction, these deep learning algorithms take inputs of genotype data and their corresponding phenotypes as learning parameters. During the testing phase, only the genotype data is used as input and the trained model is validated through the prediction of corresponding phenotypes. These deep learning methods have performed equally, or better than, existing statistical methods (Ma et al., 2018; Jubair and Domaratzki, 2019; Khaki and Wang, 2019). For an overview of deep learning algorithms and their application in genomic prediction, we refer the readers to this review (Montesinos-Lopez et al., 2021).

Neural networks such as feed-forward neural networks (Rachmatia et al., 2017; Khaki and Wang, 2019) and Convolutional Neural Networks (CNNs) (Ma et al., 2018; Jubair and Domaratzki, 2019) have been applied in genomic prediction. The feed-forward network can be compared to n linear regressions where these n linear regressions are the hidden neurons of the feed-forward network. The output neurons of the CNN also represent multiple linear regression models each based on a small subset of markers. CNN also uses a sliding window allowing it

to slide through the whole input space. Neither feed-forward network nor CNN does reflect the polygenic interactive effects of markers as the relationship between markers is not considered in these algorithms.

Transformers are a family of deep learning algorithms that have been initially applied to Natural Language Processing (NLP) tasks such as classification, next sentence prediction, and topic identification (Devlin et al., 2018; Raffel et al., 2019; Radford et al., 2019; Brown et al., 2020). Historically, these methods perform well when trained on a large amount of data (Devlin et al., 2018; Raffel et al., 2019; Radford et al., 2019; Brown et al., 2020) used for transfer learning. Apart from NLP, transformer architecture has been successfully applied to other fields such as image processing (Bazi et al., 2021; Dosovitskiy et al., 2020). In this work, we proposed a transformer-based genomic prediction model for predicting FHB and DON for barley. The transformer consists of three main components: self-attention, feed-forward networks, and layer normalization. The self-attention mechanism calculates the attention score for all genetic markers concerning a specific genetic marker (Vaswani et al., 2017), which helps to find the relationship among markers. The layer normalization function converts each input marker to zero mean and unit variance. The transformer network mainly identifies the inter-relation among markers. Hardy-Weinberg equilibrium is a principle that states the allele frequency of a population will remain constant from generation to generation in the absence of disturbing factors (Acquaah, 2009). The principle can be applied to the marker frequency and provide alternative information about the population alongside genotype data (Acquaah, 2009).

Hundreds of genes may contribute to a phenotype, such that identifying the top genes and related markers that contribute to the phenotype is a challenging task. Feature selection algorithms identify essential features for a specific task (Saeys et al., 2007; Tang et al., 2014) and have been successfully applied in many classification problems of bioinformatics (Saeys et al., 2007). Mutual information is a filter-based feature selection algorithm that identifies top features based on a set of classes and features. Top features identified using mutual information may represent targets that have biological value.

In this work, we evaluate a transformer-based deep learning method: GPTransformer, that uses genotypic and phenotypic data to predict FHB and DON levels in a two-row barley population. Our specific objectives were to (i) compare the accuracy of the GPTransformer model to

existing genomic prediction methods, (ii) study the outcomes of the model if categorical encoding was used, or marker frequency-based encoding was used, and (iii) investigate the effect of feature selection on genomic prediction using mutual information and examine the biological relevance of the top markers identified by the mutual information method. The Transformer network is trained using a graphical processing unit (GPU). As the internal mechanism of the Transformer creates a four-dimensional matrix of size (batch size, number of heads, number of markers, number of markers) which requires a large amount of GPU memory, the feature selection process also helps us to solve the GPU memory issue of the Transformer.

4.3 Materials and Methods

4.3.1 Genotyping

The seed for a genetic panel was collected for a total of 400 spring habit two-row barley genotypes of malt (171) and feed (229) types. Pure seed was provided by the Crop Development Centre, University of Saskatchewan, Saskatoon, Canada (CDC) for a diversity panel of barley (92) breeding lines tested in the Western Canadian Cooperative Two-Row Barley Registration Trials (WCTBRT) 1994-2006 (Beattie et al., 2010). Additional elite lines (176) were selected from 2001-13 WCTBRT based on past performance, with the majority from three breeding programs: CDC; Field Crop Development Centre, Olds College; Agriculture and Agri-Food Canada (AAFC), Brandon Research and Development Centre. In addition, breeding lines (105) targeting FHB resistance and involving crosses to exotic sources, were selected from these programs.

Two seeds were germinated on moist cotton balls for a week. At the two-leaf stage (Zadoks et al., 1974), leaves were cut from a single plant and flash-frozen in liquid nitrogen, then freeze-dried in a lyophilizer (Labconco Corporation, Kansas City, MO, USA). Genomic DNA was extracted from 100 mg of tissue using Qiagen, DNeasy 96 Plant Kit (Qiagen, Canada). The isolated DNA was evaluated by a NanodropTM 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) for quality and concentration then normalization to 50 μL^{-1} . Samples were assayed on an Illumina iScan (Illumina, San Diego, CA, USA) using a custom iSelect - 50K SNP microarray (Bayer et al., 2017) at AAFC, Morden Research and Development Centre, Morden, MB. A custom cluster file provided by M. Ganai (TraitGenetics

GmbH, Germany) was used to call SNP alleles using Illumina GenomeStudio V2.0.5 software (Please see data availability statement). Data were filtered for $\geq 5\%$ minor frequency alleles and $\leq 20\%$ missing data.

4.3.2 Field Studies

FHB nurseries were grown in 2014 and 2015 at 3 locations: Brandon, Manitoba (49°51'56.0" N 99°58'57.7" W); Carman, Manitoba (49°29'52.9"N 98°02'19.2"W), and Carberry, Manitoba (49°54'16.6"N 99°21'19.0"W). The experiments followed a randomized complete block design at all locations (n = 2). Plots were sown with approximately 30-40 seeds and consisted of 0.9 m rows, 30 cm row spacing (Brandon, Carberry), or 1 m rows, 34 cm row spacing (Carman). Two inoculation methods were used. Brandon and Carberry's experiments were inoculated by the grain spawn method, where maize kernels infected with two isolates each of 3ADON- and 15ADON-producing strains of *F. graminearum* were spread on the soil surface at 5 g m⁻² at flag leaf then weekly for a total of three applications. Irrigation was applied after the first inoculum application until all plots were rated. Experiments at Carman were sprayed with a macroconidia suspension of 3ADON and 15ADON isolates in equal proportions and standardized to 5 x 10⁴ spores ml⁻¹. Plots inoculated the times, the first at 75% spike emergence and then at intervals of two to three days. Mist irrigation was applied for 10 minutes per hour for 10 hours after each inoculation.

Plots were rated at the soft dough (Zadoks - Z85) stage. A visual scale (0-5) was used to evaluate a composite measure of incidence and severity (A. Tekauz, personal communication), where 0 = no infection. 1 = incidence low, up to 5% of spikes; severity low, 1 or 2 kernels per spike affected (up to 7% of spike). 2 = incidence low to moderate, 5 to 15% of spikes infected; severity low to moderate, 1 to 4 kernels (up to 15% of spike). 3 = incidence moderate, 15 to 30% of heads; severity moderate, 2 to 8 kernels (up to 25% of spike). 4 = incidence moderate to high, 30 to 50% of spikes infected; severity moderate to high, 4 to 12 kernels (up to 40% of spike). 5 = incidence high, 50% or more spikes affected; severity high, 5 to 15+ kernels (up to 50%+ of spike diseased). Additional data were collected on days to heading (date 50% of row headed minus seeding date) at Brandon and Carberry and plant height (distance of soil surface to the tip of spike excluding awns) at all locations.

Grains were harvested at maturity, using stationary research combined with a low wind speed setting, then dried in a high capacity drier at 30°C for a few days. Grains were cleaned using an SLN4 sample cleaner (Pfeuffer GmbH, Kitzingen, Germany). A 20 g subsample was removed, cleaned free of debris and/or chaff, then ground using a Perten 3610 lab mill with a fine particle disc set (PertenElmer Inc. Waltham, MA, USA). Deoxynivalenol content was analyzed by enzyme-linked immunosorbent assay (ELISA) technique using Veratox ® 5/5 (Neogen Corporation, Lansing, MI, USA) as per kit protocol (limit of detection = 0.1 mg kg⁻¹). Samples were each tested in sub-sample pairs, where samples deviating by > 10% were repeated.

4.3.3 Allele Frequency Based Encoding

As the barley population is diploid, a locus *A* can have two alleles, *A* and *a*, and three genotypes *AA*, *Aa*, and *aa*. We replaced the classical representation of the genotype of a marker (1, 0, -1) with genotypic frequency by applying Hardy-Weinberg equilibrium (Acquaah, 2009). The frequency of alleles is calculated for each genetic marker. Suppose the genotypic frequency of *AA* and *Aa* is *D* and *H* respectively. If the population size is *N*, the total number of alleles will be 2*N*. Then the frequency of allele *A* for a specific genetic marker is calculated by applying equation 1.

$$p = (2D + H) / 2N \quad (1)$$

The frequency of allele *a* is $q = 1 - p$. After calculating the allele frequency of a genetic marker, the expected genotypic frequencies of genotype *AA*, *Aa*, and *aa* for a specific marker are obtained from p^2 , $2pq$, and q^2 . These expected genotypic frequencies are used as marker values for machine learning algorithms.

4.3.4 Transformer

In this paper, we examine the application of transformer, a variety of neural networks. Deep neural networks have been applied to genomic selection (GS) in the past. See the survey of Montesinos-Lopez et al. (Montesinos-Lopez et al., 2021) for details on previous applications. The transformer is a family of deep learning algorithms successfully applied to various NLP tasks such as classification, sequence to sequence modeling, and next sentence prediction. The transformer architecture has two major components: i) encoder and ii) decoder Vaswani et al. (2017). In this work, we use the encoder part of the transformer along with an additional feed-

forward network (Figure 4.1). The encoder of the transformer architecture contains an embedding layer, a multi-head self-attention layer followed by a feed-forward neural network.

The purpose of this embedding vector is to obtain an n -dimensional distributed representation of each marker. The input of the embedding layer is m markers and the output is an $m \times n$ -dimensional vector. The vector is then reshaped to an (m, n) -matrix to obtain m expanded representation of the markers. In this work, a feed-forward neural network is applied as the embedding layer. The output of the embedding layer is then passed to a multi-head attention network.

The multi-head attention network is based on the self-attention mechanism. The input of this layer is the embedded representation of the markers obtained in the embedded layer. The main building block of the multi-head-attention is the self-attention mechanism that calculates the attention score for all other markers concerning a specific marker. To calculate the self-attention, at first, each embedded marker creates three vectors: a query vector q , a key vector k , and a value vector v by applying a linear transformation on the embedding. The query vector is the candidate marker to which the attention is measured while the keys are the set of markers where the importance scores are assigned. The attention score is calculated as:

$$Attention(Q, K, V) = softmax(\frac{QK^T}{\sqrt{d_k}})V \quad (2)$$

In equation 2, Q is a matrix of all the queries, K is a matrix of all the keys and V is a matrix of all the values and d_k is the dimension of keys. The last step is to generate a summation of the previous step, which produces the self-attention layer's output. In a multi-head attention setting, the Transformer model creates h independent linear representation from queries, keys, and values. These h representations are then concatenated and passed through a linear projection layer to obtain the final output.

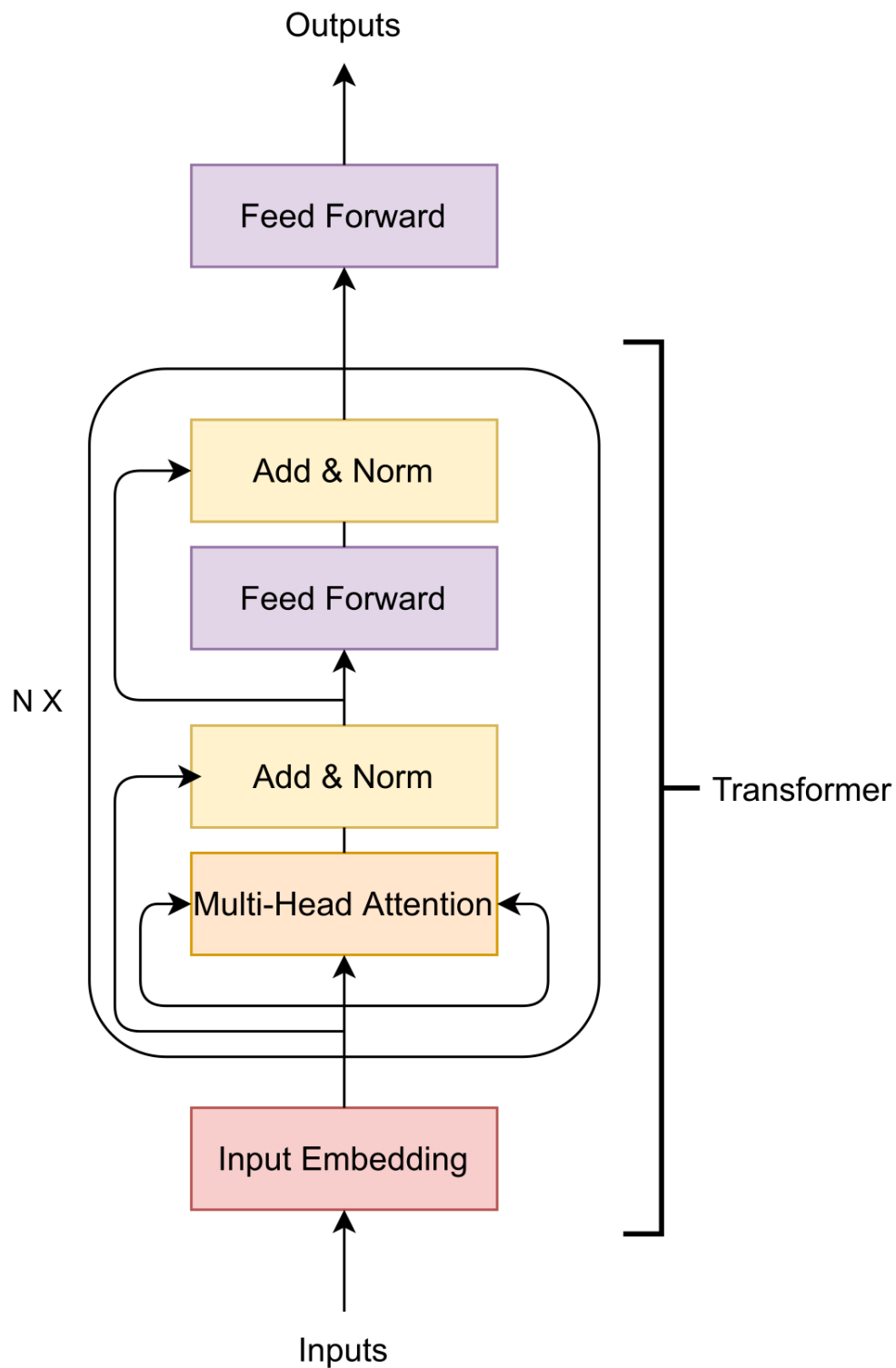


Figure 4.1. Schematic flow diagram of the Transformer architecture and associated components.

In Figure 4.1, a residual connection from the output of the input embedding layer is added to the output of multi-head attention. A layer-normalization is applied to the output of the residual connection. The Transformer block contains another feed-forward network and a layer of normalization after the feed-forward layer. The N in Figure 4.1, indicates that this transformer block can be stacked N times and the output of the N th encoder block will be the input of the feed-forward layer that predicts the phenotypes.

4.3.5 Residual Fully Connected Neural Network

The second deep learning model is based on a feed-forward network with a residual connection. In general terms, a residual neural network is a neural network that has one or more residual connections. In our implementation, the first layer of the Residual Fully Connected Neural Network (RFCNN) is a feed-forward neural network that takes M markers as the input and performs a linear transformation to produce an n -dimensional hidden representation of the markers. This hidden representation is the input of the batch-normalization layer. The batch-normalization layer normalizes the current batch by its mean and standard deviation. As the feed-forward network and batch normalization performs a linear operation on the input data, we apply the activation function ReLU to the batch-normalization layer's output. ReLU will return 0 if the input is ≤ 0 ; otherwise, it will return x where x is the input and thus, introduces the non-linearity. The output of ReLU is going to be the input of the next feed-forward network. In Figure 4.2, the residual connection is shown as the arrow on the left side of the figure skipping over the intermediary layers. A residual connection is added from the output of each odd ReLU layer to each odd batch-normalization layer (except for the first batch-normalization). The residual block can be stacked N times. The output layer is the feed-forward network that predicts the phenotypes.

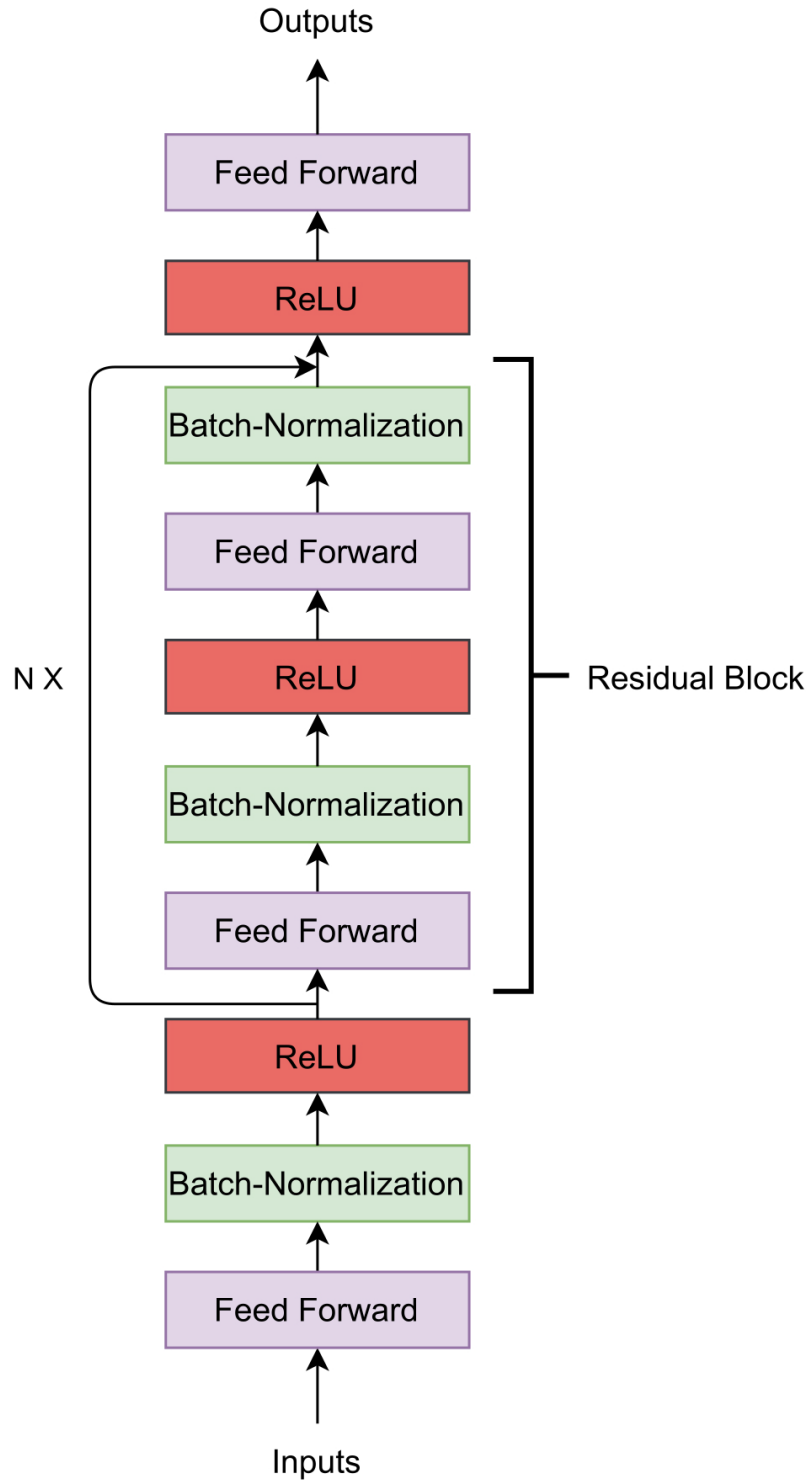


Figure 4.2. Schematic of residual fully connected network architecture and associated components.

4.3.6 Other statistical and Machine Learning Models for Baseline Comparison

4.3.6.1 Decision Tree

Decision Trees (DTs) are common statistical and machine learning methods used for predictive modelling. The baseline DT regressor models used in this analysis were built with the Scikit-learn (Pedregosa et al., 2011) Python module under default settings. To train the models, an input dataset of an $n \times p$ matrix of encoded genotypes and a n -dimensional vector of known corresponding phenotype responses were supplied. At each node, splits based on the SNP markers are considered and the highest quality split; in this case, the quality measure of the split is the mean squared error. This process continues recursively, splitting the data into subsets of instances at each internal node until the branches terminate with the leaf node and produce a response value. The values at the leaf nodes are the arithmetic mean of the known associated response variables to the instances that are present in the adjoining edge. With a fitted model, genotypes with unknown phenotype responses can be predicted by iteratively testing the SNP marker values against the trees decision procedure until a leaf node is reached.

4.3.6.2 Linear Regression

Like the DT models, the linear regression (LR) models used in this analysis were built with the Scikit learn Python module using default settings. LR fits a linear model by calculating coefficients on the independent terms that minimize the sum squared error between the known observations responses and the approximated predictions. The following is the form of the linear models:

$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \boldsymbol{\varepsilon} \quad (4)$$

where $\mathbf{y}$ is the n -dimensional column vector containing one response variable (phenotype) for each of n input p -dimensional row vectors (genotypes), $\mathbf{X}$ is the $n \times p$ regressor design matrix (n encoded genotypes), $\boldsymbol{\beta}$ is a p -dimensional column vector of unknown coefficient parameters, and $\boldsymbol{\varepsilon}$ is the n -dimensional unknown random error column vector.

2.6.3 Ridge Regression Best Linear Unbiased Prediction

The ridge regression (RR) models were built using JMP Genomics 9.1 (JMP Genomics, 1989-2021). This process computes Best Linear Unbiased Predictions (BLUPs) that linearly

correlate the genotypes, based on the input marker encodings, to a trait variable of interest. RR-BLUPs are linear mixed-models of the following form:

$$\mathbf{y} = \mathbf{F}\boldsymbol{\delta} + \mathbf{Z}\boldsymbol{\gamma} + \boldsymbol{\varepsilon} \quad (5)$$

where $\mathbf{y}$ is the n -dimensional column vector containing one response variable (phenotype) for each of n input p -dimensional row vectors (genotypes), $\mathbf{F}$ is the $n \times q$ matrix of known fixed-effects, $\boldsymbol{\delta}$ is a q -dimensional column vector of unknown fixed-effects parameters, $\mathbf{Z}$ is the $n \times p$ matrix of known random-effects (n encoded genotypes), $\boldsymbol{\gamma}$ is the p -dimensional column vector of unknown random-effects parameters, finally, $\boldsymbol{\varepsilon}$ is the n -dimensional unknown random error column vector. It is assumed that the residuals $\boldsymbol{\varepsilon}$ and random-effects $\boldsymbol{\gamma}$ are normally distributed, $\boldsymbol{\varepsilon} \sim N(0, I\sigma^2_{\boldsymbol{\varepsilon}})$ and $\boldsymbol{\gamma} \sim N(0, \sigma^2_{\boldsymbol{\gamma}})$ where I is the identity matrix and $\sigma^2_{\boldsymbol{\varepsilon}}$ and $\sigma^2_{\boldsymbol{\gamma}}$ are assumed equal for all SNP markers. The unknown model parameters are estimated from the solution of the mixed-model equations (Henderson, 1984). A scoring file is produced that contains an equation of a linear combination of SNP markers for predicting phenotype response from the testing set data.

4.3.7 Train-Test-Validation Split

To divide the data into train, test, and validation sets, we follow the recommendation of Runcie and Cheng (2019). We randomly split the data into 85% - 15%, where 85% is the training component. From the training data, we again perform three random splits of 85% - 15%. The first 85% is the training set and the remaining 15% is the validation set. Thus from the data, we created three training sets, three validation sets, and one test set.

4.3.8 Feature Selection

Feature selection methods identify features that contribute to a specific expression. In our work, the purpose of the feature selection is to identify those genetic markers that contribute to low FHB or low DON levels. We applied mutual information feature selection, a filter-based method, where the input is the genetic markers and the phenotypes and the output is a mutual information score (ranging from 0-1). Discretization was performed where we divided the genotypes into three bins based on phenotypes (lowest 25%, middle 50%, highest 25%). The categories are the labels and the genetic markers are the features of the mutual information

algorithm which produces a mutual information score for each marker. The final mutual information score for each marker is the average mutual information score over the three training sets. Markers with average mutual information of ≥ 0.02 were selected.

4.3.9 Training Transformer

The input embedding of the transformer network converts each marker to an eight-dimensional vector (hidden dimension, $n = 8$). Thus, if each genotype contains m genetic markers ($m = 25,000$ before performing feature selection), the input embedding layer's output will be an (m, n) matrix. This (m, n) matrix is the input of the multi-head attention. As the multi-head attention computes pairwise attention between each marker, the operation will result in an (h, m, n) matrix where h is the number of heads. This operation has significant memory requirements for the GPU. For instance, this dataset requires over 48 GB of memory.

To circumvent memory limitations, only selected features of mutual information are inputted. We also pass only one genotype at each batch for training. Thus, the input of the embedding layer is all the markers of a genotype selected by the mutual information algorithm. The output is an (f, n) dimensional matrix where f is the number of markers. This (f, n) matrix will be the input of the transformer encoder. Our transformer neural network contains two transformer encoder blocks ($N = 2$). We use two heads ($h = 2$) for each multi-head attention layer and each feed-forward block inside the transformer encoder contains 256 hidden neurons. The final transformer encoder's output is also an (f, n) matrix which is flattened to create a vector that contains $f \times n$ elements. This vector is the input of the last feed-forward network which contains one output neuron. We use the mean square error (MSE) loss function along with the Adam optimizer. The learning rate of the optimizer is $1e^{-5}$. The training was ceased if there was no improvement in MSE loss in the validation set for ten consecutive epochs.

4.3.10 Training Residual Fully Connected Neural Network

The first feed-forward layer takes m markers (all the markers) or f markers (feature selected) as input and produces a 512-dimensional hidden representation. Each subsequent feed-forward layer takes the previous layer's input and produces a 512-dimensional hidden representation. The last feed-forward layer contains only one output neuron. We stack five residual blocks (N

= 5) one after another. We also use MSE as the loss function and Adam as the optimizer with a learning rate of $1e^{-5}$.

4.4 Results

Pearson Correlation Coefficient (PCC) was calculated to measure the predictive performance of modelling approaches. The PCC calculates the linear relation between the true output and predicted output. The PCC value ranges from -1 to 1 where 1 indicates a perfect linear relation between the predicted phenotypes and the true phenotypes, whereas -1 indicates a perfect opposite relationship between the true and predicted phenotypes. Values close to 0 represent no relationship between the true and predicted phenotypes. In the remainder of the paper, especially in the figure, we will denote genotype-based encoding as HW, categorical encoding as CAT, Decision Tree algorithm as DT, Linear Regression as LR, and Residual Fully Connected Neural Network as RFCNN.

4.4.1 Phenotype Assessment

The distribution of FHB and DON is shown in Figure 4.3. It is observed that the FHB values are distributed over the 0.3 to 4.8 range (Mean = 1.75 ± 0.04), while the DON values range from 4.9 to 36.9 mg kg⁻¹ (Mean = 13.96 ± 0.21) (Appendix 2.1). For both phenotypes, the distribution curve is similar to a normal distribution, however, a degree of positive skewness was observed in FHB (0.881) and DON (0.976). Shapiro-Wilk W tests conducted on FHB (W=0.947, Prob <0.0001) and DON (W=0.963, Prob <0.0001) indicated a degree departure from normality. Departures from normality were most obvious in tail regions of the distributions.

Though from Figure 4.4, it seems there may be a linear relation between FHB and DON, it is found that the Pearson Correlation Coefficient (PCC) between the two phenotypes is 0.381 (P < 0.0001). Figure 4.4 shows a scatterplot of FHB vs. DON for each genotype. From Figure 4.4, it is observed that there is very little correlation between the two phenotypes which is reflected by the PCC score. Fusarium head blight (FHB) and DON were also examined for relationships with days to heading ($r = -0.18$, P < 0.004; 0.18 , P < 0.0003) and height ($r = -0.60$, P < 0.0001; -0.21 , P < 0.0001).

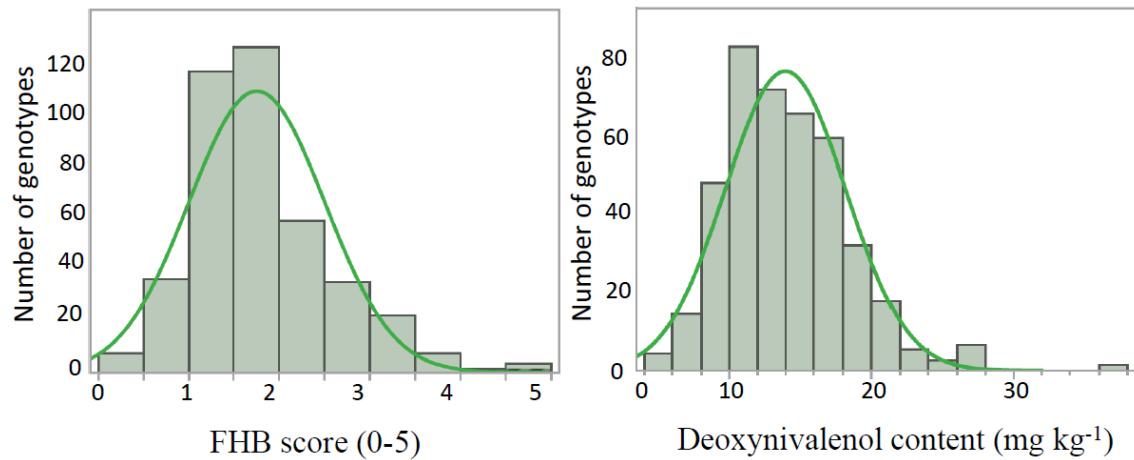


Figure 4.3. Distribution of phenotypes for Fusarium head blight (FHB, 0-5) and deoxynivalenol content (DON, mg kg⁻¹) of 400 two-row barley genotypes tested over six site years.

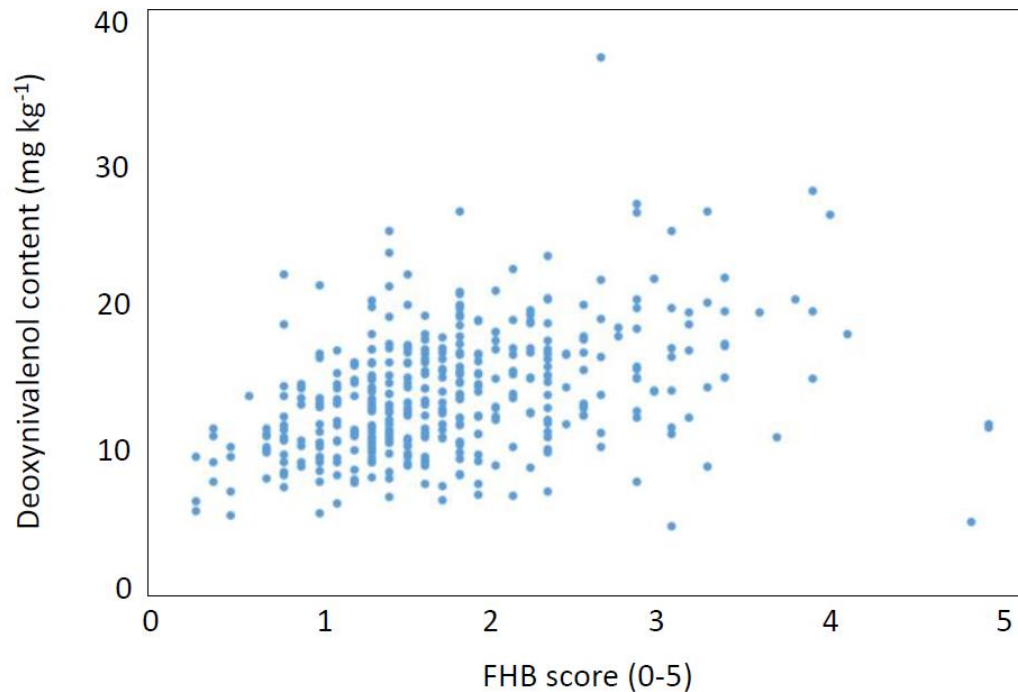


Figure 4.4. Fusarium head blight (FHB, 0-5) vs. deoxynivalenol content (DON, mg kg⁻¹) for each of the barley genotypes tested. The correlation between FHB and DON is 0.381.

4.4.2 Effect of Encoding Technique

Two encoding techniques were implemented: i) categorical encoding (-1, 0, 1) and ii) genotype frequency-based encoding that follows Hardy-Weinberg equilibrium. Table 4.1 shows the comparison of PCC between two encoding schemes for various models. In most of the models, the categorical encoding outperforms the genotype frequency-based encoding. In the BLUP, the correlation is very close only deviating by 0.001 to 0.003 for each trait. For transformer, Hardy-Weinberg encoding improves the correlation by 6.9% for FHB and 9.6% for DON. This can be explained by noting that, with the categorical encoding, the values of genotypes are 1, 0, and -1, the heterozygous alleles will be considered neurons that do not have any effects. In particular, Figure 4.5 shows the effect of categorical encoding in the embedding layer. As the embedding layer's output is the input of the multi-head attention, multi-head attention also ignores any effect of heterozygous alleles. When applying genotype frequency-based encoding, different alleles of a specific gene have different values and these values even differ from gene to gene. For example, allele A for gene X and allele A for gene Y may appear in different frequencies and will have different Hardy-Weinberg values. Thus, the embedding layer does not suffer from multiplying by zero problems and improves the performance of the Transformer.

Table 4.1. Comparison of Pearson Correlation Coefficient (PCC) based on encoding techniques. HW and CAT represent Hardy-Weinberg and categorical encoding, respectively. The correlation is measured between the target and predicted phenotypes. Decision Tree (DT), Linear Regression (LR), Best Linear Unbiased Prediction (BLUP), Residual fully connected neural network (RFCNN), and Transformer models are applied for each encoding technique.

Model	<u>FHB score</u>		<u>DON content</u>	
	HW	CAT	HW	CAT
DT	0.535	0.527	0.149	0.191
LR	0.513	0.549	0.431	0.488
BLUP	0.619	0.616	0.591	0.592
RFCNN	0.337	0.380	0.567	0.590
Transformer	0.621	0.552	0.605	0.509

4.4.3 Effect of Feature Selection

No single molecular marker dominantly explained significant portions of the variation for FHB or DON. However, specific markers could be identified as ‘top features’ which may be associated with genes of interest that may operate closer to oligogenic vs. polygenic fashion (Appendices 2.2-2.3). Biological endorsement of genomic features for FHB and DON were investigated through analysis of SNP effect annotations of markers on the 50K SNP chip (Bayer et al., 2017). The top molecular markers with the highest mutual information are displayed (Appendices 2.2-2.3). Gene annotations generally concurred with resistance patterns.

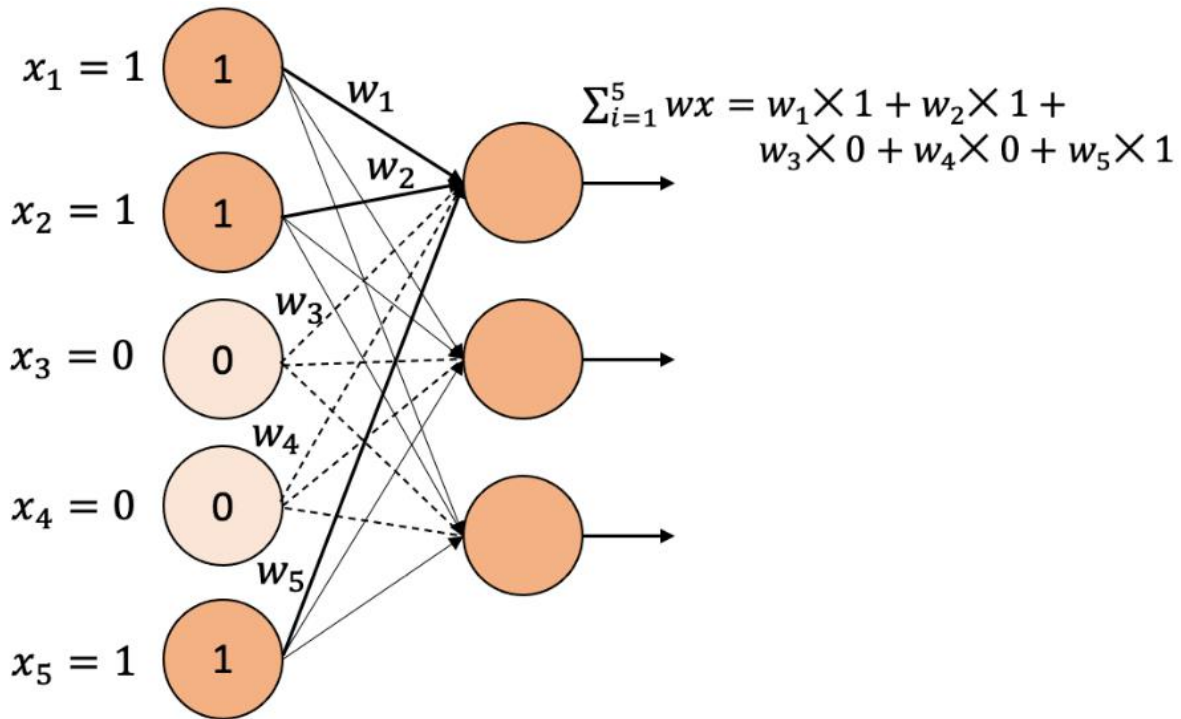


Figure 4.5. Categorical encoding when applied to a fully connected layer, where the heterozygous condition ‘0’ has no contribution to prediction.

In most of the experiments, when the machine learning or statistical methods are trained using all the markers, they performed better. Table 4.2 shows the comparison of correlation scores between models trained on all markers and models trained on selected markers for DON. Using marker frequency as features for the DON, only the BLUP method with reduced markers shows minor improvement (0.4%) over the BLUP method that uses all the markers. When the models were applied to categorical features for DON, only the Decision Tree shows

a 2.1% improvement when reduced marker sets were used as features. Recall that due to memory issues, the Transformer model with the full set of markers was not executed, and thus is absent from Table 4.2.

Table 4.2. Comparison of Pearson Correlation Coefficient (PCC) when applying all markers as features vs. selected markers for deoxynivalenol (DON) content. The PCC is measured between target and predicted DON. Decision Tree (DT), Linear Regression (LR), Best Linear Unbiased Prediction (BLUP), Residual fully connected neural network (RFCNN), and Transformer models are applied for each encoding technique.

Model	<u>Hardy-Weinberg</u>		<u>Categorical</u>	
	All	Selected	All	Selected
DT	0.149	0.116	0.191	0.212
LR	0.431	0.408	0.488	0.458
BLUP	0.567	0.507	0.590	0.572
RFCNN	0.591	0.595	0.592	0.589
Transformer	-	0.605	-	0.509

In Figure 4.6, we show the comparison of correlation scores between models trained on all markers and models trained on selected markers for FHB. From the figure, we observe a similar pattern for FHB that we observed for DON. In contrast to categorical encoding when using marker frequency as a feature value, selected markers improve the performance of the FHB RFCNN model by 27.6%. For categorical encoding of features, Linear Regression with selected features shows a 4.2% improvement in overall features.

Table 4.3. Comparison of Pearson Correlation Coefficient (PCC) when applying all markers as features vs. selected markers for Fusarium head blight (FHB). The PCC is measured between target and predicted FHB. Decision Tree (DT), Linear Regression (LR), Best Linear Unbiased Prediction (BLUP), Residual fully connected neural network (RFCNN), and Transformer models are applied for each encoding technique

Model	<u>Hardy-Weinberg</u>		<u>Categorical</u>	
	All	Selected	All	Selected
DT	0.535	0.349	0.527	0.452
LR	0.513	0.450	0.549	0.591
BLUP	0.337	0.510	0.499	0.380
RFCNN	0.619	0.602	0.616	0.622
Transformer	-	0.621	-	0.552

Though there is a significant increase in correlation score when all the markers are used for machine learning models, none of the BLUP models show any significant difference in performance when all markers or selected markers are used. Due to GPU memory limits, it was not possible to use all the features for Transformer architectures.

4.4.4 Best Performing Models

Overall, BLUP and Transformer models that use genotype frequency as features obtained better correlation scores than other models. Table 4.3 shows the comparison among the best models for FHB and DON. BLUP and Transformer's performance is similar in predictive ability, as we observe only a 1% improvement over BLUP for DON and the same correlation score for FHB.

Figure 4.7 shows the true versus predicted phenotype score using Transformer. From the figures, we observed a linear relationship between the target and predicted score, which also shows that the Transformer architecture performs well to predict phenotypes.

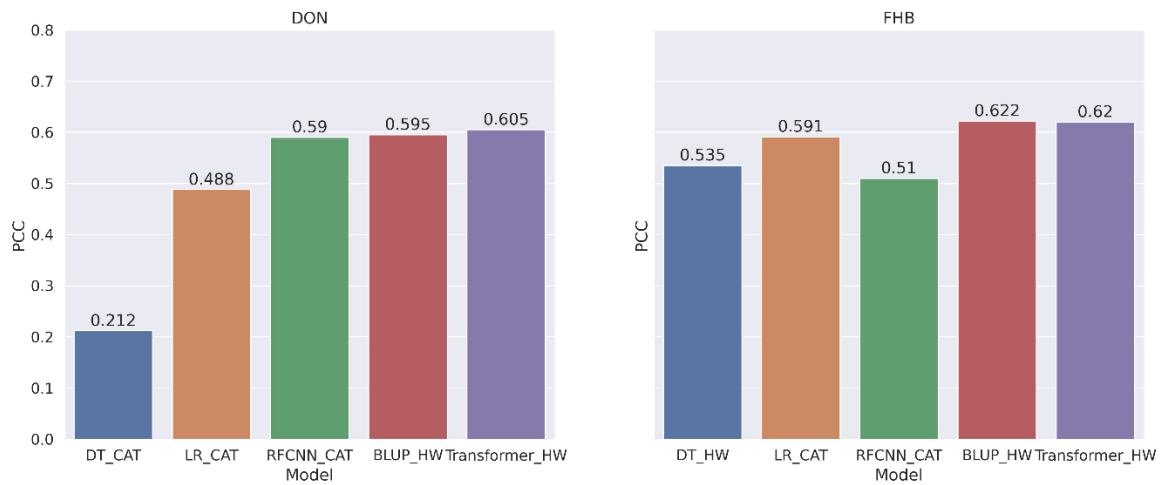


Figure 4.6. Comparison among the best models for each machine learning or statistical method for DON. The Pearson Correlation Coefficient is measured between the target and predicted values of the phenotype. Decision Tree (DT), Linear Regression (LR), Best Linear Unbiased Prediction (BLUP), Residual fully connected neural network (RFCNN), and Transformer models are applied for Fusarium head blight (FHB) and deoxynivalenol (DON) content.

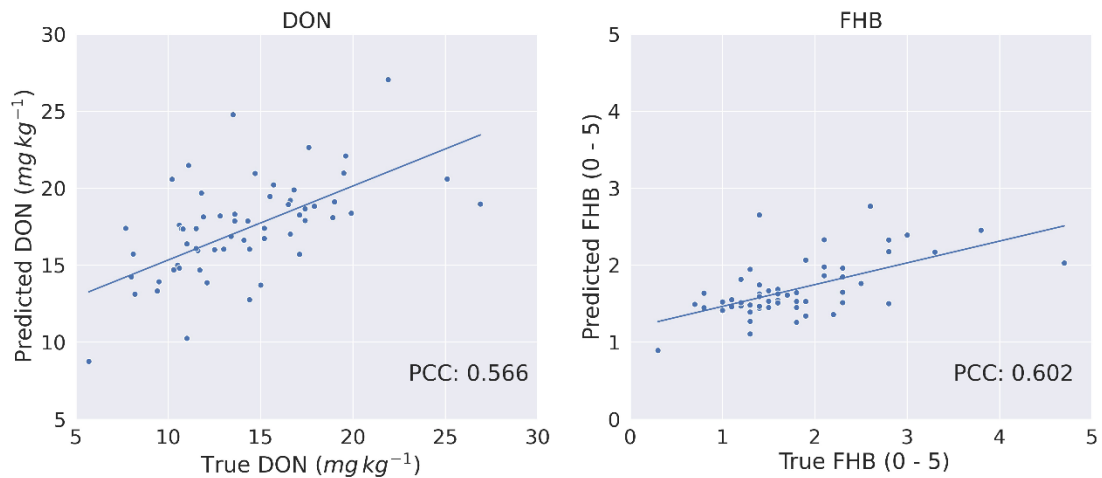


Figure 4.7. True vs. predicted phenotypes for DON (mg kg⁻¹) and FHB (0 - 5) on the test set of 60 genotypes for the Transformer model using an 85:15 % (training: validation) split.

4.5 Discussion

Transgressive segregation is common for FHB and DON in barley, where it is typical for progeny to be found outside of the range of the parental values. Such complexities in multiple resistance genes which form near-continuous distributions may not be accounted for under the assumptions of statistical models examined herein. While the machine learning approach did not substantially surpass the statistical models in prediction, the ML approaches we have used here are capable of capturing non-additive genetic components. Thus, the predictions given maybe incorporating some of these interactions in their prediction. However, we are not able to tell what effects are being modeled in the ML models because of their opacity.

The current study confirms previous association mapping analysis of FHB and DON in barley (Massman et al., 2011), where quantitative trait loci (QTL) effects were small. While minor, genes may additively contribute to resistance thereby lowering FHB and DON content. The advantage of such genes is that they do not typically carry issues seen when incorporating larger QTLs from exotics which tend to have tall stature or extreme heading date (Rudd et al., 2001). In the current study, the number of days to heading was not strongly associated with either FHB or DON content. Height was also weakly associated with DON content, however, it did demonstrate a moderate, negative relationship with FHB. Top feature molecular markers and genes identified for FHB and DON did not overlap, as one might predict based on their moderate-to-low trait correlation. Within barley, the relationship between FHB disease and DON content is not as robust as seen in other cereals such as wheat. Application of GEBVs based on DON content may offer a better target for developing resistance since it is the primary factor monitored by the industry.

Markers identified in the top features associated with FHB resistance were found on chromosomes 1H, 3H, and 7H and all chromosomes for DON content, excluding 1H. Annotations of associated genes generally displayed the direct biological function of resistance mechanisms. For instance, FHB was associated with auxin transporter (HORVU1Hr1G073490) and response factor (HORVU7Hr1G033820), where this plant hormone has been associated with FHB severity and yield loss in barley (Petti et al. 2012). Also identified were genes involved in β -glucan synthesis cell (HORVU7Hr1G003460, HORVU7Hr1G003460), which may contribute to resistance via wall reinforcements or anti-

oxidant properties (Martin et al. 2018). The molecular marker BOPA2-12_31203 in the top features group in this study previously identified by Huang et al. (Huang et al., 2018) is a flanking marker for a QTL for FHB severity in the centromeric region of chromosome 7H. As a result of this toxic function, DON may induce programmed cell death (PCD, i.e. apoptosis). Development and Cell Death (DCD) domain protein (HORVU3Hr1G017930) and autophagy-related protein 18 (HORVU3Hr1G017150) which functions in the removal of damaged cells may be involved in this process. Such top genomic features only explain a small percentage of total phenotypic variation for FHB and DON and could not be individually implemented under a marker-assisted selection program. However, biological functions associated with genes and markers highlighted above, amongst others, may help explain why feature selections of a reduced marker subset may facilitate predictions with similar proficiency as when using all markers.

The proposed GPTransformer model takes the relationship among genetic markers into account within the model. The self-attention mechanism of the transformer assigns high weight to those markers that are associated with another specific marker. After applying the self-attention module, each obtained neuron is a combined representation of the genetic markers that are related to a specific marker. As many markers contribute towards a specific phenotype, GPTransformer has a unique attribute compared to other machine learning and statistical methods that take marker relationships into account.

The frequency-based marker representation technique we applied for representing each allele carries more information as it indicates the zygosity and the frequency of the allele. The traditional categorical encoding(1, 0, -1) only indicates the zygosity and remains the same for all the genetic markers. As each allele of a genetic marker is represented by its fixed frequency value, the frequency-based encoding provides us the information about the frequency as well as the zygosity. Though the frequency value remains the same within the same genetic marker for a specific allele, it may differ between different genetic markers. Thus, when the GPTransformer is combined with the frequency-based encoding, it performs better than the traditional categorical encoding-based model. The frequency-based representation is in the range of 0-1 and minimizes issues of vanishing gradient that may occur when training the GPTransformer or other neural network models.

The stability of the proposed model is tested with three different training and test data and the result shows the deviation from the average PCC score for each different training and test set is very low. The highest deviation from the average PCC score among three different runs for FHB is 0.02 and for DON is 0.039 while the highest deviation for the BLUP model for FHB is 0.027 and for DON is 0.031. The deviation from the average PCC for three different runs is higher for other machine learning methods we experimented with. The deviation varies from 0.082 to 0.147 based on different models and traits. This shows that the GPTransformer model is stable compared to other machine learning methods and as good as the popular BLUP model.

While the time commitment is higher and takes up to an hour to train the GPTransformer, it only took 24 epochs to complete training. The time complexity of the RFCNN is much lower than the transformer though it took approximately 200 epochs to train. The most expensive task in the transformer is self-attention which requires substantial time and memory to complete. We ran both machine learning models on Intel Xeon E5-2690 v4 processor and NVIDIA Tesla P40 GPU, which contains 24 GB of memory. With our transformer architecture, we can fit all the markers that have mutual information ≥ 0.02 . To fit all the genetic markers, a larger memory or multi-GPU instance is needed.

Though the performance of most of the machine learning methods improves when all the markers are used for prediction, the transformer architecture outperforms other methods with selected markers. To the best of our knowledge, this is the first method that uses transformer architecture for genomic prediction. This work showed that this method could outperform existing machine learning methods with fewer data and obtain state-of-the-art performance. Based on the performance in the language model domain, it is expected that with an increased amount of data, the performance of the transformer model will also increase.

Our work shows the potential of the transformer-based method for genomic prediction. Though transformer generally performed well with a large amount of data in other fields, in this work, we showed that when trained on a small dataset, the transformer encoder performs equally or better compared to the existing machine learning and statistical methods. As the genotype data generally contains many markers, calculating self-attention in a GPU will require a large amount of GPU memory that may not be available. Our feature selection step in the model

addresses the memory issue of the transformer method. This step reduces the number of markers and identifies the biologically relevant markers for a specific phenotype. We also applied genotype frequency-based encoding for each genotype. This encoding performs better when combined with the transformer. If a large amount of data is available, the number of transformer encoder blocks can be increased which may increase the overall performance.

Preamble: Chapter 5

The expanse of genomic science has not only allowed the sequencing of DNA of organisms but has provided a tool to monitor gene expressions over time. Ribonucleic acid (RNA) sequencing, has emerged as a new technology applicable to studies that investigate alterations in transcriptional patterns in response to treatment. Messenger RNA (mRNA), intended for translation into proteins, is isolated and sequenced to measure alterations in gene expression. This is particularly well suited for studies involving plant disease in contrast with the non-infected state.

Barley resistance sources for FHB, generally have poor agronomics (extreme heading, tall stature) and have poor malting quality. Lack of adequate resistance has driven breeders to explore the use of *in vitro* selection (selection on growth media with mycotoxins) to identify novel resistance. The value of this approach is that somaclonal variants can be selected from a parental line in a single generation. Therefore, variants with resistance to mycotoxin accumulation can be identified, with suitable malting quality. Several resistant genotypes had been identified at the Agriculture and Agri-Food Canada, Brandon Research and Development Centre. The exact mechanisms of *in vitro* selection are not well understood. A breeding line selected through this method from malting variety CDC Kendall was registered as Norman, which is the subject of the following chapter.

Dynamic shifts in populations of *Fusarium graminearum* have been observed in North America. It has been observed that the 3ADON form has largely replaced traditionally isolated 15ADON chemotypes in western Canada. Likewise, NIV-producing forms are common in regions of the southern United States. Population shifts may be the result of adaptive advantage, and 3ADON forms have been documented to produce higher levels of DON content during infection.

The current study employed RNA-sequencing methodologies to evaluate complex patterns of gene expression in barley under infection with three chemotypes of *F. graminearum*.

CHAPTER 5

Transcriptome alterations of an *in vitro*-selected, moderately resistant, two-row malting barley in response to 3ADON, 15ADON, and NIV chemotypes of *Fusarium graminearum*.

Tucker, J.R., Legge, W.G., Maiti, S., Hiebert, C.W., Simsek, S., Yao, Z., Xu, W., Badea, A. Fernando, W.G.D. 2021. Transcriptome alterations of an *in vitro*-selected, moderately resistant, two-row malting barley in response to 3ADON, 15ADON, and NIV chemotypes of *Fusarium graminearum*. *Front Plant Sci.* 12: 701969. doi: 10.3389/fpls.2021.701969 (IF=5.753)

5.1 Abstract

Fusarium head blight caused by *Fusarium graminearum* is a devastating disease of malting barley. Mycotoxins associated with the contaminated grain can be transferred from malt to beer and pose a health risk to consumers. In western Canada, *F. graminearum* has undergone an adaptive shift from 15ADON constituency to dominance by virulent 3ADON-producers; likewise, NIV-producers have established in regions of southern United States. The lack of adapted resistance sources with adequate malting quality has promoted the use of alternative breeding methodologies, such as *in vitro* selection. We studied the low-deoxynivalenol characteristic of *in vitro* selected, two-row malting barley variety ‘Norman’ by RNAseq in contrast to its parental line “CDC Kendall,” when infected by 15ADON-, 3ADON-, and NIV-producing isolates of *F. graminearum*. The current study documents higher mycotoxin accumulation by 3ADON isolates, thereby representing an increased threat to barley production. At 72–96-h post-infection, significant alterations in transcription patterns were observed in both varieties with pronounced upregulation of the phenylpropanoid pathway and detoxification gene categories (UGT, GST, CyP450, and ABC), particularly in 3ADON treatment. Defense response was multitiered, where differential expression in ‘Norman’ associated with antimicrobial peptides (thionin 2.1, defensin non-specific lipid-transfer protein) and stress-related proteins, such as late embryogenesis abundant proteins, heat-shock, desiccation related, and a peroxidase (*HvPrx5*). Several gene targets identified in ‘Norman’ would be useful for the application of breeding varieties with reduced deoxynivalenol content.

Keywords: malt, Fusarium head blight, *Fusarium graminearum*, RNA-Seq, *in vitro* selection, deoxynivalenol

5.2 Introduction

Fusarium head blight (FHB), caused by *Fusarium graminearum* Schwabe [teleomorph: *Gibberella zeae* (Schwein.) Petch], is an economically important disease of barley (*Hordeum vulgare* L.) that has resulted in massive commercial losses (Wilson et al., 2018). *Fusarium graminearum* is capable of producing multiple, type B trichothecene mycotoxins [Deoxynivalenol (DON), 3-acetyldeoxynivalenol (3ADON), 15-acetyldeoxynivalenol (15ADON), nivalenol (NIV), and 4-acetylnivalenol (4-ANIV)], which can be commonly detected in contaminated grain (Miller et al., 1991). Type B trichothecenes are harmful to eukaryotic organisms through the interference of ribosomal function and protein translation (Desjardins, 2006). Mycotoxin content in grain is highly scrutinized by the malting and brewing industries due to their potential human health concerns. Arising from their water-soluble nature, mycotoxins, such as DON, can be transferred from malt to beer (Habler et al., 2017), which may be of elevated concern for the increasingly popular all-malt, craft brewing industry (Peters et al., 2017). Extremely strict, industrial limits on mycotoxins ($<0.5 \text{ mg kg}^{-1}$) commonly result in loss of premiums, which would be received below prime sale value in the lucrative malting barley market.

The immune reaction has not been reported within barley, and resistance sources are extremely limited (Bai and Shaner, 2004). Furthermore, resistance breeding of malting barley through back-cross methodologies has been difficult due to strict requirements of numerous quality parameters. *In vitro* selection (IVS) of somaclonal variations, which employs tissue culture and growth media, containing selective agents, offers an alternative option, for example, to rapidly improve disease resistance in crops without linkage drag (Rao and Sandhya, 2016). This method is an acceptable biotechnological tool based on changes resulting from internal variations/mutations. Examples of IVS, using fusaric acid or mycotoxins as selective agents in barley (Chawla and Wenzel, 1987) and wheat (Eudes et al., 2008) have been reported. ‘Norman’ is an IVS two-row, malting variety developed *via* anther culture, using media,

containing 1.71-mg kg⁻¹ DON, which displays reduced DON accumulation, without change in agronomics and malting quality. Based on DON content, it was evaluated with a moderately resistant reaction to FHB in the Western Cooperative Two-Row Barley Registration Test in 2005 and 2006, as part of the western Canadian registration recommending process under the auspices of the Prairie Registration Recommending Committee for Oat and Barley (Legge et al., 2011).

Several studies on wheat have indicated that 3ADON-producers are more aggressive and generally produce more DON than 15ADON-producers (Ward et al., 2008; Puri and Zhong, 2010; von der Ohe et al., 2010). Field-based studies of barley correlate 3ADON with higher DON content, implicating chemotype-associated virulence (Clear et al., 2013; Tucker et al., 2019). In a pan-genome study of 60 North American *F. graminearum* isolates, Kelly and Ward (2018) confirmed signatures of selection in the *TRI* (trichothecene) gene cluster region, signifying the importance of this region in genomic divergence. Moreover, analysis of gene expression through RNA-sequencing (RNA-Seq) further demonstrated differential gene expression between 3ADON and 15ADON-producers, notably in metabolic function and trichothecene production (Walkowiak et al., 2015; Puri et al., 2016). Amarasinghe and Fernando (2016) observed elevated *TRI* gene expression in 3ADON-producers in contrast to 15ADON or NIV strains during infection of wheat. Al-Taweel et al. (2014) documented differential transcription patterns in wheat challenged by 15ADON or 3ADON strains, where 3ADON-producers suppressed host resistances. Comparative studies can help elucidate underlying mechanisms of aggressiveness and contribute toward the understanding of plant defense response.

Fusarium graminearum displays a complex lifestyle (hemi-biotrophism), where it initially lives in a biotrophic stage, asymptotically within the apoplast for a few days, followed by a prolific stage under a switch to necrotrophy accompanied by elevated mycotoxins used to kill host tissues for consumption (Trail, 2009). DON is not essential for infection, as demonstrated by loss-of-function trichothecene non-producing mutants (*TRI5*-), which are still capable of infecting barley (Jansen et al., 2005; Boddu et al., 2007). Yet, DON is a virulence factor where

DON-expressing wild-type strains cause higher disease severity than *TRI5*- mutants (Boddu et al., 2007). A critical phase of host-response occurs at ~72 h post-infection (hpi), associated with an upsurge in the rate of DON production (Boddu et al., 2006, 2007). A study of *F. graminearum* displays a reciprocal peak of gene activity at 72 hpi, where genes of cell wall-degrading enzymes and trichothecene biosynthesis are highly expressed (Güldener et al., 2006). This unique phase of the host-pathogen interaction is distinguished by pathogen transition to necrotrophy.

While quantitative disease resistance may contribute to the deployment of durable varieties, this form of resistance remains poorly understood (Poland et al., 2009). Herein, we study complex disease resistance through a contrast of moderately resistant (MR) variety ‘Norman’ to its parent line ‘CDC Kendall’ (intermediately resistant, MR-MS), which carries both resistant and susceptible features. Compared with major-gene resistance, less is known about mechanisms of quantitatively inherited disease resistance. The transcriptome differentiation landscape would provide insight into the FHB resistance responses. Although in recent years, RNA-Seq has become a powerful tool for the identification of genes associated with differential transcription patterns (Wang et al., 2009), this technology has not yet been applied to analyze the resistance response of two-row barley to *F. graminearum*. The objective of this study was to detect and quantify transcriptional differences between moderately resistant, *IVS* variety “Norman,” and its intermediately FHB resistant parent variety ‘CDC Kendall’ when challenged by differentially virulent *F. graminearum* chemotypes. Together, the study provides insight into the expression of genomic features of DON resistance in ‘Norman’ and its interaction with pathogens characterized by differential production of secondary metabolites, such as trichothecenes.

5.3 Materials and Methods

5.3.1 Long-Term DON Content Evaluation of ‘Norman’ vs. ‘CDC Kendall’

The varieties evaluated in this study were: ‘Norman’ and ‘CDC Kendall’. ‘Norman’ is an *IVS* variety developed under a joint release between Agriculture and Agri-Food Canada,

Brandon Research and Development Centre (AAFC-Brandon), and Crop Development Centre (CDC), University of Saskatchewan (Legge et al., 2011). ‘Norman’ was derived from “CDC Kendall,” a malting variety developed by CDC (<https://beerandbrewing.com/dictionary/14mIxiZeCz/> accessed January 13, 2022). The data used for this evaluation were generated for 20 years, from 2001 to 2020. Briefly, the varieties were grown in 0.9-m rows in an irrigated FHB nursery at AAFC-Brandon, MB, as outlined in Legge et al. (2011). DON content was determined on a 20-g subsample of matured grain that was ground, using a Perten 3600 laboratory mill. From this, a 1-g sample was used in the assay of DON content determination, following the enzyme-linked immunosorbent assay (ELISA) method (Sinha and Savard, 1996). The least-squares means were calculated for DON content of each variety within the site year. To normalize data, a logarithmic transformation (LOG_{10}) was applied to the DON data before analysis. A matched pairs procedure was applied to test for the mean difference of DON content between varieties (SAS JMP® 13.2.1, SAS Institute Inc. Cary, NC, USA, SAS Institute Inc, 1989-2019).

5.3.2 Genotyping

Approximately 100 mg of fresh leaf tissue was sampled from seedlings of each variety, flash-frozen in liquid N_2 , and then freeze-dried. DNA was extracted, using Qiagen, DNeasy 96 Plant Kit (Qiagen, Canada), as per the handbook of the manufacturer, and then normalized to 50 μl ml^{-1} . Samples were genotyped with an Illumina iScan (Illumina, San Diego, CA, USA), using an Infinium HTS iSelect–50 K SNP custom microarray (Bayer et al., 2017). Chromosome graphics were produced, using the R package “chromoMap” (Anand and Rodriguez Lopez, 2020).

5.3.3 Fungal Cultures

Three chemotypes were chosen to be used in this study: 15ADON, 3ADON, and NIV, with four single-spore isolates per chemotype group. The *F. graminearum* isolates were obtained from the Department of Plant Science, University of Manitoba, and AAFC-Brandon. Chemotypes of isolates were determined by *TRI3* and *TRI12* gene-specific PCR assays as

detailed by Amarasinghe et al. (2019) (Appendix 3.1). Isolates were initially grown on 10-cm diameter potato-dextrose agar (PDA) solid growth media plates and grown to capacity (16-h light: 8-h dark) at 20°C over 2 weeks. For the production of liquid media, tomatoes were cut into 1-cm³ cubes, and 900 g were placed in 9 L of distilled water for 3 h and then strained through cheesecloth. Fifteen grams of NaCl l⁻¹ were dissolved in the filtrate and autoclaved for 20 min. One PDA culture plate from each isolate was cut into 1-cm³ cubes, added to 500 ml of suspension in 1 l flasks, and agitated on an orbital shaker for 2 weeks under natural light at room temperature. The suspension was strained through cheesecloth, and then conidia spores were counted under 40X magnification, using a hemocytometer.

5.3.4 Growth Cabinet—Experimental Design

For ‘Norman’ and “CDC Kendall,” two seeds of the same source were planted per pot (a 20-cm diameter; 30-cm depth), containing a Pro-Mix Mycorrhizae growing medium (Premier Horticulture Inc., Quakertown, PA, USA) and 15 g of slow-release granular fertilizer (18-6-8 NPK). One plant was removed from each pot, following germination. Plants were grown in a growth chamber with a 16-h light at 16°C: an 8-h dark at 18°C cycle. Starting at the fourth-week post-seeding, plants were fertilized with 20 g l⁻¹ of (20-20-20 NPK) and every 2 weeks thereafter.

Macroconidia suspensions of *F. graminearum* were diluted to 5 × 10⁴ spores ml⁻¹ and mixed by chemotype with Tween20® at 0.2% vol/vol. Mock inoculation treatment included identical suspension treatment but excluded fungus. Once spikes had emerged, 75% of three spikes were gently fully removed from the leaf sheath and entirely sprayed on either side until run-off, using an atomizer under 70 kPa of pressure. Following inoculation, spikes were covered by a translucent glycine bag for up to 96 h.

A total of three biological replicates were grown per treatment and time point. Pots were arranged in the growth cabinet in a split-plot design where the treatment group (3ADON, 15ADON, NIV, or Mock inoculated) represented the main plot treatment (Figure 5.1). Within each main treatment, pots were completely randomized for the position of variety and time

point sampling (72 or 96 hpi). All plants were grown together in the same chamber. For both time points (72 or 96 hpi), spikes were cut at the peduncle at the same time of the day, wrapped in aluminum foil, and flash-frozen by submerging in liquid N₂ and then held at –80°C until processed further. The remaining spikes were rated for the proportion of diseased kernels at 3 weeks post-infection (diseased kernels/total kernels, Appendix 3.2). Plants were grown to maturity in the growth cabinet, after which spikes were harvested for mycotoxin assay. All kernels from two spikes per variety/treatment/replicate were ground together using a handheld coffee grinder to uniform flour consistency, and then subsampled to 1 g. All mycotoxins were assayed at North Dakota State University by gas chromatography-mass spectrometry (GCMS) or in the case of deoxynivalenol-3-O-glucoside (DON3G) by the quad time of flight (QTOF).

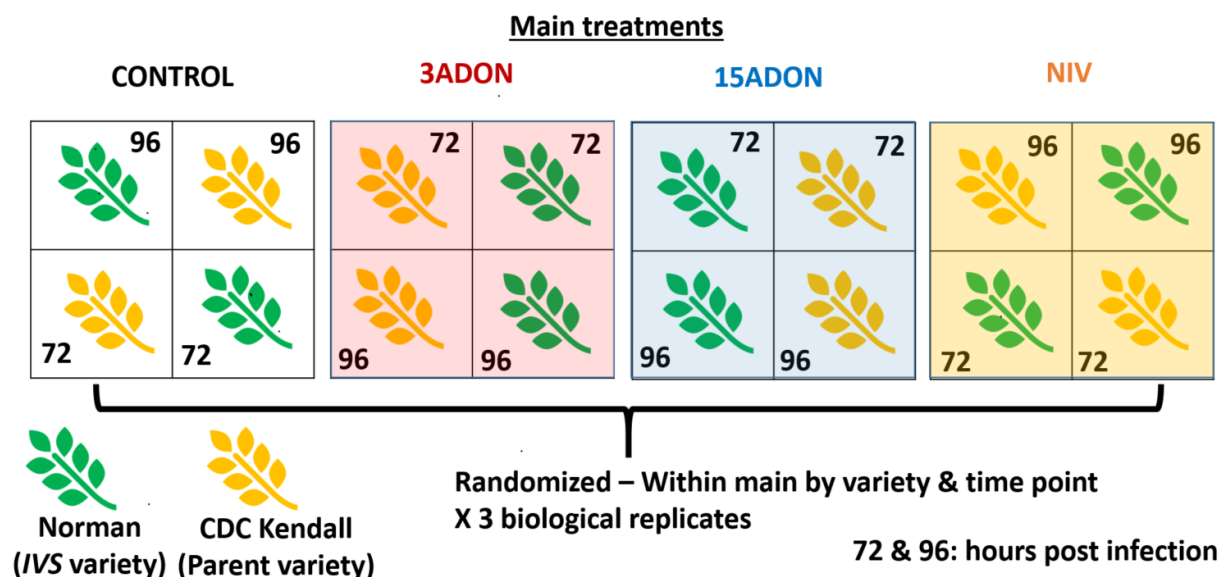


Figure 5.1. Split-plot experimental design. Main plot treatments: Control, mock control; 3ADON, 3-acetyldeoxynivalenol producer; 15ADON, 15-acetyldeoxynivalenol producer; NIV, nivalenol producer. Genotype (Norman or CDC Kendall) and sampling time points (72- and 96-h post-infection) were randomized within the main plot.

5.3.5 Statistical Analysis of FHB and Mycotoxin Content

Data were analyzed by the generalized linear mixed model (GLMM) approach. The proportion of *Fusarium* damaged kernels (FDK) was analyzed by PROC GLIMMIX in SAS® 9.4 (SAS Institute Inc., Cary, NC, USA, SAS® 9.4, SAS Institute Inc, 2013) by using a beta distribution and logit link function to test for significant differences in the proportion FDK. Likewise, PROC GLIMMIX was used to test for differences in mycotoxin content, under the lognormal distribution identity link function. Variety and chemotype treatment were considered fixed, while replicate was considered a random factor. Tukey-Kramer honestly significant difference was used to test all pairwise contrasts.

5.3.6 Total RNA Extraction and Quality

Spikes were removed from the freezer, and six kernels from the mid-spike region were rapidly dissected from the rachis, pooled, and ground to powder under liquid N₂ *via* mortar and a pestle. Total RNA was isolated by the RNAeasy Plant Mini Kit (Qiagen, Canada) as per the instructions of the manufacturer. A total of 100 mg of sample powder was used as input, followed by the RLT buffer protocol. Total RNA was first checked for quality control and determining the purity of samples, using a 2100 Agilent Bioanalyzer™ (Agilent Technologies, Santa Clara, CA, USA), where RIN scores of > 7.5 were accepted.

5.3.7 RNA-Seq Library Preparation and Sequencing and Quality

The cDNA libraries were constructed with Illumina TruSeq RNA v3 adaptors, using the Illumina first-strand TruSeq® mRNA library Prep Kit (Illumina, San Diego, CA) protocol as per instructions of the manufacturer. Briefly, this method purifies poly (A) mRNA from total RNA through applications of oligo (dT)-attached magnetic beads, followed by cDNA synthesis. Samples were sequenced on an Illumina HiSeq 4000 platform (100 bp paired-end) libraries. RNA-sequencing was conducted at the Centre de Services et d'Expertises Génome Québec (Montréal, QC, Canada). Fastq files for each sample were evaluated on an individual basis, using FastQC v0.11.5 software *via* visual graphic inspection (Andrews, 2010). Raw data

were processed, using Trimmomatic v0.36 software (Bolger et al., 2014) to remove adaptors and trim low-quality reads.

5.3.8 Sequence Alignment and Genomic Feature Evaluation

The IBSC_v2 (International Barley Sequencing Consortium) release of the barley reference genome (Mascher et al., 2017) and GTF file structural high confidence (HC) gene predictions assembly were downloaded through file transfer protocol (FTP) from the Institute of Plant Genetics and Crop Plant Research (IPK) Gatersleben (<https://galaxy-web.ipk-gatersleben.de/> accessed January 13, 2020). HISAT2 v2.0.4 software (Kim et al., 2015) was used to map clean reads to the reference genome (Appendix 3.4).

Software package StringTie (Pertea et al., 2015) was utilized to approximate the gene expression levels in each sample from the BAM files. The stringTie-merge procedure was used to input and combine lists of transcripts aligned to reference gene annotation list and newly assembled genes/transcripts. After the HC genes and newly assembled genes/transcripts were merged, the expression and the coverage at all these annotations were estimated. Coverage was converted to raw reads counts by a python script within a StringTie package. To annotate those newly assembled genes by StringTie, the protein sequences were extracted in a fasta format from the genome reference, using gene coordinates by a perl script. The protein fasta files were searched against a local NCBI nr database by the blastp program. A total of 52,266 genomic features were included in the gene matrix for expression analysis.

Package “edgeR” (Robinson et al., 2010) within the R statistical environment (R Core Team, 2020) was used for group comparison statistical analyses. The raw read counts of all samples were loaded as a gene matrix into “edgeR” and normalized. Factors included: genotype (‘Norman’ vs. ‘CDC Kendall’), treatment (Mock, 15ADON, 3ADON, or NIV), and time (72 or 96 hpi). Generalized linear models (Appendix 3.5) were applied both as: (i) pairwise single-factor contrasts or (ii) additive model design (a three-factor model with interaction) *via* likelihood ratio test (LRT). Identified differentially expressed genes (DEG)

were examined regarding the log-fold change, counts per million, and significance. Genes were determined to be significantly differentially expressed if they had a log₂-fold change >1 or < -1 and a false discovery rate ($FDR \leq 0.05$). Gene interactions of DEGs between groups were examined, using Venn diagrams, using a web-based tool (<https://bioinformatics.psb.ugent.be/webtools/Venn/> accessed January 13, 2022).

Gene ontology annotation files were downloaded from IPK Gatersleben via FTP. Genes were assigned GO terms under categories: a biological process, cellular component, or molecular function. Significant GO terms ($FDR \leq 0.05$) in each contrast were identified through GO enrichment analysis, using AgriGO v2.0 (Tian et al., 2017). ShinyGO v0.61 software (Ge et al., 2020) was used to visually evaluate relationships of DEGs in hierarchical clustering tree graphics for significant pathways where networks of GO terms were mapped, using *Arabidopsis thaliana* STRING-db v.10 with a.2 edge cut-off.

5.4 Results

5.4.1 Norman and Its Response to DON Accumulation

‘Norman’ demonstrated a significant reduction of DON content in contrast with ‘CDC Kendall’ ($P = 0.013$). ‘Norman’ possessed lower DON content than ‘CDC Kendall’ in most years [79% (15/19), averages not shown] of evaluation. DON levels varied considerably over years, ranging from 6–45 mg kg⁻¹. Reduction of DON levels in ‘Norman’ was most prominent in epidemic years characterized by high-DON production (> 10 mg kg⁻¹; Figure 2).

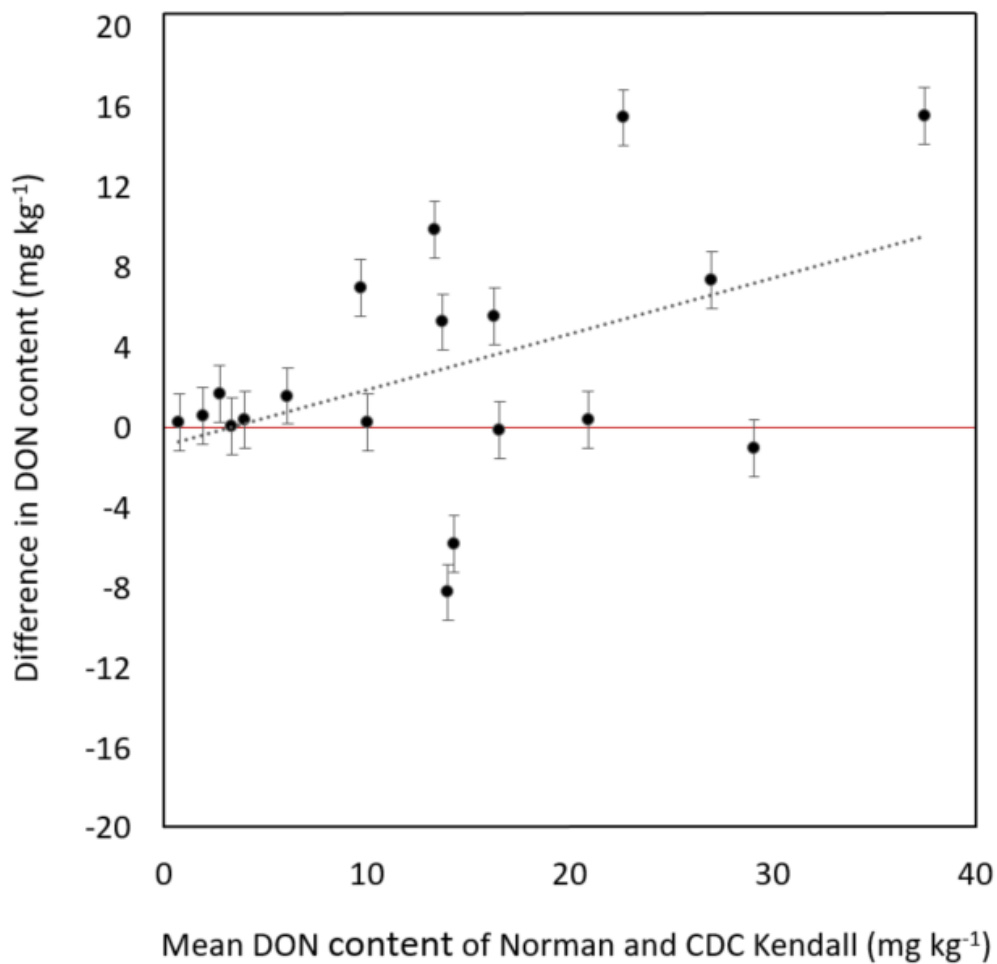


Figure 5.2. Difference in deoxynivalenol (DON) content (mg kg^{-1}) of moderately resistant ‘Norman’ from ‘CDC Kendall’ in head-to-head contrast with over 19 site years tested in an artificially inoculated disease nursery at Brandon, MB, displayed in order of mean DON content of ‘Norman’ and “CDC Kendall.” Error bars represent the standard error of difference.

The proportion of FDK was subjected to an analysis of variance. No difference in visual disease symptoms was observed between ‘Norman’ and ‘CDC Kendall’ ($P = 0.803$, Appendix 3.6). The proportion of FDK was significantly affected by chemotype treatment ($P = 0.015$), without interaction between variety and chemotype treatment ($P = 0.64$). The 3ADON treatment showed the highest disease severity (%) at 29.8 ± 4.7 , followed by NIV treatment at 18.9 ± 3.0 and 15ADON treatment at 13.3 ± 3.3 (Appendix 3.7). Tukey-Kramer group separation indicated that 3ADON treatment was found to display higher disease than 15ADON, but not NIV (Appendix 3.8). Likewise, 15ADON did not differ from NIV treatment.

The 3ADON strain demonstrated significantly elevated overall mycotoxin levels in contrast with 15ADON and NIV chemotypes (Table 5.1). The chemotype treat was significant ($P = 0.01$, Appendix 3.9). Effects of variety and interaction term variety x treatment were non-significant ($P > 0.05$). The 3ADON chemotype produced a 10-fold higher toxin in contrast with the alternative chemotypes (Table 5.1). The 15ADON and NIV chemotype produced similar levels of mycotoxin, respectively, i.e., DON = $27.4 \pm 8.6 \text{ mg kg}^{-1}$; NIV = $39.5 \pm 9.5 \text{ mg kg}^{-1}$ averaged over genotypes. The ratio of DON3G/DON trended higher in 15ADON (0.27 ± 0.1) vs. 3ADON (0.19 ± 0.1) chemotype, but this was non-significant ($P > 0.05$). The ratio did not differ between varieties within 15ADON or 3ADON treatment ($P > 0.05$).

A comparison of SNP markers of 50K SNP array between ‘Norman’ and ‘CDC Kendall’ revealed a level of variance between the varieties (98% SNP marker allele agreement, Appendix 3.10). Distributions of SNP variants were examined, where regions of three chromosomes were identified: chromosome 1H: (483–512 Mb); intermittently over regions displayed for chromosomes 4H (600–616 Mb) and 5H (621–630 Mb). Variant regions were observed on both short and long arms of chromosome 7H at multiple positions (Appendix 3.11).

Table 1. Mean $\pm$ standard error of mycotoxin content of matured barley grains for grains harvested from growth cabinet study (replication, n = 3).

Variety	Treatment ^I	DON ^{II}	NIV ^{II}	D3G ^{III}	Ratio D3G/DON
CDC Kendall	15ADON	28.1 $\pm$ 14.6 ^A	0.0 $\pm$ 0.0	5.6 $\pm$ 1.7	0.27 $\pm$ 0.1
	3ADON	207.4 $\pm$ 33.0 ^B	0.5 $\pm$ 0.1	45.3 $\pm$ 13.1	0.21 $\pm$ 0.0
	NIV	1.8 $\pm$ 0.7	36.5 $\pm$ 9.6 ^A	1.1 $\pm$ 0.9	0.54 $\pm$ 0.4
	Mock control	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	-
Norman	15ADON	26.7 $\pm$ 12.5 ^A	0.0 $\pm$ 0.0	4.6 $\pm$ 1.4	0.27 $\pm$ 0.1
	3ADON	238.5 $\pm$ 31.6 ^B	0.7 $\pm$ 0.5	40.6 $\pm$ 10.2	0.17 $\pm$ 0.0
	NIV	2.9 $\pm$ 2.2	42.5 $\pm$ 18.6 ^A	0.9 $\pm$ 0.5	0.50 $\pm$ 0.1
	Mock control	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	-

^I Tukey Grouping for Treatment Least-squares Means ($\alpha=0.05$). LS-means with the same letter are not significantly different for primary toxin – deoxynivalenol (DON) or nivalenol (NIV); ^{II} Mycotoxin content (mg kg⁻¹) of deoxynivalenol (DON) or nivalenol (NIV) as measured by gas chromatography-mass spectrometry (GC-MS); ^{III} Mycotoxin content (mg kg⁻¹) of deoxynivalenol-3-glucoside (D3G) measured by Quad time of flight (QTOF).

5.4.2 Transcriptome Differentiations

On average, sequencing produced 64×10^6 reads per library (Appendix 3.12). Following trimming, an average of 90% of cleaned reads per sample mapped to the IBSC_v2 reference genome (Appendix 3.4). Graphics produced by FastQC indicated that all sample libraries passed quality standards (Appendix 3.13).

5.4.3 Differential Genes of CDC Kendall

As summarized in Table 5.2, overall, a total of 2,973 unique DEGs were identified through contrasts of all *Fusarium* treatments (3ADON, 15ADON, NIV) to a respective control group over both time points (72, 96 hpi). Analyses under three-factor additive GLMs for *Fusarium*-treatment groups were conducted to contrast *Fusarium*-treatment groups with the mock control group. Differentially expressed genes (1,635 total unique DEGs) were identified for the three *Fusarium* treatment groups. On examination, groups of *Fusarium*-responsive DEGs (215)

with shared expression patterns were identified for all *Fusarium* treatments (Treatment intersection–15ADON $\cap$ 3ADON $\cap$ NIV; Figure 5.3), indicating common response in gene groups. A selection of DEGs commonly induced in the *Fusarium* treatments was displayed (Table 5.3).

Table 5.2. Numbers of differentially expressed genes (FDR < 0.05) identified in contrasts by chemotype: CDC Kendall vs. control; by variety Norman vs. CDC Kendall; by time point 72 vs 96 hours post-infection (hpi). Generalized linear models included: i) paired single factor or ii) additive, interactive, three-factor.

		i) Paired, single-factor contrasts						ii) Additive, interactive contrasts		
<i>Fusarium_vs_control</i> (CDC Kendall)		3ADON		15ADON		NIV		<i>Fusarium_vs_control</i>		
		72	96	72	96	72	96	3ADON	15ADON	NIV
	Up regulated	763	1022	846	122	434	556	400	842	914
	Down regulated	353	473	561	289	91	162	257	72	165
	Total	1116	1495	1407	411	525	718	657	914	1079

<i>Norman_vs_CDC Kendall</i>		3ADON		15ADON		NIV		<i>Norman_vs_CDC Kendall</i>		
		72	96	72	96	72	96			
	Up regulated	77	85	964	884	116	14	53		
	Down regulated	447	892	136	435	120	12	30		
	Total	524	977	1100	1319	236	26	83		

<i>96_vs_72 hpi</i>		3ADON		15ADON		NIV		<i>96_vs_72 hpi</i>		
		CDC Kendall		CDC Norman		CDC Kendall				
	Up regulated	478	672	1111	411	934	590	181		
	Down regulated	17	145	734	481	76	33	12		
	Total	495	817	1845	892	1010	623	193		

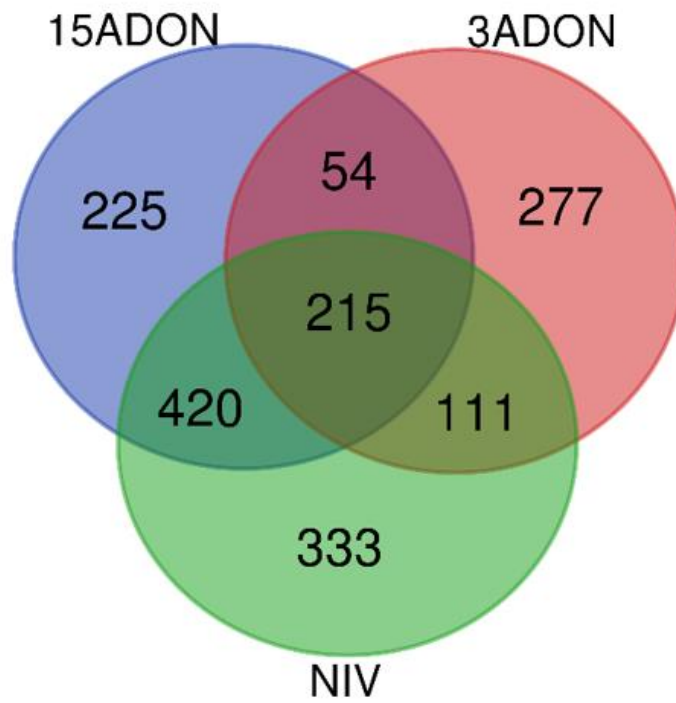


Figure 5.3. A Venn diagram of differentially expressed genes ($FDR \geq 0.05$) for contrasts of *Fusarium* treatment (3ADON, 15ADON, and NIV chemotypes) vs. mock control summarised for both time periods under the additive generalized linear model (GLM).

Table 5.3. Transcript alterations selected for greater fold change and significance level of upregulated genes expressed within *Fusarium graminearum* chemotype:15ADON, 3ADON, and NIV treatments contrasted with mock control identified in the additive, interactive generalized linear model.

Gene	Annotation	15ADON			3ADON			NIV		
		logFC	logCPM	FDR	logFC	logCPM	FDR	logFC	logCPM	FDR
HORVU2Hr1G104410	Ubiquitin 7	-3.2	6.1	3.1E ⁻¹⁹	-3.0	6.1	2.6E ⁻¹¹	-5.0	6.1	9.7E ⁻³¹
HORVU2Hr1G107100	Protein translation factor <i>SUI1</i> homolog 1	-2.3	6.5	1.3E ⁻¹⁰	-2.0	6.5	3.9E ⁻⁰⁶	-3.3	6.5	4.3E ⁻¹⁵
HORVU5Hr1G047150	UDP-Glycosyltransferase (<i>HvUGT13248</i>)	-2.4	6.1	9.7E ⁻¹²	-1.5	6.1	1.5E ⁻⁰⁴	-2.8	6.1	1.3E ⁻¹²
HORVU5Hr1G103770	Alpha/beta-Hydrolases protein	-2.1	5.1	1.8E ⁻⁰⁹	-1.1	5.1	8.1E ⁻⁰³	-2.4	5.1	1.3E ⁻¹⁰
HORVU3Hr1G065320	ABC transporter B member 4	-2.0	6.5	1.4E ⁻⁰⁸	-1.1	6.5	2.0E ⁻⁰²	-2.5	6.5	1.4E ⁻¹⁰
HORVU2Hr1G107860	Histone-lysine N-methyltransferase	-1.6	5.7	7.4E ⁻⁰⁶	-1.8	5.7	1.3E ⁻⁰⁶	-2.1	5.7	4.2E ⁻⁰⁹
HORVU5Hr1G056280	ABC transporter G family member 53	-2.2	6.6	2.3E ⁻⁰⁹	-1.5	6.6	2.0E ⁻⁰⁴	-1.9	6.6	3.1E ⁻⁰⁶
HORVU5Hr1G124650	ABC transporter G family member 48	-2.0	6.7	2.1E ⁻⁰⁸	-1.1	6.7	1.7E ⁻⁰²	-2.4	6.7	8.9E ⁻¹⁰
HORVU3Hr1G071470	ABC transporter G family member 32	-2.1	6.2	7.9E ⁻¹⁰	-1.2	6.2	8.2E ⁻⁰³	-2.2	6.2	6.8E ⁻⁰⁹
HORVU7Hr1G092510	ABC transporter G family member 44	-2.3	5.9	2.4E ⁻¹¹	-1.1	5.9	6.6E ⁻⁰³	-2.0	5.9	1.4E ⁻⁰⁷
HORVU4Hr1G026340	Transporter	-2.0	5.9	1.2E ⁻⁰⁸	-1.4	5.9	8.6E ⁻⁰⁴	-2.0	5.9	6.2E ⁻⁰⁸
HORVU1Hr1G047030	Cytochrome P450 protein	-2.2	6.1	5.3E ⁻¹⁰	-1.1	6.1	1.8E ⁻⁰²	-2.0	6.1	2.0E ⁻⁰⁷
HORVU4Hr1G067450	Sugar transporter protein 7	-2.0	6.2	1.3E ⁻⁰⁸	-1.1	6.2	2.4E ⁻⁰²	-2.3	6.2	8.4E ⁻¹⁰
HORVU5Hr1G052150	Indole-3-glycerol phosphate synthase	-2.1	6.3	5.8E ⁻⁰⁹	-1.1	6.3	2.0E ⁻⁰²	-2.0	6.3	1.2E ⁻⁰⁷
HORVU1Hr1G019750	Laccase-19	-1.8	6.6	5.8E ⁻⁰⁷	-1.2	6.6	1.0E ⁻⁰²	-2.0	6.3	8.6E ⁻⁰⁸
HORVU1Hr1G019740	Laccase-19	-1.8	6.2	3.2E ⁻⁰⁷	-1.2	6.2	6.8E ⁻⁰³	-2.1	6.2	2.9E ⁻⁰⁸
HORVU3Hr1G097860	Laccase-19	-1.7	6.3	6.7E ⁻⁰⁶	-1.1	6.3	1.1E ⁻⁰²	-2.0	6.3	8.6E ⁻⁰⁸
HORVU3Hr1G100190	Jasmonate O-methyltransferase	-1.8	5.2	1.9E ⁻⁰⁷	-1.1	5.2	9.5E ⁻⁰³	-2.1	5.2	2.8E ⁻⁰⁹
HORVU2Hr1G124930	Peroxidase superfamily protein	-1.7	5.1	8.4E ⁻⁰⁷	-1.1	5.1	1.0E ⁻⁰²	-1.8	5.1	1.5E ⁻⁰⁷
HORVU5Hr1G071140	Cellulose synthase like E1	-1.9	5.0	1.3E ⁻⁰⁸	-1.0	5.0	2.6E ⁻⁰²	-1.7	5.0	8.8E ⁻⁰⁶
HORVU5Hr1G104580	UDP-Glycosyltransferase (<i>HvUGT14077</i>)	-1.8	5.7	5.4E ⁻⁰⁷	-1.0	5.7	4.1E ⁻⁰²	-1.9	5.7	4.3E ⁻⁰⁷
HORVU5Hr1G023730	Pathogenesis-related protein <i>STH-21</i>	-1.6	5.2	1.1E ⁻⁰⁵	-1.1	5.2	5.5E ⁻⁰³	-1.6	5.2	1.2E ⁻⁰⁵
HORVU2Hr1G089440	Phenylalanine ammonia-lyase 2 (<i>PAL 2</i>)	-1.4	5.6	4.6E ⁻⁰⁵	-1.0	5.6	3.2E ⁻⁰²	-1.5	5.6	3.5E ⁻⁰⁵
HORVU3Hr1G098160	CaLB domain family protein	-1.2	5.3	1.8E ⁻⁰³	-1.1	5.3	1.3E ⁻⁰²	-1.6	5.3	2.1E ⁻⁰⁵
HORVU3Hr1G100350	Xylanase inhibitor	-1.2	6.3	5.2E ⁻⁰³	-1.1	6.3	8.5E ⁻⁰³	-1.1	6.3	1.2E ⁻⁰²

logFC = log2 (fold change); logCPM = log2 (counts per million); FDR = False discovery rate, p-value adjusted by Benjamini Hochberg procedure.

The additive interactive model identified 30 DEGs associated with ‘CDC Kendall’ in contrast with “Norman,” which were characterized by patterns of a generalized resistance motif. ‘CDC Kendall’ displayed a predominant response in detoxification resistance (Table 5.4A). These genes were largely involved in defense response and intensified in their transcript abundance at 96 hpi. Genes associated with the JA/ETH pathway were elevated, including lipolytic enzyme GDSL esterase/lipase and augmented lignification response *via* lacasse 7. Several genes of oxidative-stress response were identified: GST (glutathione S-transferase), CyP450 (Cytochrome P450), and *CBSX1* (chloroplastic, redox homeostasis). Two DUF239 carboxyl-terminal proteinase-like proteins were differentially expressed (putative *DD1A*—receptor death domain protein (*Oryza sativa*japonica group)], involved in the phagocytosis of apoptotic cells). DEGs were identified as associated with PR protein major pollen allergen Bet v 1 (*PR10*). Two Bowman-Birk-type trypsin inhibitors were identified. Differential expression patterns were displayed for receptor genes involved in pathogen recognition.

Table 5.4. (A) Transcript alterations in genes differentially expressed in ‘CDC Kendall’ identified in the additive, interactive generalized linear model. **(B)** Transcript alterations in genes differentially expressed in ‘Norman’ are identified in the additive, interactive generalized linear model.

Table 5.4-A. Transcript alterations in gene expression in ‘CDC Kendall’ in relation to ‘Norman’.

Gene_ID	Annotation [alternate species, NCBI-ProteinID]	logFC	logCPM	FDR
HORVU1Hr1G008360	Laccase 7	-2.1	3.4	3.3E ⁻⁰⁹
-	Amidase 1-like [A. tauschii, XP_020159982]	-1.8	-0.2	4.6E ⁻⁰⁷
HORVU2Hr1G095460	Glutathione S-transferase family protein	-1.5	3.0	1.3E ⁻⁰⁴
HORVU2Hr1G125690	Protein of Unknown Function (DUF239)	-1.4	3.1	1.8E ⁻⁰⁴
HORVU4Hr1G016500	Tapetum determinant 1	-1.3	4.6	1.6E ⁻⁰⁴
HORVU3Hr1G035680	Flowering promoting factor 1	-1.3	-0.2	6.4E ⁻⁰³
HORVU1Hr1G044200	Serine carboxypeptidase-like 51	-1.2	3.7	9.1E ⁻⁰⁴
HORVU6Hr1G018670	Proline-rich protein, putative, expressed	-1.2	5.6	1.3E ⁻⁰⁴
-	Glycine/proline-rich protein [D. hygrometricum, KZV44779]	-1.2	-0.3	1.2E ⁻⁰²
HORVU1Hr1G038730	GDSL esterase/lipase	-1.2	6.1	1.1E ⁻⁰³
HORVU5Hr1G104800	Acyl-CoA N-acyltransferases (NAT) superfamily protein	-1.2	3.8	1.9E ⁻⁰²
HORVU4Hr1G054870	Major pollen allergen Bet v 1-F/I	-1.1	2.3	1.3E ⁻⁰²
HORVU0Hr1G005720	Pyruvate kinase family protein	-1.1	3.5	7.9E ⁻⁰⁴
-	Glutathione S-transferase [D. villosum, ABU56005]	-1.1	3.8	3.1E ⁻⁰³
HORVU1Hr1G021180	Glutathione S-transferase family protein	-1.1	2.7	2.7E ⁻⁰²
-	Os06g0133400, partial [O. sativa Japonica, BAS96003]	-1.0	3.5	6.0E ⁻⁰³
HORVU3Hr1G117370	Glutathione S-transferase family protein	-1.0	3.4	1.5E ⁻⁰²
HORVU4Hr1G080740	Protein of Unknown Function (DUF239)	-1.0	6.3	1.5E ⁻⁰²
HORVU2Hr1G004600	Cytochrome P450 superfamily protein	-1.0	4.6	2.3E ⁻⁰²
HORVU5Hr1G047990	invertase inhibitor [C. sativus, XP_011660336]	-1.0	3.0	4.6E ⁻⁰²
HORVU7Hr1G002010	Leucine-rich repeat receptor-like protein kinase protein	-0.9	2.1	4.2E ⁻⁰²
-	Synaptic vesicle 2-related protein [T. urartu, EMS56043]	-0.9	2.8	4.5E ⁻⁰²
-	Os05g0341100 [O. sativa Japonica, BAS93469]	-0.9	4.6	2.2E ⁻⁰²
HORVU6Hr1G014600	Bowman-Birk type trypsin inhibitor	-0.9	3.9	2.7E ⁻⁰²
HORVU0Hr1G025520	Leucine-rich repeat receptor-like protein kinase protein	-0.9	3.8	4.1E ⁻⁰²
HORVU7Hr1G002930	Disease resistance protein (CC-NBS-LRR class)	-0.9	3.6	2.1E ⁻⁰²
HORVU1Hr1G068300	Somatic embryogenesis receptor kinase 1 (SERK1)	-0.9	4.7	4.6E ⁻⁰²
-	CBSX1 [A. tauschii, XP_020147453]	-0.8	3.2	4.0E ⁻⁰²
-	Pectinesterase inhibitor [H. vulgare, ACR61378]	-0.8	6.5	4.7E ⁻⁰²
HORVU0Hr1G000760	Proteinase inhibitor [T. aestivum, AAS49905]	-0.8	4.3	4.9E ⁻⁰²

logFC = log2 (fold change); logCPM = log2 (counts per million); FDR = False discovery rate, p-value adjusted by Benjamini-Hochberg procedure.

Table 5.4-B. Transcript alterations in gene expression in ‘Norman’ in relation to ‘CDC Kendall’.

Gene_ID	Annotation [alternate species, NCBI-ProteinID]	logFC	logCPM	FDR
HORVU3Hr1G075620	tRNA pseudouridine synthase D	0.8	3.9	4.2E ⁻⁰²
HORVU6Hr1G000680	Thionin 2.1	1.0	3.1	2.5E ⁻⁰²
HORVU6Hr1G000780	Thionin 2.1	1.0	4.5	7.1E ⁻⁰³
HORVU1Hr1G070310	Aldose reductase	1.0	6.2	3.5E ⁻⁰³
HORVU2Hr1G071890	Desiccation-related protein PCC13-62	1.1	5.1	4.8E ⁻⁰⁴
-	Protein <i>FAR1</i> -Related [<i>A. tauschii</i> , XP_020188798]	1.1	0.0	1.4E ⁻⁰²
HORVU1Hr1G087380	Protein of unknown function (DUF679)	1.1	4.0	4.5E ⁻⁰⁴
HORVU6Hr1G013580	F-box domain containing protein	1.2	3.4	3.3E ⁻⁰⁴
-	Zinc finger CCHC domain [<i>T. urartu</i> , EMS62713]	1.2	1.6	1.1E ⁻⁰²
HORVU4Hr1G006120	Splicing factor Prp18 family protein	1.2	1.5	3.4E ⁻⁰²
HORVU7Hr1G025460	Homeobox-leucine zipper protein ROC8	1.2	4.0	1.7E ⁻⁰⁴
-	Ribosomal protein L34Ae [<i>C. cardunculus</i> , KVI00286]	1.2	2.5	4.9E ⁻⁰⁴
HORVU7Hr1G082040	Late embryogenesis abundant protein D-34	1.2	3.1	1.7E ⁻⁰⁴
HORVU4Hr1G059260	Heat shock 70 kDa protein 3	1.3	6.1	3.2E ⁻⁰⁵
HORVU7Hr1G025490	Homeobox-leucine zipper protein <i>ROC8</i>	1.3	1.7	1.4E ⁻⁰³
HORVU1Hr1G079280	Late embryogenesis abundant protein 76	1.3	1.0	1.5E ⁻⁰³
HORVU5Hr1G091920	Copine family protein 2	1.3	1.2	1.5E ⁻⁰³
HORVU5Hr1G094680	Late embryogenesis abundant protein D-34	1.3	4.1	3.5E ⁻⁰⁵
HORVU4Hr1G005690	Antimicrobial peptides-like [<i>A. tauschii</i> , XP_020185846]	1.3	3.5	1.1E ⁻⁰⁴
HORVU7Hr1G025450	Homeodomain leucine zipper family IV protein	1.3	1.4	1.3E ⁻⁰³
HORVU7Hr1G064800	Unknown protein - Os07g0120700 [<i>O. sativa</i> , BAF20695]	1.4	0.2	4.9E ⁻⁰⁴
HORVU7Hr1G025480	Homeodomain leucine zipper family IV protein	1.4	0.7	1.1E ⁻⁰³
HORVU4Hr1G087780	Bifunctional inhibitor/LTP/seed storage 2S albumin	1.4	4.0	9.3E ⁻⁰⁷
HORVU2Hr1G077710	22.0 kDa class IV heat shock protein	1.4	3.9	3.4E ⁻⁰⁵
HORVU5Hr1G112540	Protein synthesis inhibitor II	1.5	6.9	6.6E ⁻⁰⁷
HORVU1Hr1G059900	Late embryogenesis abundant protein D-19	1.6	6.0	5.4E ⁻⁰⁷
HORVU7Hr1G083750	Protein kinase superfamily protein	1.8	2.3	3.3E ⁻⁰⁹
HORVU7Hr1G010680	Acid phosphatase 1	2.3	6.9	1.9E ⁻¹⁰
HORVU7Hr1G006260	Avenin-like a3	2.5	3.0	1.3E ⁻¹⁷
HORVU4Hr1G007680	Eukaryotic aspartyl protease family protein	2.7	2.5	1.3E ⁻¹⁴
HORVU2Hr1G116500	Avenin-like a4	3.0	2.3	1.1E ⁻²⁶
HORVU7Hr1G011180	Retinol dehydrogenase 14	3.3	1.4	7.7E ⁻²⁵

logFC = log2 (fold change); logCPM = log2 (counts per million); FDR = False discovery rate, p-value adjusted by Benjamini-Hochberg procedure.

5.4.4 Differential Genes of Norman

A total of 53 DEGs associated with ‘Norman’ were identified through the additive interactive model (Tables 5.2, 5.4B). This group of genes, which were strongly expressed in a pattern earlier in ‘Norman’ at 72 hpi and, particularly, in the 15ADON treatment. DEGs, including numerous abscisic acid (ABA) stress-responsive proteins; late embryogenesis abundance (LEA), heat-shock, desiccation related; Aldose reductase (HORVU1Hr1G070310). Multiple antifungal defenses of PR proteins were induced earlier in ‘Norman’ at 72 hpi, including thionin 2.1 (*PR13*), defensin-like protein (*PR12*), bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein (*PR14*), a protein synthesis inhibitor II (ribosome-inactivating—rRNA N-glycosidase), HORVU4Hr1G005690 (antimicrobial peptide *A. tauschii* homolog), and glucan endo-1,3beta-glucosidase GI (*PR2*). The differential response was also observed in a xylanase inhibitor.

5.4.7 Norman vs. CDC Kendall Control

A total of 473 and 580 DEGs were observed at 72 and 96 hps (hours post sprayed), respectively. Several genes appeared to be constitutively expressed earlier in “Norman,” including peroxidase class III BP1 (*HvPrx5*); ascorbate peroxidase (HORVU2Hr1G009940), cathepsin B-like cysteine, subtilisin-like protease, and chymotrypsin inhibitor, serpin (Z7), C-hordein, vicilin (HORVU5Hr1G104630) and germin-like protein (HORVU3Hr1G011990, *PR15*), LEA protein *HvA1* heat-shock protein, and disease-resistance proteins.

5.4.6 Differential Genes Between Norman and CDC Kendall by Treatment and Time Point

Paired contrasts from single-factor GLM were conducted between the two genotypes within *Fusarium*-treatments and at both time points. Total DEGs identified between ‘Norman’ and ‘CDC Kendall’ ranged from only 26 for NIV at 96 hpi to 1,319 for 15ADON at 96 hpi (Table 5.2). Under the three-factor additive GLM model, an overall total of 83 DEGs were identified between varieties (Table 5.2). The variance associated with genotype was minimal compared to *Fusarium* treatments and/or time, as might be predicted for varieties sharing 98% of their genetic code (Appendices 3.10-3.11). However, a complex resistance response occurred

where resistance factors were expressed in either variety, with differences observed over treatment and time.

5.4.7 Contrast 72 vs. 96-h Post-infection

Patterns of temporal gene expression were observed in both varieties. Paired contrasts (72 vs. 96 hpi) for single-factor GLM were conducted within ‘Norman’ and “CDC Kendall.” DEGs were identified within each treatment group (Table 5.2). Dominant patterns of elevated gene expressions over time were observed in 3ADON and more so within NIV treatments, while more balance was observed in the 15ADON treatment. Under the three-factor additive GLM for 72 vs. 96-treatment, contrast patterns indicated intensification of gene response over time, where a total of 193 DEGs were identified which were principally upregulated (Table 5.3). A selection of DEGs of greater fold change and significance are displayed, which increased over time (Table 5.5).

Table 5.5. Transcript alterations in select genes of greater fold change and significance upregulated from 72- to 96-h post-infection identified in the additive, interactive generalized linear model.

Gene	Annotation	logFC	logCPM	FDR
HORVU2Hr1G104410	Ubiquitin 7	-2.1	6.1	1.0E ⁻⁰⁹
HORVU2Hr1G107100	Protein translation factor <i>SUI1</i> homolog 1	-2.0	6.5	1.0E ⁻⁰⁹
HORVU0Hr1G038820	60S acidic ribosomal protein P0	-1.8	4.4	4.3E ⁻⁰⁷
HORVU3Hr1G006940	16.9 kDa class I heat shock protein 2	-1.7	4.0	4.0E ⁻⁰⁹
HORVU0Hr1G007340	Progesterone 5 beta reductase	-1.4	4.5	2.6E ⁻⁰⁶
HORVU1Hr1G046630	Valine--tRNA ligase	-1.4	4.5	6.0E ⁻⁰⁶
HORVU2Hr1G104390	Ubiquitin 11	-1.4	4.2	1.1E ⁻⁰⁵
HORVU7Hr1G096690	Chromosome 3B, genomic scaffold, cultivar Chinese Spring	-1.3	5.5	8.2E ⁻⁰⁶
HORVU5Hr1G056280	ABC transporter G family member 53	-1.2	6.6	1.1E ⁻⁰⁴
HORVU5Hr1G096930	Cytochrome P450 superfamily protein	-1.2	4.7	2.1E ⁻⁰⁵
HORVU1Hr1G063390	AAA-ATPase 1	-1.1	5.4	6.3E ⁻⁰⁵
HORVU5Hr1G047150	UDP-Glycosyltransferase (<i>HvUGT13248</i>)	-1.1	6.1	7.6E ⁻⁰⁴
HORVU2Hr1G032330	Sulfotransferase 17	-1.1	4.9	2.7E ⁻⁰⁴
HORVU6Hr1G034570	Receptor kinase 2	-1.1	4.1	2.0E ⁻⁰³
HORVU5Hr1G115100	GRAM domain / ABA-responsive protein-related	-1.0	4.4	4.1E ⁻⁰⁴
HORVU7Hr1G002820	alpha dioxygenase	-1.0	4.8	2.7E ⁻⁰⁴
HORVU4Hr1G065800	Inactive poly [ADP-ribose] polymerase <i>RCD1</i>	-1.0	5.1	1.3E ⁻⁰³
HORVU4Hr1G063350	Heat shock protein 21	-1.0	5.1	5.4E ⁻⁰⁴
HORVU3Hr1G052880	ATP-dependent zinc metalloprotease <i>FtsH 2</i>	-1.0	4.7	1.8E ⁻⁰³
HORVU2Hr1G094230	1-aminocyclopropane-1-carboxylate synthase 11	-1.0	4.4	1.3E ⁻⁰³
HORVU6Hr1G089560	U-box domain-containing protein	-1.0	4.4	1.0E ⁻⁰³
HORVU1Hr1G038700	Cysteine--tRNA ligase	-1.0	4.8	1.6E ⁻⁰³
HORVU6Hr1G089590	U-box domain-containing protein	-1.0	4.6	1.8E ⁻⁰³
HORVU2Hr1G012340	UDP-Glycosyltransferase superfamily protein	-1.0	4.3	7.7E ⁻⁰³
HORVU3Hr1G053350	ABC transporter G family member 36	-1.0	6.0	2.3E ⁻⁰³
HORVU5Hr1G106850	ABC transporter G family member 48	-0.9	6.9	7.3E ⁻⁰³
HORVU3Hr1G075040	L-tyrosine decarboxylase	-0.9	4.2	2.7E ⁻⁰³
HORVU3Hr1G084260	Leucine-rich repeat (LRR) family protein	-0.9	4.8	4.8E ⁻⁰³
HORVU5Hr1G112930	C2H2-like zinc finger protein	-0.9	4.8	4.0E ⁻⁰³
HORVU5Hr1G020900	GRAS family transcription factor	-0.9	4.7	2.3E ⁻⁰³
HORVU5Hr1G042030	Cytochrome P450 superfamily protein	-0.9	5.1	6.1E ⁻⁰³
HORVU2Hr1G107860	histone-lysine N-methyltransferase ATXR2	-0.9	5.7	7.4E ⁻⁰³
-	NF-X1-type zinc finger protein [<i>A. tauschii</i> , XP_020150167]	-0.9	4.9	5.5E ⁻⁰³
HORVU1Hr1G010210	Bidirectional sugar transporter N3	-0.9	4.8	4.0E ⁻⁰³
HORVU4Hr1G005500	50S ribosomal protein L18	-0.9	4.4	1.6E ⁻⁰²
HORVU5Hr1G096940	Cytochrome P450 superfamily protein	-0.9	6.5	1.2E ⁻⁰²
HORVU6Hr1G063480	Cysteine synthase D1	-0.9	4.4	1.8E ⁻⁰²
HORVU4Hr1G026340	Transporter	-0.9	5.9	1.4E ⁻⁰²
-	Fusarium resistance orphan protein [<i>T. aestivum</i> , AKZ18235]	-0.9	4.3	3.7E ⁻⁰²
HORVU2Hr1G027470	Cytochrome P450 superfamily protein	-0.9	5.9	1.1E ⁻⁰²
HORVU4Hr1G053130	Calmodulin 3	-0.9	5.1	1.2E ⁻⁰²

logFC = log2 (fold change); logCPM = log2 (counts per million); FDR = False discovery rate, p-value adjusted by Benjamini-Hochberg procedure; Annotation as per Barlex, IPK-Gatersleben

Elevated patterns of upregulation were observed at 96 hpi for genes associated with *Fusarium* reaction, including trichothecene detoxification and oxidative stress response. Extreme elevations of gene expressions were seen in ABCs (ATP-binding cassette transporters), CyP450s (Cytochrome P450s), GSTs (glutathione S-transferases), sugar transporters, and UGTs (UDP-glucuronosyltransferases) (Figures 5.4I–IV). Upregulation was observed in peroxidases, LEAs, LOXs, and subtilisin-like protease (HORVU7Hr1G024930). Promoter sequences of DEGs identified that the gibberellin-regulated transcription factor *Gamyb* of the *Myb/SANT* family was a significantly enriched motif over time (FDR = 0.04). Intensive expression of genes occurred in ‘CDC Kendall’ under the 3ADON treatment, where many DON-responsive genes were elevated by an order of magnitude at 96 hpi. Pathogen recognition increased over time marked by increased expressions in LRR proteins, MAPKKKs, protein kinases, calmodulin Ca²⁺ binding protein, and numerous WRKY transcription factors, including WRKY's 33, 50, and 75. Alterations in transcriptional patterns were evidenced by heightened expression of ubiquitination, a translation initiation factor, splicing factor Prp18 family, and ribosomal proteins. Many genes, initially lowly expressed in the NIV treatment at 72 hpi, demonstrated a significant upsurge in both varieties by 96 hpi.

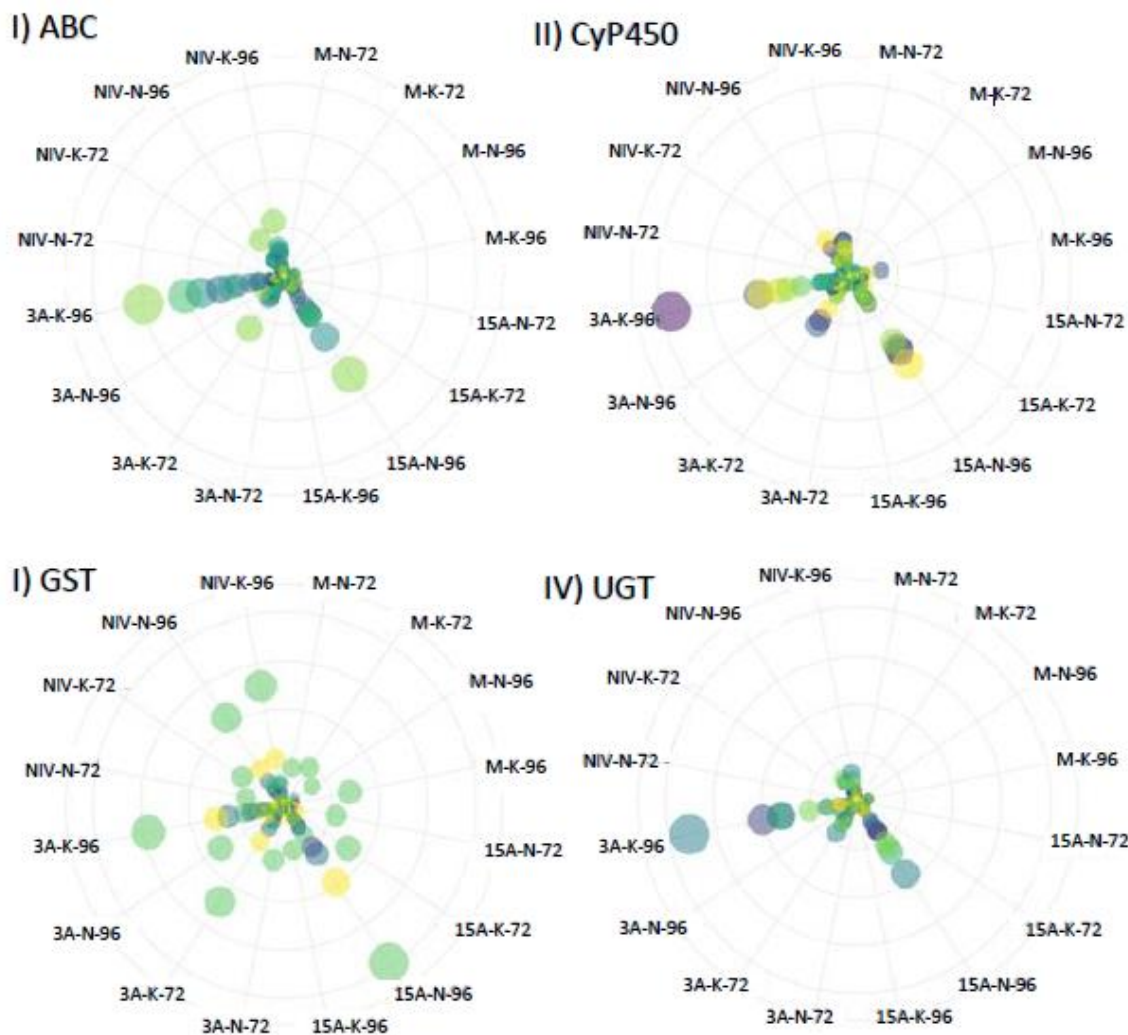


Figure 5.4. Gene expression for gene families associated with resistance to deoxynivalenol (DON) expressed in transcripts per million (TPM). Genes are identified by color, and circle size corresponds to TPM. **(I)** ABC, ATP-binding cassette transporter ($n = 24$ genes); **(II)** CyP450, cytochrome P450 superfamily protein ($n = 42$ genes); **(III)** GST, glutathione S-transferase (16 genes); **(IV)** UGT, UDP-Glycosyltransferase superfamily proteins (36 genes). M, mock control; 3ADON, 3-acetyldeoxynivalenol; 15ADON, 15-acetyldeoxynivalenol; NIV, nivalenol. 72- and 96-h post-infection. Colour coding represents different genes within gene families.

5.4.8 Unique Resistance Patterns Were Observed in Norman in 15ADON Treatment at 72 hpi

DEGs of ‘Norman’ expressed at 72 hpi within the 15ADON treatment were associated with nutrient reservoir activity of storage proteins and defense response. Genes of this kind were not transcribed in ‘CDC Kendall’ at 72 hpi but were expressed later at 96 hpi. Defense response to pathogen cell wall digestive enzymes was seen in xylanase inhibitors. Defense in ‘Norman’ was expressed *via* multiple protease inhibitors: Serine protease inhibitors: serpin (Z4 and Z7 families), subtilisin-chymotrypsin inhibitors (CI-1A and CI-1B), alpha-amylase inhibitor 28, and metalloprotease inhibitor. Plant toxins expressed, such as protein synthesis inhibitor II (HORVU5Hr1G112540), rRNA N-glycosidase (ribosome-inactivating, ricin A-chain activity). Multiple RNases were observed, which could act to repress fungal effectors. Expression was observed in pathogen responsive proteins with antifungal activity, including defensin-like protein, thionin 2.1, and chitin-binding metabolite agglutinin isolectin 3. Auxin-related genes were enhanced in ‘Norman’ at 72 hpi, as indicated by the upregulation of multiple tryptophan aminotransferase-related protein 1 (*TAR1*) genes. Many senescence-associated proteins showed an elevated response. Also observed was a high expression in ribose-binding protein 1 homolog.

Lower expression was seen in ‘Norman’ for gibberellin receptor *GID1* (HORVU1Hr1G060810, gibberellin insensitive dwarf), which binds to gibberellins to facilitate GID1-DELLA complexing and subsequent degradation *via* ubiquitination. DELLA protein scarecrow-like 5 (HORVU3Hr1G058100, *Sln1*-like) was differentially expressed earlier in “Norman.” Numerous ABA stress-related genes were induced, including LEA proteins [HORVU1Hr1G058400, HORVU1Hr1G079290 (*HVA1*), HORVU2Hr1G099870, HORVU4Hr1G051780, HORVU4Hr1G073120]; laccase 7 (HORVU2Hr1G002440); Dehydrin 6 (HORVU4Hr1G010750, HORVU6Hr1G084070); Dehydrogenase/reductase SDR 4 (HORVU1Hr1G018140, retinol dehydrogenase); heat shock protein [HORVU3Hr1G020500 (17.6 kDa class II), HORVU4Hr1G059260 (70 kDa protein 3)]; thioredoxin 2 (HORVU6Hr1G078960, mitochondrial ROS homeostasis); peroxidase superfamily protein (HORVU4Hr1G022280). A significant increase was seen in nutrient storage-related proteins like cupin seed storage proteins, including vicilin-like antimicrobial peptides (HORVU4Hr1G050210, HORVU4Hr1G070970, HORVU5Hr1G104630), globulin-2

(HORVU4Hr1G002800), tritacin, and germin-like proteins (HORVU5Hr1G010880, HORVU6Hr1G089570, *PR15*) (Appendix 3.13).

5.4.9 3ADON Treatment at 72 hpi

Under infection by the more virulent 3ADON isolate group, ‘Norman’ and ‘CDC Kendall’ also displayed differential response patterns. Numerous resistance-responsive genes demonstrated higher expression in ‘CDC Kendall’ than “Norman.” Elevated gene expression in ‘CDC Kendall’ in phenylalanine ammonia-lyase (*PAL*) genes. Elevations were observed in UGT 74F1 (HORVU2Hr1G012280), which may conjugate glucose to salicylic acid (SA), thereby inactivating it. The Octadecanoid pathway was prominent along with many JA-related metabolite genes: Lipoxygenases (*LOX2*, *LOX3*, and *LOX6*), Lipase/lipoxygenase—PLAT/LH2 proteins, Patalin-like protein 5 (HORVU4Hr1G075070), Allene oxide synthase (AOS), and Jasmonate O-methyltransferase (*MeJA*). Extremely high levels of 12-oxophytodienoate reductase (*OPR2*) were observed in ‘CDC Kendall’ at 72 hpi in contrast with “Norman”.

Tryptophan metabolism was indicated by anthranilate synthase component 1 (HORVU4Hr1G061120) and tryptophan synthase beta chain 1 (HORVU7Hr1G073460). Defense-related genes were congruently expressed: Thaumatin-like pathogenesis-related proteins (*PR5*), cysteine-rich venom protein, and allergen V5/Tpx-1-related protein (*PRB1-2*). Also, defense response was seen in chitinases (*PR3*), wound-induced protein (HORVU3Hr1G113120, *wheatwin-2*), and pathogenesis-related protein (*PR10*). Cell wall fortification was evident in upregulated lignification *via* laccase-19, peroxidases (HORVU2Hr1G026450, HORVU3Hr1G074960), cellulose synthase-like D3 (HORVU1Hr1G022900), and extension (hydroxyproline-rich glycoproteins). Detoxification response was enhanced by ABC transporters and UGT gene *HvUGT13248*. While robust gene expressions were observed in “CDC Kendall,” similar defenses also occurred in ‘Norman’ in the 3ADON treatment at 72 hpi where elevated genes expressions unique to ‘Norman’ included chromosome 3B, genomic scaffold, cultivar Chinese spring (HORVU7Hr1G006250, LEA protein wheat homolog), CyP450 (HORVU4Hr1G083930, 86B1 family involved in very-long-chain fatty acids omega-hydroxylation), glucan endo-1,3beta-

glucosidase *GI* (*PR2*), and peroxidase *BP1* (*HvPrx5*). ‘Norman’ demonstrated relatively higher expression in specific JA-induced proteins: 60 kDa—rRNA N-glycosidase; 32.7 kDa dirigent proteins and defensin-like protein (HORVU4Hr1G082400, *PR12*). Stress response was observed in upregulation of calcium-dependent lipid-binding protein (HORVU3Hr1G098160, CaLB domain) and response in homolog Fusarium resistance orphan protein, *TaFROG* (TraesCS4A02G20190).

Low expression of defense genes was observed under NIV treatment at 72 hpi. Some resistance-related genes were found to show elevated expression in ‘Norman’ at 72 hpi, including peroxidase *BP1* (*HvPrx5*), lipid-transfer proteins (*PR14*), protein synthesis inhibitor II, vicilin antimicrobial peptides, homeobox-leucine zipper protein ROC, GDSL esterase/lipase, late embryogenesis abundant protein D-19 (HORVU1Hr1G059900), *PHV A1*, and several heat shock proteins.

5.4.10 Norman Displayed Alterations in Responsive Patterns in 15ADON Treatment at 96 hpi

At 96 hpi, a significant change in resistance pattern was observed in ‘Norman’ from what was seen initially at 72 hpi. As seen in ‘CDC Kendall’ 3ADON treatment, a secondary phenylpropanoid metabolite pathway was induced at 96 hpi, demonstrated by upregulation of phenylalanine synthase (HORVU5Hr1G052150) and *PAL*, shikimate kinase 1, 4-coumarate-CoA ligase (*4CL*), dihydroflavonol 4-reductase (HORVU2Hr1G033610, HORVU6Hr1G089460, *DFRA*). Detoxification genes were induced: ABC, CyP450, GST, and UGT (Figures 4I–IV). Oxidative-stress response was seen under elevations in alternative oxidase 1a (HORVU2Hr1G101980 *AOX1A*), peroxidases, progesterone 5 beta reductase (HORVU0Hr1G007340), germin-like proteins (oxalate oxidase, *PR15*), and laccases. Elevations that occurred in resistance-responsive DEGs included Thaumatin-like pathogenesis-related protein 1 (HORVU7Hr1G122120), cysteine-rich venom protein (HORVU7Hr1G033530, *PRB1-2*), major pollen allergen Bet v 1-F/I (HORVU4Hr1G054870, *PR10*). Protease inhibitor families (Bowman-Birk type and chymotrypsin inhibitors) and xylanase inhibitors were expressed within “Norman.”

While ‘Norman’ at 96 hpi followed the patterns of the generalized resistance motif as seen in “CDC Kendall,” several resistance-responsive genes were enhanced in “Norman,” including defense-related PR proteins: *PRB1-2*, *PR3*, *PR5*, *PR10*, *PR15*, plant basic secretory (BSP) protein (*PR17*), and pathogenesis-related protein (HORVU3Hr1G111600). Another elevated defensive response was observed in GST (*GSTU6*), stress-responsive protein (HORVU2Hr1G017420), wound-induced protein (HORVU3Hr1G113120, *wheatwin*), *MYC2* transcription factor (HORVU7Hr1G038900), and NBS-LRR resistance proteins. ‘Norman’ expressed influential genes of cell function: Indole-2-monooxygenase-like (HORVU3Hr1G011850, benzoxazinoid synthesis), *WIR1A* proteins (HORVU5Hr1G010130, HORVU6Hr1G088880, cell wall structure), and water supply via aquaporin PIP2-4 (HORVU6Hr1G05893). Nitric oxide (NO)-producing nitrate reductase 1 genes (HORVU6Hr1G003300, HORVU6Hr1G079700) were also induced and preferentially in “Norman.” Cathepsin B-like cysteine proteinases (HORVU2Hr1G036930, HORVU5Hr1G095580, HORVU5Hr1G061770), boron transporter 1 (HORVU5Hr1G022140, *HvBOR1*), copper ion-binding protein (HORVU7Hr1G036180), and metacaspase 1 (HORVU3Hr1G095700) genes indicated programmed cell death response in “Norman”.

5.4.11 DON Avoidance Mechanisms Were Pronounced in CDC Kendall in 3ADON Treatment at 96 hpi

Detoxification genes initially expressed in ‘CDC Kendall’ at 72 hpi continued to rise to extremely high expression levels at the 96 hpi time point under 3ADON treatment, in dramatic contrast to “Norman,” which was more restrained (Figures 5.4I–IV). The phenylpropanoid pathway was greatly induced with genes *PAL* and *4CL*. Dihydroflavonol 4-reductase (*DFRA*) and other flavonoid genes were differentially expressed, such as chalcone synthase (HORVU2Hr1G004170, *CHS*) and chalcone-flavanone isomerase (HORVU5Hr1G046480, *CHI*). JA and ETH hormone signatures were highly apparent. Pathogen recognition was evident through the common expression of many receptors, modifiers, and signaling cascades, including leucine-rich repeat receptor (LRR) kinases [HORVU3Hr1G084260 (*BAK1*), HORVU3Hr1G104940, and HORVU1Hr1G068380, *SERK2*], together with modifiers

of *BAK1* (MOB) kinase activator-like 1A and 1B (HORVU0Hr1G018730, HORVU6Hr1G009410, and HORVU0Hr1G037740). Also evidenced were many genes of signaling cascade molecules: Numerous protein kinase superfamily proteins (MAPKKK), calmodulin-binding protein (HORVU0Hr1G002930), calcium-dependent protein kinase (*HvCPK28*), and calcium-transporting ATPase protein (HORVU1Hr1G076950). Bowman-Birk type trypsin inhibitors and *Fusarium*-specific orphan gene *TaFROG* were induced.

Heightened expression was observed in ‘CDC Kendall’ for genes associated with a respiratory burst (HORVU0Hr1G013380, HORVU4Hr1G081670, HORVU5Hr1G078630, NADPH oxidase homolog D, *HvRbohD*). Arginine decarboxylase was upregulated, which is involved in the biosynthesis of polyamine spermidine. Polyamine oxidase was also activated, which is involved in H₂O₂ production in the apoplast. Upregulation was seen in multiple 2-oxoglutarate and Fe (II)-dependent oxygenase superfamily proteins (HORVU6Hr1G088430, HORVU2Hr1G004280, HORVU6Hr1G088440, *2OGO*). Stress-responsive genes were upregulated, including NAC domain protein (HORVU5Hr1G111590, *SNAC1*) and peroxidase. Numerous CyP450 superfamily proteins that were highly expressed were involved in redox reactions. CyP450s (HORVU1Hr1G080680, CyP450 94B3, and HORVU6Hr1G001520, CyP450 94C1) are involved in the modification of jasmonoyl-L-isoleucine (JA-Ile) and attenuation of JA-response. Ubiquitination (HORVU7Hr1G109650, HORVU2Hr1G104410) and negative regulatory factor of DON-resistance, *NF-X1*-type zinc finger protein increased over time. Extremely high expression was observed in UGTproteins *HvUGT13248* and *HvUGT14077* vs. control (log₂FC = 9.6 and 6.4, respectively). Negative regulation of cell death was apparent through a display of *BON* association protein 2 (HORVU5Hr1G080820, *BAP2*) and *MLO*-like protein 1 (HORVU0Hr1G008830) and inactive poly (ADP-ribose) polymerase *RCD1* (HORVU4Hr1G065800, *radical-induced cell death 1*). A boron efflux transporter (*HvBOR1*) was activated, where boron is a mitochondrial stabilizer and inhibitor of apoptosis. Programmed cell death-associated protein metacaspase-1 was expressed in ‘Norman’, but was highly suppressed in ‘CDC Kendall’ at this time. While detoxification resistance was engaged, patterns of gene expression in ‘Norman’ under 3ADON at 96 hpi showed some similarities to those observed in ‘Norman’ at 72 hpi in 15ADON

treatment. Vicilins (HORVU4Hr1G070970, HORVU5Hr1G104630), Serpins (Z7), subtilisin-chymotrypsin inhibitors (CI-1A and CI-1B), and B hordeins storage proteins were all elevated in “Norman.” Likewise, amine oxidase 1 (HORVU2Hr1G012710), *TAR1* proteins, xylanase inhibitor (HORVU2Hr1G043890), LEA-like protein (HORVU1Hr1G058400, *NHL13*), NAC transcription factor (HORVU6Hr1G01938, *HvNAM-1*), defensin-like protein (*PR12*), and peroxidase BP1 (*HvPrx5*). A serine/threonine-protein kinase protein was expressed in ‘Norman’ at these stages (HORVU1Hr1G064110, *PBL7* homolog).

5.4.12 NIV Treatment at 96 hpi

Receptors and signaling genes were slow to be induced in the NIV treatment at 72 hpi, in contrast to DON-producers (Figure 5.5). However, by 96 hpi, the NIV chemotype demonstrated an upsurge in expression, where gene families were similar to those seen in DON-producing treatments. The varieties showed very similar gene expressions at 96 hpi; however, germin-like (*PR15*), aspartic proteinase nepenthesin and peroxidase *BP1* (*HvPrx5*) protein expressions were higher in “Norman.” *GSTs* displayed a higher induction than *UGTs* under the NIV vs. DON treatments (Figures 5.4, III, and IV).

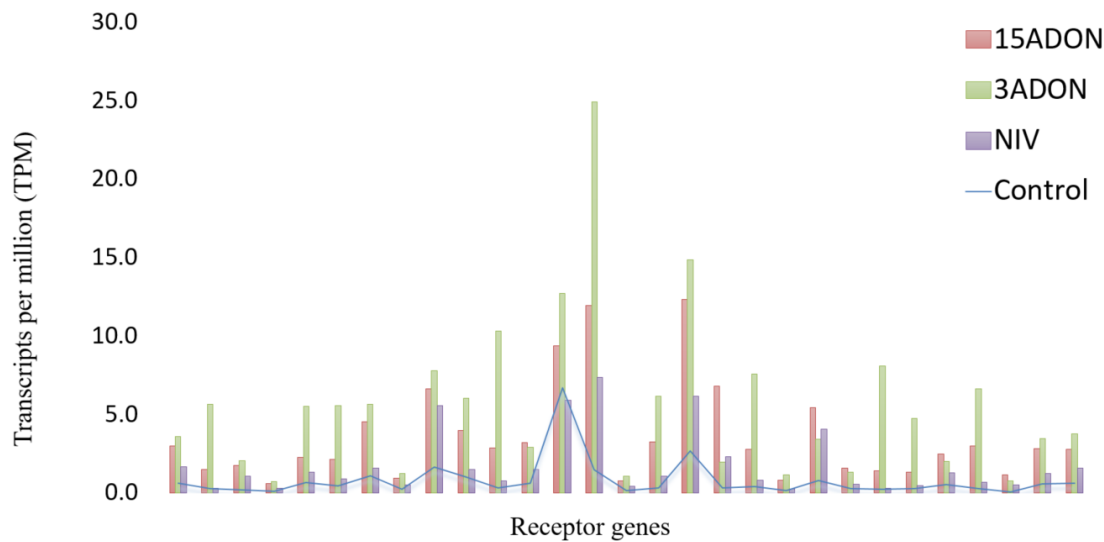


Figure 5.5. Differentially expressed genes of receptor classification ($n = 29$) for ‘CDC Kendall’ in treatment groups (15ADON, 3ADON, and NIV) at 72 hpi vs. mock control expressed in transcripts per million (TPM).

5.4.13 Gene Ontology (GO) Enrichment in *Fusarium*-Treatment vs. Control

An overrepresentation analysis was used to evaluate DEGs from treatment contrasts to mock control to identify GO (Gene Ontology) terms enriched for the *Fusarium*-barley interaction. In contrast to the mock control group, the *Fusarium* treatments displayed upregulation in a suite of genes. Groups of statistically significant GO terms ($FDR \geq 0.05$) were analyzed for all treatment groups (15ADON, 3ADON, and NIV). GO terms between treatments were compared for intersection and similarity of expression patterns. Gene processes displayed common enrichment of mechanisms among the groups (Figure 5.6).

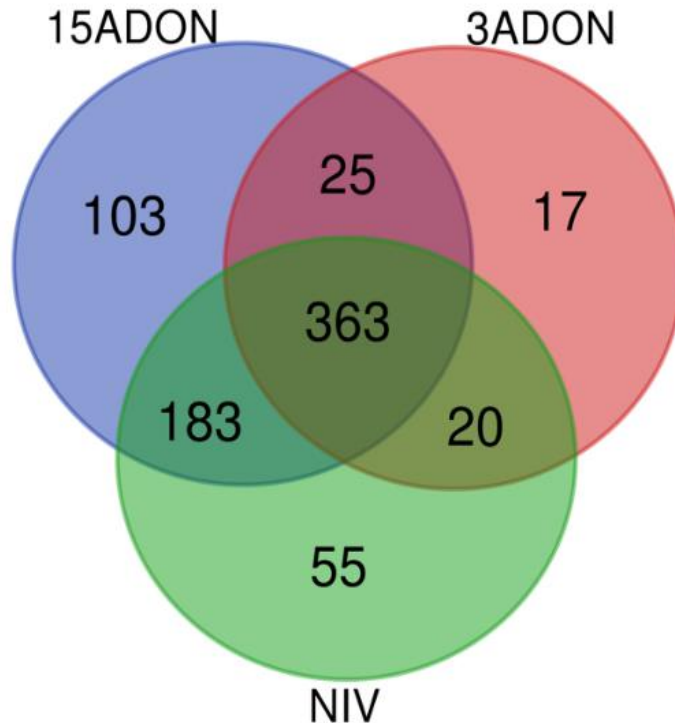


Figure 5.6. A Venn diagram of enriched categories of GO terms ($FDR \geq 0.05$) associated with differentially expressed genes in additive generalized linear model (GLM) contrast of mock control and *Fusarium* treatment (3ADON, 15ADON, and NIV chemotypes).

GO-enrichment portrayed increased unification of host response of coordinated mechanisms. Approximately half (47%) of all enriched terms were identified as common to all *Fusarium* treatment groups, including biological process terms: “L-phenylalanine metabolic/catabolic process”; “ammonium transmembrane transport”; “phenylpropanoid metabolic process”; “lignin metabolic process”; “oxidation-reduction process”; “organonitrogen compound catabolic process”; “oxoacid metabolic process”; “carboxylic acid metabolic/catabolic process”; “cellular amino acid catabolic process” (GO:0009063); “aminoacyl-tRNA ligase activity” (GO:0004812), tryptophan biosynthetic process (GO:0000162), and indolalkylamine (GO:0042430; GO:0046219), which was evidenced by strong upregulation of anthranilate synthase component 1 (HORVU4Hr1G061120), tryptophan synthase. Pathogen response was perceived in “response to biotic stimulus” (GO:0009607) and “establishment of localization” (GO:0051234).

Figure 5.7 displays a network assembly of GO terms common to the *Fusarium* treatments. Non-coding RNA metabolism generates alterations in amino acid composition, which influence a metabolic process. Transcriptional reprogramming of *Fusarium*-response was directed at the level of amino acid metabolism through activation of aminoacyl-tRNA synthetase for particular amino acids (Ala, Cys, Leu, Lys, Phe, Pro, Val). Indole-3-glycerol phosphate synthase (HORVU5Hr1G052150) was upregulated, which participates in aromatic amino acid biosynthesis. Single-organism processes stimulate oxidation-reduction processes, which, in turn, influence aromatic compound catabolism. Processes together lead to the production of secondary metabolism of phenylpropanoids and lignification response. This resistance pattern occurred in both varieties.

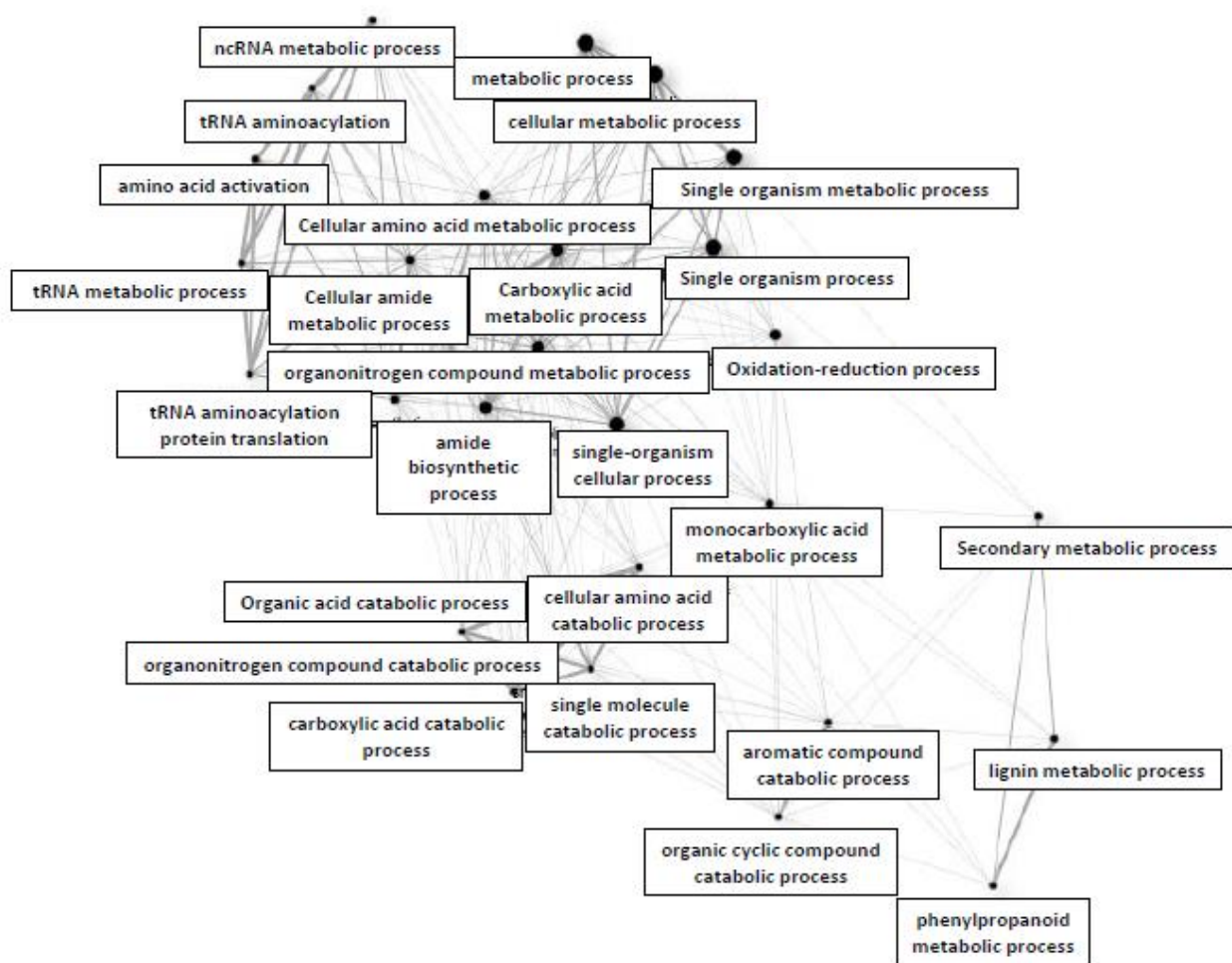


Figure 5.7. A network of significant ($FDR > 0.05$) Gene Ontology (GO) terms for biological processes common to all treatment groups (15ADON, 3ADON, and NIV).

5.4.14 GO Enrichment in 72 vs. 96 hpi

The additive GLM identified significant ($FDR < 0.05$) enrichment of biological processes over all treatments. Enrichment was observed with gene groups associated with elevated functions at 96 hpi. Changes in cellular reprogramming were apparent through augmentations in “protein ubiquitination” (GO:0016567) “ubiquitin-protein transferase activity” (GO:0004842) were observed arising from the expression of multiple U-box domain-containing proteins

(HORVU1Hr1G069990, HORVU6Hr1G089560, HORVU6Hr1G089590, HORVU7Hr1G040790). “Anion transport” was identified as a process-enriched term, including boron transporter 1 (HORVU5Hr1G022140) and high-affinity nitrate transporter 3.1 (HORVU6Hr1G053710, NAR2.3). “Sequence-specific DNA binding” (GO:0043565) was amplified, including genes encoding multiple WRKY transcription factors of the JA signaling pathway. Functional enrichment was observed in calcium ion binding (GO:0005509) in calcium-transporting ATPase (HORVU1Hr1G076950). “ATPase activity” (GO:0016887) and “ATPase activity,” coupled with the movement of substances (GO:0043492) are associated with elevations of ABC transporter activities.

Pairwise contrasts also identified common enrichment in processes over time, including a “phenylpropanoid metabolic process” (GO:0009698) and a “lignin metabolic process” (GO:0009808), such as laccases and a “cell wall macromolecule metabolic process” (GO:0044036). “Defense response” (GO:0006952), “alternative oxidase activity (GO:0009916). Regulatory functions were enriched, including “regulation of biosynthetic processes” (GO:0009889), including genes rRNA N-glycosidase, protein synthesis inhibitor II, and quinone; “regulation of gene expression” (GO:0010468) enrichments was seen in NAC, WKRY, ERF, bZIP families. Enhanced molecular function was seen in “peptidase inhibitor activity” (GO:0030414), nutrient reservoir activity (GO:0045735) seen in the increase of storage proteins. Temporal patterns of enrichment demonstrated similar progressions within individual *Fusarium*-treatment groups.

5.4.15 GO Enrichment in ‘Norman’ vs. ‘CDC Kendall’

The most significant category of gene enrichment for the biological process was “defense response” (Table 5.6). Enrichment was also seen in response to “biotic stimulus,” “response to stress,” and “response to a stimulus.” Enriched categories of DGEs displayed negative biological regulatory processes. Molecular functions were enriched in elements of pathogenic repression. Enriched elements indicated that differences in host-pathogen interaction response occur within the “extracellular region.”

Table 5.6. Gene Ontology (GO) terms of ‘Norman’ vs. ‘CDC Kendall’ contrast identified in the additive, interactive generalized linear model.

GO code	Type	Term	FDR
GO:0006952	P	defense response	4.9E ⁻²⁰
GO:0051248	P	negative regulation of protein metabolic process	4.1E ⁻¹⁰
GO:0034249	P	negative regulation of cellular amide metabolic process	4.1E ⁻¹⁰
GO:0032269	P	negative regulation of cellular protein metabolic process	4.1E ⁻¹⁰
GO:0017148	P	negative regulation of translation	4.1E ⁻¹⁰
GO:0009607	P	response to biotic stimulus	1.9E ⁻⁸
GO:0010608	P	posttranscriptional regulation of gene expression	2.5E ⁻⁸
GO:0034248	P	regulation of cellular amide metabolic process	2.5E ⁻⁸
GO:0006417	P	regulation of translation	2.5E ⁻⁸
GO:0031327	P	negative regulation of cellular biosynthetic process	4.1E ⁻⁷
GO:0009890	P	negative regulation of the biosynthetic process	4.1E ⁻⁷
GO:2000113	P	negative regulation of cellular macromolecule biosynthetic process	4.1E ⁻⁷
GO:0010558	P	negative regulation of macromolecule biosynthetic process	4.1E ⁻⁷
GO:0031324	P	negative regulation of cellular metabolic process	5.5E ⁻⁷
GO:0051172	P	negative regulation of nitrogen compound metabolic process	5.5E ⁻⁷
GO:0032268	P	regulation of cellular protein metabolic process	6.3E ⁻⁶
GO:0006950	P	response to stress	8.7E ⁻⁶
GO:0051246	P	regulation of protein metabolic process	1.7E ⁻⁵
GO:0010629	P	negative regulation of gene expression	3.3E ⁻⁵
GO:0009892	P	negative regulation of metabolic process	4.7E ⁻⁵
GO:0010605	P	negative regulation of macromolecule metabolic process	4.7E ⁻⁵
GO:0048523	P	negative regulation of cellular process	5.5E ⁻⁵
GO:0048519	P	negative regulation of the biological process	6.7E ⁻⁴
GO:0050896	P	response to stimulus	4.6E ⁻³
GO:0044712	P	single-organism catabolic process	3.4E ⁻²
GO:0030597	F	RNA glycosylase activity	2.2E ⁻¹⁰
GO:0030598	F	rRNA N-glycosylase activity	2.2E ⁻¹⁰
GO:0004867	F	serine-type endopeptidase inhibitor activity	1.4E ⁻⁹
GO:0030414	F	peptidase inhibitor activity	5.4E ⁻⁸
GO:0004866	F	endopeptidase inhibitor activity	5.4E ⁻⁸
GO:0061135	F	endopeptidase regulator activity	5.4E ⁻⁸
GO:0061134	F	peptidase regulator activity	5.4E ⁻⁸
GO:0016799	F	hydrolase activity, hydrolyzing N-glycosyl compounds	3.9E ⁻⁷
GO:0004857	F	enzyme inhibitor activity	2.5E ⁻⁶
GO:0030234	F	enzyme regulator activity	1.2E ⁻⁴
GO:0098772	F	molecular function regulator	2.7E ⁻⁴
GO:0045735	F	nutrient reservoir activity	9.9E ⁻³
GO:0005576	C	extracellular region	5.0E ⁻¹⁰

P = Biological process; F = Molecular function; C = cellular component. FDR = False discovery rate, p-value adjusted by Benjamini-Hochberg procedure.

5.4.16 Differential Genes Associated With Oxidative Stress Reduction

Fusarium treatments demonstrated a sign of oxidative stress through activation of multiple genes-encoding proteins with antioxidant genes with oxidoreductase activity included: numerous peroxidases, L-ascorbate oxidase, thioredoxin 2, ferredoxin 3, FAD-binding Berberine family protein, quinone, 2-oxoglutarate, and Fe (II)-dependent oxygenase superfamily, and lignin-associated laccase-19. Oxidative stress was apparent under *Fusarium* treatments through the expression of ubiquinol oxidase-alternative oxidases, *AOX1A* (HORVU2Hr1G101920), *AOX2* (HORVU2Hr1G101920, HORVU2Hr1G101990), and external alternative NADPH-ubiquinone oxidoreductase B3 (HORVU7Hr1G073050). These genes provide alternative oxygen reduction mechanisms within the electron transport chain and mitigate problems of excess ROS for maintaining growth. Likewise, induction of ATP-dependent zinc metalloprotease *FtsH* 2 (HORVU3Hr1G052880) indicated potential photo-damage of thylakoid membranes. Such genes were expressed in excess in “CDC Kendall,” indicating higher oxidative stress. GO terms for molecular function were enriched in activities associated with oxidative stress and detoxification: “alternative oxidase activity” (GO:0009916); “oxidoreductase activity” (GO:0016491); “oxidoreductase activity, acting on diphenols and related substances as donors, oxygen as acceptor” (GO:0016682); “iron ion binding” (GO:0005506); “heme binding” (GO:0020037). Enrichment was seen in all treatment groups in response to an oxidative-stressed state of a host, following *Fusarium*-infection.

5.5 Discussion

We document in the current study that 3ADON chemotypes created more disease and much higher mycotoxin in barley than 15ADON or NIV chemotypes. Deoxynivalenol (DON) is a virulence factor contributing to primary infection in barley (type I resistance) but, in contrast to wheat, does not contribute to increased spread from point of initial infection (Jansen et al., 2005). Resistance to spread from point of infection (type II resistance) is robust in barley, where even susceptible barleys can adequately impede internal growth through the rachis, owing to strong detoxification mechanisms. As seen in previous studies of six-row barley (Boddu et al., 2007; Gardiner et al., 2010; Huang et al., 2016), two-row barley in the current study demonstrates a coordinated defense response through a suite of DON-induced genes. Elevated

co-expression of detoxification genes was observed in the 3ADON treatment and predominantly in “CDC Kendall,” including CYP450's, which modify harmful molecules and/or facilitate oxidative reactions of phenylpropanoid production. Glutathione S-transferase (GST) and UGTs conjugate toxins to create glutathione or glucose moieties. The ABC transporters facilitate the transport of xenobiotics out of a cell or into vacuoles. Detoxification resistance was common to both varieties and was increasingly expressed to 96 hpi.

Both ‘Norman’ (moderately resistant variety) and ‘CDC Kendall’ (intermediate variety) displayed strong detoxification responsive against DON. The UDP-glucuronosyltransferase (UGT) genes like *HvUGT13248*, which converts DON to DON3G through the addition of a glucose molecule, are very well-characterized (Schweiger et al., 2010) and are essential components of type II resistance. Barley UGTs like *HvUGT13248* are very effective, where enhanced type II resistance was attained by insertion of this transgene in wheat (Li et al., 2015). Virus-induced gene silencing of *HvUGT-10W1*, an allelic variant of *HvUGT14077*, resulted in compromised type II resistance, allowing spread through the spike (Xing et al., 2017). DON conjugation transforms mycotoxins into less-toxic forms but also facilitates mobilization to apoplast/vacuoles (Audenaert et al., 2014) or binding to cell wall constituents (Zhou et al., 2008). While the enhanced rate of DON-glycosylation is associated with wheat resistance (Kluger et al., 2015), the ratio of DON3G/DON in barley was similar in mature grains of differentially resistant varieties (Tucker et al., 2019). This suggests that resistance in barley is likely not enhanced by elevated UGT gene expression. While DON conjugation effectively lowers overall toxin content, conjugates are also undesirable as they can readily be hydrolyzed to the principal toxin by common gut bacteria (Berthiller et al., 2011).

‘Norman’ displayed a more conservative response to DON under challenge by the more aggressive 3ADON-producing strains under pathogen progression. Overstated detoxification response in ‘CDC Kendall’ suggests a host-pathogen interaction of increased mycotoxin content. In environments where pathogens may demonstrate proclivities for toxin production, reliance on DON resistances involving avoidance mechanisms may not be adequate. Generation of reactive oxygen species (ROS) through an oxidative burst is observed under the first stages of cereal host defense response to *F. graminearum* (Taheri, 2018). As seen by Huang et al. (2016), a large number of receptor kinase genes were invoked in the current study linked with pathogen

recognition and resultant signaling cascades. ROS molecules associated with the oxidative burst operate directly as protective agents, trigger programmed cell death (PCD), and/or signal pathogenesis-related (PR) protein induction (Lamb and Dixon, 1997). Deoxynivalenol (DON) elicits H₂O₂ production in cereals, which, in juxtaposition, may trigger antimicrobial defense but may stimulate cell death, which ultimately benefits necrotrophic growth (Desmond et al., 2008). *Fusarium graminearum* responds reciprocally to ROS through the stress-responsive generation of DON (Ponts et al., 2007), thereby establishing a positive feedback loop, leading potentially to toxin amplification. However, the more-virulent 3ADON form demonstrated abilities to increase infection rates and mycotoxin production in either variety. The increased frequency of occurrence of these more virulent forms poses a serious threat to barley production. Focus on incorporating robust resistance to initial infection within DON-based resistance may be an increasingly important resistance mechanism for challenges to more-virulent strains.

Phenylalanine ammonia-lyase (*PAL*) genes were highly expressed in both varieties herein and the study conducted by Huang et al. (2016). *PAL* catalyzes a deamination reaction, converting L-phenylalanine to trans-cinnamic acid. It represents a shift from primary to secondary metabolic function and the first step of the biosynthetic process of the polyphenol compounds, such as flavonoids, phenylpropanoids, and lignin. *PAL* is an important component of the basal resistance mechanism of barley associated with *MYB* transcription factors. Mass spectrometry applications have confirmed the common occurrence of resistance-related compounds in *Fusarium*-infected cereals, where flavonoid phenylpropanoids are predominant (reviewed by Gauthier et al., 2015). Lignin formed through polymerized phenolic precursors contributes to resistance *via* fortification of physical barriers but is also important as a precursor of JA biosynthesis (Chamarthi et al., 2014). Boutigny et al. (2008) implicate phenolic compounds as DON suppressors through quenching oxidative stress. However, defenses of this nature are generally slow and maybe outpaced by more virulent pathogens. Phenolic acids generally inhibit *F. graminearum* growth; however, compounds with higher antioxidant properties may also stimulate increased toxins *via* stress response (Ponts et al., 2011).

Jasmonic acid/ ETH pathways were induced by *Fusarium* in either variety, where response increased over time. Kumaraswamy et al. (2012) demonstrated JA involvement in transcriptional response to DON in wild type to *TRI5*- contrast. ‘CDC Kendall’ and ‘Norman’ were

characterized with elevated JA-response in later treatment, while *PR* expressions were generally more pronounced in “Norman.” Jasmonic acid (JA) response under study was associated with lignification, detoxification mechanisms, and elevated expressions genes, such as chitinases (*PR3*) and thaumatin-like (*PR5*) proteins. Geddes et al. (2008) demonstrated these *PR* proteins and oxidative burst/stress response occurred both in moderately resistant and intermediately resistant two-row barley varieties infected by *F. graminearum*. In the current study, DEGs were primarily associated with JA vs. salicylic acid (SA) expression; however, SA signatures may also have disappeared by this time point as seen by Huang et al. (2016). Hormones are important coordinating factors in quantitative disease traits and are principally involved in response to a pathogen, where SA is typically associated with resistance to biotrophs vs. JA/ETH, which is common in necrotrophic response (Glazebrook, 2005). Basal resistance, activated early in the defense response of cereals, is associated with SA accumulation (Makandar et al., 2012; Hao et al., 2018; Wang et al., 2018). Resistance associated with JA/ETH signaling pathways was also implicated in several cereal studies (Li and Yen, 2008; Gottwald et al., 2012; Makandar et al., 2012; Qi et al., 2016; Wang et al., 2018). Antagonism between hormones is documented in cereal response to *F. graminearum*, where host compatibility is complicated by a hemibiotrophic lifestyle. Resistance occurs in an orderly sequence, where resistance coordination may be important for fully effective resistance (Ding et al., 2011; Makandar et al., 2012).

We observed antagonism between stress-responsive ABA and growth-related GA phytohormones. In cereals, ABA is a phytohormone associated with the onset of stress-related metabolites, anti-oxidative activation, stomatal closure, and lower photosynthetic efficiencies (Gietler et al., 2020). Gibberellic acid (GA) insensitive dwarf 1 (*GID1*) receptor interacts with GA and DELLA to form a protein complex (Murase et al., 2008), followed by marking of DELLAs via F-box proteins for ubiquitination and degradation. Degradation of GA-repressive DELLA-encoding *Slender1* (HORVU3Hr1G058100, *Sln1*) gene permits the GA-signaling pathway. Mutations in the DELLA domain required for interaction with *GID1* and GA-growth response result in a semi-dwarf phenotype, which is susceptible to biotrophic pathogens, but resistant to necrotrophic pathogens and *F. graminearum* (Saville et al., 2012). De Bruyne et al. (2014) discuss the role of DELLAs in providing resistance to necrotrophs through induction of compounds, which alleviate oxidative stress, repress cell wall loosening, and compete with JAZ molecules, allowing MYC2 to bind G-box and release JA-responsive genes. ‘Norman’

at 72 hpi demonstrated an elevated level of ABA-responsive genes and senescence-related proteins, while GA-signatures (*Gamyb*) dominated the defense response of DON-detoxification at 96 hpi, particularly under the 3ADON treatment. Petti et al. (2010) also observed downregulation of GA-receptor and elevated DELLA expression and disease control of *F. culmorum*, following induction of induced systemic resistance (ISR) via priming of *Pseudomonas fluorescence*. The interplay of ABA-GA phytohormones may play a role in modulating the biotrophic-necrotrophic transition. This antagonism is a basis of trophic divergence in a diversified host-pathogen interaction, which may result in both resistance and susceptibility under species-specific circumstances (Huang et al., 2020).

Oxidative stress visible in all *Fusarium* treatments likely is the result of elevated ROS production. ‘Norman’ demonstrated a reduced state of oxidative stress relative to “CDC Kendall.” Abiotic stress response of ABA phytohormones may overlap with biotic pathways that may occur via stimulus by water-stress and electrolyte leakages, arising from cell wall damage (López et al., 2008). The host needs effective means to control oxidative stress to minimize cell damage. Late embryogenesis abundant (LEA) proteins are a large group of hydrophilic, stress-induced proteins termed as such since they occur at the onset of seed desiccation. LEA proteins were associated with rachides of *Fusarium*-resistant wheat lines (Liu et al., 2019). A wheat transgene (*TdLEA3*) expressed in *A. thaliana* demonstrated (Koubaa and Brini, 2020) increased stress tolerance and oxidative stress reduction through the protection of ROS-scavenging genes. Numerous LEA genes were expressed early and at higher levels in “Norman.” While peroxidase expression was common to both varieties in *Fusarium*-treatments, the barley peroxidase class III *BP1* (*HvPrx5*) gene was highly expressed, particularly in “Norman.” This barley peroxidase is uniquely endosperm-specific (Rasmussen et al., 1991) and was also identified as an important defensive gene by Petti et al. (2010), who speculated contributes to increasing cross-linkages in cell walls.

Expression patterns in ‘Norman’ differed from ‘CDC Kendall’ for genes, which may impede successful colonization through pronounced antimicrobial expression. Multiple xylanase inhibitors were expressed in “Norman,” which combat *Fusarium* hydrolytic enzymes, which degrade cell walls. While *F. graminearum* employs toxins as virulence factors, it is also equipped with a comprehensive secretome, including over 600 proteins (Brown et al., 2012).

Secreted proteins include cell wall-degrading enzymes, which contribute as virulence factors through enhanced penetration of physical barriers. Some xylanase inhibitors are also believed to possess antifungal function, with homologous similarities to chitinase III (*PR8*) proteins (Wu et al., 2013). ‘Norman’ displayed elevated rRNA N-glycosidase (ribosome-inactivating protein, Ricin A-chain), which could either function as an antifungal toxin, or facilitate programmed cell death. In the current study, antimicrobial peptides were identified earlier and with elevated expression in “Norman,” including Thionin 2.1 (HORVU6Hr1G000680, HORVU6Hr1G000780), non-specific lipid-transfer proteins (ns-LTP2) (HORVU4Hr1G087780, *PR14*), and a defensin-like protein (HORVU4Hr1G082400, *PR12*). Hao et al. (2020) demonstrated a thionin gene in *A. thaliana*-conferred resistance to *F. graminearum* but also raised *PR*-genes through SA and JA/ET hormone stimulation. Such cysteine-rich antimicrobial peptides were differentially expressed in moderately resistant wheat variety “Dream” and recognized as key resistance factors (Gottwald et al., 2012).

‘Norman’ displayed an intensified expression of proteases, including serpins, (serine protease inhibitors, which act on both trypsin and chymotrypsin), subtilisin-chymotrypsin inhibitors, alpha-amylase inhibitors, and metallocarboxypeptidase inhibitors. Alternatively, Bowman-Birk-type inhibitors (trypsin inhibitors) were dominant in “CDC Kendall,” particularly at 96 hpi. Petti et al. (2010) also documented subtilisin/chymotrypsins and serpin proteases as key actors in barley response to *F. graminearum*. Serpins play an essential role in the regulation of programmed cell death (Dickman and Fluhr, 2013) and may represent important mechanisms for the control of *Fusarium*-induced death response. Cell death may occur as a defense mechanism, but also is induced by DON. Pekkarinen et al. (2007) conducted kinetic studies of protease kinds and found chymotrypsin/subtilisin inhibitor 2 (CI-2) to be more efficient than Bowman-Birk-type trypsin inhibitors, while both were considered capable of binding *Fusarium* proteinases which may inhibit barley *PR* defense proteins. Alpha-amylase inhibitor, which was highly pronounced in “Norman,” was identified by Zantinge et al. (2010) as a protein found of higher abundance in FHB-resistant barleys. The success of more aggressive strains of *F. graminearum* is associated with increased protease levels vs. molecular diversification (Fabre et al., 2019), so stronger expressions may be important for the control of more-virulent strains. Eggert and Pawelzik (2011) observed very little degradation of storage

proteins under high FHB infection of barley, implying strong resistance mechanisms protect the endosperm. ‘Norman’ was enriched for vicilin storage proteins, which carry antimicrobial properties.

NIV-producers incited the most similar response between the two varieties under evaluation, implying that the resistance in ‘Norman’ may be DON specific. NIV producers do not share the same mycotoxin-induced stress response to H₂O₂ as DON producers, as they have higher adaptation to oxidative stress (Ponts et al., 2009). While not as aggressive as the 3ADON-producers, the NIV producers demonstrated equal ability to infect barley and accumulate mycotoxins as the 15ADON producers. While NIV may be less toxic to plants, it is more toxic to humans (Ferrigo et al., 2016). Climate changes, which may extend northern latitudes of fungi, coupled with sexual recombinant cycles and wind-dispersed ascospores, supply conceivable threats of NIV producers in northern barley production regions of North America. Given its pathogenic capabilities on barley and potential for increased toxicity, such NIV forms should be monitored through chemotype surveillance.

In the current study, we investigated the genetic variation of a moderately resistant *IVS* variety in contrast to its parent variety, where a differential response was observed. As seen in many other genetic studies resistance to DON, accumulation was complicated, where different forms of resistance occurred. While resolution limitations exist since whole seeds were ground together, this study identifies global transcriptional alterations involved in the host defense response within developing grains. Resistance genes differentially expressed in ‘Norman’ were observed in divergent relationships to generalized resistance mechanisms associated with DON avoidance. Resistances in ‘Norman’ may impede *Fusarium* growth through antimicrobial compounds and reduce DON-inducing oxidative stress *via* antioxidants. Limitations imposed by more-virulent 3ADON forms capable of producing higher levels of DON remain challenging, where barley breeders will need to continue incorporating resistance to maintain DON content below the very low limits set by the industry. Overlap was seen in resistances in either variety, implying that control of response may shape an outcome. The study has identified many genomic features, which may contribute to the barley resistance response. New technologies, such as microlaser dissection and digital droplet PCR that combine histological and transcriptomic methodologies,

can be applied to further investigate features identified in this study at the single-cell level to illuminate host-pathogen interactions within specific organelles.

Preamble: Chapter 6

In previous chapters, the industrial importance of DON contamination of the food chain has been highlighted. The fungus produces DON as a stress response to host resistance factors and through impairment of protein, translation can limit host defense mechanisms. In the previous chapter 5, we observe that a primary defense response of the host is to counter this with detoxification strategies. Robust expression of several genes categorized as uridine 5'-diphosphoglucuronosyltransferases (UDP-glucuronosyltransferases, UGTs) was observed. These genes are enzymes that transfer a glucose sugar molecule to DON, thereby changing its molecular properties. Conjugated toxins (DON-3-glucoside, DON3G) are less toxic and can be mobilized into less disruptive areas in the cell such as vacuoles. Once the mycotoxin has been modified to DON3G, it poses a problem for industrial tracking as the molecule can no longer be detected by conventional analyses. Thus seemingly non-toxic grain may possess unknown levels of 'masked' toxins. These conjugated forms can be hydrolysed by gut bacteria, and so can regain their toxic status rapidly, thereby posing a threat to consumers of barley products. As this is a common resistance mechanism to barley, an investigation was conducted using differentially resistant barley genotypes, where the levels of mycotoxins were evaluated in immature tissues (rachis and grain) as well as in mature grains. Along with the analysis of 13 common *Fusarium* mycotoxins, the ratio of DON3G/DON was evaluated across the group of selected genotypes.

CHAPTER 6

Deoxynivalenol-3-glucoside content is highly associated with deoxynivalenol levels in two-row barley genotypes of importance to Canadian barley breeding programs

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6.1 Abstract

Barley (*Hordeum vulgare* L.) is a multipurpose crop that can be harvested as grain or cut before maturity for use as forage. Fusarium head blight (FHB) is a devastating disease of barley that reduces the quality of grain. FHB can also result in the accumulation of mycotoxins such as deoxynivalenol (DON). Breeding FHB resistant varieties has been a long-term goal of many barley-producing countries, including Canada. While the genetic basis of DON detoxification via the production of less-phytotoxic conjugates such as DON-3-glucoside (DON3G) is well documented in barley, little information exists about the varietal response. Over two years, 16 spring two-row barley genotypes of importance to western Canadian barley breeding programs, were grown as short-rows and inoculated following spike emergence with a *Fusarium graminearum* conidia suspension. Half of the plots were harvested at the soft dough stage and then dissected into rachis and grain components, whereas the remainder was harvested at maturity. Multiple *Fusarium*-mycotoxins were assayed using liquid chromatography-mass spectrometry. Mycotoxin content was elevated at the earlier harvest point, especially in the rachis tissue. DON3G constituted a significant percentage (26%) of total trichothecene content and thus its co-occurrence with DON should be considered by barley industries. DON3G was highly correlated with DON and 3-acetyl-deoxynivalenol (3ADON). The ratio of D3G/DON exhibited consistency across genotypes, however, more-resistant genotypes were characterized by a higher ratio at the soft-dough stage followed by a decrease at maturity. Plant breeding practices that use DON content as a biomarker for resistance would likely result in the development of barley cultivars with lower total DON-like compounds.

Keywords: *Hordeum vulgare*; barley; Fusarium head blight; *Fusarium graminearum*; trichothecene; deoxynivalenol; deoxynivalenol-3-glucoside; DON; FHB

6.2. Introduction

Fusarium head blight (FHB) is a devastating disease of barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) that has led to immense economic losses (Wilson et al. 2018). In the USA, Canada, China, and southern and eastern Europe, this disease is primarily incited by *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schweinitz) Petch) (Gale, 2018), which produces a range of phytotoxic, secondary metabolites. *Fusarium graminearum* is capable of producing multiple type B trichothecene mycotoxins, which are characterized by a keto (carbonyl) function at the C-8 position of the trichothecene molecule, eg. deoxynivalenol (DON), 3 and 15-acetyl deoxynivalenol (3ADON, 15ADON), nivalenol (NIV) (McCormick et al. 2011). Trichothecenes inhibit protein and ribonucleic acid synthesis, impact cellular functions of the division, membranes, and mitochondrial activity (Rocha et al. 2005), and have the potential to cause toxicoses in humans and animals (Antonissen et al. 2014). *Fusarium graminearum* also synthesizes zearalenone (ZEA) an immunotoxic compound that mimics estrogen by binding to mammalian receptors (Hueza et al. 2014). Trichothecenes such as DON are particularly damaging to agricultural production systems, due to their global distribution and the high frequency at which they occur (Ferrigo et al. 2016). Due to fact that DON is thermally stable and water-soluble, it can readily be transmitted from malt to beer (Kostelanska et al. 2009; Peters et al. 2017). Deoxynivalenol is highly regulated by the brewing and malting industry due to public safety concerns, where exceeding limits as low as 0.5 mg kg⁻¹ may result in a rejected sale (Wilson et al. 2018). Malting barley is considered a high-value crop with price premiums, where producers employ crop management strategies to minimize the risk of quality loss due to the presence of DON. Breeding genetically resistant varieties remain the most economical and environmentally-friendly means to mitigate mycotoxin occurrence in human food and animal feed.

Fusarium graminearum is considered a hemi-biotrophic pathogen. Following infection, the pathogen lives biotrophically within the host for a short period growing inter-cellularly and asymptotically, then shortly thereafter switches to necrotrophy where it derives nutrients from dead cells (Trail, 2019; Ma et al. 2013). The pathogen employs specialized infection structures

such as compound appressoria and foot structures to penetrate the host tissues which are associated with DON production; regardless, DON is not essential for the infection process (Boenisch and Schäfer, 2011). Significant quantities of DON are not observed in barley until a few days post-infection, where it accompanies a transition to the necrotrophic phase (Evans et al. 2000; Boddu et al. 2006). Host plants have evolved layers of resistance mechanisms, which have been characterized as resistance categories for breeding purposes (Mesterházy, 1995). Resistance may involve the inhibition of initial infection (Type I); or restriction of subsequent spread (Type II) during the prolific phase, where a striking contrast exists for FHB epidemiology between barley and wheat (Janssen et al. 2018). Barley generally possesses superior Type II resistance (Bai et al. 2004). In contrast to wheat, fungal spread within the spike is highly restricted at the rachis node, and defense is not enhanced for mutants with a disrupted trichodiene synthase gene (*TRI5*) and inability to produce DON (Jasen et al. 2005; Maier et al. 2006; Langevin et al. 2004). While DON does not contribute to fungal spread in barley, disrupted *tri5*-mutant strains produce lower disease severity, fungal biomass, and floret necrosis/bleaching compared to the wild-type, establishing the role of DON as a virulence factor (Boddu et al. 2007). Barley displays an extended infection period following anthesis, where later infections may be symptomless while still accumulating mycotoxin (Yoshida et al. 2007; Osborne et al. 2007). The sum of individual kernels from infections at different developmental stages collectively contributes to overall mycotoxin levels, both through mycotoxin content and grain biomass.

Following pathogen infection, plant defense may involve Type V resistance mechanisms (resistance to toxins) which may function to a) modify mycotoxins via conjugation with other molecules for consequential detoxification and; b) limit mycotoxin production by the fungus (Boutigny et al. 2008). Mycotoxins conjugated through specific reactions of host enzymes result in compounds with diminished phytotoxicity. Chemical modification of toxin structure may result in reduced toxicity by directly impeding their mode of action, or increasing their mobility and transfer to storage in organs such as vacuoles and/ or binding to cellular membranes (Berthiller et al. 2013). Once conjugated, modified mycotoxins may not be detected by routine analysis of food or feed products. Deoxynivalenol-3-glucoside (DON3G), a common conjugated form of DON found in barley grains, is readily cleaved in the intestines such that the same toxic effects are expected (EFSA CONTAM Panel, 2017).

The genetic basis of the DON detoxification mechanism has been well studied in barley, where a uridine diphosphate glycosyltransferase (UGT) barley gene *HvUGT13248* (MLOC_65675) has been functionally characterized through transgenic approaches in yeast (Schweiger et al. 2010) and *Arabidopsis thaliana* (Shin et al. 2012) model systems. Resistant wheat varieties that carry *Fhb1* QTL show greater ability for conversion of DON to DON3G in resistant genotypes (Lemmens et al. 2005; Kluger et al. 2015). However, in wheat lines with an elevated detoxification rate, levels of DON and DON3G together are generally lowered compared with susceptible varieties (Lemmens et al. 2016; Buerstmayr and Lemmens, 2015; Amarasingne et al. 2016). To date, minimal information is available regarding relative occurrence patterns of DON and its conjugated form DON3G and their interaction within barley genotypes with differential resistance. To analyze DON, its conjugated form DON3G, and the acetylated forms of 3ADON and 15ADON, the current study utilized liquid chromatography-tandem mass spectrometry (LC-MS/MS). This is a method with a high separation capacity of analytes paired with mass analysis which offers the utility of multiplex analysis within an individual sample (Soleimany et al. 2012). With an increased understanding of the mechanisms that underlie resistance to DON accumulation, barley breeders can make informed decisions during the development of FHB resistant varieties with the result of lowered toxin in the field. This study was conducted to evaluate the effect of genotypic differences in barley on mycotoxin production, with a specific interest in DON3G and its relative occurrence to DON.

6.3 Materials and Methods

6.3.1 Inoculum Preparation

An equal mixture of 3-ADON-producing (M2-06-01, Q-06-11, S3BS-06-01, WRS-2065, and WRS-2067) and 15-ADON-producing *F. graminearum* isolates (S3AN-06-01, NB-06-18, A1-06-01, WRS-1915, and WRS-1918) from multiple provinces across Canada was used to initiate inoculum. Mycelium-infected filter paper discs were placed on 10 cm plastic Petri plates of potato dextrose agar (PDA). Petri plates were placed in a growth chamber for 10 days at 20 °C; 16 h D: 8 h L where they were grown to full colonization of the PDA media. Fresh tomatoes were cut into 1 cm³ cube and 100 g L⁻¹ were soaked in distilled water for 4 h. Solids were strained out through cheesecloth, and 15 g L⁻¹ NaCl was added to the suspension. The suspension was divided into 500 mL aliquots into 1 L Erlenmeyer flasks, autoclaved for 20 min, and then allowed to cool. In a

biosafety cabinet, individual-isolate PDA plates were cut into 1 cm³ cube and placed into flasks. Flasks were placed on an orbital shaker for 2 weeks under natural light cycle. Following, conidia were counted on a hemocytometer under 100X microscopy, then diluted to 5×10^4 spores mL⁻¹. Tween 20 surfactant was added to the suspension at 0.04% (v/v). Standardized spore suspensions were mixed in equal proportions for either chemotype set.

6.3.2 Field Experiment

A total of 16 two-row, spring barley varieties were selected for their differential patterns of resistance to FHB and DON accumulation, as previously assessed over multiple years in an FHB nursery at Brandon, MB. This set included ten cultivars adapted to western Canada (AAC Synergy, AC Metcalfe, CDC Austenson, CDC Bold, CDC Copeland, CDC Kendall, CDC Mindon, Conlon, Norman, and Xena), two elite resistant Canadian breeding lines (TR04282 and TR05287) and four exotic barleys (Harbin, HDE84194-622-1, Kutahya and Shenmai 3). All varieties were grown together at the Agriculture and Agri-Food Canada, Brandon Research and Development Centre in 2016 (49°51'59.5" N, 99°58'46.6" W) and 2017 (49°51'55.5" N, 99°58'52.9" W). Research plots consisted of 0.9 m single rows with 0.3 m spacing. Plots were sown at a density of 40 seeds per row. The experimental design was a split-split plot arrangement randomized as a randomized complete block design. Whole plot treatments either consisted of inoculated plots of *F. graminearum* conidia suspensions or a mock spray of water and Tween 20. Sub-plots were barley genotypes, where the entire experiment was replicated three times. The entire experiment was surrounded by a double guard-row of tall, forage barley CDC Cowboy.

A polyvinyl chloride irrigation system was employed, consisting of a 5 cm main tube and 2 equispaced risers (10 cm) positioned above the canopy. Fine water droplets were applied on a 7 m radius via NAAN 501-U 2.2 mm nozzle sprinkler heads. Moisture was applied before inoculations until spikes were saturated. Heading notes (75% of the row, spikes emerged) were taken on each plot. Following the heading, plots were inoculated with 40 mL of conidia suspension or water as applied by a spray wand from a stainless-steel canister pressured to 250 kPa. A second application was applied to each plot, three days following the first application.

Three weeks post-infection, FHB incidence (% of spikes infected) and FHB severity (% of infected grains per infected spike) were recorded, based on observations of 10 spikes. At the soft-dough stage, (Z85; Zadoks scale for growth stages of cereals [Zadoks et al. 1974]) half of the row was

cut with a sickle and placed in a mesh harvest bag and held in a high capacity drier at 37 °C for three days to arrest fungal development. The remaining plot was allowed to ripen in situ and subsequently harvested at maturity (Z92) by a small-plot combine under a minimal wind speed setting.

6.3.3 Mycotoxin Analysis

6.3.3.1. Sample Preparation

The spikes cut at the soft-dough stage were further dissected into rachis and grain portions. Harvested grains from both developmental stages were sub-sampled by scooping 20 g and ground to flour using a Perten 3610 laboratory mill. Likewise, rachis portions (1 g) were ground.

6.3.3.2. Extraction and Assay

Samples were analyzed for mycotoxins following the method reported by Tittlemier et al. (Tittlemier et al. 2019). Grain samples were extracted using a 1:4 (*m/v*) ratio of extraction solution consisting of acetonitrile/Milli-Q water/acetic acid (74/25/1). Rachis samples were extracted using a 1:8 (*m/v*) ratio of extraction solution. Samples were placed on a flatbed shaker for 20 min on high. After samples settled, the extract was transferred to a centrifuge tube and centrifuged at 20 °C, 2534× *g* for 10 min. A total of 62.5 uL of the extract was diluted to a final volume of 1000 uL with 5 mM ammonium Acetate in water containing 0.5% acetic acid. Sample extracts were fortified with ¹³C-labeled internal standards to mitigate matrix effects during analysis. Mycotoxins were separated using reverse-phase liquid chromatography and analyzed using tandem mass spectrometry in the electrospray ionization mode. Two transitions were monitored for each analyte. Analytes were considered to be identified and were quantified in samples when their retention time was within 0.1 min of the mean retention time in external standards used to construct the calibration curve, the ratio of qualifier to quantitation transition was within ±30% of the mean ratio in external standards used to construct the calibration curve, and the peak area signal-to-noise ratio was at least 10:1. A panel of fourteen mycotoxins (six produced by *F. graminearum* in Canada) were quantified within grain and rachis tissues (Appendix 4.3). Mycotoxins were quantified using a calibration curve constructed from seven external standards. Peak areas from quantitation transitions were normalized to the peak area of ¹³C-labeled internal standard during data analysis to mitigate matrix effects on quantitation before interpolation of analyte concentration from the

calibration curve. Specifically, ^{13}C -labeled internal standards were available and used for DON and DON3G.

Several quality control samples were processed and analyzed aside from the study samples throughout the study to ensure good method performance throughout the multi-year study. Matrix blanks were used to monitor for contamination. Portions of matrix blanks were also fortified before extraction with all mycotoxin analytes and were used with commercially-available and in-house reference materials to monitor the accuracy and precision of the method.

6.3.3.3. Quality Control during Analysis

Calibration curves ($R^2 > 0.99$) were used to calculate the concentration of the analyte. Analyte concentrations were recovery corrected using the percent recovery of analyte from a matrix-matched fortified blank, which was run with each batch of samples. Matrix-matched blanks, matrix-matched fortified blanks (spiked with a solution of standards), and certified reference materials were extracted and analyzed to monitor method performance throughout this study.

6.3.4. Statistical Analysis

A generalized linear mixed model (GLMM), was used to analyze the data over years. PROC GLIMMIX in SAS (version 9.4; SAS Institute, Cary, NC, USA) was used to test for significant differences in genotypes across years for FHB incidence and FHB severity, and mycotoxin content. Replicate was nested in the year as a random factor and modeled using a Poisson distribution and log link function. Mycotoxin distributions were positively skewed. The lowest AIC was used to choose the best transformation. Mycotoxin content (DON, DON3G, 3ADON, DON3G/DON) was modeled using a log-normal distribution and identity link function. Tukey-Kramer honestly significant difference was used to test for all pairwise comparisons.

Data were converted from mass to molar concentrations through division by the molar mass of DON3G and DON (458.5 g mol^{-1} and 298.3 g mol^{-1} respectively) to facilitate a comparison of DON3G/ DON ratios amongst genotypes. This allowed DON3G/ DON ratios to be reported on a molar basis, as opposed to a mass basis. Ratios were calculated as $\text{DON3G mol/ DON mol} \times 100$.

The ‘cor’ function in the R computing environment (R core team) was used to generate Pearson correlations between days to heading, FHB incidence, FHB severity, DON, DON3G, 3ADON,

DON3G/DON for 16 genotypes. Packages ‘ggcorrplot’ and ‘ggplot2’ were used to produce graphics of the correlation matrix heatmap and scatterplot.

6.4 Results

This study evaluated a set of barley varieties that represent cultivars, elite breeding lines, and FHB resistance sources relevant to the western Canadian barley breeding activities for developing barley cultivars with improved resistance to FHB (Table 6.1).

Table 6.1. Pedigree and origin of 16 two-row, hulled spring barley genotypes evaluated for reaction to *Fusarium* head blight in 2016 and 2017 at Brandon, Manitoba.

Name (Alias ¹)	Pedigree	Origin ²	Type ³	Release ⁴
AC Metcalfe (TR232)	AC Oxbow/Manley	CDN	MALT	1997
CDC Copeland (TR150)	WM861-5/TR118	CDN	MALT	1999
CDC Bold (SD422)	SB88403/Tyra	CDN	GP	1999
CDC Kendall (TR133)	Manley/SM85221	CDN	MALT	1999
Xena (TR975)	Stark/Baroness	USA	GP	2002
Conlon (TR982)	Bowman*2/Birgitta dwarf//ND10232	USA	MALT ⁵	2004
CDC Mindon (TR04378)	TR339/TR251	CDN	GP	2008
Norman (TR05915)	<i>In vitro</i> selection from CDC Kendall	CDN	MALT	2009
CDC Austenson (TR06389)	TR358/94Ab12271	CDN	GP/F	2010
AAC Synergy (TR09208)	TR 02267/Newdale	CDN	MALT	2012
TR05287	Svanhals/AC Metcalfe//TR253	CDN	MALT	-
TR04282	Harbin/TR253//TR253	CDN	MALT	-
Kutahya		NLD	-	-
Shenmai 3	Gobernadora /Humai 10	CHN	-	-
HDE84194-622-1		CHN	-	-
Harbin		CHN	-	-

¹ Coding under evaluation in Western Canadian Cooperative Barley Registration Test.

² CDN = Canada; CHN = China; NLD = Netherlands; USA = United States of America.

³ MALT = Malting; GP = General Purpose; GP/F = General Purpose, Forage.

⁴ Year of rights granted by the Canadian Food Inspection Agency. Conlon was registered in the USA in 1996.

⁵ Accepted for malting quality in the USA, but not in the Canadian market.

6.4.1. Heading, Incidence, and Severity

The number of days to heading differed significantly amongst genotypes (F-value = 74.0, $p \leq 0.0001$), while no significant interaction was observed between genotypes with year (F-value 1.62, $p = 0.142$). Genotypes demonstrated a 10-day range of average heading dates, where exotic breeding line HDE84194-622-1 headed at 45.0 ± 0.4 days in contrast with 55.3 ± 0.2 days for Canadian forage cultivar CDC Austenson. Control and inoculated treatment groups did not differ within any year for days to heading (2016: $t = 0.14$; $p = 0.889$; 2017: $t = 0.52$; $p = 0.600$).

Fusarium head blight was present at low levels in the control group each year, equivalent to background infection. A contrasting FHB epidemic was incited in the treatment group by the conidial spray applications. Differences in magnitude were observed between control and inoculated treatments ($p \leq 0.0001$), for both incidence (3.1% control vs. 33.5% treatment) and severity (1.8% control vs. 17.8% treatment). While FHB symptoms of control and Fusarium treatment groups differed by an order of magnitude, average incidence and severity of barley genotypes demonstrated a positive association between the groups ($R^2 = 0.74$; 0.61 respectively).

Fusarium head blight symptoms showed differentiation amongst barley genotypes with wide ranges in average percent incidence and severity, with less variation observed in the latter (Figure 6.1). Significant differences were observed amongst genotypes for incidence ($F = 85.63$, $p \leq 0.001$) and severity ($F = 27.08$, $p \leq 0.001$). A significant interaction was observed between genotype and year for incidence ($F = 7.94$, $p \leq 0.001$) and severity ($F = 6.11$, $p \leq 0.001$). A significant effect of year was seen for severity ($F = 15.76$, $p = 0.017$), but not for incidence ($F = 4.23$, $p = 0.109$). The highest incidence occurred in HDE84194-622-1, whereas semi-dwarf, Canadian variety CDC Bold displayed the highest severity. The lowest incidence was observed for Chinese accession Harbin and the lowest severity occurred in Kutahya, a cultivar from the Netherlands. Earlier heading genotypes such as Conlon, Shenmai 3, and HDE84194-622-1 have typically demonstrated higher resistance in past trials when inoculated using surface-applied, Fusarium-infected corn kernels (US Wheat and Barley Scab Initiative, North American Barley Scab Evaluation Nursery report).

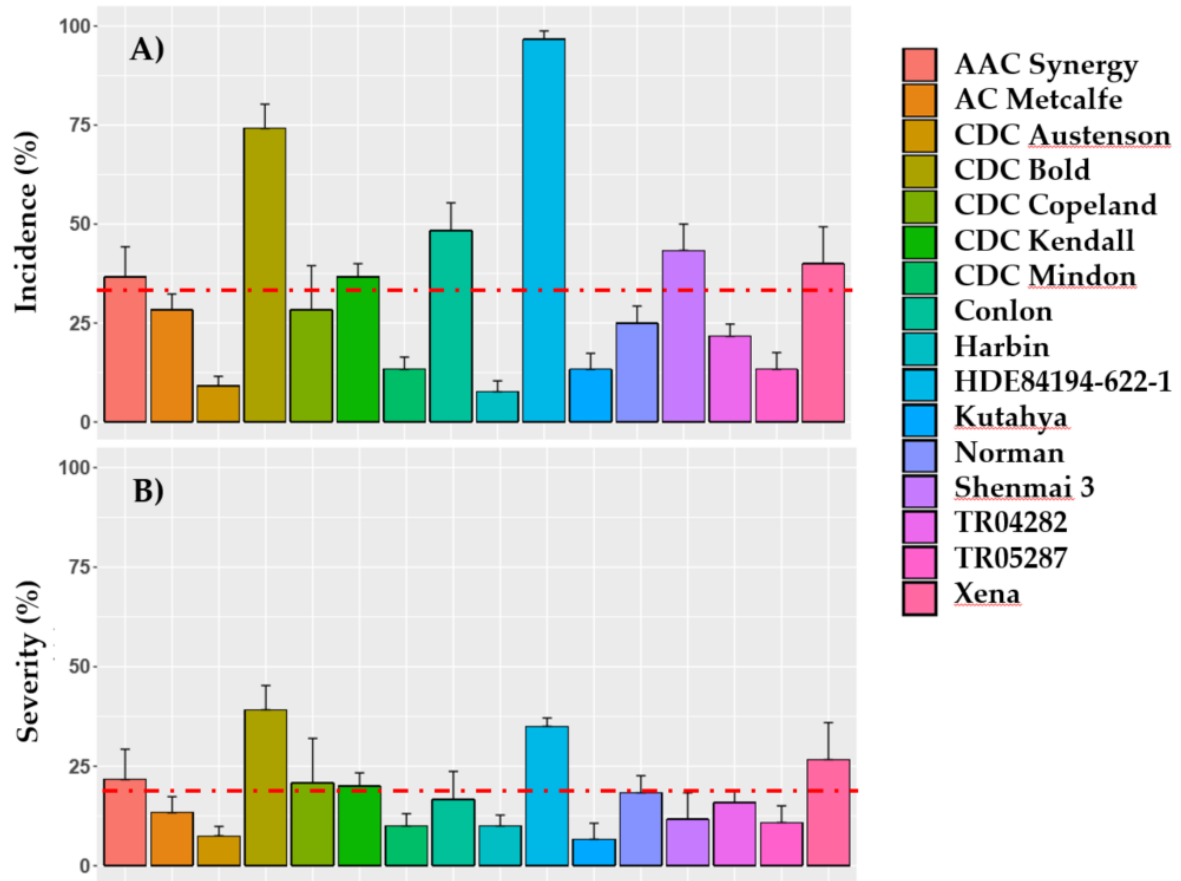


Figure 6.1. Mean Fusarium head blight symptoms of 16 inoculated barley genotypes over 2016–2017 at Brandon, MB. **(A)** Percent incidence **(B)** Percent severity. Error bars represent standard error. The red dashed line represents the overall mean.

6.4.2 Mycotoxins

Significant increases in mycotoxins were observed in the inoculated treatment group. Mycotoxins demonstrated a wide range of magnitude and frequency of occurrence (Appendices 4.1–4.2). Zearalenone (ZEAR) was found at very low levels, where a trend was observed for higher content at maturity (0.007 vs. 0.024 mg kg⁻¹, $p = 0.27$). NIV was detected at low levels overall, but with higher concentration at the early vs. mature stage (0.8 vs. 0.3 mg kg⁻¹, $p < 0.00001$) and in rachis vs. grain tissues (2.1 vs. 0.8 mg kg⁻¹, $p < 0.0001$). Culmorin (CUL) was only evaluated in 2017, where it showed low content in all tissues and stages (<1 mg kg⁻¹ on average). 15ADON was only detected in the 2016 samples and at very low levels (<0.1 mg kg⁻¹). Acetylated forms of DON i.e.

effectively 3ADON, were found at low levels representing approximately 4% of total DON for both control and inoculated treatments. In a few samples, 3ADON occupied a relatively larger proportion of total mycotoxin composition, where it was found to represent up to 15% of DON-like compounds. 3ADON showed higher content on soft dough ($p < 0.0001$) and in rachis vs. grain tissue ($p < 0.01$). As generally seen in commercial samples, the majority of non-DON Fusarium-related mycotoxins were found at levels of insignificance, even under epidemic conditions.

Of all the Fusarium-associated mycotoxins evaluated, DON and its conjugated form DON3G were detected commonly and at the highest concentration levels, particularly for DON. The effect of genotype was significant for DON and DON3G in all tissues (Table 6.2A–C). DON and DON3G genotype means demonstrated a moderate correlation between control and Fusarium treatment groups ($R = 0.76$ and 0.64 , respectively). For inoculated materials, the highest concentrations were observed at the earlier developmental stage and within rachis tissue (Table 6.3A–C). DON was detected above the limit of quantification (0.03 mg kg^{-1}) for every inoculated sample, and in over 93% of the ripe grains of the control treatment. Of the naturally infected control samples, 15% (2016) and 48% (2017) would have failed the industry malting acceptance standard of $\leq 0.5 \text{ mg kg}^{-1}$ (Wilson et al. 2018).

Table 6.2. Analysis of variance for deoxynivalenol (DON), deoxynivalenol-3-glucoside (DON3G), 3-acetyl-deoxynivalenol (3ADON) concentration, and the ratio (DON3G/DON) for different sample types using sixteen genotypes of two-row barley.

Source	DON		DON3G		3ADON		DON3G/DON	
	F	Pr > F	F	Pr > F	F	Pr > F	F	Pr > F
A) Grain – Soft-dough								
Genotype	4.09	<0.0001	4.72	<0.0001	5.27	<0.0001	2.21	0.02
Year	2.96	0.16	5.58	0.08	16.92	0.01	6.74	0.01
Genotype x Year	2.17	0.02	2.51	0.01	9.15	<0.0001	1.11	0.37
B) Grain – Rachis								
Genotype	3.63	0.0002	2.43	0.01	2.12	0.03	4.72	<0.0001
Year	5.81	0.07	1.38	0.30	3.43	0.07	12.01	<0.001
Genotype x Year	1.50	0.13	2.10	0.02	5.24	<0.0001	3.51	<0.001
C) Grain – Mature								
Genotype	4.31	<0.0001	5.56	<.0001	3.14	0.001	9.51	<0.0001
Year	5.36	0.08	10.07	0.03	16.39	0.02	23.04	<0.0001
Genotype x Year	3.91	<0.0001	2.53	0.01	4.32	<0.0001	3.09	<0.001

Table 6.3. Least-squares means of 16 barley genotypes for deoxynivalenol (DON), deoxynivalenol-3-glucoside (DON3G), 3-acetyl-deoxynivalenol (3ADON) concentration (mg kg⁻¹) and the percent molar ratio (DON3G/DON) for different sample types over years 2016–2017.

Genotype	DON		DON3G		3ADON		DON3G/DON	
A) Grain - Soft-dough								
AAC Synergy	19.5	abc	11.2	a	1.5	a	0.6	a
AC Metcalfe	12.9	abc	4.9	ab	1.4	a	0.4	ab
CDC Austenson	4.2	bc	2.1	ab	0.4	a	0.5	ab
CDC Bold	33.2	ab	14.0	a	2.8	a	0.4	ab
CDC Copeland	9.0	abc	3.3	ab	0.6	a	0.4	ab
CDC Kendall	11.0	abc	4.4	ab	1.0	a	0.4	ab
CDC Mindon	4.0	c	2.7	b	0.5	a	0.5	ab
Conlon	17.3	abc	6.0	ab	0.9	a	0.3	ab
Harbin	9.2	c	2.5	b	0.8	a	0.4	ab
HDE84194-622-1	32.8	a	10.9	a	1.8	a	0.3	ab
Kutahya	5.8	abc	1.8	b	0.5	a	0.3	b
Norman	3.8	c	1.8	b	0.3	a	0.4	ab
Shenmai 3	17.8	abc	5.7	ab	1.4	a	0.4	ab
TR 04282	14.4	abc	6.0	ab	1.5	a	0.4	ab
TR 05287	14.7	abc	6.4	ab	1.3	a	0.5	ab
Xena	11.4	abc	4.4	ab	1.0	a	0.4	ab
Overall Mean	13.8		5.5		1.1		0.4	

Genotype	DON		DON3G		3ADON		DON3G/DON	
B) Rachis - Soft-dough								
AAC Synergy	34.0	ab	40.1	a	2.3	a	1.4	abc
AC Metcalfe	17.7	abc	15.2	ab	1.8	a	1.1	abc
CDC Austenson	6.1	bc	7.4	ab	0.8	a	1.5	ab
CDC Bold	28.7	abc	37.7	a	2.5	a	1.4	abc
CDC Copeland	13.0	abc	13.7	ab	0.8	a	1.1	abc
CDC Kendall	15.8	abc	16.3	ab	1.3	a	1.4	abc
CDC Mindon	8.6	abc	8.0	ab	1.0	a	1.4	abc
Conlon	26.5	abc	10.1	ab	1.1	a	0.7	cd
Harbin	12.8	c	11.8	ab	1.1	a	1.7	a
HDE84194-622-1	48.8	a	22.9	ab	3.7	a	0.4	d
Kutahya	5.6	bc	5.9	b	0.3	a	0.9	bcd
Norman	5.1	c	5.6	ab	0.3	a	1.4	abc
Shenmai 3	17.2	abc	9.2	ab	1.0	a	0.6	cd
TR 04282	18.1	abc	16.5	ab	1.9	a	0.9	abcd
TR 05287	20.3	abc	17.8	ab	1.7	a	1.0	abc
Xena	16.6	abc	15.8	ab	1.5	a	0.9	abc
Overall Mean	18.4		15.9		1.5		1.1	

Genotype	DON		DON3G		3ADON		DON3G/DON	
C) Grain - Mature								
AAC Synergy	10.3	abc	4.9	abcd	0.6	ab	0.5	a
AC Metcalfe	12.9	abc	4.4	abcd	0.7	ab	0.3	bcd
CDC Austenson	5.7	bc	1.6	cde	0.3	b	0.3	f
CDC Bold	19.2	ab	6.9	abc	1.2	a	0.3	bcd
CDC Copeland	11.8	abc	4.5	abcde	0.8	ab	0.4	ab
CDC Kendall	10.1	abc	4.1	abcde	0.6	ab	0.4	abc
CDC Mindon	4.9	c	1.4	e	0.3	ab	0.3	df
Conlon	15.5	ab	5.8	ab	0.6	ab	0.4	abcd
Harbin	6.5	bc	1.5	d	0.3	ab	0.2	f
HDE84194-622-1	26.0	a	8.2	a	1.1	a	0.4	abcd
Kutahya	7.2	abc	2.4	abcde	0.3	ab	0.3	bcd
Norman	4.1	c	1.6	bcde	0.2	b	0.4	abcd
Shenmai 3	18.6	ab	7.1	a	0.7	ab	0.4	abc
TR 04282	7.8	abc	3.2	abcde	0.6	ab	0.4	abcd
TR 05287	9.9	abc	3.9	abcde	0.5	ab	0.4	abcd
Xena	9.7	abc	3.2	abcde	0.5	ab	0.3	dc
Overall Mean	11.3		4.0		0.6		0.4	

Note: The letter following represents the significance group from the Tukey-Kramer Honest Significant Difference test), where shared letters indicate values that do not differ significantly. Means have been back-transformed to the original scale.

6.4.3. Relationships between Characters

Percent incidence and severity demonstrated a moderately-high positive association (Figure 6.2A–C). The number of days to heading was negatively associated with incidence and DON content. Both incidence and severity were moderately associated with DON content, where the strongest relationship was observed between visual symptoms and DON content in grains at the soft-dough stage. Incidence and severity demonstrated a moderate association with 3ADON.

Visual symptoms were not associated with NIV and ZEA. DON3G demonstrated a robust linear relationship with DON, which was highest in grains vs. rachis tissues ($R^2 = 0.92$, Figure 6.3).

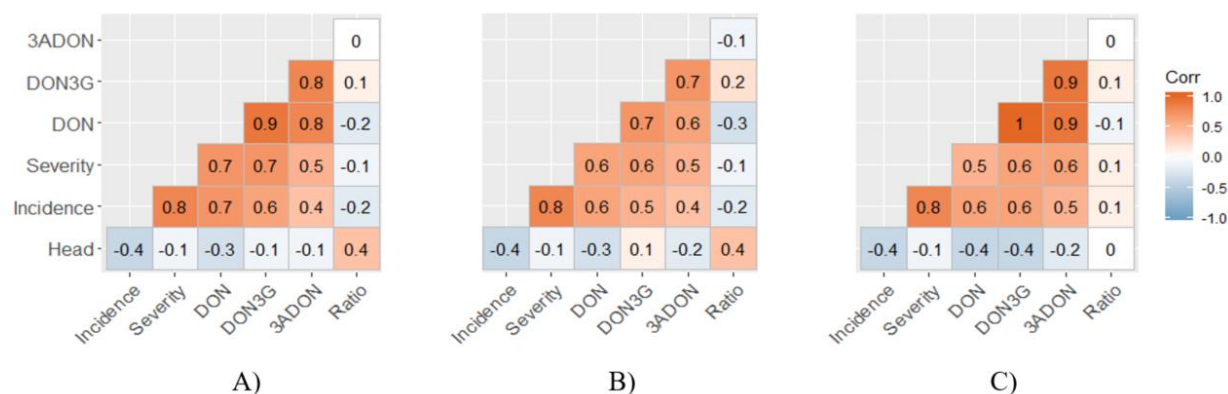


Figure 6.2. Pearson correlation coefficients for visual symptoms and mycotoxin content (mg kg⁻¹), where Head = Days to 75% of spike emergence in a row; Incidence = Percentage of infected spikes per plot; Severity = Percentage of infected grains per infected spike; DON = deoxynivalenol; DON3G = deoxynivalenol-3-glucoside; 3ADON = 3-acetyl-deoxynivalenol; Ratio = DON3G/DON. **(A)** grain tissue harvested at soft-dough stage. **(B)** rachis tissue harvested at soft-dough stage. **(C)** grain tissue harvested at maturity.

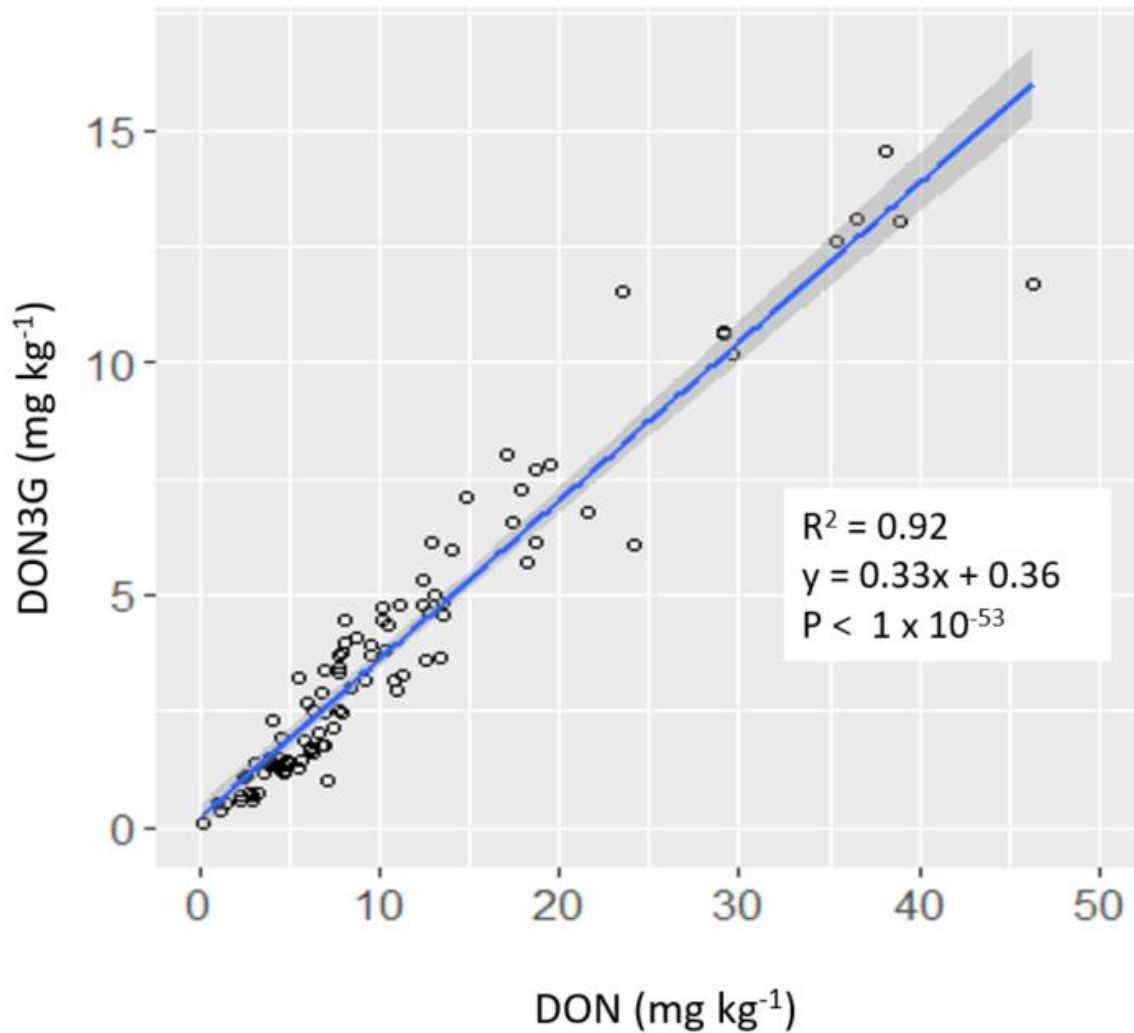


Figure 6.3. Linear relationship of deoxynivalenol-3-glucoside (DON3G) with deoxynivalenol (DON) content in matured grains. The shaded area represents a 95% confidence interval for the fitted line.

Genotypes in this study were chosen to represent a range of resistance reactions based on past performance. Rankings of genotypes by FHB disease symptoms or DON content did not appear to be strongly associated with the ratio of DON3G to DON (Figure 6.2A–C). This ratio proved consistent between soft-dough (27 mol%) and maturity harvest dates (23 mol%) (Figure 6.4). While the genotypes in this study displayed a wide range of resistance, the ratio generally displayed subtle differences over genotypes. However, some of the lowest-DON-producing genotypes such as Norman, CDC Mindon, CDC Austenson, and Harbin demonstrated an above-average ratio at the soft dough, but a below-average ratio at maturity (Figure 6.4). At soft-dough harvest, AAC

Synergy and CDC Mindon displayed the highest DON3G/ DON ratio (37 mol% and 35 mol% respectively), whereas Kutahya and HDE84194-622-1 showed the lowest ratio (20 mol% and 22 mol% respectively). At maturity, AAC Synergy and CDC Copeland displayed the highest DON3G/ DON ratio (31 mol% and 29 mol%), while Harbin and CDC Austenson showed the lowest ratio (15 mol% and 17 mol%). DON and DON3G were both found at elevated levels in the rachis tissue, where they were more equally represented (DON3G/ DON ratio = 71 mol%). Unlike in grain tissues, greater differentiation was seen over varieties where a negative relationship was observed for DON content at maturity and the ratio of DON3G/ DON ($R^2 = 0.37$). HDE84194-622-1 and Shenmai 3 showed the lowest ratio (29 mol% and 39 mol% respectively), whereas Harbin and CDC Austenson displayed a very high ratio of (111 mol% and 95 mol%, respectively).

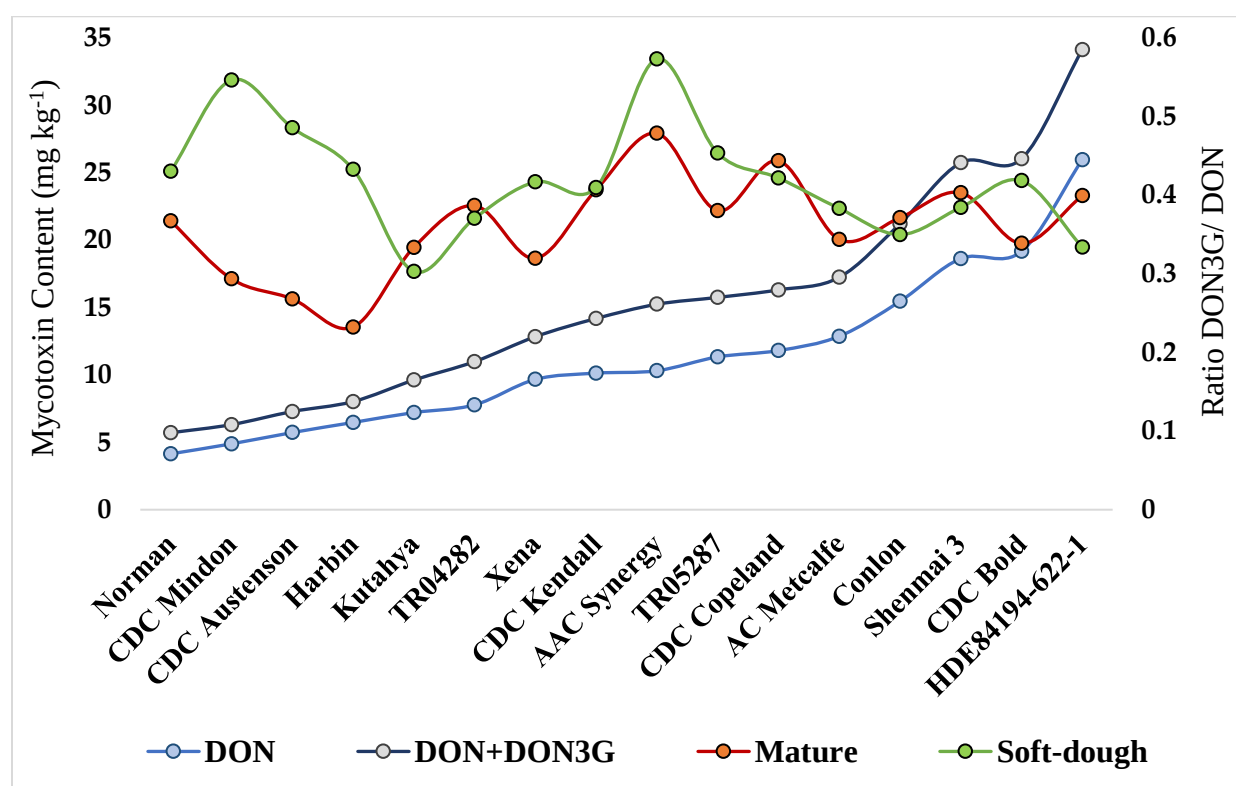


Figure 6.4. Left axis: Deoxynivalenol (DON) concentration (light blue line) and total DON + deoxynivalenol-3-glucoside (DON3G) concentration (black line) in grain. Right Axis: Ratio of DON3G to DON at soft-dough (green line) or mature stage (red line) for 16 barley genotypes organized in ascending order for mean DON content at maturity over 2016–2017.

3. Discussion

A series of FHB epidemics of immense economic proportions occurred in the Red River basin of North America during the mid-1990s (McMullen et al. 1997). In Canada, FHB quickly arose from an insignificant concern to the most economically damaging disease of barley in only a period of a few years (Tekauz et al. 2000). Since its initial occurrence in Manitoba, production issues associated with *F. graminearum* have become increasingly common in neighboring provinces of Saskatchewan and Alberta, which are the major regions of barley production in Canada. Moderately-resistant barley germplasm has been identified in FHB nurseries under Canadian environmental conditions and developing FHB resistance with low DON accumulation has been a long-term goal maintained by the Canadian barley breeding programs (Legge et al. 2004). The use of FHB resistant varieties that accumulate less DON in combination with proper crop management practices may increase the probability of barley being selected and sold with malting premium. This study investigated host response and mycotoxin accumulation of 16 two-row barley genotypes relevant to FHB resistance breeding activities in western Canada.

In Canada, 3ADON-producers of *F. graminearum* have rapidly displaced the traditional 15ADON population, where an east-to-west geographical gradient has been observed (Ward et al. 2008). However, the native 15ADON chemotype remains dominant in Ontario potentially due to stable maize production among other factors (Crippin et al. 2020). Several studies of wheat have shown that 3ADON chemotypes produce higher DON during pathogenesis (Puri and Zhong, 2010; Foroud et al. 2012; Von der Ohe et al. 2010). A field-based barley study at the same location found higher DON content associated with the proportion of 3ADON-producers as measured by DNA markers (Clear et al. 2013). Under the current study, chemotypes were applied in equal proportion, however, 3ADON was by far the dominant acetylated form detected. DON content demonstrated a strong association with 3ADON under the study conditions. While many other virulence factors may be of equal importance, the competitiveness of 3ADON-producing strains may be associated with elevated DON production.

The relationship between infection and mycotoxin formation has not been extensively studied in barley (Kostelanska et al. 2009). The developing barley caryopsis is protected by the thick-walled epidermis of the palea and lemma, but the pathogen evades this barrier by entry via the crevice between these structures or apical mouth before fusion occurs approximately 8 days post-

emergence of the spike (Lewandowski et al. 2006; Skadsen and Hohn, 2004). Grains harvested at the soft-dough stage were observed to possess 22% higher DON content than those harvested at maturity. This is consistent with previous results for barley, where it has been demonstrated that DON content peaks during seed development, followed by reductions to maturity (Prom et al. 1999). Rachis tissue had the highest concentration of mycotoxins, 33% more DON than grains from the same spikes. In wheat, elevated DON concentration has also been documented in rachis tissues (Cowger and Arellano, 2013). In the current study, the ratio of DON3G to DON was much higher in the rachis (71 mol%) vs. grains tissue (27 mol%), indicating heightened resistance response in this tissue, which is in agreement with the strong Type II resistance seen in barley. While resistant to spread, barley is susceptible for an extended period following spike emergence, where cleistogamous (closed-flowered) genotypes accumulated more mycotoxins when inoculated 10 or 20 days after anthesis (Yoshida et al. 2007). Recently, trichomes of palea and lemma have been highlighted as structural targets for susceptibility in later-stage infections (Imboden et al. 2018). Trichomes of rachis tissues have been previously hypothesized as an invasion point that could be used to overcome the strong Type II resistance of barley through external growth of mycelia over the epidermis (Jansen et al. 2005). Total-DON forms were elevated in rachis tissue both by higher DON levels and increased proportion of DON3G, however, limitations of tissue biomass and the impact of microbial activity in silage should be considered when determining the risk of toxicosis when feeding animals forages produced using whole-plant tissues. Elevated DON and DON3G levels observed within the rachis may be the result of translocation from infected kernels or direct colonization. Regardless, this rachis tissue is highlighted for further study of host response in barley.

Transcriptomic analysis experiments of six-row barley which used contrasts of *tri5* mutants vs. wild type (Boddu et al. 2007) or exogenous application of DON solution vs. mock (Gardiner et al. 2010) have identified a range of mycotoxin-specific genetic defense in barley. Induced genes were generally up-regulated during trichothecene accumulation, which included functions such as transport, programmed cell death, ubiquitination, transcription factors, and cytochrome P450s and UGT genes. Wheat plants transformed with a barley UGT gene (*HvUGT13248*), exhibited elevated FHB resistance accompanied by reduced DON content through hastening elevation of DON3G/DON ratio (Li et al. 2015). Barley demonstrates a strong ability to induce an enzymatic system to convert DON to DON3G. An RNA-Sequencing analysis of near-isogenic lines (NILs)

of Chevron-derived resistant alleles confirmed that *F. graminearum* infection-response in barley may involve both constitutive and inducible defense mechanisms involving earlier induction of genes such as *HvUGT13248* (Haung et al. 2016). DON is known to interfere with ribosomal function and thereby the production of defensive pathogen-related proteins, such that earlier onset of DON-related resistance may allow for the expression of other resistance mechanisms. Fusarium resistance response is complex where defense compounds, typically associated with restricting fungal spread such as reactive oxygen species (ROS) such as H₂O₂, may ultimately be antagonistic due to positive regulation of trichothecene production (Ponts et al. 2007). For hemibiotrophic organisms, the functionality of defense response may depend on the trophic status of the fungus and if it has shifted to necrotrophy for nutrient acquisition from dead tissue. Resistant plants may not only need resistance genes, but also the ability to regulate the defense response to the pathogen's condition.

In matured grains, DON3G on average constituted one quarter (26%) of total-DON-like compounds (DON3G + 3ADON + DON), whereas a similar ratio was also observed in naturally infected controls. DON3G is known to be an important DON-form and has been detected in commercial samples from the upper mid-western USA. However, its relationship with DON has been inconsistent and subject to annual conditions (Schwartz et al. 2014). The relationship between DON3G and DON has not been studied in barley within the context of varietal resistance. In this study, DON was found to be a robust predictor for the total content of DON-like compounds and thus an adequate bio-indicator of quality for grain commerce while considering that it only constitutes a portion of total concentration. A very strong relationship was seen in the ratio of DON3G to DON, particularly within grain tissue. While both DON and DON3G content varied by resistance level of barley genotypes, their relative amounts appeared generally consistent. Genotypes with the highest level of resistance were characterized by an elevated ratio of DON3G to DON at the soft-dough stage, followed by a reduced representation of DON3G at maturity, implying that timing on the expression of resistance response may be important. DON content used as a biomarker of resistance for selection, would likely facilitate lowering total toxicity. DON3G constituted a significant portion of the total concentration of DON-like compounds in plant tissues and this should be taken into consideration by barley industries.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

This novel research has provided an increased understanding of complex host-pathogen interactions in the *F. graminearum* – barley pathosystem. Since its emergence as a disease of major economic importance in the mid-1990s, the threat of FHB to barley production has worsened with pathogen variants producing higher levels of the mycotoxin deoxynivalenol and increased spread and establishment throughout the major barley-production regions of western Canada. The studies herein, provide insights that will be extremely useful in designing genetic resistance and development of varieties to ensure sustainable production with reduced need for pesticides to uphold sector resiliency and maintain the status of barley as a competitive crop choice for western Canadian producers. The research provides a greater understanding of quantitatively inherited disease resistance, which is generally considered to be complex and challenging. The dissertation explores applications of biotechnologies for disease resistance involvements in barley, and contributes to the knowledge in this research field as listed below:

- Validation and implementation of new technology (Illumina 50K SNP beadchip array, Bayer et al. 2017) for application within western Canadian barley germplasm. This array proved to be highly useful under application to western Canadian germplasm, where a high level of polymorphism (> 50%) was observed for SNP markers. The current thesis sets a foundation for the application of this technology to future breeding and genetics applications to improve other traits of economic importance to Canadian barley producers (agronomics, malting quality, and many other diseases). The analysis provides an understanding of the genetic structure and relatedness of elite breeding lines and current two-row barley varieties, adapted for production in western Canada.
- Provides insights into resistance to Fusarium head blight (FHB) and deoxynivalenol (DON) accumulation, within the two-row barley kind, which has been far less studied than six-row barley. Through association mapping (linkage disequilibrium), it has identified genomic regions associated with these traits of economic importance. Knowledge from the current study showed that barley resistance genes in Canadian germplasm are likely numerous, each contributing a small breeding value to the phenotype of lowered FHB and DON accumulation. Numerous marker-trait-associations

were identified (FHB [51 SNPs over 22 regions]; DON [29 SNPs over 13 regions]), which may provide useful information for barley breeders. While a few loci associated with FHB were coincident with heading date or height as seen in bi-parental mapping, the majority identified through GWAS were independent. Regions associated with DON generally were not common with those associated with heading or height such that while breeding values were generally low they may not pose the same issues as those derived from bi-parental QTL mapping of exotic barleys. These results are similar to previous association mapping study of germplasm from American breeding programs (Massman et al. 2011), which also found low-effect QTLs in a study primarily focused on six-row barley. While several loci identified in this dissertation had been previously highlighted in that or other GWAS studies, many novel marker-trait associations have been identified in the current study of two-row barley, primarily of Canadian origin.

- Demonstrated application of genome-wide, single nucleotide polymorphism (SNP) molecular markers for use in prediction of genomic estimated breeding values (GEBVs) for FHB and DON content. Prediction methods employed and contrasted included more-established procedures based on complex statistical modelling of ridge regression (genomic best linear unbiased prediction, GBLUP) and also a novel approach with machine learning. While these approaches apply diversified mechanisms for genomic prediction, the results of this study indicate that genomic prediction is possible with moderate success ($\sim r = 0.6$) for the prediction of FHB and DON content. At this level, it will be possible to impose selection on populations in the absence of field nurseries. The genomic prediction approach is demonstrated to be a valuable tool for plant breeders to use independently or in parallel with field-based assessment of breeder lines. This dissertation has documented that while two-row barley is limited by a lack of adequate resistance sources (Steffenson et al. 2003; Bai and Shaner, 2004), the promise remains for the development of resistant barley varieties through genomics-based selection procedures and accumulation of minor resistance genes. This is a major finding of the thesis, and following a recommendation is justified to pursue this avenue to apply such methodologies in the contemporary practice of barley breeding.
- Messenger ribonucleic acid (mRNA) -sequencing was used to describe transcriptional alterations for novel resistance of *in vitro* selected variety Norman, where differentially

expressed genes were identified through contrast to parent variety ‘CDC Kendall’. Genetic resistance factors were identified which could be useful to plant breeders for selecting resistance to DON accumulation. These resistance factors (antimicrobial peptides i.e. thionin 2.1, defensin non-specific lipid-transfer protein; stress-related proteins i.e. late embryogenesis abundant proteins, heat-shock, desiccation-related, and a peroxidase [*HvPrx5*]) may be further explored and exploited for developing breeding targets to enhance resistance through development and application of molecular markers.

- The analysed ratio of conjugated deoxynivalenol (DON-3-glucoside, DON3G) vs. DON for a select group of two-row barley varieties with differentially expressed resistance to FHB and DON accumulation. From this study it was found that: i) DON3G can be present in contaminated grains and represent a significant proportion of the total amount of mycotoxin. ii) The ratio of DON3G/DON was relatively stable over the varieties tested. Deoxynivalenol content is thus considered a good bio-indicator of total mycotoxin content produced by *F. graminearum*. Industry practice does not use chemistries capable of detecting ‘masked’ toxins, which can easily be hydrolyzed back to their toxic form by microbes in human (Dall’Erta et al. 2013) or livestock gut (Nagl et al. 2014). While overall DON-like compounds in barley grains may be found at a greater level than indicated by an analysis of DON, the additional toxin in DON3G form may be comparable for different varieties. iii) While two-row barley can display variation for Type II resistance (resistance to spread from initial infection) in disease-concussive environments (Zhu et al. 1999), focus for two-row breeding FHB resistant barley should likely not be placed on mechanisms associated with detoxification response as it is adequate even in susceptible barleys.

The botanical tribe *Triticeae*, which includes crops such as wheat, rye, and barley, is characterized by extremely large genomes. Beyond the immense size, their DNA code is complex involving a high degree of repetitive elements. Recently high-quality reference genomes have become available for these life-sustaining crops. With the ongoing development of new sequence-based technologies, genetics experiments can be conducted with elevated proficiencies. The wheat genome is further complicated by polyploidy (2n=6X=42 chromosomes). Barley plays an important role as a representative model for plant pathology for the *Triticeae* (Harwood, 2019).

Recent advancements in genomic sequencing technologies i.e. Next-Generation sequencing (NGS) e.g. Genotyping-by-sequencing (GBS) or sequenced-based, whole-genome array (50K SNP chip), allow for applications of advanced genetic studies. Bi-parental QTL mapping (linkage mapping) or Genome-wide association mapping (GWAS – linkage disequilibrium mapping) are applications that have proven successful studies of complex, quantitatively-inherited traits in barley (Alqudah et al. 2019). A major drawback to bi-parental QTL mapping is that it can only detect loci with major effects, whereas many disease resistances are complex and controlled by many minor-effect loci. Of the many bi-parental QTL studies conducted on barley in the past, very few have identified loci free of negative morphological linkages (Steffenson, 2016).

As seen in previous studies, genetic resistance was under quantitative control in two-row barley. The third chapter of the thesis applies GWAS methodologies to identify genomic regions associated with FHB resistance and DON content in barley. Numerous marker-trait associations were identified for FHB and DON content, however, these were generally small in effect with each explaining a limited percentage of the phenotypic variance for either trait ($\leq 5\%$). Marker-trait associations were identified on all chromosomes for FHB and all chromosomes for DON content with exception of 1H and 6H. Interestingly, genomic regions associated with FHB and DON content generally did not overlap, implying resistance functions may be partially independent of these traits. This is not surprising, as FHB and DON content generally display a weak to moderate level of association in barley (Buerstmayr et al., 2004; Choo et al., 2004; Buerstmayr et al., 2004; He et al., 2015; Huang et al., 2018; Tucker et al., 2019).

Genomic regions of trait-marker associations were generally found in proximity to genes annotated with potential resistance function. Cell walls are the primary barriers to pathogens. This study has identified genes linked to markers identified in association with FHB and tied to building and strengthening cell walls, the first barrier to pathogens. Genes included a hexosyltransferase (HORVU5Hr1G081520) which produces pectin and Caffeoyl-CoA 3-O-methyltransferase (HORVU5Hr1G072070) is involved in phenylpropanoid biosynthesis and cell wall lignification. GDSL esterase/lipase (HORVU4Hr1G001420); Non-Specific Lipid-Transfer Proteins (*PR14*) (HORVU7Hr1G102030); UDP-Glycosyltransferases, like HORVU5Hr1G071940 demonstrate the ability to convert DON to non-toxic form DON-3-

glucoside, were identified as families of resistance factors, expressed in ‘Norman’ under RNA-sequencing under infection of *F. graminearum* (Chapter 5).

Marker-DON associations regions were linked with genes associated with pathogen detection as Leucine-rich receptor-like protein kinase family proteins (HORVU2Hr1G010490 & HORVU5Hr1G001920) which are involved in recognizing Pathogen Associated Molecular Patterns (PAMPs); WRKY 50 (HORVU3Hr1G067510) and WRKY 74 (HORVU5Hr1G014710) transcription factor; Calmodulin-binding Ca^{2+} messenger protein-protein (HORVU3Hr1G066840). Cytochrome P450 superfamily proteins, like HORVU5Hr1G096940 were highly induced in the resistance response within the RNA-Seq study (Chapter 5).

Quantitative disease resistance (incomplete or partial) has been poorly understood (Poland, 2009). In the current study, marker-trait associations were identified for lowered DON content at regions linked with genes involved in resistance response. While some regions associated with FHB were coincident with heading date or height, most regions were not. Unlike QTLs identified through bi-parental QTL mapping, regions associated with DON were generally independent of these negative agronomic traits. As associations only explain a small amount of the variance seen for FHB and DON content the SNPs identified in associations under this study will likely be best applied under the design of reduced-representation SNP panels or in a combination of a GWAS-assisted genomic prediction approach, where loci may be modelled as a fixed effect covariate.

In chapter 4, an alternative strategy is used in the exploration of genome-wide SNP markers, for the development of FHB resistance and lowering DON in two-row barley. Genomic selection is a modern breeding method that capitalizes on low genotyping costs which now enables researchers to collect thousands of SNP markers at a fraction of the cost from a decade ago. All SNP markers are used collectively to establish predictions of genomic estimated breeding values (GEBVs). The underlying theory assumes that under adequate marker density, all genes which contribute to a trait will be in linkage disequilibrium with at least one marker. In this regard, very small additive effects may be summed through in their overall contribution to a trait. Several models were investigated in the current study with statistical and machine learning-based methodologies. The best predicting methods used in the study were founded on statistical modelling (ridge regression, BLUP) and a novel machine learning (GPTransformer) procedure

that is not dependent on assumptions of statistics. Either approach demonstrated that it is possible to moderately predict FHB and DON content.

It is thus possible to use this method in developing FHB resistance through selection using genotype alone. This technology may lessen demands for labor-intensive field evaluation and specialized chemistries e.g. enzyme absorbent immune assay (ELISA). Benefits may be best realized in combination with contra-season nurseries. While genomic selection may be a useful tool for breeding resistance, practical applications must consider the costs of the SNP array along with the requisite for skilled laboratory personnel for DNA extraction and analysis. While genomics-based technologies have dropped significantly in price over the past decade, significant costs are still required. While genomic selection may carry its limitations, this method shows promise and offers an alternative approach for breeding quantitative disease resistance and may lessen the dependence of burden for field evaluation.

Resistance sources for barley are very limited in the fact that they only possess partial resistance and have low applicability in variety development due to many undesirable characteristics (extreme heading, tall stature, poor quality). This has driven breeders to explore the use of *in vitro* selection (IVS, selection of soma-clonal variants via growth media containing mycotoxins). Norman is an IVS that displays a 25% reduction in DON content, in contrast with its parent variety CDC Kendall. Another complexity for breeding resistance to *F. graminearum* arises from the dynamics of populations where rapid changes have been observed in North America with shifts from 15ADON- to 3ADON-producing chemotypes in western Canada, and the appearance of NIV-producing strains in the southern USA. In chapter 5, resistance within this variety is explored through the application of next-generation sequencing (NGS) technology in the form of RNA-sequencing. The existence of high-quality reference genomes, with strong gene annotation models allowed for comprehensive analysis of the expression data. A complex model was used to analyze the two varieties, in contrast, subjected treatments of 3ADON, 15ADON, or NIV chemotypes. The 3ADON chemotypes produced the highest levels of DON, identifying them as an elevated threat in the challenge of existing resistance and overall toxicity of grains. The study illustrated complexities in resistances where different types of resistances were seen with anti-fungal and protease inhibition properties or as DON-detoxifying resistance, whereas such resistances displayed a level of incompatibility. This study provides insight into the complex

interactions of a hemibiotrophic pathogen in transition to necrotrophy and the underlying quantitative host resistance. On one hand, detoxification resistances appear to be strongly inducible in barley including phenylpropanoid pathway and detoxification gene categories (UGT, GST, CyP450, and ABC), while an alternative defense response observed in *IVS* variety ‘Norman’ was associated with antimicrobial peptides (thionin 2.1, defensin and non-specific lipid transfer protein), stress-response like LEA, heat-shock desiccation and a peroxidase (*HvPrx5*).

Barley differs in response to *F. graminearum* infection from the closely-related, cereal crop wheat. In wheat, the infection progresses from the initial point through the rachis, to infect asipetal and basipetal spikelets. DON is an important factor for fungal penetration of the rachis. *Fusarium graminearum* mutants (*TRI5* -) which cannot produce DON can infect kernels, but cannot spread through the spike. Thus in wheat, resistance to higher expression of uridine 5'-diphospho-glucuronosyltransferase (UDP-glucuronosyl transferase, UGT) genes which convert DON to DON-3-glucoside is associated with resistant varieties. The ratio of DON-3-glucoside/DON is higher in varieties categorized with resistance. Along with plants, DON-3-glucoside is less toxic to humans, however, such conjugated forms of toxins are considered a risk as they are not conventionally detected by routine industrial screening. Gut bacteria possess enzymes that readily cleave the glucose moiety from DON-3-glucoside, thereby restoring its toxicity. From a breeding perspective, it is important to understand the total toxic load of a variety. Barley displays a much higher level of resistance to spread than wheat, due to strong UGT expressions, where even highly susceptible varieties resist spread through the spike. In chapter 6 varieties of differential resistance to DON accumulation were evaluated, where the DON-3-glucoside ratio was similar to overall varieties. The ratio was elevated in rachis tissues over that of grains. DON was found at higher content in tissues before maturation. In barley, DON content was a strong predictor of total toxin content.

Overall the studies in the dissertation have used a combination of biotechnological tools to help further understanding of barley-*Fusarium* interaction. Barley has received but less attention than related crop wheat, and further crop-specific knowledge is required to understand this pathosystem. Several minor genes may collectively contribute to the quantitatively based resistance seen in two-row barley (Chapter 3). While the biological function of these gene

families is copasetic with many gene expression studies of FHB in cereals in literature and current thesis (Chapter 5), they do not hold enough strength to confer full resistance to *F. graminearum*. Alternatively, the whole-genome approach of SNP markers was demonstrated to provide moderate prediction (chapter 5). The thesis documents the acceptable application of prediction in both statistical (BLUP ridge regression) and machine learning (GPTransformer) methods, where a further study on more expansive populations may help further establish the value of specific models. RNA-Sequencing illustrated the complexities of resistances induced by *F. graminearum*, and several selection targets are highlighted (chapter 5). Evaluation of DON-conjugation as a resistance response across a range of two-row barley genotypes revealed relative consistencies in response. This dissertation has filled knowledge gaps of FHB resistance for the two-row barley, which has received less attention than six-row barley for this disease. There are large economic pressures to reduce levels of mycotoxins in the food chain, and requirements to maintain a level of genetic resistance in the face of a pathogen with a high propensity to evolve. Information provided herein will assist breeding in developing varieties with resistance to FHB and DON accumulation.

CHAPTER 8

LIMITATIONS AND FUTURE DIRECTIONS

Domestication of barley occurred over 10,000 years ago. Currently, barley is one of the most important cereal crops grown worldwide, contributing raw materials for use in global food and feed industries. Advancements in barley breeding are required to keep pace with the needs of future generations. As we move into the near future, we do so under very exciting times where genomics technologies can be increasingly leveraged to make improvements in traits that were previously considered extremely difficult and realistically overwhelming in past decades.

While the genome-wide association study (GWAS) did not identify any major quantitative trait loci (QTL) that would warrant independent molecular marker-assisted selection (MAS), information of significant SNP markers will be of worth for choice within reduced-representative genetic assays. Future studies may benefit from more complicated genetic designs such as nested-association mapping (NAM) that share benefits common both to bi-parental QTL mapping and GWAS. While these genetic designs may have better resolutions for identifying marker-trait association regions, they do require more planning and advanced production of germplasm through specific crosses.

The dissertation specifically focussed on elite Canadian, *Fusarium*-resistant germplasm, which is more likely to benefit in genomic selection applied within populations adapted materials of a breeding program. However, this expectedly resulted in a narrowing of additive genetic diversity due to selection for adaptability. Future directions could involve a different approach to using highly diversified germplasm. While this approach may be less likely to identify alleles without conflict with desirable agronomic traits, through diversification it may identify less common resistances but with a larger additive effect. Such genetic variants which may explain higher levels of the phenotypic variation may be more useful in application to molecular MAS.

The large amount of genotypic data generated for the barley populations assembled for this thesis holds high value for many future studies, which could be designed for other complex/ difficult-to-measure traits of economic importance (i.e. leaf disease, yield, malting quality traits). As outlined in previous chapters, barley is host to several pathogens which the can cause economic harm under epidemic conditions. For example, further genetic experiments could be designed

and conducted to supply the required phenotypic information. Accuracies of these phenotyping data could be improved by deploying various ‘phenomics’ tools. This is an emerging trans-discipline where various devices and systems such as hand-held meters, pheno-carts, drones, satellites are being used to collect image information for measurements of changes in plants/organisms over time.

While the investigation of transcript alterations in the *IVS* variety ‘Norman’ was successful at identifying resistance factors, these were measured at the level of the whole seed. Particular tissues may be more involved with the resistance, and a focussed approach could be of benefit. As gene expression technologies continue to improve, high resolutions are possible with very minute RNA inputs. Tissue-specific analysis through laser dissection and/or single-cell sequencing are now possibilities that could provide a platform for a more intimate investigation of host-pathogen interaction.

Triticeae genomes like that of barley with high repeat content and immense size have posed a significant obstacle for genomic research, however recent advances in sequencing technologies coupled with several high-quality reference genome assemblies provide unique opportunities for barley improvements of economic traits. Long-read sequencing technologies are useful tools for minimizing alignment errors for such complex genomes. The barley pan-genome enables the application of both core and dispensable genomes (Kamal et al. 2022). With the release of a Canadian reference genome for the two-row, malting variety ‘AAC Synergy’ (Xu et al. 2021) targeted genomic applications within Canadian germplasm (including Norman and CDC Kendal) are now possible.

It was demonstrated within a disease nursery with disease-promoting conditions, DON-3-glucoside can constitute a significant proportion of toxin levels of *Fusarium*-damaged barley kernels. This result needs to be further characterized over natural infections, across environments, and under different weather conditions. Confirmation through an industrial survey is recommended to further evaluate the threat of conjugated mycotoxins.

CHAPTER 9

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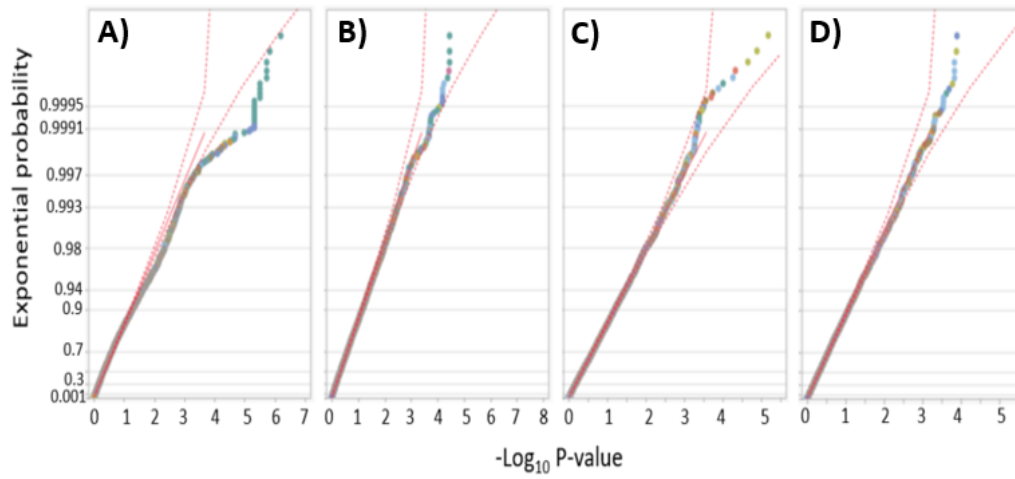
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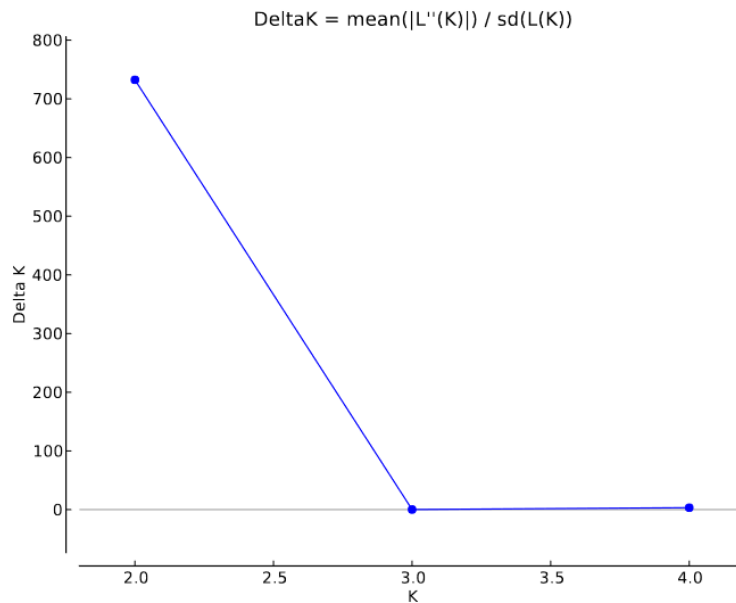
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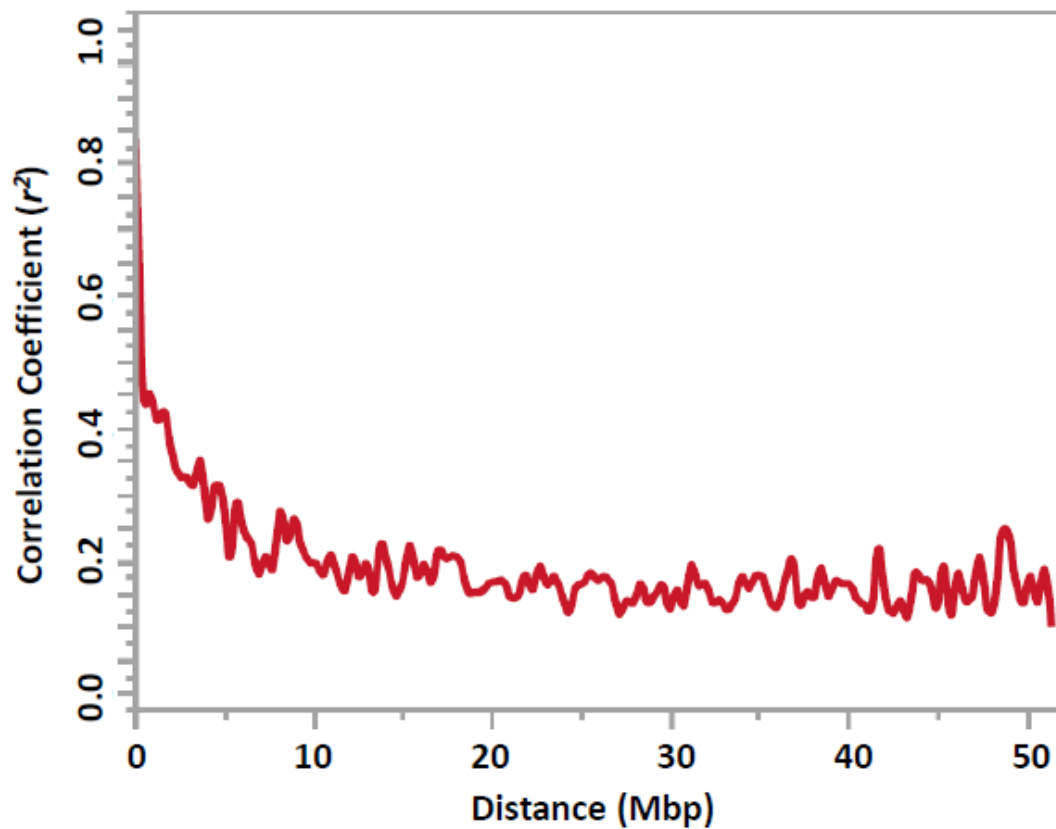
APPENDIX 1 – Chapter 3: Supplementary Figures and Tables



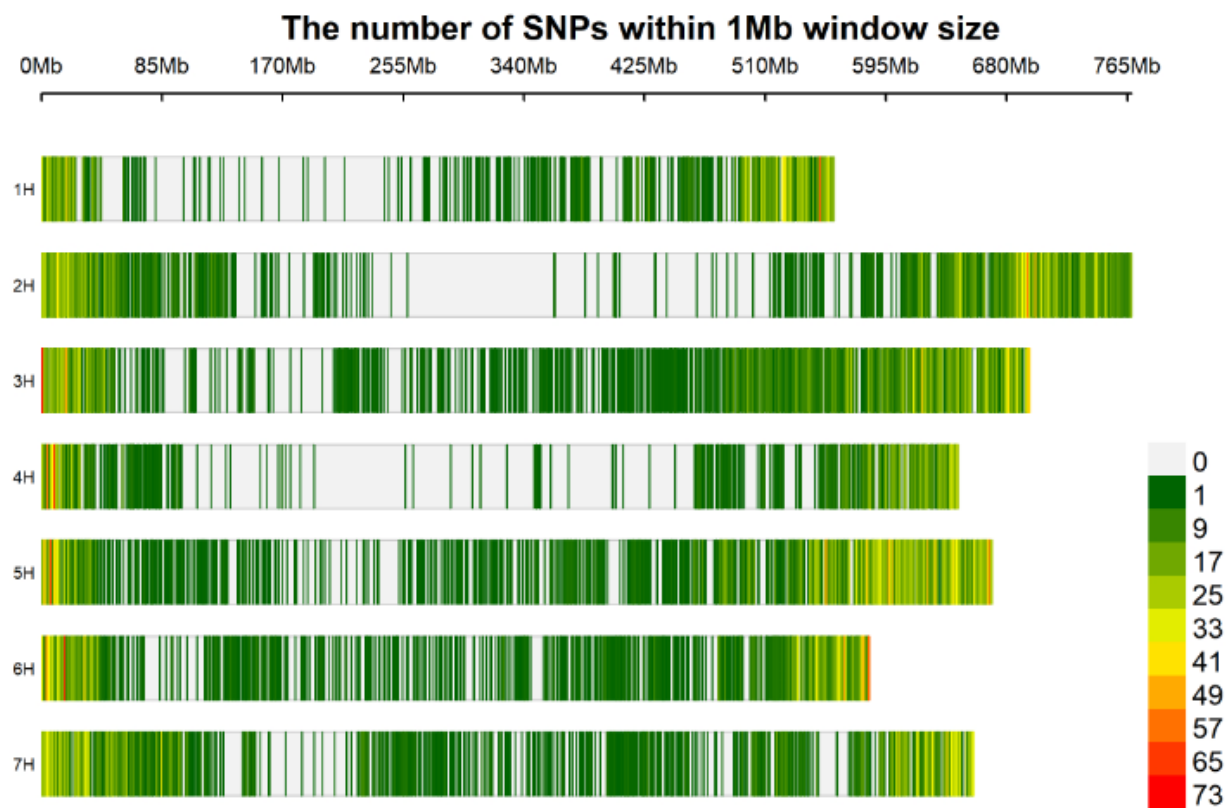
1.1. Q-Q plots for A) fifty-percent heading date (HD50), B) plant height (HT), C) FHB (Fusarium head blight), and D) deoxynivalenol content (DON) over chromosomal position. GWAS was performed using the *MLM* ($Q + K$) model for six site years, except for HD50 which was based on four site years.



1.2. Representation of optimal number of subpopulations clusters (ΔK) in the population of the barley genomic panel ($n=400$) with cluster potentials set to $K=5$. An optimal sub-population structure is indicated as $K=2$.

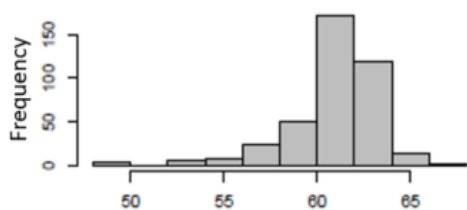


1.3. Intra-chromosomal linkage disequilibrium (r^2 , squared correlation coefficient) decay plot of paired markers on all seven chromosomes as a function of increasing mega base pair (Mbp) distance. The red line represents a local smoothing function.

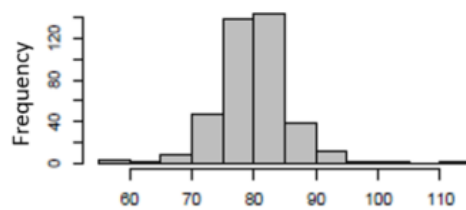


1.4. The concentration of single nucleotide polymorphic molecular markers by chromosome position as per Illumina iSelect 50K SNP Barley beadarray. The density of markers is indicated by colouration, where green = low marker density region; red = high marker density region.

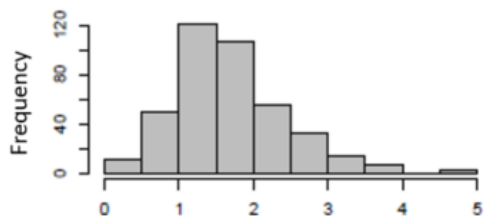
I) Days to heading



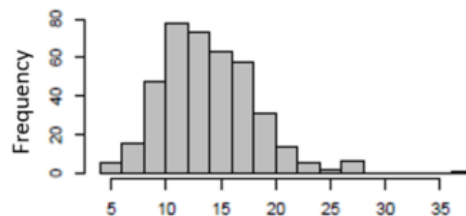
II) Plant height (cm)



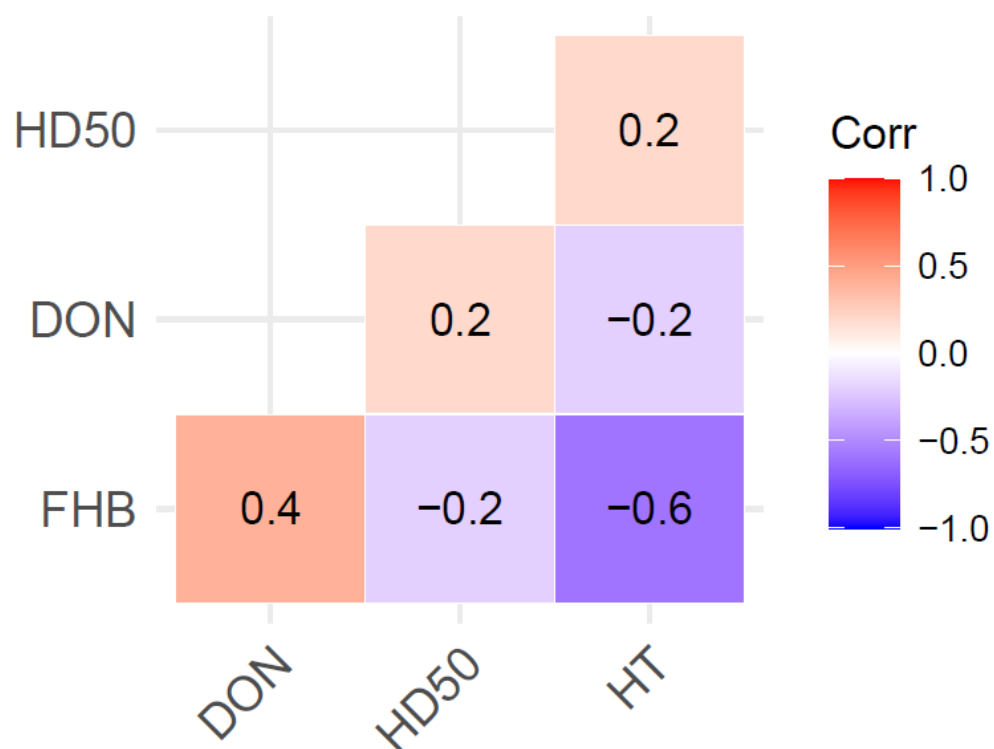
III) FHB rating (0-5)



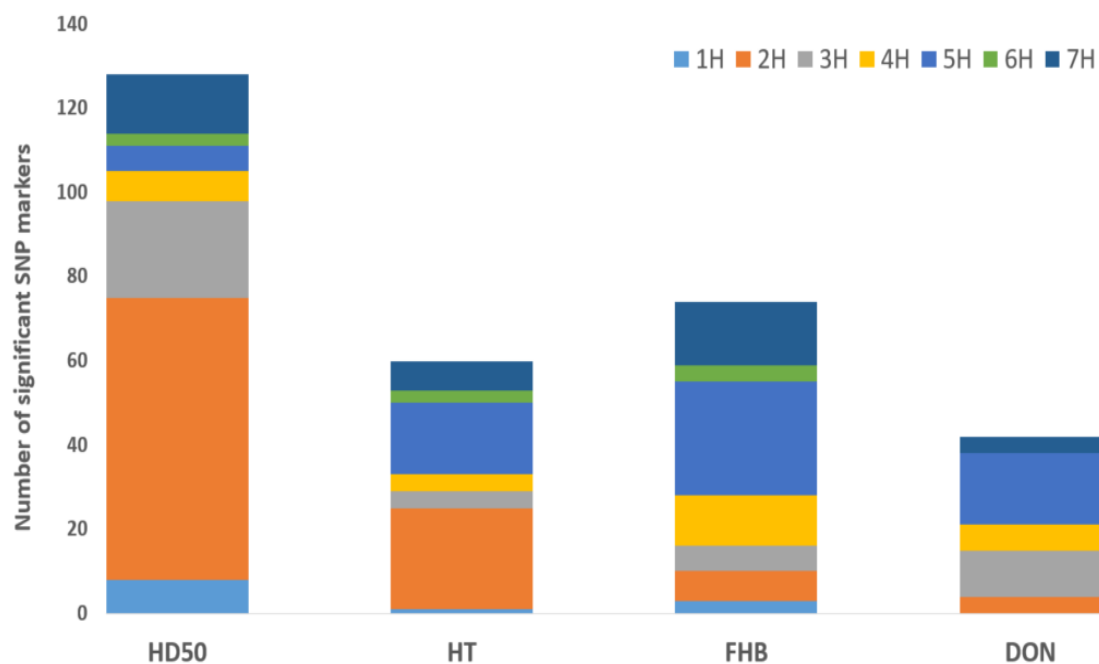
IV) Deoxynivalenol content (mg kg⁻¹)



1.5. Histograms for traits I) Days to heading; II) Plant height; III) Fusarium head blight rating; IV) Deoxynivalenol content of mature grain. Data represent grand means of six site years, with exception of heading date representing four site years.



1.6. Pearson correlation coefficients (r) for height (HT) in cm, days to 50% heading (HD50), fusarium head blight score (FHB) 0-5, and deoxynivalenol (DON) content in mg kg⁻¹ over site years (n=6). The heatmap colouration indicates correlation strength, where red indicates positive relationship and blue indicates a negative relationship.



1.7. The number of significant markers by trait ($-\text{Log}_{10} [\text{P value}] \leq 3$) per trait: Days to fifty percent heading (HD50); height (HT); Fusarium head blight (FHB); deoxynivalenol content (DON) based on grand mean analysis of six site years, except for HD50 which is based on four site years. Colouration refers to chromosome number.

APPENDIX 2 – Chapter 4: Supplementary Figures and Tables

2.1. Least-squares means of traits measured on 400 barley genotypes over site years.

Genotype	HD50*	HT	FHB	DON
Genotype_1	62.0	76.0	3.2	20.2
Genotype_2	62.5	74.5	3.0	16.5
Genotype_3	63.3	77.8	2.1	15.5
Genotype_4	58.9	67.8	3.0	25.1
Genotype_5	61.9	75.8	3.0	14.2
Genotype_6	62.9	67.4	3.1	16.9
Genotype_7	60.9	81.0	1.8	10.0
Genotype_8	63.5	84.8	1.4	11.9
Genotype_9	61.6	74.7	2.6	10.3
Genotype_10	62.6	72.8	2.8	15.1
Genotype_11	60.1	69.8	2.3	12.0
Genotype_12	58.5	70.3	1.8	13.8
Genotype_13	53.4	76.8	1.4	6.9
Genotype_14	63.9	74.8	2.2	16.8
Genotype_15	64.1	78.5	2.2	15.0
Genotype_16	62.0	78.5	2.8	19.8
Genotype_17	61.6	78.8	2.7	17.9
Genotype_18	63.0	75.0	2.0	12.4
Genotype_19	61.0	83.1	3.3	17.4
Genotype_20	63.3	84.3	2.4	14.4
Genotype_21	62.3	76.3	2.8	12.3
Genotype_22	59.8	79.4	2.8	15.8
Genotype_23	61.3	78.3	2.1	17.1
Genotype_24	62.6	79.6	2.8	26.9
Genotype_25	61.3	72.7	3.0	17.1
Genotype_26	61.9	83.1	2.6	16.5
Genotype_27	62.8	76.0	2.5	16.8
Genotype_28	62.0	75.9	2.2	17.1
Genotype_29	60.4	74.7	2.8	12.8
Genotype_30	62.3	79.8	2.2	19.4
Genotype_31	61.4	74.1	2.8	15.0

Genotype_32	60.5	80.4	1.9	11.2
Genotype_33	64.5	82.5	2.0	18.2
Genotype_34	61.4	74.6	1.8	17.4
Genotype_35	64.1	86.8	1.5	17.3
Genotype_36	59.3	74.7	2.4	16.7
Genotype_37	62.3	84.0	1.3	16.0
Genotype_38	62.9	81.1	1.6	9.1
Genotype_39	63.4	84.1	1.0	13.2
Genotype_40	61.8	84.4	1.4	11.0
Genotype_41	62.0	81.5	1.3	11.8
Genotype_42	61.6	73.5	3.5	19.5
Genotype_43	61.8	85.8	1.4	15.2
Genotype_44	60.9	82.8	2.0	15.0
Genotype_45	60.9	80.4	0.9	10.8
Genotype_46	61.9	84.1	1.3	10.3
Genotype_47	63.5	82.3	1.8	11.8
Genotype_48	61.1	73.8	2.5	13.2
Genotype_49	61.9	79.7	2.3	14.8
Genotype_50	64.0	84.2	1.1	10.7
Genotype_51	62.4	82.0	1.3	20.3
Genotype_52	62.5	77.8	1.0	10.6
Genotype_53	63.5	75.7	1.9	12.4
Genotype_54	61.8	84.4	2.3	15.2
Genotype_55	62.6	73.3	3.3	17.2
Genotype_56	61.5	83.7	1.7	16.3
Genotype_57	62.6	75.8	1.7	15.1
Genotype_58	63.1	78.1	1.6	9.7
Genotype_59	63.0	78.3	1.8	15.3
Genotype_60	61.5	78.0	2.1	14.0
Genotype_61	60.6	85.8	2.2	19.7
Genotype_62	63.0	82.9	0.8	13.8
Genotype_63	61.4	78.8	1.8	10.6
Genotype_64	61.8	78.8	2.3	20.5
Genotype_65	62.3	78.5	1.8	16.6
Genotype_66	60.8	82.6	1.9	16.6
Genotype_67	62.6	76.0	1.8	16.7
Genotype_68	62.1	79.1	1.0	10.6
Genotype_69	61.4	78.7	1.2	11.1
Genotype_70	58.0	77.3	1.6	17.7
Genotype_71	62.6	80.6	0.8	9.8

Genotype_72	61.0	83.6	0.8	11.5
Genotype_73	63.1	76.1	1.3	14.2
Genotype_74	60.9	73.6	2.6	19.1
Genotype_75	59.9	82.1	1.8	12.3
Genotype_76	62.4	75.0	2.9	14.1
Genotype_77	62.4	79.3	1.9	7.8
Genotype_78	60.9	80.3	2.3	18.8
Genotype_79	63.3	88.9	1.9	14.1
Genotype_80	60.6	87.8	1.7	14.1
Genotype_81	59.9	87.6	2.6	11.3
Genotype_82	60.5	84.1	1.8	10.5
Genotype_83	62.5	89.5	2.6	13.9
Genotype_84	61.5	80.3	3.1	19.5
Genotype_85	58.6	79.8	2.0	12.2
Genotype_86	60.4	79.1	1.5	15.1
Genotype_87	61.5	80.9	1.3	11.0
Genotype_88	61.0	79.8	2.2	18.8
Genotype_89	61.8	81.3	2.8	18.4
Genotype_90	62.4	86.2	1.2	10.1
Genotype_91	62.9	83.4	1.3	8.3
Genotype_92	57.8	87.7	0.9	9.3
Genotype_93	64.0	81.2	1.6	15.5
Genotype_94	62.0	86.0	1.4	11.2
Genotype_95	61.6	87.1	1.9	9.8
Genotype_96	53.5	81.3	0.3	6.0
Genotype_97	61.5	85.9	1.5	16.8
Genotype_98	61.6	86.0	1.6	12.5
Genotype_99	61.1	80.0	1.5	16.0
Genotype_100	63.0	85.4	1.0	5.8
Genotype_101	58.0	85.6	1.3	11.7
Genotype_102	61.9	81.0	1.3	9.3
Genotype_103	61.9	84.8	1.4	8.2
Genotype_104	63.1	84.3	1.7	7.7
Genotype_105	57.4	84.3	1.5	17.1
Genotype_106	65.0	89.7	0.8	9.3
Genotype_107	61.5	78.0	1.6	16.7
Genotype_108	61.8	83.9	2.0	13.0
Genotype_109	63.1	87.5	1.0	9.6
Genotype_110	62.5	75.6	1.7	12.7
Genotype_111	63.1	83.4	1.6	9.1

Genotype_112	60.9	77.4	2.8	15.7
Genotype_113	62.4	74.8	1.1	13.3
Genotype_114	61.3	84.2	1.6	16.3
Genotype_115	59.1	84.4	1.8	20.8
Genotype_116	59.4	86.8	1.1	15.4
Genotype_117	61.4	79.7	2.6	21.7
Genotype_118	61.9	82.9	1.5	10.9
Genotype_119	61.9	77.3	3.1	18.7
Genotype_120	60.5	84.0	1.5	12.8
Genotype_121	60.9	78.0	2.9	21.8
Genotype_122	65.4	83.0	1.3	11.4
Genotype_123	63.5	85.1	1.1	10.8
Genotype_124	61.8	93.9	1.3	13.3
Genotype_125	59.6	78.3	1.3	9.6
Genotype_126	61.8	86.4	1.6	19.3
Genotype_127	60.9	83.0	1.4	11.4
Genotype_128	60.3	87.1	1.0	10.3
Genotype_129	62.0	87.5	0.8	8.6
Genotype_130	63.6	80.3	1.3	14.9
Genotype_131	65.0	76.6	1.1	9.6
Genotype_132	64.3	78.6	0.8	10.8
Genotype_133	62.3	83.3	1.7	12.9
Genotype_134	64.1	86.4	1.4	13.0
Genotype_135	60.6	82.7	1.5	10.4
Genotype_136	60.4	81.3	1.7	13.4
Genotype_137	59.6	73.3	1.6	13.5
Genotype_138	64.0	80.9	1.6	15.3
Genotype_139	63.5	79.7	1.5	12.6
Genotype_140	57.9	77.3	1.5	9.1
Genotype_141	61.6	80.3	1.4	13.2
Genotype_142	58.4	76.5	2.1	16.6
Genotype_143	60.9	78.5	1.8	13.9
Genotype_144	61.9	73.4	2.5	17.7
Genotype_145	63.0	79.8	2.2	12.7
Genotype_146	62.8	82.8	1.4	10.9
Genotype_147	61.5	81.3	1.8	8.5
Genotype_148	61.0	79.3	1.6	14.3
Genotype_149	62.8	82.8	0.7	10.1
Genotype_150	62.3	82.6	1.0	9.3
Genotype_151	61.8	78.5	1.8	20.9

Genotype_152	60.9	82.1	1.3	15.2
Genotype_153	64.4	78.6	1.8	17.9
Genotype_154	62.3	79.3	1.1	14.6
Genotype_155	61.5	78.2	2.3	15.8
Genotype_156	62.1	78.7	1.8	14.0
Genotype_157	61.6	77.5	2.1	22.5
Genotype_158	59.9	79.9	1.3	10.0
Genotype_159	62.3	85.5	1.5	17.2
Genotype_160	60.6	76.8	1.8	20.0
Genotype_161	62.6	73.4	2.8	26.3
Genotype_162	62.4	81.1	1.4	16.1
Genotype_163	62.3	81.1	1.4	17.4
Genotype_164	62.6	79.2	1.7	16.8
Genotype_165	62.6	72.8	2.3	16.3
Genotype_166	62.9	71.8	1.0	21.4
Genotype_167	61.6	71.3	2.3	23.4
Genotype_168	62.1	84.0	0.8	22.1
Genotype_169	63.0	80.2	2.0	17.0
Genotype_170	62.1	76.1	1.9	14.6
Genotype_171	61.8	83.5	2.5	17.9
Genotype_172	62.3	81.8	2.5	13.0
Genotype_173	67.0	85.6	0.7	11.6
Genotype_174	59.9	73.5	1.3	18.0
Genotype_175	64.8	78.4	1.6	13.2
Genotype_176	61.9	74.7	3.8	27.8
Genotype_177	64.4	74.6	1.8	11.9
Genotype_178	61.9	82.2	1.5	20.0
Genotype_179	61.5	68.6	3.3	21.9
Genotype_180	62.1	83.4	1.6	14.3
Genotype_181	63.1	79.8	1.4	12.7
Genotype_182	62.3	75.1	2.3	17.6
Genotype_183	62.3	75.8	2.3	20.4
Genotype_184	56.1	73.8	1.3	11.0
Genotype_185	55.9	84.5	1.2	7.9
Genotype_186	61.5	90.1	1.3	10.7
Genotype_187	60.9	80.3	1.4	11.2
Genotype_188	58.4	74.9	1.2	8.1
Genotype_189	52.8	84.3	1.1	11.8
Genotype_190	62.9	83.8	1.5	12.5
Genotype_191	57.0	74.3	1.3	13.0

Genotype_192	57.5	84.0	1.9	11.2
Genotype_193	58.3	82.5	1.5	10.8
Genotype_194	61.6	85.7	1.7	6.7
Genotype_195	60.8	78.3	3.1	12.3
Genotype_196	58.8	80.7	1.5	9.7
Genotype_197	61.3	82.8	1.6	7.8
Genotype_198	57.6	75.3	2.3	13.0
Genotype_199	61.5	80.8	1.9	9.4
Genotype_200	61.4	80.8	1.6	11.0
Genotype_201	55.9	75.9	1.5	9.1
Genotype_202	63.9	76.1	1.8	8.4
Genotype_203	62.3	110.6	0.7	11.2
Genotype_204	60.1	84.1	0.7	11.1
Genotype_205	61.0	83.2	1.5	10.4
Genotype_206	61.3	84.4	1.1	8.4
Genotype_207	62.1	84.2	2.3	11.4
Genotype_208	55.6	70.5	3.2	9.0
Genotype_209	58.5	86.7	2.3	10.0
Genotype_210	49.4	56.1	4.8	11.7
Genotype_211	48.3	55.0	4.8	11.9
Genotype_212	64.3	65.7	2.9	14.2
Genotype_213	57.5	80.5	1.4	21.3
Genotype_214	58.4	73.6	3.6	11.0
Genotype_215	59.5	79.6	2.3	13.9
Genotype_216	58.4	77.3	1.4	10.6
Genotype_217	61.6	77.8	1.8	18.6
Genotype_218	60.8	73.1	2.0	17.6
Genotype_219	60.5	76.3	2.0	9.1
Genotype_220	60.4	75.5	1.6	11.1
Genotype_221	62.3	74.6	3.3	19.6
Genotype_222	61.5	75.0	3.2	14.4
Genotype_223	61.1	58.5	3.8	19.6
Genotype_224	61.1	67.3	3.2	26.4
Genotype_225	60.6	75.3	2.2	18.9
Genotype_226	60.9	80.0	1.5	22.1
Genotype_227	62.0	83.6	1.3	13.6
Genotype_228	62.4	82.2	0.9	13.2
Genotype_229	62.3	81.3	1.5	16.1
Genotype_230	62.0	83.2	1.1	13.5
Genotype_231	63.3	77.3	1.6	17.0

Genotype_232	61.4	81.1	1.7	17.8
Genotype_233	59.8	77.3	1.4	14.7
Genotype_234	58.5	79.4	1.8	12.3
Genotype_235	60.4	81.5	1.5	13.0
Genotype_236	59.5	85.5	1.2	16.1
Genotype_237	63.6	78.3	1.9	12.5
Genotype_238	63.1	81.0	1.7	15.5
Genotype_239	63.5	81.3	1.4	11.5
Genotype_240	62.4	82.3	1.7	12.6
Genotype_241	60.1	76.2	2.3	17.0
Genotype_242	61.9	81.8	1.5	13.4
Genotype_243	61.6	79.9	1.0	13.4
Genotype_244	62.0	74.4	1.9	16.2
Genotype_245	62.5	78.7	1.6	17.7
Genotype_246	48.3	75.6	2.2	8.9
Genotype_247	62.8	80.2	1.3	12.9
Genotype_248	60.9	77.9	1.0	13.1
Genotype_249	60.6	80.3	1.4	12.7
Genotype_250	59.1	83.0	0.8	11.0
Genotype_251	61.1	78.1	1.2	8.8
Genotype_252	61.6	80.4	1.7	17.1
Genotype_253	61.3	79.6	1.5	14.4
Genotype_254	61.3	81.0	1.4	12.2
Genotype_255	55.3	89.6	0.5	7.3
Genotype_256	61.3	81.1	0.9	14.6
Genotype_257	63.1	81.6	1.6	9.4
Genotype_258	63.0	87.0	1.3	13.6
Genotype_259	61.6	79.1	1.2	11.4
Genotype_260	61.0	76.0	1.2	11.2
Genotype_261	60.6	81.8	1.3	13.1
Genotype_262	62.0	78.5	1.2	11.5
Genotype_263	64.5	75.6	1.1	13.3
Genotype_264	61.4	75.8	2.0	18.2
Genotype_265	60.5	81.2	1.9	19.0
Genotype_266	59.8	85.7	1.8	19.4
Genotype_267	60.3	73.8	2.3	16.7
Genotype_268	63.5	77.2	1.9	18.9
Genotype_269	62.5	77.4	2.8	20.4
Genotype_270	63.0	74.5	1.6	13.4
Genotype_271	62.6	79.1	1.8	10.7

Genotype_272	61.3	84.8	1.8	26.4
Genotype_273	59.6	82.7	1.4	25.1
Genotype_274	61.8	81.3	1.5	14.7
Genotype_275	61.6	83.8	0.7	10.0
Genotype_276	63.3	82.3	0.8	8.4
Genotype_277	63.0	80.4	2.3	11.0
Genotype_278	59.9	94.8	0.8	18.7
Genotype_279	62.3	85.1	1.1	9.3
Genotype_280	60.3	77.0	1.8	14.8
Genotype_281	59.0	90.3	0.8	12.4
Genotype_282	61.9	80.8	1.2	14.9
Genotype_283	59.0	87.3	0.4	11.1
Genotype_284	59.8	91.0	1.0	8.7
Genotype_285	62.0	92.3	0.5	10.3
Genotype_286	60.6	80.3	1.8	14.7
Genotype_287	61.9	78.4	1.3	17.0
Genotype_288	59.1	75.9	1.5	13.4
Genotype_289	62.5	84.6	0.7	8.2
Genotype_290	57.6	76.7	2.3	10.2
Genotype_291	63.9	79.3	1.4	10.6
Genotype_292	58.5	81.5	2.3	12.2
Genotype_293	61.1	75.7	2.3	15.2
Genotype_294	58.1	73.7	2.5	12.5
Genotype_295	60.8	76.7	1.8	16.8
Genotype_296	58.3	80.3	0.8	11.8
Genotype_297	63.6	86.0	1.4	8.6
Genotype_298	58.3	88.1	1.4	19.2
Genotype_299	57.8	75.1	2.1	19.0
Genotype_300	63.0	75.3	1.6	18.0
Genotype_301	60.1	81.3	1.8	19.8
Genotype_302	59.5	80.7	1.6	9.4
Genotype_303	61.3	104.2	0.9	13.7
Genotype_304	62.5	76.1	2.2	12.6
Genotype_305	62.1	83.3	2.1	15.3
Genotype_306	62.5	98.3	0.9	14.4
Genotype_307	62.3	74.6	2.2	15.1
Genotype_308	61.3	69.9	3.9	26.2
Genotype_309	62.9	79.0	1.3	16.1
Genotype_310	61.6	80.3	1.8	15.5
Genotype_311	60.5	78.4	2.5	15.6

Genotype_312	57.9	76.1	1.1	6.5
Genotype_313	57.8	84.3	1.0	11.4
Genotype_314	60.6	80.7	1.9	16.7
Genotype_315	62.8	79.3	1.9	15.4
Genotype_316	60.6	85.8	1.9	15.3
Genotype_317	62.3	81.7	1.7	15.7
Genotype_318	62.8	78.8	1.6	16.1
Genotype_319	61.8	77.8	2.5	20.0
Genotype_320	60.1	74.1	2.7	18.5
Genotype_321	62.0	80.8	2.1	10.3
Genotype_322	56.0	67.4	3.0	4.9
Genotype_323	57.6	78.2	1.0	8.0
Genotype_324	61.0	81.3	1.0	9.7
Genotype_325	60.3	79.4	2.5	13.3
Genotype_326	57.9	80.8	2.4	11.9
Genotype_327	55.6	72.0	3.8	15.0
Genotype_328	60.3	79.6	2.3	7.3
Genotype_329	64.6	77.6	1.0	13.1
Genotype_330	55.8	75.2	2.8	8.0
Genotype_331	57.5	77.3	1.9	7.1
Genotype_332	55.0	67.7	3.3	15.1
Genotype_333	57.1	73.3	3.0	11.7
Genotype_334	60.0	81.0	3.0	11.2
Genotype_335	63.0	63.8	3.7	20.4
Genotype_336	60.0	76.3	1.3	19.9
Genotype_337	62.4	76.1	1.2	14.7
Genotype_338	60.9	77.1	1.8	15.8
Genotype_339	61.1	80.3	3.0	19.8
Genotype_340	61.8	84.0	2.6	36.9
Genotype_341	59.9	70.8	2.4	16.6
Genotype_342	56.8	79.9	1.6	11.6
Genotype_343	62.8	78.9	1.3	17.0
Genotype_344	58.9	81.8	1.3	15.1
Genotype_345	59.4	74.9	1.9	14.5
Genotype_346	59.1	75.1	1.4	23.6
Genotype_347	58.3	79.6	1.7	11.0
Genotype_348	61.8	80.2	1.1	12.1
Genotype_349	62.4	81.3	1.8	9.5
Genotype_350	63.1	83.1	1.3	14.4
Genotype_351	61.8	84.9	0.6	13.8

Genotype_352	61.5	90.0	1.0	11.9
Genotype_353	61.3	78.1	1.2	16.0
Genotype_354	57.4	82.2	1.0	16.7
Genotype_355	60.6	81.5	2.0	21.0
Genotype_356	61.8	81.9	0.8	14.5
Genotype_357	61.8	77.0	1.3	10.6
Genotype_358	61.6	78.4	1.7	11.9
Genotype_359	61.6	79.0	1.5	10.8
Genotype_360	61.8	82.2	1.2	13.8
Genotype_361	61.8	77.7	1.1	14.3
Genotype_362	57.0	79.6	1.5	9.2
Genotype_363	58.9	81.0	1.3	14.1
Genotype_364	62.8	85.3	0.9	10.4
Genotype_365	62.8	80.6	1.4	14.0
Genotype_366	61.3	76.4	1.3	13.1
Genotype_367	63.0	77.7	1.2	15.9
Genotype_368	59.6	77.9	1.7	11.5
Genotype_369	62.1	76.4	1.4	9.9
Genotype_370	62.1	81.3	1.3	10.2
Genotype_371	61.8	77.8	1.1	16.9
Genotype_372	62.0	74.6	1.8	13.6
Genotype_373	59.9	77.5	1.5	9.9
Genotype_374	60.1	83.2	1.3	9.3
Genotype_375	61.5	84.6	0.9	14.3
Genotype_376	62.9	84.8	1.4	17.1
Genotype_377	61.3	84.2	1.4	14.0
Genotype_378	63.6	80.3	0.5	9.7
Genotype_379	59.1	71.3	1.3	12.8
Genotype_380	60.8	76.1	2.1	13.7
Genotype_381	56.1	78.9	2.5	12.5
Genotype_382	57.5	79.5	1.0	16.4
Genotype_383	60.3	74.8	1.1	13.6
Genotype_384	61.5	81.4	1.0	13.7
Genotype_385	62.3	94.3	0.3	9.7
Genotype_386	66.1	96.8	0.4	8.0
Genotype_387	64.1	93.2	0.4	11.6
Genotype_388	50.0	55.4	4.7	5.2
Genotype_389	59.9	77.8	1.6	11.3
Genotype_390	62.8	80.0	1.7	10.9
Genotype_391	53.0	93.6	0.3	6.6

Genotype_392	61.3	101.4	0.4	9.3
Genotype_393	56.6	93.5	0.5	5.7
Genotype_394	52.8	83.6	0.7	10.3
Genotype_395	52.8	64.5	0.9	9.0
Genotype_396	62.1	88.8	2.1	7.0
Genotype_397	61.1	81.0	0.8	7.6
Genotype_398	59.4	81.3	4.0	18.0
Genotype_399	61.3	80.2	2.0	13.1
Genotype_400	60.5	91.1	1.8	19.2
Standard error	0.7	1.9	0.3	1.9

* All lsmeans calculated on 6 site years, except HD50 which was calculated using 4 site years.

HD50 = days to 50% headed (based on 2 locations)

HT = plant height (cm); FHB = Fusarium head blight score (0-5);

DON = deoxynivalenol content (mg kg⁻¹)

2.2. Selection of top molecular markers associated with fusarium head blight, and accompanying information for chromosomal position, annotation, gene ontology terms and protein family domain.

Molecular marker ^A	Chr ^B	SNP position ^C	Gene ^D	Annotation ^D	GO Terms	Pfam domains
BOPA2_12_30948	chr1H	12554357	HORVU1Hr1G005790	3-ketoacyl-CoA synthase 5	none	none
JHI-Hv50k-2016-39263	chr1H	472103486	HORVU1Hr1G073490	Auxin transporter-like protein 2	none	PF01490
JHI-Hv50k-2016-39281	chr1H	472144650	HORVU1Hr1G073500	Arginyl-tRNA--protein transferase 1	GO:0004057, GO:0016598	PF04376, PF04377
JHI-Hv50k-2016-39526	chr1H	472980576	HORVU1Hr1G073720	Solute carrier family 35 member F2	GO:0006810, GO:0016021	PF06027
JHI-Hv50k-2016-41873	chr1H	480634875	HORVU1Hr1G075830	Histidine-containing phosphotransfer protein 4	GO:0000160, GO:0004871	none
JHI-Hv50k-2016-43487	chr1H	484418096	HORVU1Hr1G077230	Cellulose-synthase-like C6	none	PF13641
JHI-Hv50k-2016-44295	chr1H	486523175	HORVU1Hr1G077830	phospholipase D beta 1	none	none
SCRI_RS_176644	chr1H	504460083	HORVU1Hr1G073760	Solute carrier family 35 member F2	GO:0006810, GO:0016021	PF06027
JHI-Hv50k-2016-198658	chr3H	543331459	HORVU3Hr1G082650	undescribed protein	none	none
JHI-Hv50k-2016-201950	chr3H	560236083	HORVU3Hr1G087110	Lung seven transmembrane receptor family protein	GO:0016021	PF06814
JHI-Hv50k-2016-439637	chr7H	4146852	HORVU7Hr1G002210	Ferredoxin--NADP reductase	GO:0016491, GO:0055114	PF00175
JHI-Hv50k-2016-441961	chr7H	6026456	HORVU7Hr1G003460	glucan synthase-like 8	GO:0000148, GO:0003843, GO:0006075, GO:0016020	PF02364, PF14288
JHI-Hv50k-2016-468035	chr7H	65733784	HORVU7Hr1G033820	Auxin response factor 19	GO:0005515, GO:0005634, GO:0006355	PF02309
JHI-Hv50k-2016-468129	chr7H	65968923	HORVU7Hr1G033990	NAC domain containing protein 103	GO:0003677, GO:0006355	PF02365
BOPA1_2669-1012	chr7H	70162054	HORVU7Hr1G033900	10 kDa chaperonin	GO:0005524, GO:0005737, GO:0006457, GO:0046914, GO:1901671	PF00166
JHI-Hv50k-2016-470256	chr7H	79954551	HORVU7Hr1G036460	glucan synthase-like 8	GO:0000148, GO:0003843, GO:0006075, GO:0016020	PF02364, PF14288
JHI-Hv50k-2016-474079	chr7H	111314648	HORVU7Hr1G041940	undescribed protein	none	none
BOPA2_12_31203	chr7H	126827168	HORVU7Hr1G043620	receptor-like protein kinase 2	GO:0004672, GO:0005524, GO:0006468	PF00069
JHI-Hv50k-2016-476379	chr7H	217471841	HORVU7Hr1G055560	Polyamine aminopropyltransferase	GO:0003824	PF01564
SCRI_RS_147689	chr7H	251402098	HORVU1Hr1G059010	pectinesterase family protein	GO:0004857, GO:0005618, GO:0030599, GO:0042545	PF01095, PF04043
JHI-Hv50k-2016-488609	chr7H	487704917	HORVU7Hr1G083410	Protein of unknown function (DUF594)	none	PF04578, PF13968
JHI-Hv50k-2016-488642	chr7H	487977868	HORVU7Hr1G083490	Acyl-coenzyme A oxidase 3	GO:0003995, GO:0003997, GO:0005777, GO:0006631, GO:0006635, GO:0008152, GO:0016627, GO:0050660, GO:0055114	PF00441, PF01756, PF02770
JHI-Hv50k-2016-488665	chr7H	489294629	HORVU7Hr1G083700	undescribed protein	none	none
JHI-Hv50k-2016-489020	chr7H	496054209	HORVU7Hr1G084970	Argonaute family protein	GO:0003676	PF02171

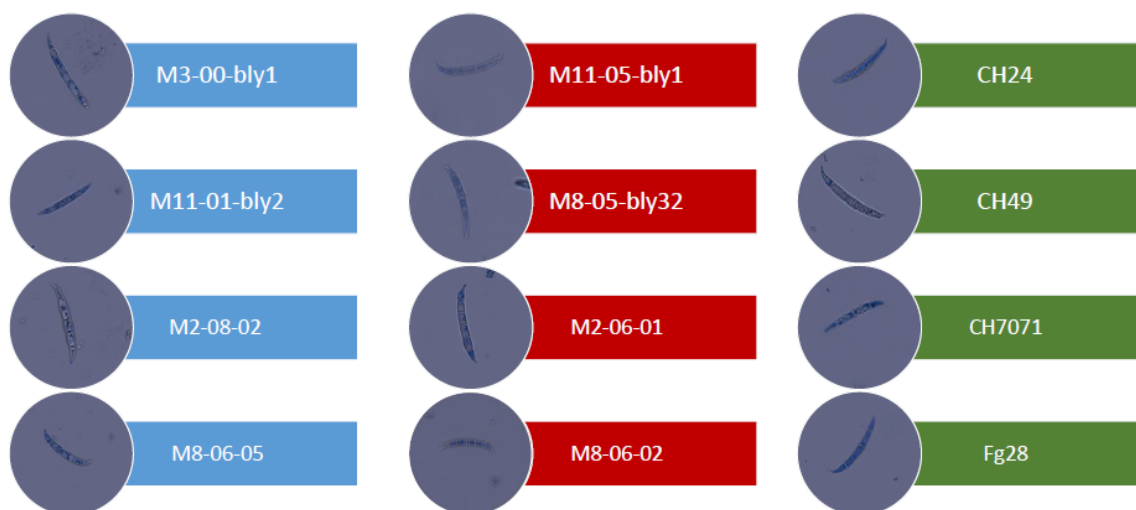
^A Molecular marker of 50K SNP array (Bayer et al. 2017); ^B Chr = Chromosome; ^C Genomic position in base pairs (bp); ^D Reference genome IBSC v2 'Morex v1' gene models (Mascher et al., 2017).

2.3. Selection of top molecular markers associated with deoxynivalenol content, and accompanying information for chromosomal position, annotation, gene ontology terms (GO Terms) and protein family domain (Pfam domains).

Molecular marker^A	Chr^B	SNP position^C	Gene^D	Annotation^D	GO Terms	Pfam domains
JHI-Hv50k-2016-111132	chr2H	603325638	HORVU2Hr1G097220	Vesicle-associated membrane protein-associated protein A	none	PF00635
JHI-Hv50k-2016-163731	chr3H	34589754	HORVU3Hr1G016890	Chromosome 3B, genomic scaffold, cultivar Chinese Spring	none	none
JHI-Hv50k-2016-163796	chr3H	35301935	HORVU3Hr1G017000	Tetratricopeptide repeat 6	GO:0005515	none
JHI-Hv50k-2016-163896	chr3H	35571360	HORVU3Hr1G017150	Autophagy-related protein 18	GO:0005515	none
JHI-Hv50k-2016-164364	chr3H	38093200	HORVU3Hr1G017930	DCD (Development and Cell Death) domain protein	GO:0005515	PF10539
JHI-Hv50k-2016-165100	chr3H	61396035	HORVU3Hr1G021600	Lipid A export ATP-binding/permease protein MsbA	GO:0005524, GO:0006810, GO:0016021, GO:0016887, GO:0042626, GO:0055085	PF00005, PF00664
SCRI_RS_114566	chr3H	439956640	HORVU3Hr1G063220	Splicing factor 3A subunit 3	GO:0003676, GO:0005634, GO:0008270	PF11931, PF12108, PF13297, PF16837
JHI-Hv50k-2016-182803	chr3H	440388699	HORVU3Hr1G063300	Katanin p60 ATPase-containing subunit A1	GO:0005524, GO:0008017, GO:0008568, GO:0051013	PF00004, PF09336
JHI-Hv50k-2016-183100	chr3H	446467087	HORVU3Hr1G064100	unknown protein	none	none
JHI-Hv50k-2016-192744	chr3H	511731917	HORVU3Hr1G075480	Core-2/I-branching beta-1,6-N-acetylglucosaminyltransferase family protein	GO:0008375, GO:0016020	PF02485
JHI-Hv50k-2016-200100	chr3H	550063195	HORVU3Hr1G084430	Transcription initiation factor TFIID subunit A	GO:0005669, GO:0006352, GO:0046982	PF03847
JHI-Hv50k-2016-205399	chr3H	572541569	HORVU3Hr1G090990	receptor kinase 2	GO:0004672, GO:0005524, GO:0006468	PF00069
JHI-Hv50k-2016-232769	chr4H	25203323	HORVU4Hr1G009620	GDP-mannose transporter	none	PF03151
JHI-Hv50k-2016-345309	chr5H	557586295	HORVU5Hr1G105870	60S ribosomal protein L4-1	GO:0003735, GO:0005840, GO:0006412	PF00573, PF14374
BOPA2_12_30673	chr6H	22584391	HORVU6Hr1G012010	Eukaryotic translation initiation factor 3 subunit J-A	GO:0003743, GO:0005737, GO:0005852	PF08597
JHI-Hv50k-2016-391014	chr6H	120502561	HORVU6Hr1G029710	tubby like protein 8	none	PF01167
JHI-Hv50k-2016-456227	chr7H	28704563	HORVU7Hr1G021490	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	GO:0016491, GO:0055114	PF03171, PF14226
JHI-Hv50k-2016-463338	chr7H	49461533	HORVU7Hr1G028460	BSD domain-containing protein	none	PF03909
JHI-Hv50k-2016-493623	chr7H	573534631	HORVU7Hr1G097760	purple acid phosphatase 28	GO:0016787	PF00149

^A Molecular marker of 50K SNP array (Bayer et al. 2017); ^B Chr = Chromosome; ^C Genomic position in base pairs (bp); ^D Reference genome IBSC v2 'Morex v1' gene models (Mascher et al., 2017).

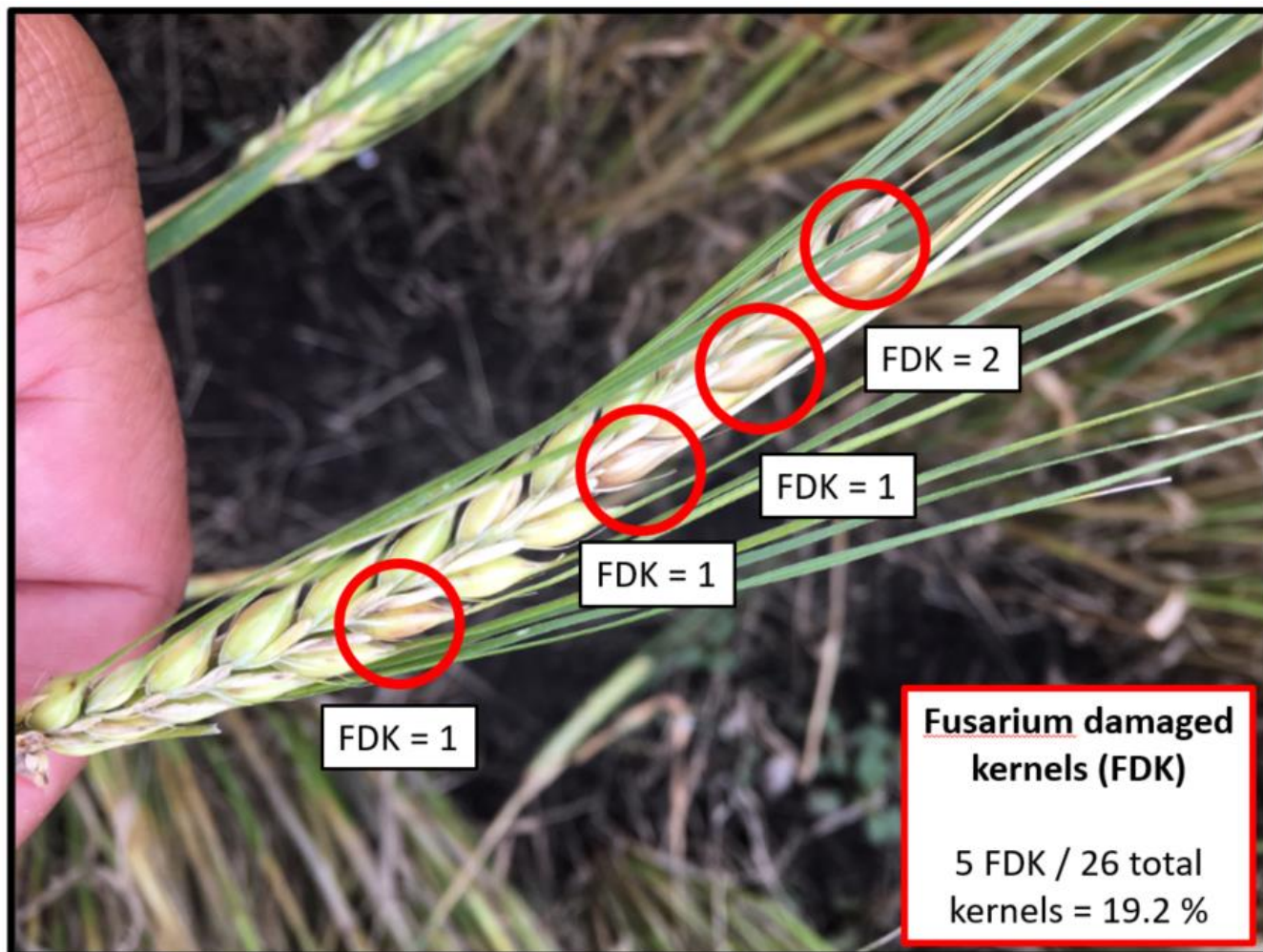
APPENDIX 3 – Chapter 5: Supplementary Figures and Tables



Isolate Code	CGC Code	NRRL code	Chemotype
WRS1915	M3-00-bly1	43162	15ADON
WRS1918	M11-01-bly2	43165	15ADON
M2-08-02	M2-08-02		15ADON
M8-06-05	M8-06-05		15ADON
WRS2065	M11-05-bly1	43217	3ADON
WRS2067	M8-05-bly32	43216	3ADON
M2-06-01	M2-06-01		3ADON
M8-06-02	M8-06-02		3ADON
Fg28			NIV
CH24			NIV
CH49			NIV
CH7071			NIV

CGC = Canadian Grain Commission; NRRL = United States Department of Agriculture, Agricultural Research Service (NRRL) collection.

3.1. *Fusarium graminearum* isolates and chemotype classification used in the study.



3.3. Fusarium head blight symptoms on a two-row barley spike. Fusarium-damaged kernels are highlighted in red circles.

3.4. Percent (%) alignment by sample to the barley reference genome (IBSC_v2) using HISAT2.

Sample_ID	Percent (%)	TIME	VARIETY	TREAT	REP
1	92.7	72	N	mock	1
2	90.8	72	K	mock	1
5	91.8	72	N	mock	2
6	87.3	72	K	mock	2
11	90.9	72	N	mock	3
12	93.3	72	K	mock	3
3	87.5	96	K	mock	1
4	90.5	96	N	mock	1
7	93.9	96	K	mock	2
8	90.2	96	N	mock	2
9	91.5	96	K	mock	3
10	89.3	96	N	mock	3
16	92.5	72	K	15ADON	1
17	90.6	72	K	15ADON	2
23	92.9	72	K	15ADON	3
15	92.5	72	N	15ADON	1
18	91.3	72	N	15ADON	2
24	81.1	72	N	15ADON	3
14	82.4	96	K	15ADON	1
19	87.5	96	K	15ADON	2
22	90.7	96	K	15ADON	3
13	89.1	96	N	15ADON	1
20	75.9	96	N	15ADON	2
21	92.2	96	N	15ADON	3
28	90.5	72	K	3ADON	1
30	92.7	72	K	3ADON	2
35	91.0	72	K	3ADON	3
27	92.6	72	N	3ADON	1
29	92.0	72	N	3ADON	2
33	89.6	72	N	3ADON	3
25	92.5	96	K	3ADON	1
32	85.2	96	K	3ADON	2
34	86.9	96	K	3ADON	3
26	88.3	96	N	3ADON	1
31	91.4	96	N	3ADON	2
36	90.9	96	N	3ADON	3
38	90.9	72	K	NIV	1
42	88.7	72	K	NIV	2
45	91.6	72	K	NIV	3

37	92.7	72	N	NIV	1
41	90.0	72	N	NIV	2
46	89.3	72	N	NIV	3
40	83.7	96	K	NIV	1
43	91.6	96	K	NIV	2
47	89.8	96	K	NIV	3
39	87.2	96	N	NIV	1
44	91.2	96	N	NIV	2
48	85.6	96	N	NIV	3

Time: 72 & 96 hours post infection; Variety: N = Norman & K = CDC Kendall; Treat: mock = control; 15ADON = 15-acetyldeoxynivalenol, 3ADON = 3-acetyldeoxynivalenol, NIV = nivalenol.

3.5. A list of 15 analyses conducted for differential gene expression and overrepresentation of Gene Ontology (GO) terms.

Paired, single-factor GLM:

Fusarium treatment contrasts (2x $n = 3$ samples):

1. 15ADON Fusarium vs. mock control for CDC Kendall (72 and 96 hpi)
2. 3ADON Fusarium vs. mock control for CDC Kendall (72 and 96 hpi)
3. NIV Fusarium vs. mock control for CDC Kendall (72 and 96 hpi)

Varietal contrasts (2 x $n = 3$ samples):

4. CDC Kendall vs. Norman, within mock control (72 and 96 hpi)
5. CDC Kendall vs. Norman, within 15ADON Fusarium (72 and 96 hpi)
6. CDC Kendall vs. Norman, within 3ADON Fusarium (72 and 96 hpi)
7. CDC Kendall vs. Norman, within NIV Fusarium (72 and 96 hpi)

Time contrasts (2x $n = 3$ samples):

8. 72 vs. 96 hpi, within 15ADON Fusarium (Norman and CDC Kendall)
9. 72 vs. 96 hpi, within 3ADON Fusarium (Norman and CDC Kendall)
10. 72 vs. 96 hpi, within NIV Fusarium (Norman and CDC Kendall)

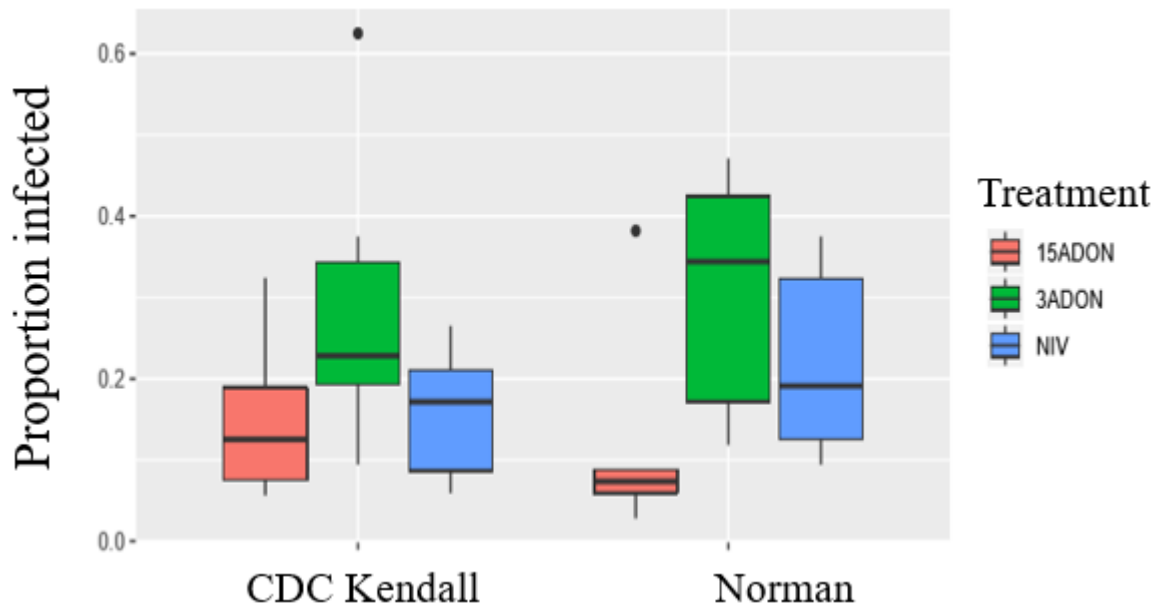
Additive, three-factor GLM:

11. 72 vs. 96 hpi (2 x $n = 24$ samples)
12. Norman vs. CDC Kendall (2 x $n = 24$ samples)
13. 15ADON (2 x $n = 12$ samples; 15ADON Fusarium vs. mock control)
14. 3ADON (2 x $n = 12$ samples; 3ADON Fusarium vs. mock control)
15. NIV (2 x $n = 12$ samples; NIV Fusarium vs. mock control).

3.6. Analysis of variance for the proportion of *Fusarium* damaged kernels and Tukey-Kramer groups for chemotype treatment.

ANOVA – Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F value	Pr>F
Variety	1	30	0.06	0.803
Treatment	2	30	4.82	0.015
Variety x Treatment	2	30	0.45	0.643

Tukey-Kramer Grouping for Chemotype Treatment ($\alpha=0.05$)



3.7. Percent *Fusarium* damaged kernels per spike over treatments: 15ADON, 3ADON, NIV chemotypes by variety (n=6) in the growth cabinet study. No disease was observed under the mock treatment.

3.8. Proportion of *Fusarium* damaged kernels by chemotype treatment.

Treatment	FDK
3ADON	0.30 a
NIV	0.19 ab
15ADON	0.13 b

FDK = Proportion of *Fusarium* damaged kernels; LS-means with the same letter are not significantly different.

3.9. Analysis of variance for the content of primary mycotoxin (DON or NIV) for a composite sample of two barley spikes ground together.

ANALYSIS OF VARIANCE (ANOVA)				
Type III Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Variety	1	6	0.02	0.90
Treatment	2	6	12.63	0.01
Variety*Treatment	2	6	0.08	0.92

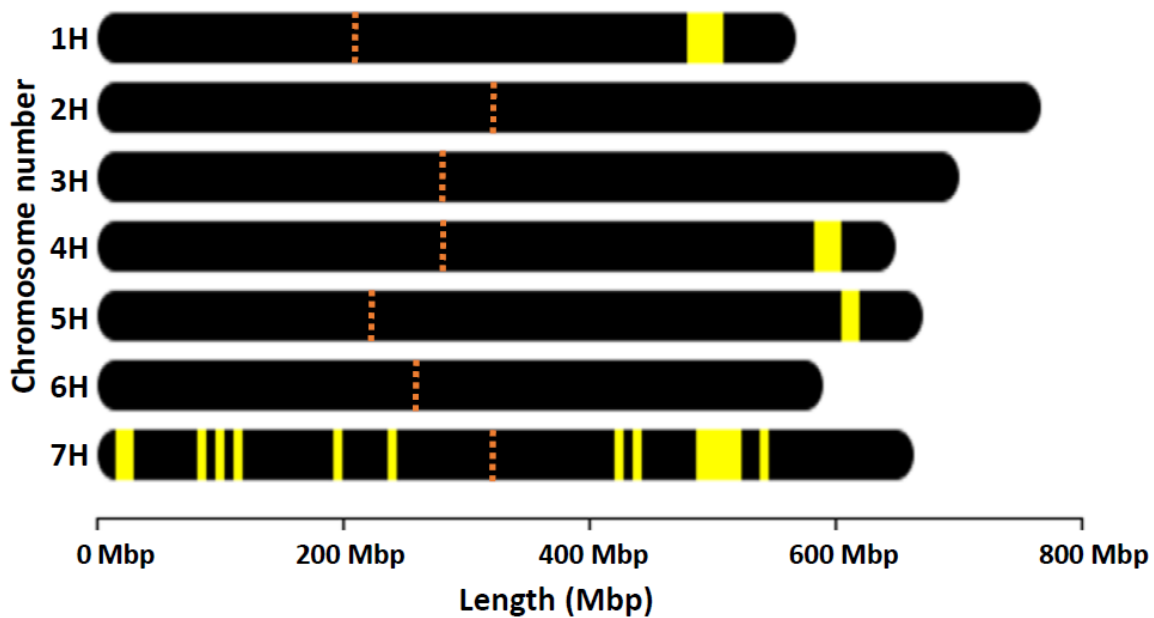
3.10. Percent variant single nucleotide polymorphic molecular markers contrasted between ‘Norman and ‘CDC Kendall’ by chromosome as determined by Illumina 50K SNP array.

Chr¹	Total SNP markers	Variant markers	Variance (%)
1H	3468	251	5.9
2H	6168	2	0.0
3H	5803	1	0.0
4H	4480	76	1.7
5H	6930	67	1.0
6H	4678	0	0.0
7H	5515	527	9.6
Total	39363	1095	1.8

¹Chromosome assignment as per Bayer et al. (2017) ;

0 = Position unassigned.

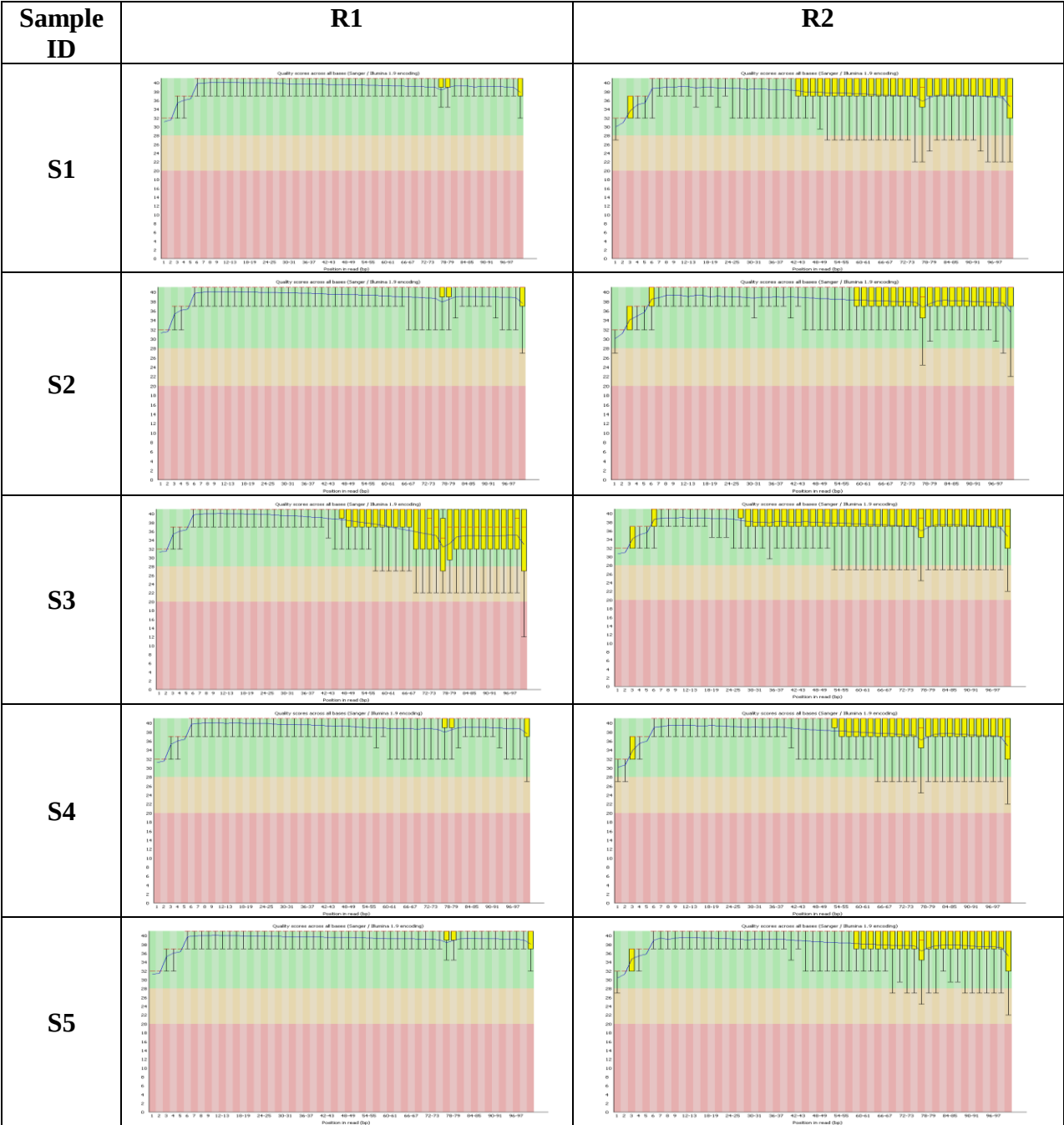
3.11. Chromosomal regions of variation are depicted in yellow for single nucleotide polymorphic markers between ‘Norman’ and ‘CDC Kendall’. The Orange dashed line indicates the location of the centromere.

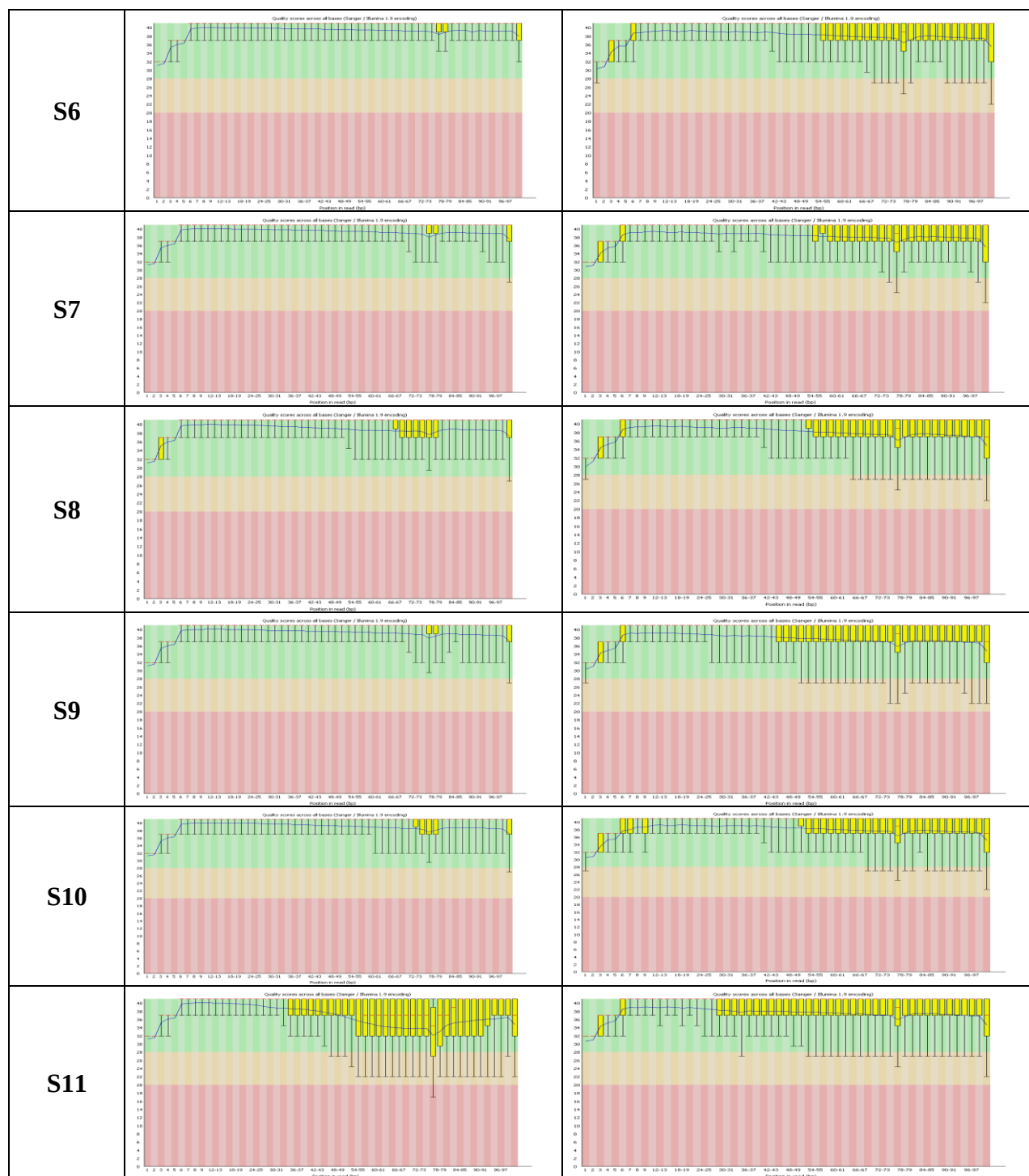


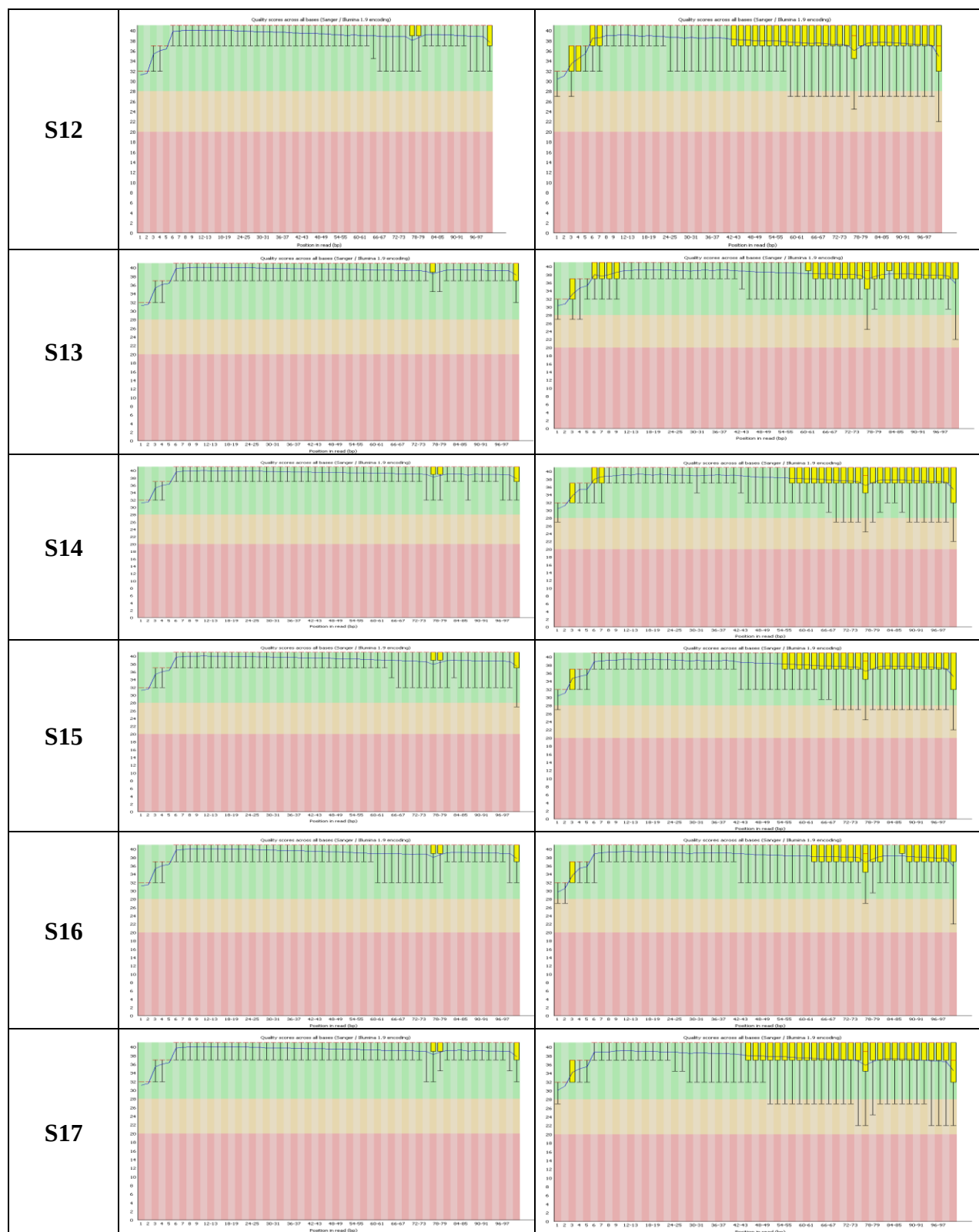
3.12. The number of reads per sample. R1 and R2 indicate forward and reverse strands respectively.

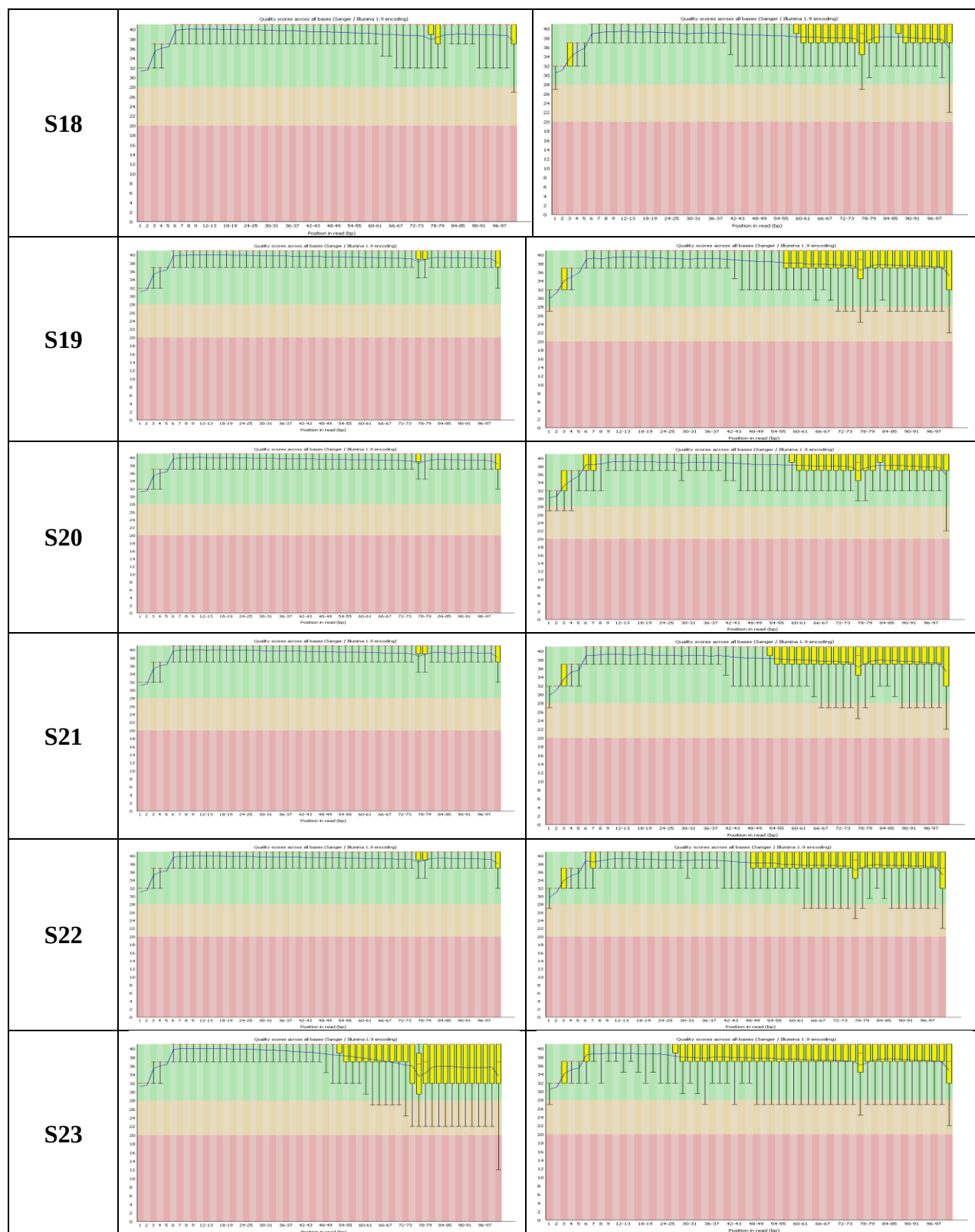
Sample ID	Total Read		Sample ID	Total Read		Sample ID	Total Read	
	R1	R2		R1	R2		R1	R2
S1	66246622	66246622	S17	51154954	51154954	S33	56109140	56109140
S2	59419800	59419800	S18	57396646	57396646	S34	59567586	59567586
S3	81109352	81109352	S19	75121255	75121255	S35	66677063	66677063
S4	61503028	61503028	S20	56986465	56986465	S36	93660519	93660519
S5	63546185	63546185	S21	70749856	70749856	S37	66019322	66019322
S6	53884500	53884500	S22	52514161	52514161	S38	57926516	57926516
S7	64127086	64127086	S23	67187185	67187185	S39	72710657	72710657
S8	47372468	47372468	S24	56900161	56900161	S40	71891982	71891982
S9	60177791	60177791	S25	65038296	65038296	S41	82634553	82634553
S10	66192463	66192463	S26	68519005	68519005	S42	59095659	59095659
S11	48478933	48478933	S27	83807875	83807875	S43	61836822	61836822
S12	61132880	61132880	S28	54561216	54561216	S44	63049737	63049737
S13	54380771	54380771	S29	57362755	57362755	S45	72641217	72641217
S14	66093056	66093056	S30	68754397	68754397	S46	55727719	55727719
S15	66560484	66560484	S31	66534392	66534392	S47	55807395	55807395
S16	66452389	66452389	S32	59562623	59562623	S48	56200520	56200520

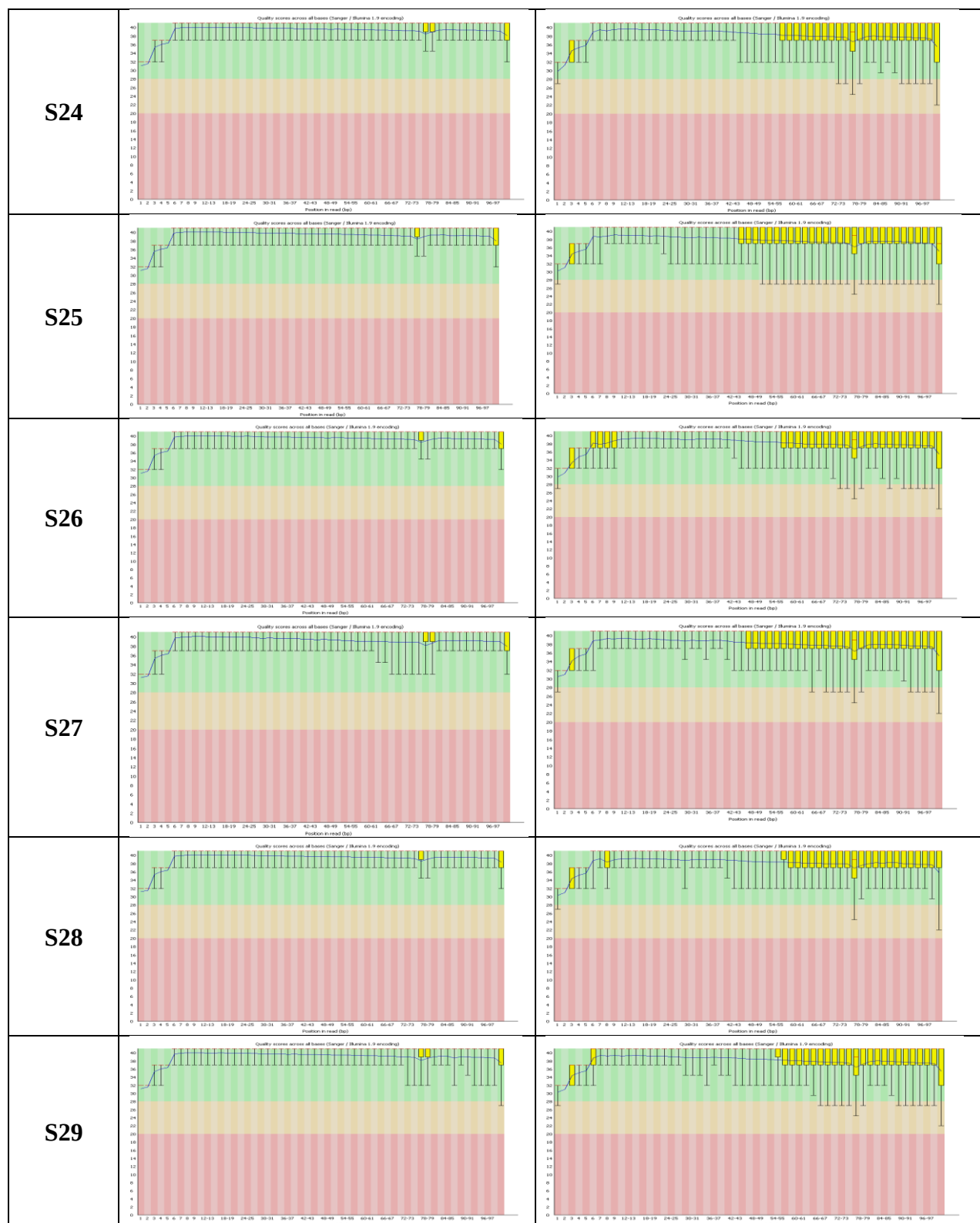
Appendix 3.13. Read quality analysis from FastQC summary report for samples (1 to 48). Box-and-whisker plots of quality scores (Y-axis, 1-40) by the position of read (X-axis, 1-100). R1 & R2 indicate forward and reverse strand respectively. Yellow bars represent the inter-quartile range (25-75%) with upper and lower whiskers representing 10% and 90% points. The background of the graph indicates good (green), reasonable (orange), and poor (red) quality.

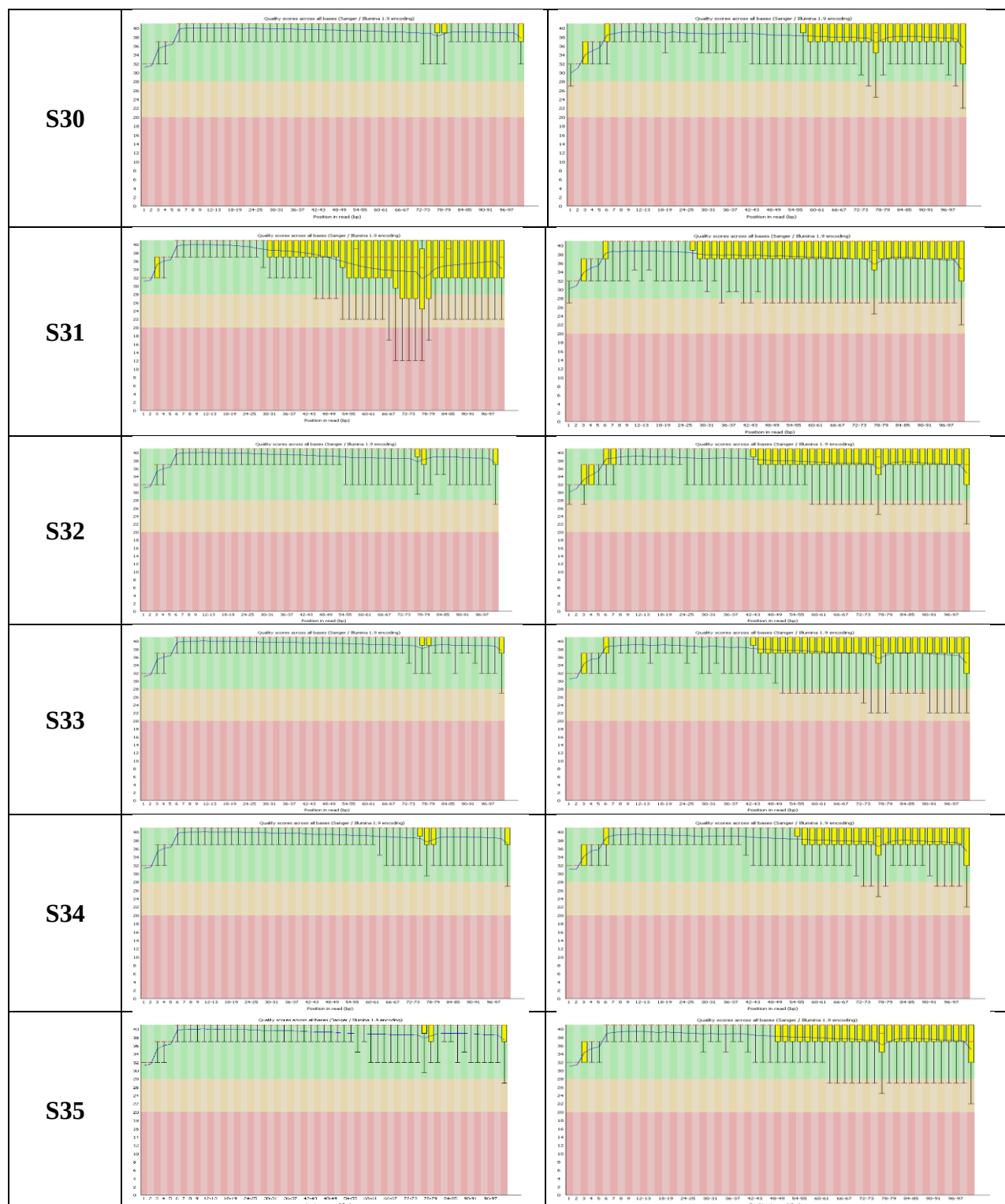


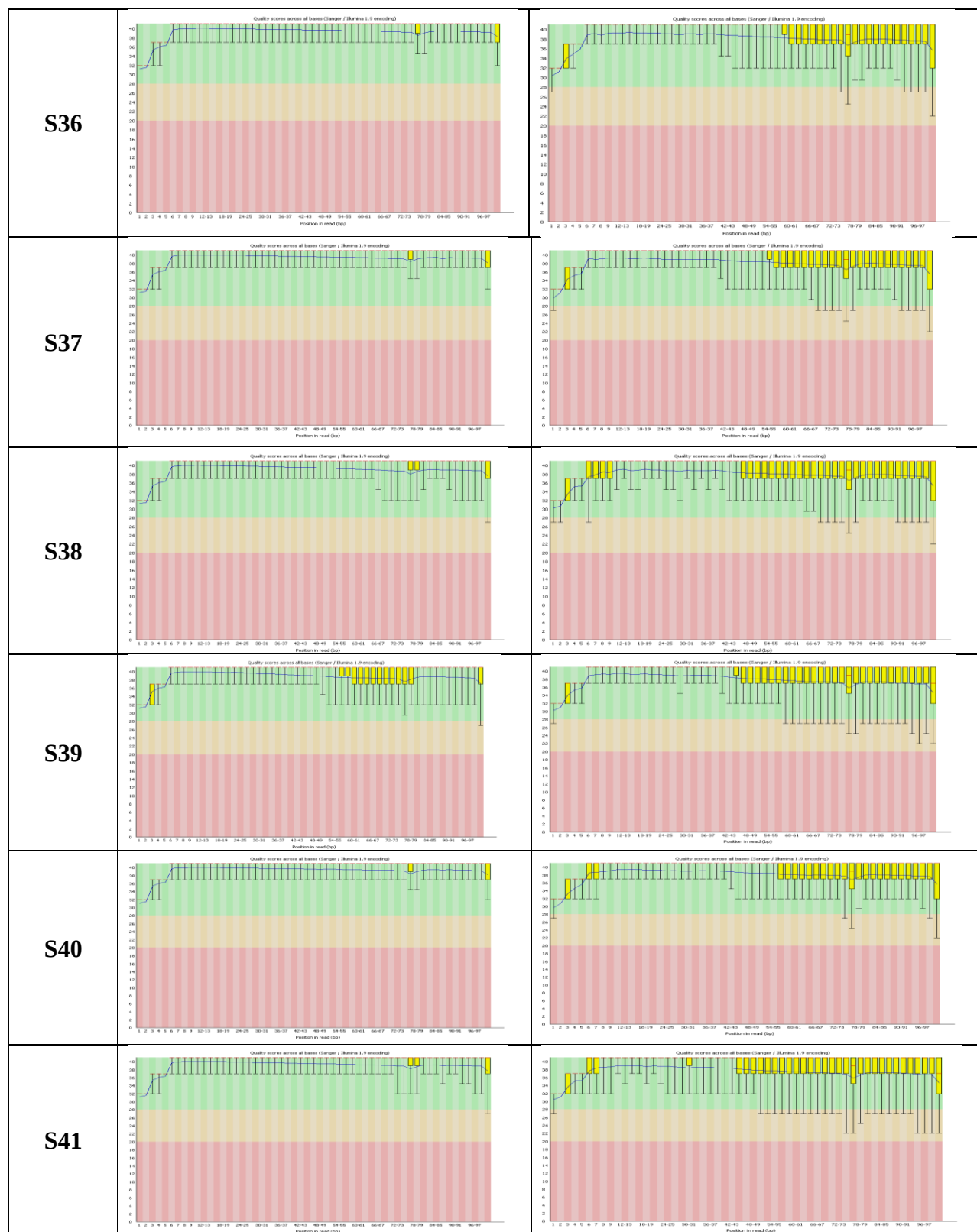


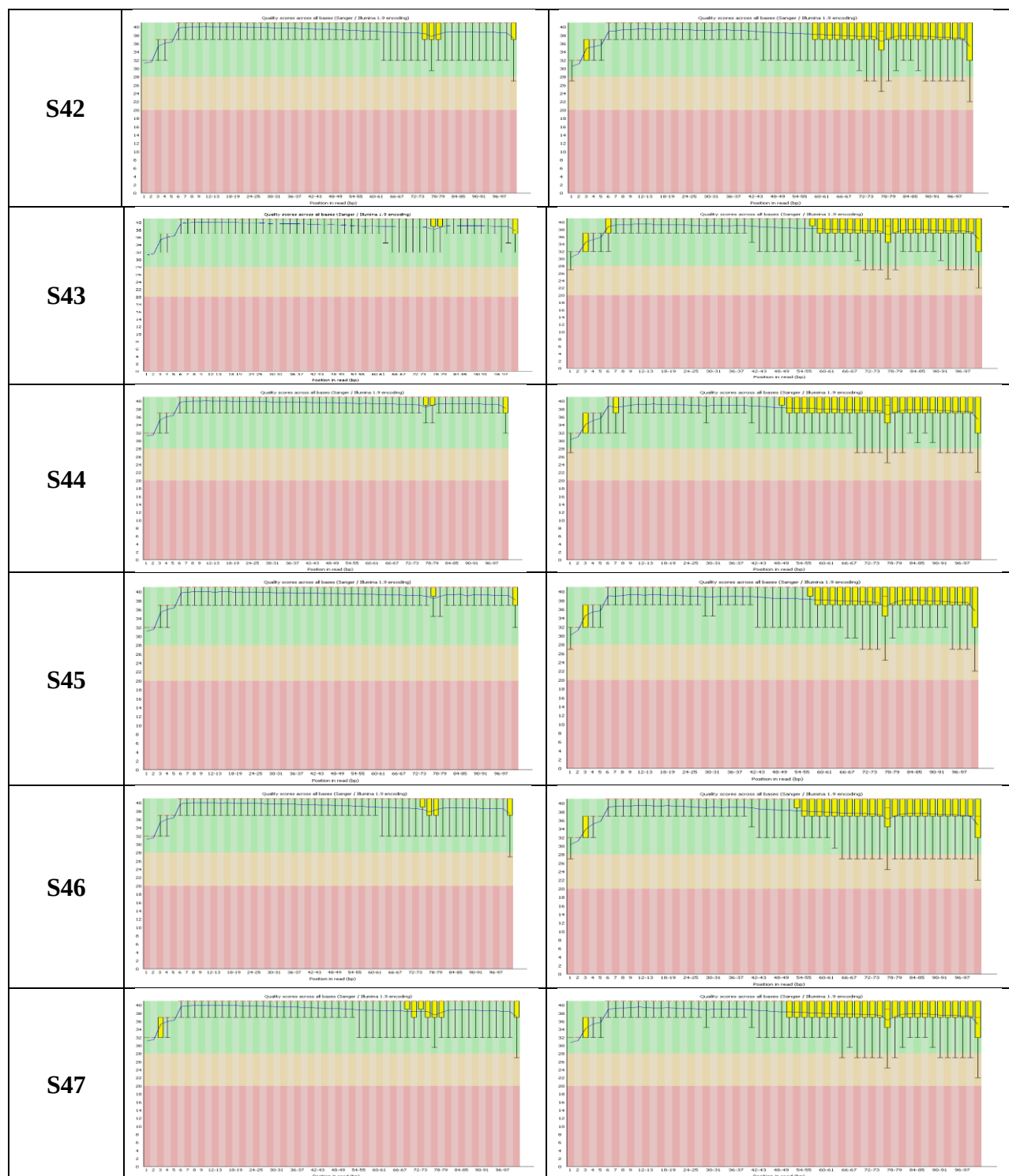


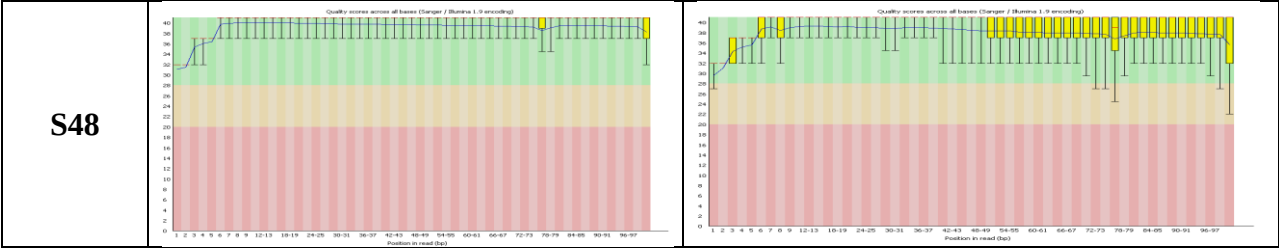


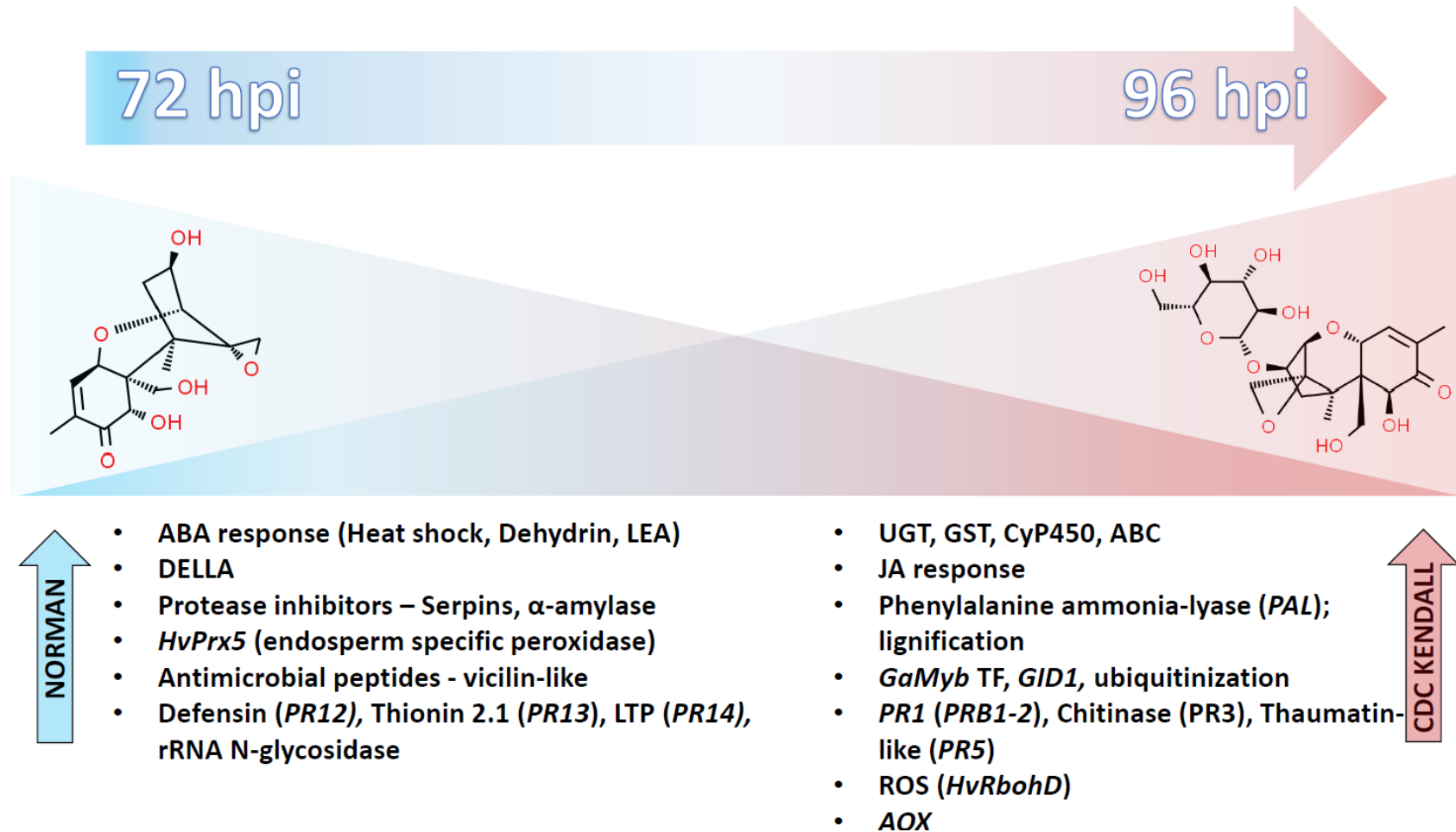












3.14. Schematic of differentially expressed genes between ‘Norman’ (Blue) and ‘CDC Kendall’ (Pink) between 72 hpi and 96 hpi.

APPENDIX 4 – Chapter 6: Supplementary Figures and Tables

4.1. Descriptive statistics of mycotoxin concentrations (mg kg⁻¹) of mock-inoculated (water) barley plots by developmental stage at harvest and tissue type in 2016 and 2017 (n=48).

Soft-dough (grain)	2016				2017			
Mycotoxin¹	mean	se	min	max	mean	se	min	max
HT2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
T2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
15ADON	0.00	0.00	0.00	0.12	0.00	0.00	0.00	0.00
DON3G	0.09	0.02	0.00	0.53	0.25	0.05	0.00	1.72
NIV	0.83	0.13	0.00	4.02	0.91	0.13	0.00	5.07
DON	0.24	0.07	0.00	2.89	0.54	0.11	0.00	4.23
ZEAR	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03
3ADON	0.01	0.00	0.00	0.18	0.05	0.01	0.00	0.62
CUL	-	-	-	-	0.06	0.01	0.00	0.46

Soft-dough (rachis)	2016				2017			
Mycotoxin	mean	se	min	max	mean	se	min	max
HT2	0.01	0.01	0.00	0.26	0.00	0.00	0.00	0.00
T2	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.00
15ADON	0.01	0.01	0.00	0.36	0.00	0.00	0.00	0.00
DON3G	0.36	0.09	0.00	2.98	0.70	0.09	0.00	2.34
NIV	2.54	0.44	0.00	15.81	0.95	0.24	0.00	7.26
DON	0.35	0.09	0.00	3.10	0.63	0.20	0.00	9.37
ZEAR	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3ADON	0.02	0.01	0.00	0.32	0.05	0.02	0.00	0.42
CUL	-	-	-	-	0.13	0.02	0.00	0.61

Mature (grain)	2016				2017			
Mycotoxin	mean	se	min	max	mean	se	min	max
HT2	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00
T2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
15ADON	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
DON3G	0.10	0.02	0.00	0.44	0.19	0.03	0.00	0.92
NIV	0.25	0.03	0.00	1.05	0.28	0.04	0.00	1.40
DON	0.29	0.04	0.00	1.46	0.61	0.07	0.00	2.27
ZEAR	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00
3ADON	0.00	0.00	0.00	0.08	0.03	0.01	0.00	0.19
CUL	-	-	-	-	0.07	0.01	0.01	0.30

¹ HT2 = HT2 toxin; T2 = T2 toxin; 15ADON = 15-acetyl-deoxynivalenol; DON3G = deoxynivalenol-3-glucoside; NIV = nivalenol; DON = deoxynivalenol; ZEAR = zearalenone; 3ADON = 3-acetyl-deoxynivalenol; CUL = culmorin. se = standard error; min=minimum; max=maximum.

4.2. Descriptive statistics of mycotoxin concentrations (mg kg⁻¹) of *Fusarium*-inoculated barley plots by developmental stage at harvest and tissue type in 2016 and 2017 (n=48).

<u>Soft-dough (grain)</u>	<u>2016</u>				<u>2017</u>			
Mycotoxin¹	mean	se	min	max	mean	se	min	max
HT2	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.35
T2	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.31
15ADON	0.09	0.02	0.00	0.83	0.00	0.00	0.00	0.00
DON3G	6.91	0.83	0.10	22.77	4.12	0.63	0.07	22.38
NIV	0.77	0.12	0.00	4.20	0.83	0.08	0.00	2.21
DON	16.09	1.89	0.11	57.99	11.59	1.87	0.15	62.98
ZEAR	0.01	0.01	0.00	0.31	0.01	0.00	0.00	0.07
3ADON	1.50	0.20	0.00	5.35	0.72	0.15	0.00	4.51
CUL	-	-	-	-	0.89	0.17	0.02	5.59

<u>Soft-dough (rachis)</u>	<u>2016</u>				<u>2017</u>			
Mycotoxin	mean	se	min	max	mean	se	min	max
HT2	0.04	0.02	0.00	1.11	0.00	0.00	0.00	0.00
T2	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00
15ADON	0.10	0.02	0.00	0.54	0.00	0.00	0.00	0.00
DON3G	19.47	2.32	0.17	58.35	12.35	2.13	0.00	71.81
NIV	3.30	0.59	0.00	17.22	0.92	0.17	0.00	4.27
DON	23.92	2.93	0.11	101.92	12.93	2.24	0.33	63.45
ZEAR	0.02	0.01	0.00	0.26	0.01	0.01	0.00	0.18
3ADON	1.89	0.23	0.00	5.38	1.02	0.27	0.00	10.19
CUL	-	-	-	-	1.86	0.33	0.18	10.13

<u>Mature (grain)</u>	<u>2016</u>				<u>2017</u>			
Mycotoxin	mean	se	min	max	mean	se	min	max
HT2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11
T2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
15ADON	0.01	0.01	0.00	0.18	0.00	0.00	0.00	0.00
DON3G	4.59	0.43	1.17	13.10	3.49	0.55	0.34	15.51
NIV	0.24	0.02	0.06	1.15	0.30	0.03	0.00	1.05
DON	11.89	1.13	3.79	38.91	10.67	1.75	0.97	54.62
ZEAR	0.03	0.01	0.00	0.20	0.02	0.01	0.00	0.52
3ADON	0.74	0.09	0.21	3.27	0.43	0.07	0.00	2.72
CUL	-	-	-	-	0.64	0.10	0.06	2.74

¹ HT2 = HT2 toxin; T2 = T2 toxin; 15ADON = 15-acetyl-deoxynivalenol; DON3G = deoxynivalenol-3-glucoside; NIV = nivalenol; DON = deoxynivalenol; ZEAR = zearalenone; 3ADON = 3-acetyl-deoxynivalenol; CUL = culmorin. se = standard error; min=minimum; max=maximum.

4.3. A panel of fourteen mycotoxins and their limit of quantification for samples evaluated in 2016 and 2017 for content in barley tissues.

Abbreviation	Mycotoxin	LOQ¹
DON	Deoxynivalenol	0.03
DON-3G	deoxynivalenol-3-glucoside	0.05
15-ADON	15-acetyl deoxynivalenol	0.05
3-ADON	3-acetyl deoxynivalenol	0.05
ZEAR	Zearalenone	0.02
NIV	Nivalenol	0.06
CUL ²	Culmorin	0.05
HT-2	HT-2 toxin	0.06
T-2	T-2 toxin	0.03
AFG1	aflatoxin G2	0.001
AFG2	aflatoxin G1	0.001
AFB1	aflatoxin B2	0.001
AFB2	aflatoxin B1	0.001
OTA	ochratoxin A	0.0006

¹Limit of quantification (mg kg⁻¹)

²Only evaluated in 2017

Appendix II List of abbreviations

3ADON, 3-acetyl-deoxynivalenol

15ADON, 15-acetyl-deoxynivalenol

AAFC, Agriculture and Agri-Food Canada

ABA, abscisic acid

CDC, Crop Development Centre

CNN, convolutional neural network

DON, deoxynivalenol

DON3G, deoxynivalenol-3-glucoside

FCDC, Field Crop Development Centre

FHB, fusarium head blight

GA, gibberellic acid

GEV, genomic estimated breeding value

GLM, general linear model

GO term, gene ontology term

GS, genomic selection

GWAS, genome-wide association study

IVS, *In vitro* selection

JA, jasmonic acid

LC-MS/MS, liquid chromatography-tandem mass spectrometry

LD, linkage disequilibrium

MAPK, mitogen-activated protein kinase

NIV, nivalenol

PAMP, pathogen-associated molecular pattern

PCA, principal component analysis

PCD, programmed cell death

PCR, polymerase chain reaction

PR, pathogenesis-related protein

PTI, PAMP-triggered immunity

QTL, quantitative trait loci

RFCNN, Residual Fully Connected Neural Network

ROS, reactive oxygen species

SA, salicylic acid

SNP, single nucleotide polymorphic