

GENETICS OF RESISTANCE TO FUSARIUM HEAD
BLIGHT AND SPOT BLOTCH IN *HORDEUM*

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Dedication

This thesis is dedicated to my family: my parents, Paul and Claudia Haas; my sister, Kirsten Haas-Camitsch, and brother-in-law, Devon Camitsch.

Abstract

Fusarium head blight (FHB) and spot blotch are two important diseases of barley (*Hordeum vulgare*) both in the Upper Midwest region of the USA and globally. Given conducive weather conditions, both diseases can drastically reduce the yield of susceptible barley crops. The principal fungus responsible for FHB in the Upper Midwest is *Fusarium graminearum*, which also produces mycotoxins such as deoxynivalenol (DON) that have major implications for growers and end-users alike. Genetic resistance to FHB in barley is rare and quantitative in nature with most loci explaining a small proportion of the total phenotypic variance. Further, 'resistance' is associated with a variety of agro-morphological traits including heading date and plant height. Tall, late-heading plants tend to have less disease than short, early-heading plants. Immunity to the disease does not exist. Wild barley (*H. vulgare* ssp. *spontaneum*) is known to be a highly diverse reservoir of novel alleles, including those for disease resistance. From a previous global survey of over 20,000 diverse wild and landrace barley accessions, 78 were identified as having a moderate level of FHB resistance. Three of these accessions, wild barleys PI 466423 and W-365 and landrace Kutahya were used as donor parents in advanced backcross populations with six-rowed Minnesota malting barley cultivars in order to pyramid novel quantitative trait loci (QTL) for reduced FHB severity and DON accumulation into germplasm and recover transgressive segregants that possess a greater level of resistance than is presently available in commercial cultivars. The largest effect QTL identified in the populations derived from a wild donor (PI 466423 and W-365) maps at or near the photoperiod response gene *Ppd-H1*, which affects heading date and plant height, suggesting that the QTL for reduced FHB and DON is a pleiotropic effect of that locus. There were no major effect QTL identified in the Kutahya-derived population. In all three populations, minor effect QTL were not consistently detected across all environments. Given the results of these mapping projects and similar results from other populations created with diverse 'resistant' parents, improvements in resistance to FHB and DON accumulation can be best achieved through the use

of genomic selection. For over 50 years, spot blotch, caused by *Cochliobolus sativus*, has been controlled in the Upper Midwest production region through the deployment of resistant cultivars. This durable resistance is conferred by three QTL derived from breeding line NDB112. Recently, *C. sativus* isolates such as ND4008 have been recovered in North Dakota and adjacent areas of Canada that possess virulence for the NDB112 resistance. Routine screening of germplasm revealed that PI 466423 possesses a high level of resistance to isolate ND4008. Therefore, the population created with PI 466423 as a parent (Rasmusson/PI 466423) to map QTL for reduced FHB severity was also used to map QTL for resistance to isolate ND4008. Four resistance QTL were identified on chromosomes 1H, 2H, 4H, and 5H, explaining 10.3%, 7.4%, 6.5%, and 8.4% of the variance, respectively. The QTL on chromosomes 1H, 4H, and 5H were contributed by PI 466423, while the one on chromosome 2H was contributed by Rasmusson, which carries the NDB112 resistance. The genetics of spot blotch resistance was also explored in a gamma radiation-induced susceptibility mutant identified in Morex, a cultivar with the same QTL for durable resistance contributed by NDB112. When inoculated at the seedling stage, this mutant exhibits highly compatible lesions in response to the spot blotch pathogen. In the absence of the pathogen, the mutant produces very large necrotic lesions in maturing plants that closely mimic the symptoms of spot blotch. An RNAseq experiment was conducted to study the seedling response of the mutant over time (12, 24, and 36 hours after inoculation) compared with the resistant wild-type Morex. Differential expression analysis between the two genotypes revealed a role for lipid signaling in the resistance response. These signaling events may activate the jasmonic acid pathway. The mutant is defective in this pathway, resulting in a susceptible spot blotch reaction.

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Chapter 1

General Introduction

Introduction

Barley (*Hordeum vulgare* ssp. *vulgare*) is an economically important crop both in the United States and worldwide. Globally, barley is the fifth most-produced cereal crop and is grown for a variety of uses, including animal feed (55-60%), food (2-3%), malting/brewing (30-40%), and seed (5%) (Ullrich 2010). The widespread production of barley is attributed to its adaptability to extreme environments including a wide range of latitudes and altitudes as well as tolerance for cool, dry, and saline environments (Ullrich 2010). Disease is a major limiting factor for barley production in terms of both yield and quality. Two of the many diseases that afflict barley are Fusarium head blight (FHB) and spot blotch. The primary objectives of this thesis were to map quantitative trait locus/loci (QTL) for resistance to FHB and spot blotch and to characterize the transcriptomic response of the spot blotch-resistant cultivar Morex and a derived susceptible mutant in order to understand the molecular pathways responsible for resistance.

Introduction to Fusarium head blight (FHB)

Fusarium graminearum Schwabe is the main causal agent of FHB in North America, although other *Fusarium* species are also known to cause the disease including *F. pseudograminearum* O'Donnell & Aoki, *F. avenaceum* (Fr.:Fr.) Sacc., *F. sporotrichioides* Scherb, *F. culmorum* (W.G. Sm.) Sacc., and *F. poae* (Peck) Wollenweb. (Steffenson 2003). In Minnesota, barley production has

decreased dramatically from over 1 million acres/year prior to the FHB epidemics of the 1990s to around 100,000 acres today according to the National Agricultural Statistic Service (NASS). Although this decline in acreage is not strictly due to FHB and may also be attributed to changes in cropping patterns in favor of more profitable crops such as maize, these epidemics resulted in losses exceeding \$3 billion throughout the United States and Canada (McMullen et al. 1997, Ward et al. 2008, Windels 2000). FHB is particularly devastating to growers and consumers because it affects the spike, whose seed is ultimately consumed and may be contaminated with fungus-produced trichothecene mycotoxins that impact the health of humans and animals. Trichothecenes are secondary metabolites produced by *Fusarium* species that act as virulence factors for the pathogen and include the Type B trichothecenes deoxynivalenol (DON) and its relatives: 3-acetyl-deoxynivalenol (3-ADON), 15-acetyl-deoxynivalenol (15-ADON), and nivalenol (NIV).

***Fusarium graminearum* biology**

Like *C. sativus*, *F. graminearum* is also an ascomycete. The fungus reproduces both sexually and asexually. Since 2013, the genus *Fusarium* has been used exclusively to refer to both the asexual (anamorph) and sexual (teleomorph) stages (Geiser et al. 2013). The asexually-produced spores are referred to as macroconidia and are multi-septate, elongated, and clear in color. The sexually-produced ascospores form in dark blue perithecia, but themselves are clear or

light brown in color. Most isolates of *F. graminearum* that have been characterized are homothallic (self-fertile), but are also capable of reproducing sexually with other isolates (Yun et al. 2000).

Population structure of *Fusarium graminearum*

Populations of *F. graminearum* are defined differently than other groups of plant pathogens. Rather than describing populations based on virulence or avirulence on differential host sets, populations of *F. graminearum* are frequently categorized by the primary type of trichothecene toxin produced by constituent members (Moss and Thrane 2004). Trichothecene toxins include deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3ADON), 15-acetyl-deoxynivalenol (15ADON), and nivalenol (NIV). 3ADON and 15ADON are biosynthetic precursors to DON (Varga et al. 2015). Figure 1.2 shows the chemical structures of these common type B trichothecenes.

Introduction to spot blotch

Spot blotch is a foliar disease of barley caused by the hemibiotrophic ascomycete fungal pathogen *Cochliobolus sativus* (Ito & Kurib.) Drechs. ex. Dastur [anamorph: *Bipolaris sorokiniana* (Sacc.) Shoem.]. Hemibiotrophs begin the infection cycle as a biotroph (i.e., feeds on living tissue) before switching to a necrotrophic lifestyle (i.e., feeds on dead or dying tissue). *Cochliobolus sativus* also causes common root rot, seedling blight, head blight, and black point of wheat and barley (Kumar et al. 2002). To aid in virulence, *C. sativus* produces a

number of toxins that act as virulence factors, including prehelminthosporol, helminthosporol, and sorokinianin (Figure 1.1). Prehelminthosporol inhibits H⁺-ATPase, as well as Ca²⁺-ATPase and β -1,3-glucan synthase (Kumar et al. 2002). Helminthosporol inhibits β -1,3-glucan synthase in addition to disrupting membrane integrity, preventing oxidative phosphorylation in mitochondria and chloroplasts of the host plant (Kumar et al. 2002). Sorokinianin inhibits seed germination (Kumar et al. 2002). The focus of *C. sativus*-related research presented in this thesis is on the foliar phase of spot blotch, but the pathogen was also used in the Minnesota barley breeding program for developing cultivars with resistance to kernel discoloration (KD) (Steffenson 2003). The research on KD using *C. sativus*, along with other causal *Fusarium* and *Alternaria* species, may have increased the level of FHB resistance in Minnesota barley germplasm through selection for improved resistance to general seed discoloration, including FHB (Steffenson 2003).

Spot blotch of barley has been kept in check in the Upper Midwest region of the United States for over 50 years through the deployment of cultivars with durable resistance contributed by breeding line NDB112 (Bilgic et al. 2005 & 2006, Wilcoxson et al. 1990, Zhou and Steffenson 2013). This durable resistance is conferred by three QTL on chromosomes 1H, 3H, and 7H that are collectively referred to as the Midwest Six-row Durable Resistance Haplotype (MSDRH). This resistance is present in every six-rowed cultivar released from the

Minnesota barley breeding program in the past 50 years including cultivar 'Morex' (Rasmusson and Wilcoxson 1979). However, there have been recent reports of *C. sativus* isolates with virulence for NDB112 and its derivatives (Ghazvini and Tekauz 2007, Gyawali 2010). One such isolate is ND4008, which was first cultured from the roots of barley in North Dakota. Characterization of the genetic architecture of resistance to isolate ND4008 is the focus of Chapter 4 of this thesis.

Pathotypes of *Cochliobolus sativus*

The virulence spectra of *C. sativus* populations have been characterized by a number of research groups (Ghazvini and Tekauz 2007, Valjavec-Gratian and Steffenson 1997). Ghazvini and Tekauz (2007) found that, generally, there is no race structure in *C. sativus* populations, but isolates may vary in aggressiveness. For race nomenclature of *C. sativus*, Valjavec-Gratian and Steffenson (1997) used a coded triplet system (Limpert and Müller 1994). According to this system, differential host lines, set in groups of three, are placed in a specific order and a binary value is assigned to each based on its reaction to the pathogen. A susceptible reaction on the host indicates the pathogen isolate in question is virulent on that genotype and is given a value of 1. In contrast, a resistant host response indicates avirulence of the isolate and is given a value of 0. Each of these binary values is subsequently converted to a decenary value using the conversion factors of 2^0 , 2^1 , and 2^2 for the first, second, and third genotype in the

differential set, respectively (Valjavec-Gratian and Steffenson 1997). Three barley differential lines (ND 5883, Bowman, and ND B112), set in this specific order, were used to designate pathotypes of *C. sativus* in Minnesota and North Dakota. For example, an isolate with virulence for ND 5883 and avirulence for Bowman and ND B112 would be designated as pathotype 1 according to the following:

$$1) \text{ ND 5883: } (1 \times 2^0=1)$$

$$2) \text{ Bowman: } (0 \times 2^1=0)$$

$$3) \text{ ND B112: } (0 \times 2^2=0)$$

$$\text{SUM} = 1$$

Similarly, an isolate with virulence for Bowman but avirulence for ND 5883 and ND B112 would be designated as pathotype 2, and an isolate with avirulence for all three differential lines would be designated pathotype 0. ND4008 has been reported to carry virulence for all three differentials, giving it a pathotype designation of 7; however, its virulence toward Bowman is variable. If ND4008 is indeed avirulent on Bowman, the correct pathotype designation would be 5. Finally, it should also be noted that research groups in Canada (Ghazvini and Tekauz 2007) have used other nomenclatural systems to assign *C. sativus* isolates to specific virulence groups.

Genetic resistance to plant disease

The classical view of plant-pathogen interactions is based on the work of Harold Flor who demonstrated that resistance to an invading pathogen is conferred by resistance (*R*) genes in the host that correspond to genes for avirulence (*Avr*) in the pathogen in a 'gene-for-gene' manner (Flor 1942). The importance of Flor's work is not to be understated; however, the simplicity and elegance of his results may be partially attributed to the pathosystem he used to conduct his work. Flor studied the flax-flax rust (*Linus usitatissimum-Melampsora lini*) pathosystem. As a biotroph, the flax rust pathogen requires living plant tissue to complete its life cycle. Therefore, a major host gene that acts as a switch to activate the hypersensitive response (HR) will be more effective at stopping the invading pathogen than the same host response to a necrotrophic pathogen. In addition, host resistance and pathogen avirulence proteins interact directly in the flax-flax rust system (Ellis et al. 2007). Genetic resistance to many plant pathogens is not controlled by single major genes, but instead by the inheritance of quantitative trait loci (QTL). The current understanding of these molecular interactions has become more complicated as new data have accumulated. Jones and Dangl (2006) proposed that host proteins encoded by *R* genes may sense the modification of other host proteins, rather than sensing the presence of a pathogen avirulence protein directly.

According to the model of Jones and Dangl (2006), plants initially sense the presence of a pathogen through broadly conserved molecules called pathogen-associated molecular patterns (PAMPs). Recognizing that many of these components are not unique to pathogens, but are also common among non-pathogenic microbes, the term microbe-associated molecular patterns (MAMPs) is also appropriate. Chitin, a major constituent of fungal cell walls, is a clear example of a PAMP since all fungi have chitin in their cell walls, and it is essential for their survival. Plants sense PAMPs such as chitin through pattern recognition receptors (PRRs) and activate an appropriate defense response known as PAMP-triggered immunity (PTI). For non- or weakly pathogenic microbes, this defense response is sufficient for arresting further colonization of the plant. For specialized pathogens, this is only the beginning. Pathogens commonly produce effectors in order to silence PTI, which is accomplished by masking the presence of the pathogen. Plants have evolved the capacity to recognize these effectors through proteins encoded by *R* genes.

Both pathogens that are the focus of this thesis (*C. sativus* and *F. graminearum*) are known to produce toxins that promote pathogenicity, meaning they can be called effectors under the broad definition of Kamoun (2007).

Genomic resources in barley

The ability to assign a specific genetic locus to a given phenotype depends on the ability to unequivocally associate a trait with a specific gene or region of a genome. The barley genome is very large at 5.1 Gb with approximately 80% consisting of repetitive sequences (IBGSC 2012). Therefore, the size and complexity of the barley genome must be reduced in order to be studied efficiently. One method of doing this is through the use of molecular markers, which are patterns of sequence variation that occupy a specific physical position in the genome and can be assigned a relative position (in centiMorgans or cM) based on recombination frequency in a segregating bi-parental population or an absolute position (in nucleotides) based on the location on a physical map, although the latter is not yet universally established for barley. Another method of complexity reduction is whole-genome sequencing (WGS), which has numerous applications, including RNAseq, which was used in Chapter 5 of this thesis.

Genotyping resources

Molecular markers have greatly advanced the study of plant genetics since most agronomically important traits are polygenic in nature and are not able to be studied in a traditional manner (Bernardo 2010). Restriction Fragment Length Polymorphism (RFLP) markers were the first to be utilized in QTL mapping (Botstein et al. 1980). Other types of genetic markers include Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeat (SSR), and Single Nucleotide Polymorphism (SNP)

markers. SNP markers were used to genotype the three advanced backcross populations (Tanksley and Nelson 1996, Matus et al. 2003) that are the focus of Chapters 2 and 3 of this thesis. SNP markers became the genotypic marker system of choice with the release of the genotyping array featuring SNPs developed by Close et al. (2009). This array was the first high-density SNP array available for barley. Briefly, two Barley Oligo Pool Arrays (BOPA 1 and BOPA2) comprised of 1,536 SNPs, each derived from expressed sequence tags (ESTs) and sequenced amplicons from cultivated barley, were generated (Close et al. 2009). ESTs are short sequences (300-600 bp) derived from cDNA; thus, they are a valuable resource for studying genes that are expressed under a variety of conditions (Ullrich 2010). As of April 2016, there are 858,963 barley ESTs in GenBank (<http://www.ncbi.nlm.nih.gov/>). The barley iSelect chip includes 2,832 SNPs from the existing BOPAs as well as 5,010 new SNPs that were discovered through next-generation sequencing (NGS) data by Comadran et al. (2012) for a total of 7,842 SNP markers. A comprehensive review of how these SNPs were generated is beyond the scope of this review; however, a detailed description of how the SNPs were selected may be found online at bioinf.hutton.ac.uk/iselect/app/. Next-generation sequencing technologies have also led to the widespread adoption of genotype-by-sequencing (GBS), for which SNP calls are made based on population-specific genetic variants, rather than through hybridization with sequences on an array (Nielsen et al. 2011).

Quantitative trait locus/loci (QTL) mapping

The earliest recorded genetic research was performed by Gregor Mendel in the middle of the 19th century using pea plants. He demonstrated that certain traits, such as pea and peapod shape and color, are controlled by discrete factors that are inherited from each parent and that the expression of a particular trait depended on which phenotype is 'dominant' or 'recessive'. Mendel also demonstrated that different traits are controlled by different factors that are inherited independently (Mendel 1866). Many agriculturally important traits, including disease resistance, are controlled by multiple genetic loci of small effect that do not follow regular Mendelian segregation ratios. Sax (1923) was the first to associate a quantitative trait (seed size) with phenotypic markers (seed color and pattern). The full potential of QTL mapping was not realized at the time because of the limited number of markers and the lack of powerful statistical techniques necessary for analyzing most agronomic traits. Still, Sax (1923) laid the foundation for the future of QTL analysis. The principle idea behind QTL mapping is that trait means for each marker class can be compared to each other using statistical tests with the null hypothesis (H_0) of these statistical tests being that the examined marker is not linked to a QTL. Several different methods of QTL analysis exist, but three basic methods are: single marker analysis, simple interval mapping, and composite interval mapping. The next few paragraphs will describe the benefits and drawbacks to each method, beginning with a brief description of how genetic linkage is calculated.

There are two common methods for determining genetic linkage: the Haldane function and the Kosambi function. For both mapping functions, d represents the distance between markers and r represents the recombination fraction. The Haldane function was the first to be published and accounts for double cross-over events between loci. The equation for calculating genetic distance using the Haldane function is:

$$d = -\left(\frac{1}{2}\right) \ln(1 - 2r)$$

The Kosambi function is an improvement upon the Haldane function because it accounts for cross-over interference and is calculated using the following equation:

$$d = -\left(\frac{1}{4}\right) \ln\left[\frac{(1 + 2r)}{(1 - 2r)}\right]$$

Single marker analysis is the simplest method of QTL analysis, and basic statistical software can be used to perform the analysis. Another advantage is that a complete linkage map is not required since only a single marker is considered at a time. The t -test can be used for single marker analysis, but so can an F -test combined with Analysis of Variance (ANOVA) or linear regression. The latter two are particularly useful if also considering heterozygous loci in addition to the two homozygous marker classes (Bernardo 2010). However,

single marker analysis is also the least powerful method for QTL detection. This is because as the distance between a marker and a QTL increases, the likelihood that a recombination occurred between them increases leading to incorrect estimations of QTL effects. In addition, because statistical tests are conducted independently for each marker, even if a genetic map is used it is not possible to definitively state whether neighboring markers are detecting a single QTL or multiple linked QTL.

Lander and Botstein (1989) proposed interval mapping as an improved method of QTL analysis. This approach uses one marker interval at a time and conducts a likelihood ratio test at every position in that interval. One option for determining the likely position of a QTL is to use a maximum likelihood algorithm.

Alternatively, regression analysis may also be used in interval mapping to determine the position of a QTL. Regression analysis calculates the residual sums of squares, and a QTL is declared where these are minimized. One advantage of using regression over maximum likelihood is that it is computationally simpler (Bernardo 2010). A drawback of interval mapping is that QTL identification can be biased when there is more than one QTL on a chromosome (Lander and Botstein 1989, Haley and Knott 1992, Zeng 1994).

An improvement to interval mapping came in 1993 and 1994 when Jansen (1993) and Zeng (1993, 1994) independently proposed what is now known as

composite interval mapping (CIM), a term coined by Zeng (1994). This approach combined interval mapping with multiple regression analysis. A subset of markers are used as co-factors in the regression analysis. Background variation due to the presence of other QTL is reduced, but not eliminated entirely. One weakness of CIM is that any potential QTL in an interval immediately adjacent to an interval containing a putative QTL will be missed (Bernardo 2010).

Draft reference genome

Another early genomic resource includes bacterial artificial chromosome (BAC) libraries such as the one created by Yu et al. (2000), which facilitated genomic studies such as the Sanger sequencing of target regions and positional cloning (Brueggeman et al. 2002, Halterman et al. 2001, Zhou et al. 2001). More recently, 6,287 BACs (5,341 gene-containing and 937 randomly selected) from that library were used to aid in the assembly of the 2012 draft assembly of the barley genome, which was released by a consortium of scientists led by the Leibniz Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK). Whole-genome shotgun sequencing (Illumina GAllx technology) was used to generate a *de novo* assembly of approximately 1.9 Gb. The draft genome has 376,261 contigs larger than 1 kb with an N50 of 1,425 bp (IBGSC 2012). In addition to this work, Muñoz-Amatriaín et al. (2015) sequenced 15,622 BACs, which are believed to contain two-thirds of all Morex barley genes. Release of this reference genome was critical to the success of the RNAseq experiment

described in Chapter 5. The availability of a reference genome allows for short reads to be aligned to the genome without the complex task of first constructing a *de novo* assembly from the RNAseq data (Wang et al. 2009). Many software programs have been developed to map short reads generated from next-generation sequencing to reference genomes. Short reads are typically about 100 bp in length, so scanning an entire genome for proper alignment would consume a large amount of computational resources. Bowtie (Langmead et al. 2009), a software tool for short read alignment, is used by another software tool TopHat2 (Kim et al. 2013), which was used in this thesis. The University of Minnesota Genomics Center (UMGC) utilizes Illumina technology (HiSeq2000 and HiSeq2500) for sequencing projects. The Illumina technology is described by Bentley et al. (2008). Briefly, forward and reverse primers are attached to a flow cell at a high density and DNA (or cDNA) samples are fragmented into segments of 100-300 bp before sequence adapters are ligated to both the 5' and 3' ends of the fragments. These adaptors allow the fragmented DNA to be physically attached to the flow cell through complementary binding of the adapters to the primers on the surface of the flow cell. Through solid-phase amplification, the immobilized DNA is amplified to millions of copies which form clusters approximately 1 μm in diameter. Fluorescently labeled nucleotides are incorporated during a sequencing-by-synthesis process. Signals generated by this incorporation are captured by charge-coupled device (CCD) cameras and base calls are measured during each cycle. The dye terminator and fluorescent

label is cleaved after each cycle. These cycles run independently for millions of clusters, resulting in very high level of throughput.

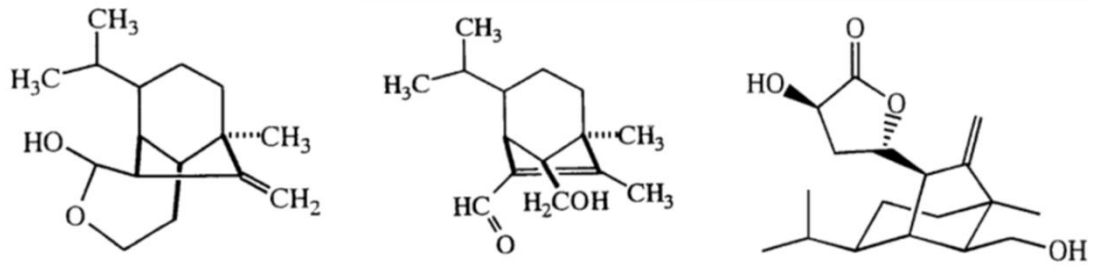


Figure 1.1. Three common toxins produced by the hemibiotrophic fungus *Cochliobolus sativus*. From left to right: prehelminthosporol, helminthosporol, sorokinianin. Source: Kumar et al. (2002).

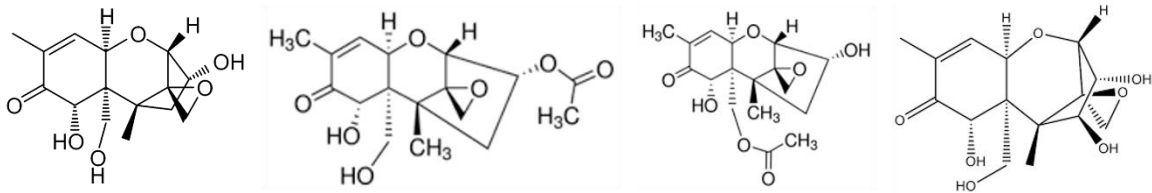


Figure 1.2. Type B trichothecenes toxins produced by *Fusarium graminearum*. From left to right: deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3ADON), 15-acetyl-deoxynivalenol (15-ADON), and nivalenol (NIV).

Chapter 2

Mapping QTL for Reduced Levels of Fusarium Head Blight Severity and Deoxynivalenol in Wild and Landrace Barley Accessions

Introduction

Fusarium head blight (FHB) is one of the most devastating diseases of barley (*Hordeum vulgare* ssp. *vulgare*) due to its significant impact on both yield and quality (Steffenson 2003). It is primarily caused by *Fusarium graminearum* Schwabe, but a number of other *Fusarium* species including *F. avenaceum* (Fr.:Fr.) Sacc., *F. sporotrichioides* Scherb., *F. poae* (Peck) Wollenweb., and others are also capable of causing the disease. In the Upper Midwest region of the United States, FHB re-emerged as a major concern in 1993 (McMullen et al. 1997, Windels 2000), but is of global consequence wherever the climate is moist during the growing season. Prior to 1993, sporadic epidemics of FHB occurred during the 1930s and 1940s in this region (Steffenson 2003). FHB may be partially controlled by cultural practices that reduce pathogen inoculum surviving in crop residue (e.g. deep plowing, crop rotation, etc.), use of fungicides, and host resistance. Many growers have adopted no-till or reduced-till practices in order to prevent soil erosion, but this has contributed to greater survival of pathogen inoculum (Steffenson 2003). Studies in both wheat and barley show that fungicide application is only partially effective with the best chemistries reducing FHB severity by only 60% (Jones et al. 2000, Mesterházy et al. 2003, Wegulo et al. 2015). The partial efficacy of fungicides, combined with their additional cost and unpredictability with regards to optimal application time, mean that growers cannot rely solely on fungicide treatment to protect their barley crop. Moreover, the class of fungicides known as quinone inhibitors (QoI) can actually

increase DON concentrations in grain and should not be used (Wegulo et al. 2015). Host resistance is therefore the most economical and environmentally-friendly method of controlling FHB; however, since genetic resistance to FHB in barley is only partial, it should be considered as just one component of a broader FHB management strategy.

To reduce the overall impact of FHB as much as possible, all management strategies should be used in concert (Steffenson 2003). Plowing and crop rotation away from congenial hosts for the pathogens will decrease the amount of viable inoculum on the soil surface, application of fungicides will protect the plant from new infections as well as stop those already in progress, and planting of cultivars with the highest level of resistance possible will further mitigate the risk of a severe FHB outbreak. The combination of all of these practices should prevent epidemics on the scale of the 1993 event and reduce the chances for future disease outbreaks.

Resistance to FHB is rare in the *Hordeum* gene pool, and true immunity likely does not exist. Moreover, one major difficulty in resolving “true genetic resistance” against FHB in barley is that a number of agro-morphological traits (e.g. row type, plant height, heading date, spike density, and spike angle) are correlated with the level of FHB severity sustained by the crop in the field (Steffenson 2003). Therefore, it is extremely important to exploit and genetically

characterize any accessions that possess moderate FHB resistance. A number of studies focused on the mapping of FHB resistance QTL have been published on barley. The resistance sources used in these studies include: Chevron (CIho 111), a six-rowed landrace from Switzerland (Canci et al. 2004, de la Peña et al. 1999, Ma et al. 2000, Nduulu et al. 2007); CIho 4196, a two-rowed landrace from China (Horsley et al. 2006); Frederickson, a two-rowed accession from Japan (Mesfin et al. 2003); Gobernadora, a two-rowed accession developed in Mexico and widely cultivated in China (Zhu et al. 1999); Harbin, a two-rowed variety from China (Hori et al. 2006); Russia 6, a two-rowed accession from China (Hori et al. 2005); Zhedar 2, a two-rowed accession from China (Dahleen et al. 2003); Atahualpa, a two-rowed hull-less accession from Ecuador; and HOR211, a six-rowed hull-less accession from the Ukraine (Sallam 2005). Each of these sources was used to develop populations (i.e. F₂, doubled haploid, and recombinant inbred lines) for mapping FHB resistance and/or DON accumulation. Selection of resistant parents for mapping studies in the early phase of this research was based primarily on their geographical origin. In more recent studies, haplotype data for known genomic regions (“bins”) harboring FHB resistance QTL and comparisons of genetic similarity were used for the selection of resistant parents in mapping population development (Huang et al. 2013).

Collectively, the results of these mapping studies suggest that populations segregating for various agro-morphological traits can confound the clear

delineation of FHB resistance QTL. For example, FHB resistance QTL were only detected at the *Vrs1* (the gene controlling the fertility of spikelets) locus (Komatsuda et al. 2007) in bi-parental (Frederickson/Stander, Russia 6/H.E.S., Clho 4196/Foster; and Atahualpa/M81; Hori et al. 2005; Horsley et al. 2006; Mesfin et al. 2003; K. Smith *unpublished data*), tri-parental (Zhedar 2/ND9712//Foster; Dahleen et al. 2003), and association mapping panels (Ethiopian and Eritrean Barley Collection; Mamo and Steffenson 2015) segregating for row type. Similarly, FHB resistance QTL were only detected at the cleistogamy locus *cly1/Cly2* in populations segregating for that gene in chromosome 2H (Hori et al. 2005 Hori et al. 2006, Sato et al. 2008). Finally, both the Atahualpa/M81 and HOR211/Lacey populations were segregating for the hull-less locus *nud*. The studies featuring Atahualpa/M81 and HOR211/Lacey populations were the only studies where FHB resistance QTL have been mapped to this genomic position in chromosome 7H (Sallam 2005; K. Smith *unpublished data*). However, in one study (Nduulu et al. 2007), the major-effect heading date QTL in chromosome 2H bin 8 (*HD-qtl-2H-8*) was dissected in near-isogenic lines (NILs) derived from F₂- and F₄-derived segregating populations from a cross of the susceptible breeding line M69 and a BC₅ individual carrying the Chevron allele for delayed heading date in the *HD-qtl-2H-8* region. The results suggest tight linkage between delayed heading date and FHB resistance rather than pleiotropy.

Accessions of wild barley (*H. vulgare* ssp. *spontaneum*) and other diverse barley accessions are reported to carry partial resistance to FHB, but have not been utilized as parents in any mapping studies to date (Dahl et al. 2009, Tucker et al. 2009). A previous global screening effort of 23,255 barley accessions led to the identification of 78 accessions with moderate resistance to FHB (Huang et al. 2013). Three of these accessions (PI 466423, W-365, and Kutahya) were selected in order to exploit potentially novel QTL for FHB resistance. Their selection was based on three criteria: 1) lowest disease severity from multiple years and locations of evaluation in the field, 2) distinct haplotypes for genomic “bins” previously reported to harbor FHB resistance QTL, and 3) genetic uniqueness (based on unweighted pair group method with arithmetic mean (UPGMA)) from other previously used resistance sources (Huang et al. 2013). Mean FHB severities for each accession were calculated from five (PI 466423 and W-365) or six (Kutahya) field experiments and normalized relative to the severity of the six-rowed Minnesota malting cultivar Stander (Rasmusson et al. 1993), which is susceptible to FHB. Compared with Stander, which was assigned a severity of 100%, the mean FHB severities (%) were: 46.9 ± 10.9 , 53.2 ± 7.3 , and 53.6 ± 16.6 for PI 466423, W-365, and Kutahya, respectively (Huang et al. 2013). Since the actual severity (not normalized) of Stander typically ranges from 18-25% (*data not shown*), this means that PI 466423, W-365, and Kutahya all have average FHB severities of approximately 9-12%. PI 466423 and W-365 are wild barley (*Hordeum vulgare* ssp. *spontaneum*) accessions from Israel and Iraq,

respectively, and Kutahya (known as CN 43457 and PGR 16807 at Plant Gene Resources of Canada (PGRC) and CGN 460 at the Center for Genetic Resources at Wageningen University) is a landrace from Turkey. This study is unique because two out of the three donor parents are wild barley accessions and because the advanced backcross population structure was used, which allows for the simultaneous identification and introgression of resistance QTL in agronomically advanced germplasm. The QTL mapping results from three advanced backcross populations are presented here.

Materials and Methods

Plant materials

PI 466423 was donated by the United States Department of Agriculture-Agricultural Research Service, National Small Grains Collection (USDA-ARS NSGC) (Aberdeen, ID), W-365 by the N. I. Vavilov Research Institute of Plant Industry (VIR) (St. Petersburg, Russia), and Kutahya by the PGRC (Saskatoon, Canada). Kutahya was initially collected in Turkey by Louis J. M. van Soest of the Centre for Plant Breeding and Reproduction at Wageningen University in the Netherlands and subsequently donated to PGRC (J. Tucker *personal communication*). The recurrent parents were either Rasmusson (Smith et al. 2010) or Quest (Smith et al. 2013). Both are six-rowed Minnesota malting barley cultivars, but they differ in their general reaction to FHB. Rasmusson is considered moderately susceptible (MS) (Smith et al. 2010) with a mean FHB severity relative to Stander of 96.8 ± 16.6 (Huang et al. 2013). In contrast, Quest is

considered moderately resistant (MR). Quest was the first six-rowed malting barley specifically bred for FHB resistance in the Upper Midwest region (Smith et al. 2013). Quest (PI 663183) was not included in the study by Huang et al. (2013), so the same comparisons cannot be made to a standard susceptible across the same years of testing. In our nurseries, the mean FHB severity of Quest ranged from 4.67% in St. Paul 2013 (STP13) to 18.65% in Nanjing 2013 (NJG13) (Tables 2.2-2.3), compared to a typical range of 18-25% for Stander (*data not shown*).

Population Development

Three advanced backcross populations (Tanksley and Nelson 1996) were developed using the three resistance sources (PI 466423, W-365, and Kutahya) as donor parents and two different recurrent parents (Rasmusson and Quest). The three populations were; Rasmusson/PI 466423, Quest/W-365, and Quest/Kutahya. Methodology for population development was according to Matus et al. (2003). All crosses and single-seed descent increases were made in a greenhouse (18-25°C with a 16-hr photoperiod supplemented by sodium vapor lights emitting $>300 \mu\text{mol photons s}^{-1} \text{ m}^{-2}$) at the Plant Growth Facility on the Saint Paul campus of the University of Minnesota from 2005-2014. Plants were grown in square plastic pots (13.3 x 13.3 x 10.2 cm, l x w x h) filled with a 50:50 mixture of steam-sterilized field soil and Sunshine MVP potting mix (Sun Gro Horticulture, Quincy, MI). At planting, all pots were fertilized with Osmocote slow-

release fertilizer (14-14-14 N-P-K) as well as water-soluble Peters Dark Weather fertilizer (15-0-15) (Scott's Company, Marysville, OH). After these initial treatments, plants were fertilized every two weeks with another water-soluble formulation (20-20-20) (J.R. Peters Inc., Allentown, PA). At physiological maturity, spikes were harvested, dried in a forced air dryer for two days at 35°C and subsequently threshed and cleaned for the next generation advance and testing. In total, 244 BC₂F₄ progeny were generated for the Rasmusson/PI 466423 population; 379 BC₂F₅ progeny for the Quest/W-365 population, and 360 BC₂F₅ progeny for the Quest/Kutahya population.

DNA extraction, SNP genotyping, and data analysis

To obtain DNA for genotyping, lyophilized leaf tissue from the three populations and respective parents was ground to a fine powder using the Qiagen TissueLyser II (Qiagen, Valencia, CA) and 3 mm tungsten carbide beads (one bead per microtube). Then, genomic DNA was extracted using the Qiagen BioSprint 96 workstation and the BioSprint 96 DNA Plant Kit (Qiagen). Genotyping was performed using the Illumina Infinium assay at the USDA-ARS Biosciences Research Laboratory in Fargo, North Dakota. The Illumina genotyping array is capable of interrogating 7,842 SNPs within the barley genome simultaneously. Genotype calls for all SNP markers were visually inspected and validated using Illumina's GenomeStudio Genotyping Module v.2011. Of the 7,842 potential SNPs, 2,554 were polymorphic between PI

466423 and Rasmusson; 2,161 were polymorphic between W-365 and Quest; and 2,081 were polymorphic between Kutahya and Quest.

Genetic map construction

JoinMap 4.1[®] was used to create genetic linkage maps for QTL analysis. All polymorphic loci in each individual population were considered for map construction. Polymorphic markers were sorted into groups based on recombination frequency beginning with a frequency of 0.250 and ending with 0.050 in increments of -0.05. The maximum likelihood algorithm was used for ordering loci and calculating genetic distances for each linkage group. Markers that were uninformative or flagged in suspect linkages (defined as having a recombination frequency with another marker greater than 0.5) were removed from consideration. Barley chromosome designations (i.e. 1H to 7H) were assigned based on markers in common with the consensus map of Muñoz-Amatriaín et al. (2014).

Phenotypic evaluations of Advanced Backcross populations for Fusarium head blight

FHB evaluations were conducted at several locations from 2010-2014. The Rasmusson/PI 466423 population was screened at Hangzhou, China in 2011; the University of Minnesota Northwest Research and Outreach Center (NWROC) in Crookston, MN from 2010-2012; and the Minnesota Agricultural Experiment

Station (MAES) in St. Paul from 2010-2012. The Quest/Kutahya population was screened at the Agriculture and Agri-Food Canada (AAFC) station in Brandon, MB in 2012 and 2013; the Jiangsu Academy of Agricultural Sciences (JAAS) in Nanjing, China in 2013; the NWROC in 2013 and 2014; and the MAES in 2013 and 2014. The Quest/W-365 population was screened at JAAS in 2013; the NWROC in 2013 and 2014; and the MAES in 2013 and 2014. A Randomized Complete Block Design (RCBD) was used in all experiments except those in 2014. In the 2014 experiments, a Modified Augmented Design (MAD II) was used to account for spatial variation (Lin and Poushinsky 1985).

The grain spawn method of inoculation (Prom et al. 1997) was used at all locations, except for the St. Paul nurseries where the conidial spray method was used (Dill-Macky 2003). For the grain spawn method, maize (*Zea mays*) kernels were placed in a stainless steel pan and soaked in water overnight. Excess water was drained the following morning, and then the pans were covered with aluminum foil and autoclaved twice for 20 min at 121°C to sterilize the grain. After cooling, pieces of Potato Dextrose Agar (PDA) containing cultures of *F. graminearum* were placed on the sterile grain to produce infested maize kernels as inoculum for the field. Once the grain was inoculated with *F. graminearum*, it was stored in the dark for 14 d at 25°C before being dried at 32°C in burlap bags. After drying, the *F. graminearum*-infested grain was stored at room temperature until needed.

The conidial spray method was used for inoculation in the St. Paul nurseries. A composite of 35-50 isolates of *F. graminearum* collected from commercial fields across the state between 2005-2013 was used for inoculum. The composite of isolates used varied from year to year. Detailed information about the mixture of isolates used in the St. Paul nurseries may be found in Appendix A (B. Zargaran *personal communication*). The same information is not available for the other nurseries, however the isolates used in the Nanjing 2013 nursery were actually *F. asiaticum* (X. Zhang *personal communication*). In St. Paul, a macroconidial suspension of the isolates given in Appendix A was applied to plants using a CO₂-pressurized backpack sprayer. Entries were first inoculated as they headed, i.e. when 90% or more of the spikes had emerged from the boot. A second inoculation was performed 3 d after the first inoculation. The spore concentration was 100,000 macroconidia per liter and applied at a rate of 1 linear foot per second. This resulted in the application of approximately 40 ml of inoculum per row for each inoculation. Inoculations were conducted in the afternoon and followed by a misting period to provide an environment conducive to infection. An overhead irrigation system provided the misting period needed for infection and subsequent development by the pathogen.

At every nursery except AAFC, the kernel count method was employed to assess disease severity. For the kernel count method, 10 spikes from each row were

arbitrarily selected, and the number of symptomatic kernels was recorded. Symptomatic kernels were those with at least one-fourth of their surface showing the tan to brown lesions and/or watersoaking typical of FHB infection. The average number of symptomatic kernels in these 10 spikes was divided by the total number of kernels per spike (assessed on three samples within a row) and multiplied by 100 to derive a FHB severity. At the MAES, disease pressure was lower, so data were collected from 20 spikes instead of 10. The FHB assessment scale used at AAFC was as follows: a score of 0 denotes no infection; 1 denotes low incidence (up to 5% of spikes infected) and low severity (1 or 2 kernels per spike, up to 7% of the spike); 2 indicates low to moderate incidence (5-15% of spikes infected) and low to moderate severity (1 to 4 kernels per spike, up to 15% of the spike); 3 denotes moderate incidence (15-30% of spikes infected) and moderate severity (2-8 kernels per spike, 15-30% of the spike); 4 denotes moderate to high incidence (30-50% of spikes infected) and moderate to high severity (4-12 kernels per spike, up to 40% of the spike); and 5 denotes high incidence (50% or more of spikes affected) and high severity (5 to 15+ kernels per spike, up to 50%+ of the spike). In nurseries where the grain spawn method was used, assessments of FHB severity were made at the soft-dough stage of development. Late-heading progeny were assessed twice during the growing season. In St. Paul, severity assessments were made 18-21 d after the first inoculation (soft dough stage). Terminal disease severities were used for all QTL analyses.

Deoxynivalenol concentration

At maturity, spikes from each plot were harvested for subsequent deoxynivalenol (DON) assays in the grain. Harvested spike samples were dried in a forced air dryer at 35°C and then threshed and cleaned. Ten gram samples of seed from each plot were assayed for DON concentration measured in parts per million (ppm) using the gas chromatography assay described by Tacke and Casper (1996). DON concentration was not determined for the Hangzhou and Nanjing nurseries.

Agronomic and morphological traits

Traits such as row type (RT), heading date (HD), plant height (HT), spike density (SD), and spike angle (SA) are all believed to have possible pleiotropic effects on the amount of FHB infection observed on barley. Therefore, data were also collected for these traits. RT was assessed as the number of rows of fertile spikelets in a spike. Since RT can have such a marked effect on FHB severity, only six-rowed lines in each population were considered for analysis. HD was recorded as the number of days after planting that 50% of the plants in a given row had 50% or more of the spikes emerged from the boot. HT was measured as the distance (in cm) from the base of the plant at the ground to the tip of the spike, excluding awns. SD was calculated by counting the number of rows with fertile spikelets in a spike and dividing by the length of the spike (excluding awns) in cm. SA was measured at maturity on a 1 to 3 scale. A score of 1 denotes

spikes that deviate from the vertical by less than 45°; a score of 2 denotes spikes that deviate from the vertical by 45 to 90°; and a score of 3 denotes spikes that deviate from the vertical by more than 90°.

Statistical analysis of traits

Pearson correlation coefficients were calculated using Microsoft Excel® in order to assess correlation among traits. Statistical analysis was conducted using SAS (v.9.3, SAS Institute 2011). Broad-sense heritability was determined using the PROC IML procedure which uses the formula $H^2 = \text{Var}(G) / \text{Var}(P)$ where $\text{Var}(G)$ is the genotypic variance and $\text{Var}(P)$ is the phenotypic variance (Holland et al. 2003). St. Paul and Brandon nurseries were not replicated and therefore different years were considered as replicates. The same could not be done for the Chinese nurseries since in the Hangzhou and Nanjing nurseries the experiments were not replicated and not repeated over multiple years. The Crookston nurseries were replicated, so heritability was calculated using both replicates from each year separately. In order to test for significant variation in distribution of traits in each population, Analysis of Variance (ANOVA) was conducted using the PROC GLM procedure in SAS (v.9.3, SAS Institute 2011),

QTL analysis

QGene (Joehanes and Nelson 2008) was selected for QTL analysis because it is capable of handling the unbalanced population structure of advanced backcross

populations. The composite interval mapping (CIM) maximum likelihood estimator algorithm of Lander and Botstein (1989) was used for all QTL analyses. Each population was segregating for row-type, which is known to markedly affect FHB severity; therefore, QTL analysis was conducted only on the six-rowed subset of each population as this was the spike type preferred by end users at the time. Second-pass scanning was performed using heading date and plant height as a covariate in the QTL analyses to account for the effect of heading date and plant height on disease severity. QTL were named by the following convention: 1) the trait for which a QTL was identified (FHB, DON, HD, HT), 2) *qtl* for quantitative trait locus, 3) chromosome number (1H to 7H), and 4) the bin number from the consensus maps of Kleinhofs et al. (2005) and Ahmad Sallam (*unpublished*). Both maps are in agreement with each other in regards to marker order and genetic distance. Bin numbers for both maps were assigned based on the principle that a bin should represent 10 cM. Bin numbers were used to mark the position in the genome of identified QTL in order to facilitate comparisons with previous FHB QTL mapping studies. The bin numbers presented were estimated based on recombination distances (cM) and, where possible, common anchoring markers. Significant markers that correspond to these bin positions are given in Tables 2.4-2.6.

Results

Genetic map construction

The genetic map for the Rasmusson/PI 466423 population contains 1,083 (from 2,554) polymorphic SNP markers, spanning a genetic distance of 1,147 cM. Overall, the map was well-saturated with markers every 1-3 cM. However, there was one to several large gaps in each of the seven chromosomes as follows: 9 and 15 cM on chromosome 1H; 8, 14, and 15 cM on chromosome 2H; 7 and 19 cM on chromosome 3H, 7 cM on chromosome 4H; 8, 10, and 20 cM on chromosome 5H; 6 cM on chromosome 6H; and 7 cM on chromosome 7H. A graphical representation of the genetic map for the Rasmusson/PI 466423 population is given in Appendix B.

The genetic map for the Quest/W-365 population contains 1,710 (from 2,161) polymorphic markers and spans a genetic distance of 1,079 cM. This map was also well-saturated with markers spaced every 1-3 cM. There were several large gaps on almost every chromosome as follows: 10, 9, and 15 cM on chromosome 1H; 17 and 10 cM on chromosome 2H; 9 cM on chromosome 4H; 13 cM on chromosome 5H; and 9 cM on chromosome 7H. A graphical representation of the genetic map for the Quest/W-365 population is given in Appendix C.

The genetic map for the Quest/Kutahya population contains 607 (from 2,081) polymorphic markers and spans a genetic distance of 1,023 cM. As was the case with the other two populations, the Quest/Kutahya genetic map was well-saturated with markers spaced every 1-3 cM. There were several large gaps on

each of the chromosomes except 4H as follows: 10, 11, and 14 cM on chromosome 1H; 8, 11, 12, and 12 cM on chromosome 2H; 7, 7, and 9 cM on chromosome 3H; 8,10, and 10 cM on chromosome 5H; 9, 9 and 16 cM on chromosome 6H; and 11 and 16 cM on chromosome 7H. A graphical representation of the genetic map for the Quest/Kutahya population is available in Appendix D.

Correlation coefficients among traits for the Rasmusson/PI 466423, Quest/W-365, and Quest/Kutahya populations are given in Tables 2.1, 2.2, and 2.3. The traits of Fusarium head blight severity (FHB) and deoxynivalenol concentration (DON) were weakly to moderately positively correlated. FHB and DON were negatively correlated with heading date (HD) and plant height (HT). Summary statistics for the Rasmusson/PI 466423, Quest/W-365, and Quest/Kutahya populations are given in Tables 2.4, 2.5, and 2.6, respectively. These tables include parental means and standard deviations as well as population means and ranges for each trait. Trait values for the wild barley donor parents, PI 466423 and W-365, could not be determined in each environment due to the difficulties in growing these wild and unadapted accessions to the heading stage in the field. Data that are available for those parents come from a limited number of samples. Heritabilities were generally very low for FHB, ranging from 0 in the Rasmusson/PI 466423 population (STP 10, 11, and 12) to 0.38 in the Quest/W-365 population (CRK13). For DON, heritabilities were similarly low, ranging from

0 in the Quest/Kutahya population (BRD12 and 13) to 0.38 in the Quest/W-365 population (Tables 2.4-2.6). Heritabilities for well-characterized traits such as HD and HT were much higher than for FHB and Don, ranging from 0.23 to 0.89 (Tables 2.4-2.6).

FHB QTL

In the Rasmusson/PI 466423 population, two QTL were detected for reduced FHB severity. One was identified in chromosome 2H bin 4 (*FHB-qt1-2H-4*) in five of seven environments where the trait was measured (Fig. 2.1.1). The most significant marker identified in four of the five environments where this QTL was detected was SCRI_RS_182408, while BOPA2_12_10219 was detected in one of five environments (CRK11). Both markers are in close proximity to one another (Table 2.7, Fig. 2.1.1). The second QTL identified resides in chromosome 5H bin 8 (*FHB-qt1-5H-8*) and was detected in one of seven environments (QTL trace not shown). *FHB-qt1-2H-4* explained a minimum of 9% of the variation in CRK11 and a maximum of 38% in CRK10 (Table 2.7). Interestingly, the allelic effect (α) was inconsistent between environments. Rasmusson contributed the beneficial allele in three environments (CRK10, CRK11, and STP10), while PI 466423 contributed the beneficial allele in the other two (STP11 and STP12) environments (Table 2.7). *FHB-qt1-5H-8*, detected only in CRK12, explained 10% of the variation with Rasmusson contributing the beneficial allele. CRK12 was one of two environments where *FHB-qt1-2H-4* was not detected. The other one was HGZ11 (Table 2.7). Second-pass scanning did not reveal additional QTL.

Results for the Quest/W-365 population were similar to those of the Rasmusson/PI 466423 population. A QTL was again identified in the chromosome 2H bin 4 region and was detected in three out of five environments where the trait was measured (Fig. 2.2.1). Several significant markers were detected across environments in the Rasmusson/PI 466423 population. SCRI_RS_207144 and BOPA2_12_30145 were each the most significant marker found in one of four environments, while SCRI_RS_207399 was significant in two of four environments, however these are all neighboring markers (Table 2.8 Fig. 2.2.1). In this population, *FHB-qtl-2H-4* explained a minimum of 8% of the variation in STP13 and a maximum of 28% in CRK13 (Table 2.8). The favorable allele was contributed by Quest in all environments. In one out of five environments (STP13), a QTL (*FHB-qtl-2H-2*) for reduced FHB severity was detected in the chromosome 2H bin 2 region that explained 8% of the variation. Finally, a single QTL (*FHB-qtl-4H-4*) was detected in the bin 4 region of chromosome 4H in NJG13 that explained 8% of the variation with the beneficial allele contributed by Quest. The QTL trace of chromosome 2H is also shown for comparison (Table 2.8, Fig. 2.2.2). Second-pass scanning did not yield additional QTL.

The results for the Quest/Kutahya population were markedly different than the Rasmusson/PI 466423 and Quest/W-365 populations, which both have wild

barley donor parents. The most noticeable difference was the lack of a major effect QTL at or near *FHB-qtl-2H-4* in the Quest/Kutahya population (*data not shown*). Instead, only two small effect QTL were detected, each in a single environment. One QTL (*FHB-qtl-1H-4*) was identified in chromosome 1H bin 4 in BRD13 explaining 9% of the variation. The second QTL (*FHB-qtl-6H-10*) was detected in bin 10 of chromosome 6H and explained 9% of the variation. Both QTL were contributed by Quest (Table 2.9).

DON QTL

A single QTL (*DON-qtl-2H-4*) for DON accumulation was detected in the Rasmusson/PI 466423 population that was coincident with *FHB-qtl-2H-4*. It was detected in five of six environments where DON was assayed (Fig. 2.1.2). In four of these five environments, the most significant marker was SCRI_RS_182408, while BOPA2_10715 was significant in one of five environments (CRK10). This QTL explained a minimum of 15% of the variation in STP10 and a maximum of 26% in CRK11. In all cases, the favorable allele was contributed by PI 466423 (Table 2.7).

In the Quest/W-365 population, a QTL coincident with *DON-qtl-2H-4* was again detected in all four environments where the trait was measured, explaining a minimum of 6% of the variation in CRK13 and a maximum of 62% in STP14. (Table 2.8). The allelic effect was inconsistent with the favorable allele coming

from Quest in three out of four environments, while W-365 provided the favorable allele in CRK14 (Table 2.8).

In the Kutahya/Quest population, a single QTL (*DON-qt1-2H-4*) for reduced DON accumulation was identified in BRD13, explaining 7% of the variation with the beneficial allele contributed by Quest (Table 2.9).

HEIGHT QTL

In the Rasmusson/PI 466423 population, a single QTL for plant height was detected in the bin 4 region of chromosome 2H (*HT-qt1-2H-4*) in six out of six environments where the trait was measured. In all environments, SCRI_RS_182408 was the most significant marker identified (Table 2.7, Fig. 2.1.3). This QTL explained from 13% of the variation in STP11 to as much as 38% in CRK11. In all cases, Rasmusson contributed the allele for increased plant height (Table 2.7). As in the Rasmusson/PI 466423 population, plant height was controlled by a single QTL (*HT-qt1-2H-4*) in the bin 4 region of chromosome 2H in the Quest/W-365 population. In all environments, the most significant marker identified was BOPA2_12_30145 (Table 2.8). *HT-qt1-2H-4* was detected in four out of four environments where the trait was measured (Fig. 2.2.4), explaining from 9% of the variation in STP13 to as much as 34% in CRK14 (Table 2.8).

In the Quest/Kutahya population, two primary QTL were detected for plant height: one in chromosome 3H and the other in chromosome 6H. The exact position of the chromosome 3H QTL varied among environments, but the most frequently detected marker was BOPA1_11657-398 (Table 2.9). This QTL mapped to bin 5 in BRD12, bin 6 in STP13, and bin 9 in three other environments. It was not detected in CRK13 (Fig. 2.3.1). These QTL were designated as *HT-qt1-3H-5-6* and *HT-qt1-3H-9*, respectively. *HT-qt1-3H-5-6* explained 5% and 6% of the variation in BRD12 and STP13, respectively. *HT-qt1-3H-9* explained 6% of the variation in STP14 and CRK14, but 17% of the variation in BRD13 (Table 2.9). *HT-qt1-6H-6* was detected in five of six environments where the trait was measured (all except CRK13), explaining from 5% of the variation in STP13 to 13% in CRK14. The most significant marker in each case was SCRI_RS_208399 (Table 2.9, Fig. 2.3.2).

HEADING DATE QTL

The primary determinant of heading date in the Rasmusson/PI 466423 population was found in the bin 4 region of chromosome 2H (*HD-qt1-2H-4*). This QTL was detected in six out of six environments for which data were collected (Fig. 2.1.4), explaining from 39% (CRK10) to 64% (STP11 and CRK11) of the variation across environments. The most significant marker identified was SCRI_RS_182408, and the Rasmusson allele consistently increased the days until heading (Table 2.7).

HD-qt1-2H-4, identified the Rasmusson/PI 466423 population, was also detected in the Quest/W-365 population in four out of four environments where data were collected. In all environments, BOPA2_12_30145 was the most significant marker identified (Table 2.8, Fig. 2.2.5), explaining from 54% (STP13) to 66% (CRK13) of the variation across environments (Table 2.8). In all cases, the Quest allele increased the days until heading (Table 2.8).

In the Quest/Kutahya population, two heading date QTL were detected: one residing in chromosome 2H and the other in 7H. The chromosome 2H QTL (*HD-qt1-2H-4*) was detected in three of six environments where the trait was measured. The most significant marker identified was SCRI_RS_191136 (Table 2.9, Fig. 2.3.3). Traces are not shown for BRD12 or BRD13 since no QTL were detected in either environment. *HD-qt1-2H-4* explained a minimum of 8% of the variation in STP14 and a maximum of 13% in CRK14 (Table 2.9). The chromosome 7H QTL (*HD-qt1-7H-SCRI_RS_193044*) was also detected in three out of six environments where the trait was measured (Table 2.9, Fig. 2.3.4). Traces are not shown for BRD12 or BRD13 since no QTL were detected in either environment. *HD-qt1-7H-SCRI_RS_193044* explained a minimum of 22% of the variation in CRK14 to a maximum of 32% in CRK13 (Table 2.9). With respect to *HD-qt1-2H-4*, the Kutahya allele increased the days until heading, whereas with *HD-qt1-7H-SCRI_RS_193044* the Quest allele increased the days until heading.

Discussion

The three donor parents used in this research (PI 466423, W-365, and Kutahya) were selected from more than 23,000 *Hordeum* accessions evaluated for FHB resistance over the past 15 years. These accessions exhibited among the lowest FHB severities within the screened germplasm and possessed different alleles at one or more previously identified QTL for resistance (Huang et al. 2013). To simultaneously identify resistance QTL and advance them into more agronomically suitable parental material for the breeding program, we utilized the advanced backcross QTL method (Tanksley and Nelson 1996) with the recurrent parents of Rasmusson and Quest (Smith et al. 2013). Multiple QTL were detected for reduced FHB severity and DON accumulation in each of the three populations under study, but other than *DON-qt1-2H-4* which explained 62% of the variation in the Quest/W-365 population (STP14), none of the others explained more than 38% of the variation nor were detected in all of the environments. *FHB-qt1-2H-4* was detected most frequently in five of seven environments in Rasmusson/PI 466423, four of five environments in Quest/W-365, and none in Quest/Kutahya). To be useful in breeding, a QTL should explain a significant amount of the variation for a trait and be consistently detected in multiple environments. Further, a majority of the FHB resistance QTL identified in this study were contributed by the recurrent parent, Rasmusson or Quest (Tables 2.7-2.9). This is not completely surprising since Quest is the first Midwestern six-rowed barley released that was bred for FHB resistance (Smith et al. 2013).

Rasmusson, although considered moderately susceptible to FHB, may carry some alleles for FHB resistance since it is a product of a long-term breeding project that sought to reduce the discoloration of kernels caused by an array of different fungi, including *Fusarium* species. As for the donor parents, the resistance QTL contributed by wild barley accession PI 466423 was detected in two environments (STP11 and STP12). Interestingly, this same resistance QTL associated with SNP marker SCRI_RS_182408 was also detected in STP10 and CRK10, but in these cases the positive allele was contributed by the recurrent parent of Rasmusson. Variable allelic effects have been previously reported (Dahleen et al. 2003, Mesfin et al. 2003, Zhu et al. 1999). The basis for this phenomenon is not known, but may be a result of a trait that is not under direct selection in nature. The most frequently detected QTL for reduced FHB severity (*FHB-qt1-2H-SCRI_RS_182408*) that also explains the greatest amount of the variation for DON accumulation (*DON-qt1-2H-SCRI_RS_182408*) is in the bin 4 region of chromosome 2H (broadly, *FHB-qt1-2H-4* and *DON-qt1-2H-4*). Both *FHB-qt1-2H-4* and *DON-qt1-2H-4* were coincident with each other having the same significant SNP marker association (SCRI_RS_182408). Moreover, major effect QTL for heading date and plant height (*HD-qt1-2H-SCRI_RS_182408* and *HT-qt1-2H-182408* or, more broadly, *HD-qt1-2H-4* and *HT-qt1-2H-4*) were also identified at this same coincident locus, suggesting that the reduced disease and mycotoxin levels may be a pleiotropic effect of increased plant height and delayed heading date. In general, tall and late heading plants often have lower

FHB and DON levels than short and early heading plants under Midwest conditions, although exceptions to this trend do occur (Steffenson 2003). Since inoculations at St. Paul are done by applying a prescribed amount of inoculum directly onto spikes at the heading stage, one might expect this to negate pleiotropic effects of increased height and/or delayed heading. However, there are likely other factors that may result in tall plants having less disease than short plants. For example, tall plants, due to their greater exposure to sunlight and wind, may retain dew and moisture for shorter periods than short plants. Since FHB pathogens require moist conditions for infection, this could be one possible explanation for lower disease on tall plants. There is a major gene (*Ppd-H1*) controlling heading time in this region of chromosome 2H. It is expressed only under long-day conditions as would be the case under the spring sowing conditions in the Midwest (Turner et al. 2005). The coincident QTL for *FHB-qtl-2H-4*, *DON-qtl-2H-4*, *HD-qtl-2H-4*, and *HT-qtl-2H-4* at SCRI_RS_182408 (in the Rasmusson/PI 466423 population) or BOPA2_12_30145 (in the Quest/W-365 population) were only detected in the two populations with a wild barley donor parent and not in the Quest/Kutahya population. Most, if not all, wild barleys have a facultative growth type and would therefore be segregating for photoperiod sensitivity (spring type habit) from the Minnesota recurrent parents. This is apparently not the case for the Quest/Kutahya population because the landrace Kutahya is a spring type like cultivar Quest and heads within three days of it; however, in most environments (4 of 6), Kutahya actually heads within two days.

The fact that the heading date and plant height QTL were possibly conferred by *Ppd-H1* and were only found for the populations segregating for these traits is further evidence to support the contention that *FHB-qtl-2H-4* and *DON-qtl-2H-4* are actually pleiotropic effects of photoperiod response. Growth habit is determined by *Ppd-H1* plus several other genes. These other genes include vernalization-responsive genes (*VRN-H1*, *VRN-H2*, and *VRN-H3*), *CONSTANS*-like genes (*HvCO*) that promote flowering under long-day conditions in *Arabidopsis* by activating the *FLOWERING LOCUS T* genes (*HvFT*), and *GIGANTEA*-like genes (*HvGI*) that act upstream of *CO* genes (Wang et al. 2010). Interestingly, the effect of the QTL in the *Ppd-H1* region was not detected in either the Rasmusson/PI 466423 or Quest/W-365 population when they were grown in Hangzhou, China (2011) or Nanjing, China (2013), respectively. These two cities reside at much lower latitudes (30° N and 32° N, respectively) than St. Paul, MN (45° N), Crookston, MN (48° N), or Brandon, MB (50° N). Day length at these lower latitudes varies less throughout the year than those in Minnesota or Manitoba. The nurseries in China were planted in the late fall (November or December) with disease scoring in the spring (May). The day length in the Chinese nurseries is likely not sufficient for the expression of *Ppd-H1*, thereby eliminating the differences in plant height and heading date. Such an effect on FHB severity has not previously been reported for *Ppd-H1*. But *Ppd-H1* has been shown to affect traits (i.e., yield) relating to flowering (Wang et al. 2010) and FHB

severity fits into this category. A QTL in the interval ABG008-HVM36 has been reported at this locus in the Frederickson/Stander population, although it was only detected in a greenhouse environment (Mesfin et al. 2003). The possibility that *Ppd-H1* affects FHB severity is not entirely surprising. A survey of previous FHB mapping studies revealed that most of the previously detected QTL are coincident with major genes or large-effect QTL for agro-morphological traits that are segregating in the population of interest including *Vrs1*, the primary determinant of row type; *INTERMEDIUM-C (Int-c)*, which controls lateral floret size; the *cly1/Cly2* locus, which controls cleistogamy in the floret, and *nud*, which controls the presence or absence of the hull. Even though data collectively suggest that *Ppd-H1* is responsible for the observed reduction in FHB severity and DON accumulation in the Rasmusson/PI 466423 and Quest/W-365 populations, the low disease pressure observed in Hangzhou (2011) could also explain the lack of QTL detection in Rasmusson/PI 466423 population rather than the absence of *Ppd-H1* expression. In addition, *FHB-qt1-2H-4* was not detected in CRK12, even though both *HD-qt1-2H-4* and *HT-qt1-2H-4* were detected in this environment (Fig. 2.1.1). QTL × environment interactions are commonly reported for FHB (Dahleen et al. 2003, Horsley et al. 2006, Ma et al. 2000) and may explain these observations. Finally, the absence of detection of QTL in the Quest/Kutahya population could be attributed to the absence of significant variation for FHB severity and DON accumulation as shown by non-

significant *P*-values (Table 2.6). If there is no difference in phenotypes among progeny, then the power to conduct QTL analysis is diminished.

The results of these experiments reaffirm how difficult it has been to obtain useful levels of FHB resistance in barley. In fact, the cultivar ‘Quest’ was named as such because it was a *quest* to achieve even the moderate level of FHB resistance it possesses (K. Smith *personal communication*). Other potentially useful QTL for FHB resistance likely exist in unadapted *Hordeum* accessions. Extensive research has been done to identify new sources of FHB “resistance” and to map the underlying QTL in order to pyramid them into agronomically advanced lines. One lesson to take from the collective knowledge generated by FHB research on barley over the past two decades is that we can keep trying to identify new sources of FHB resistance, but we may never recover genetic resistance to the disease that will have the same success in ameliorating losses as the NDB112-derived Midwest Six-rowed Durable Resistance Haplotype (MSDRH) for spot blotch and durable resistance gene *Rpg1* for stem rust (*Puccinia graminis* f. sp. *tritici*). In wheat, a major effect QTL for FHB resistance (*Fhb1*) was identified on chromosome 3BS from the breeding line Sumai 3. When the center floret of susceptible wheat is inoculated with *F. graminearum*, the infection can spread upward and downward killing the entire spike. *Fhb1* acts to reduce spread in the spike—the so called Type II resistance--leading to disease reductions from 32.0 to 43.3% depending on the genetic background of

the breeding line (Xie et al. 2007). Barley is unique in that it appears to have an innate level of Type II resistance. Thus, the large gains made in wheat for FHB resistance have come from augmenting levels of Type II resistance. In both crops, efforts are now directed at augmenting the level of resistance to initial infection, the so called Type I resistance. This may prove to be a more difficult undertaking given the experiences learned from barley.

Genomic selection (GS) may offer the greatest chance for significantly reducing FHB severity and DON accumulation, while still maintaining all of the QTL needed to develop a high yielding malting barley with an acceptable quality profile since GS takes into account the effect of all markers across the genome. Still, since no immunity exists for the disease (in wheat or barley), the best possible host resistance must also be combined with cultural practices that would reduce pathogen inoculum on the surface and treatments of the most effective fungicides (Wegulo et al. 2015) applied with best technology (Hofman et al. 2000) timed to coincide with the greatest risk of a FHB epidemic, according to disease forecasting models (Prandini et al. 2009).

Table 2.1. Pearson correlation coefficients among traits from the Rasmusson/PI 466423 mapping population.

		FHB						DON						HD						HT						
		HZO11	CRK10	CRK11	CRK12	STP10	STP11	STP12	CRK10	CRK11	CRK12	STP10	STP11	STP12	CRK10	CRK11	CRK12	STP10	STP11	STP12	CRK10	CRK11	CRK12	STP10	STP11	STP12
FHB	HZO11	0.062	0.003	0.119*	0.167*	0.171**	0.061	0.140*	0.152**	0.202*	0.005	0.157**	0.088	0.128	0.123	0.053	0.086	0.095	0.063	-0.072	-0.056	-0.006	-0.124	-0.046	-0.052	
	CRK10		0.228***	0.296***	0.452***	-0.128	-0.198**	-0.192**	-0.377**	-0.135**	-0.199***	-0.166**	-0.105	-0.474**	-0.561**	-0.579**	-0.439*	-0.513**	-0.459**	-0.575**	-0.533**	-0.518**	-0.361**	-0.320**	-0.263**	
	CRK11			0.084	0.094	-0.032	-0.093*	0.043	-0.248**	-0.117*	-0.080	-0.083*	-0.085	-0.151**	-0.342**	-0.334**	-0.164**	-0.210**	-0.251**	-0.239**	-0.1567*	-0.197**	-0.141**	-0.169**	-0.155**	
	CRK12				0.227***	0.075	0.059	-0.066	-0.112	0.147	0.027	-0.052	0.12	-0.25	-0.273**	-0.206**	-0.223**	-0.178**	-0.202**	-0.376**	-0.349**	-0.423**	-0.262**	-0.247**	-0.178**	
	STP10					0.014	-0.043	-0.109	-0.096	0.008	0.044	0.115	0.061	-0.084	-0.167**	-0.207**	-0.124*	-0.205**	-0.098	-0.382**	-0.413**	-0.401**	-0.446**	-0.251**	-0.287**	
	STP11						0.117	0.171*	0.362***	0.297***	0.191**	0.538***	0.073	0.294***	0.380***	0.302***	0.248***	0.489***	0.235***	0.067	0.116	0.08	0.075	-0.156**	0.079	
	STP12							0.390***	0.213***	0.277***	0.307***	0.188***	0.377***	0.306***	0.309***	0.314***	0.299***	0.293***	0.491***	0.102	0.028	0.125*	0.034	0.052	-0.159*	
DON	CRK10								0.369***	0.316***	0.295***	0.293***	0.188*	0.463***	0.400***	0.358***	0.340***	0.423***	0.364***	0.139*	0.171*	0.236**	0.104	0.069	0.087	
	CRK11									0.428***	0.304***	0.515***	0.116*	0.488***	0.729***	0.583***	0.449***	0.540***	0.645***	0.308***	0.381***	0.311***	0.231***	0.068	0.028	
	CRK12										0.275***	0.308***	0.278***	0.288***	0.398***	0.435***	0.322***	0.352***	0.370***	0.026	0.021	0.037	0.088	-0.039	-0.119	
	STP10											0.299***	0.263***	0.320***	0.348***	0.335***	0.298***	0.340***	0.313***	0.084	0.1	0.078	0.001	-0.001	-0.087	
	STP11												0.232***	0.379***	0.455***	0.364***	0.334***	0.512***	0.413***	0.076	0.141	0.145	0.055	0.019	-0.035	
	STP12													0.131	0.152	0.207	0.199	0.159	0.169	0.07	0.04	0.118*	0.015	0.127*	-0.041	
HD	CRK10															0.807***	0.674***	0.747***	0.614***	0.595***	0.328***	0.275***	0.350***	0.188**	0.004	0.166*
	CRK11															0.792***	0.754***	0.737***	0.745***	0.463***	0.453***	0.467***	0.297**	0.108	0.153*	
	CRK12															0.663***	0.645***	0.677***	0.48**	0.448***	0.458***	0.272**	0.168**	0.143*		
	STP10																0.578***	0.568***	0.350***	0.340***	0.382***	0.142*	0.076	0.12*		
	STP11																	0.585***	0.414***	0.424***	0.390***	0.301***	0.105*	0.162*		
	STP12																		0.396***	0.387***	0.370***	0.220***	0.135*	-0.176*		
HT	CRK10																				0.762***	0.698***	0.625***	0.558***	0.305***	
	CRK11																					0.699***	0.637***	0.537***	0.325***	
	CRK12																						0.509***	0.553***	0.361***	
	STP10																							0.417***	0.274***	
	STP11																								0.226**	
	STP12																									

Traits: FHB = Fusarium head blight; DON = deoxynivalenol; HD = heading date; HT = plant height
 Locations: HZO = Hangzhou, China; CRK = Crookston, MN; STP = St. Paul, MN
 * Significant at $p = 0.05$
 ** Significant at $p = 0.01$
 *** Significant at $p = 0.001$

Table 2.2. Pearson correlation coefficients among traits from the Quest/W-365 population.

		FHB				DON				HD				HT				
		STP13	STP14	NJG13	CRK13	CRK14	STP13	STP14	CRK13	CRK14	STP13	STP14	CRK13	CRK14	STP13	STP14	CRK13	CRK14
FHB	STP13	0.236***	0.184**	0.343***	0.291	0.383***	0.297***	0.259**	-0.055	-0.219**	-0.249**	-0.212**	-0.215**	-0.292**	-0.249**	-0.296**	-0.255**	
	STP14	0.168*	0.393***	0.252**	0.301***	0.542***	0.336***	-0.036	-0.402**	-0.466**	-0.375**	-0.387**	-0.380**	-0.259**	-0.342**	-0.371**		
	NJG13	0.119**	0.135**	0.266**	0.108*	0.169**	0.055	-0.136*	-0.100	-0.143*	-0.104	-0.191*	-0.174*	-0.128	-0.161*			
	CRK13	0.483***	0.411***	0.491***	0.558***	0.042	-0.386**	-0.432**	-0.375**	-0.415**	-0.500**	-0.537**	-0.604**	-0.552**				
	CRK14	0.312***	0.367***	0.439***	0.033	-0.343**	-0.339**	-0.345**	-0.397**	-0.300**	-0.392**	-0.473**	-0.453**					
DON	STP13	0.235**	0.517***	0.242**	-0.074	-0.116*	-0.037	-0.030	-0.536**	-0.471**	-0.458**	-0.476**						
	STP14	0.296**	-0.158*	-0.642**	-0.713**	-0.647**	-0.639**	-0.322**	-0.232**	-0.397**	-0.413**							
	CRK13	0.210*	-0.105	-0.156*	-0.080	-0.125	-0.525**	-0.478**	-0.508**	-0.457**								
	CRK14	0.189*	0.209*	0.269**	0.239**	-0.143	-0.164*	-0.076	0.016									
HD	STP13	0.818***	0.873***	0.821***	0.166*	0.161*	0.084	0.092										
	STP14	0.820***	0.806***	0.209**	0.177*	0.112	0.166*											
	CRK13	0.873***	0.280**	0.333**	0.223**	0.258**												
	CRK14	0.347***	0.370***	0.291**	0.319***													
HT	STP13	0.653***	0.640***	0.588***														
	STP14	0.628***	0.583***															
	CRK13	0.594***																
	CRK14																	

Traits: FHB = Fusarium head blight; DON = deoxynivalenol; HD = heading date; HT = plant height

Locations: NJG = Nanjing, China; CRK = Crookston, MN; STP = St. Paul, MN

* Significant at $p = 0.05$

** Significant at $p = 0.01$

*** Significant at $p = 0.001$

Table 2.3. Pearson correlation coefficients among traits from the Quest/Kutahya mapping population.

		FHB						DON						HD						HT						
		BRD12	BRD13	NJG13	STP13	STP14	CRK13	CRK14	BRD12	BRD13	STP13	STP14	CRK13	CRK14	BRD12	BRD13	STP13	STP14	CRK13	CRK14	BRD12	BRD13	STP13	STP14	CRK13	CRK14
FHB	BRD12		0.218*	0.214*	0.155*	0.102	0.227*	0.171*	0.358**	0.145*	0.149*	0.195*	0.145*	-0.039	-0.347**	-0.192*	-0.177*	-0.301**	-0.231*	-0.270**	-0.081	-0.338**	-0.095	-0.151*	-0.309*	-0.202*
	BRD13			0.092	0.055	-0.077	0.362***	0.374***	0.029	0.323***	0.079	0.126*	0.334***	0.043	-0.172*	-0.164*	-0.127*	-0.214**	-0.306***	-0.244**	-0.298**	-0.430***	-0.221**	-0.271**	-0.328***	-0.339***
	NJG13				-0.011	0.057	0.123*	0.082	0.250**	0.232**	0.073	0.109	0.199*	0.153*	-0.065	-0.173*	-0.005	-0.015	-0.111	-0.015	-0.170	-0.221**	-0.349***	-0.218**	-0.222**	-0.209**
	STP13					0.077	0.221*	0.181*	0.054	0.079	0.395***	0.063	-0.022	0.025	-0.196*	-0.122*	-0.261**	-0.215**	-0.158*	-0.266**	0.122*	-0.092	0.051	-0.052	-0.130*	-0.083
	STP14						0.021	-0.038	0.030	-0.043	-0.114	0.016	0.021	0.032	-0.024	0.042	-0.006	0.080	-0.037	0.099	-0.016	0.038	-0.085	0.106	-0.009	0.038
	CRK13							0.383***	0.095	0.169*	0.023	0.258**	0.456***	-0.005	-0.295**	-0.322***	-0.294**	-0.329***	-0.459***	-0.322***	-0.224**	-0.456***	-0.215**	-0.316***	-0.459***	-0.335***
CRK14								0.065	0.203**	0.190*	0.170*	0.296**	0.124	-0.054	-0.151	-0.086	-0.155	-0.145	-0.188	-0.250*	-0.347***	-0.177*	-0.207**	-0.370***	-0.422***	
DON	BRD12								0.071	0.148	-0.090	0.227**	0.161	-0.093	-0.030	0.118	0.066	0.071	-0.002	-0.049	-0.183	-0.045	-0.085	-0.175	-0.130	
	BRD13									0.173*	0.051	0.211*	0.153*	0.126	-0.020	0.033	0.012	0.045	0.010	-0.190*	-0.146*	-0.180*	-0.108	-0.091	-0.181	
	STP13										0.040	0.055	-0.043	-0.034	0.041	-0.231**	0.009	-0.030	-0.181*	-0.080	-0.172*	-0.199*	-0.191*	-0.139*	-0.177*	
	STP14											0.006	-0.070	-0.178	-0.230**	-0.547***	-0.580***	-0.534***	-0.464***	-0.086	-0.247**	-0.257**	-0.122	-0.262**	-0.166	
	CRK13												0.177*	-0.110	-0.198*	-0.008	-0.048	-0.248**	-0.105	-0.340***	-0.402***	-0.151	-0.199*	-0.403***	-0.294**	
CRK14													0.164	0.032	0.136	0.112	0.086	0.095	-0.125	-0.067	-0.002	-0.050	-0.077	-0.107		
HD	BRD12														0.416***	0.330***	0.448***	0.448***	0.458***	-0.222**	0.192**	0.143	0.114	0.247**	0.137	
	BRD13															0.314***	0.451***	0.567***	0.465***	-0.001	0.262**	0.158	0.142	0.287**	0.204**	
	STP13																0.588***	0.699***	0.673***	-0.010	0.337***	0.255**	0.252**	0.303**	0.162*	
	STP14																	0.693***	0.656***	-0.060	0.276**	0.173*	0.002	0.278**	0.155*	
	CRK13																		0.701***	0.018	0.389***	0.273**	0.258**	0.404***	0.223**	
CRK14																				-0.040	0.311***	0.185*	0.198*	0.348***	0.208**	
HT	BRD12																				0.443***	0.281**	0.320***	0.376***	0.419***	
	BRD13																					0.471***	0.481***	0.588***	0.582***	
	STP13																						0.355***	0.357***	0.356***	
	STP14																							0.359***	0.364***	
	CRK13																								0.454***	

Traits: FHB = Fusarium head blight; DON = deoxynivalenol; HD = heading date; HT = plant height
 Locations: NJG = Nanjing, China; BRD = Brandon, MB; CRK = Crookston, MN; STP = St. Paul, MN
 * Significant at $p = 0.05$
 ** Significant at $p = 0.01$
 *** Significant at $p = 0.001$

Table 2.4. Parental and population means plus standard deviations and population range for Fusarium head blight (FHB) severity, DON concentration, and related agronomic and morphological traits in the Rasmusson/PI 466423 advanced backcross population (six-rowed subset only).

Trait ^a	Environment ^b	PI 466423 ^c	Rasmusson	Population Mean	Population Range	H ²	P-value
FHB	HZO11	0.6±3.0	0.6±0.5	0.6±0.4	0.0-3.8	ND	ND
	STP10	ND	8.9±1.1	13.1±7.1	1.0-37.1	0	0.74
	STP11	ND	11.9±6.3	10.7±7.3	0.1-34.0	0	0.74
	STP12	ND	ND	16.5±9.7	2.3-58.1	0	0.74
	CRK10	ND	14.2±4.7	21.3±11.9	3.3-90.0	0.370	<0.0001
	CRK11	13.2±10.2	39.0±23.2	32.4±10.5	10.9-69.0	0.040	0.290
	CRK12	8.2±6.4	8.8±2.3	12.2±4.8	2.3-34.9	0.085	0.10
DON	STP10	ND	19.4±8.5	20.8±8.9	3.4-54.4	0.251	0.0008
	STP11	ND	13.0±5.1	8.5±6.2	0.3-29.3	0.251	0.0008
	STP12	ND	16.2±4.7	9.5±7.2	0.7-49.5	0.251	0.0008
	CRK10	3.0±2.7	23.2±7.1	15.1±8.1	1.0-56.9	ND	ND
	CRK11	28.5±13.6	36.5±8.2	34.1±15.0	3.7-83.0	0.55	<0.0001
	CRK12	ND	32.6±5.3	13.6±6.1	2.7-76.8	0.232	0.0005
HD	STP10	ND	58±1.7	55±3.7	41-69	0.523	0.04
	STP11	73±1.4	65±2.3	64±3.5	52-71	0.523	0.04
	STP12	ND	59±1.0	57±6.1	45-75	0.523	0.04
	CRK10	58±1.7	52±1.0	51±1.9	46-57	0.753	<0.0001
	CRK11	57±2.1	50±1.0	50±3.2	42-57	0.894	<0.0001
	CRK12	63±3.4	59±1.9	59±4.7	49-77	0.629	<0.0001
HT	STP10	ND	84.3±5.0	82.9±9.7	58.0-114.0	0.272	0.548
	STP11	ND	60.3±5.2	62.0±7.7	34.5-81.0	0.272	0.548
	STP12	ND	65.5±3.2	67.1±8.1	39.0-86.0	0.272	0.548
	CRK10	72.0±5.6	75.8±2.1	82.6±9.6	59.0-110.0	0.441	<0.0001
	CRK11	81.0±4.3	62.7±5.4	70.0±9.6	48.0-92.0	0.566	<0.0001
	CRK12	80.0±6.1	80.1±6.0	78.7±9.7	39.4-105.4	0.572	<0.0001
SD	STP10	ND	1.2±0.1	1.4±0.2	0.8-1.9	0.131	0.01
	STP11	ND	1.3±0.2	1.4±0.2	0.9-1.8	0.131	0.01
	STP12	ND	1.7±0.1	1.6±0.3	0.4-2.3	0.131	0.01
	CRK10	1.2±0.2	1.3±0.1	1.5±0.1	1.2-1.8	0.145	0.04
	CRK11	1.7±0.1	1.5±0.1	1.4±0.1	1.1-2.0	0.100	0.09
	CRK12	1.4±0.1	1.6±0.2	1.5±0.2	1.1-3.2	0.117	0.08
SA	STP10	ND	1±0.0	1±0.3	1-3	0.072	0.16
	STP12	ND	1±0.0	1±0.3	1-3	0.072	0.16
	CRK10	1±0.0	1±0.0	1±0.3	1-3	0.152	0.001
	CRK12	1±0.0	1±0.0	1±0.3	1-3	0.05	0.32

^a FHB=FHB severity (% of infected kernels), DON=deoxynivalenol (in ppm), HD=heading date (days from sowing to when 50% of spikes in a row emerged from the boot), HT=plant height (in cm), SD=spike density (# of nodes per cm rachis), SA=spike angle (1=completely erect spike, 3=nodding spike more than 90° from the vertical)

^b HZO=Hangzhou, China; STP=St. Paul, MN; CRK=Crookston, MN

^c Due to the difficulty in growing wild barley in the field at the Minnesota locations, data for PI 466423 were obtained from a limited number of samples; ND = no data

Table 2.5. Parental and population means plus standard deviations and population range for Fusarium head blight (FHB) severity, DON concentration, and related agronomic and morphological traits in the Quest/W-365 advanced backcross population (six-rowed subset only).

Trait ^a	Environment ^b	W-365 ^c	Quest	Population Mean	Population Range	H ²	P-value
FHB	NJG13	12.1±14.1	18.7±9.6	30.7±16.2	1.0-100.0	ND	ND
	STP13	1.8±1.3	4.7±3.9	7.6±5.1	0.2-32.1	0.168	<0.0001
	STP14	3.0±1.5	5.6±2.6	9.2±6.4	1.8-54.4	0.168	<0.0001
	CRK13	19.5±13.8	6.4±3.1	14.9±12.5	1.2-68.4	0.385	<0.0001
	CRK14	26.0±15.9	14.1±5.3	24.0±12.1	6.5-100.0	0.116	<0.0001
DON	STP13	2.0 ^d	3.3±2.4	6.5±4.8	0.1-33.8	0.191	<0.0001
	STP14	1.1±0.7	13.0±8.3	5.9±10.4	0.1-51.7	0.191	<0.0001
	CRK13	43.2±35.5	13.7±6.8	20.0±10.7	3.0-56.8	0.384	<0.0001
	CRK14	62.4±21.6	39.4±30.1	43.7±25.4	11.7-149.6	0.089	0.02
HD	STP13	50±2.8	54±2.1	54±4.3	40-60	0.670	<0.0001
	STP14	66±1.7	50±2.4	55±4.7	41-68	0.670	<0.0001
	CRK13	60±7.2	61±6.9	60±3.9	49-67	0.818	<0.0001
	CRK14	ND	50±1.9	53±4.2	39-60	0.814	<0.0001
HT	STP13	66.5±10.8	83.3±6.3	80.5±11.8	42.5-109.3	0.631	<0.0001
	STP14	63.3±14.3	70.0±4.4	66.8±12.8	32.5-96.0	0.631	<0.0001
	CRK13	74.5±11.2	84.4±10.2	79.5±15.7	40.5-108.6	0.752	<0.0001
	CRK14	92.9±11.2	86.2±7.4	86.7±14.4	41.5-113.0	0.907	<0.0001
SD	STP13	1.1±0.3	1.4±0.3	1.2±0.2	0.9-1.7	0.079	0.10
	STP14	1.3±0.3	1.3±0.3	1.3±0.2	1.0-1.9	0.079	0.10
	CRK13	1.2±0.2	1.3±0.3	1.4±0.1	1.0-2.1	0.219	<0.0001
	CRK14	1.4±0.3	1.4±0.3	1.4±0.1	0.7-2.1	0.165	0.002
SA	STP13	1±0.0	1±0.0	1±0.4	1-3	0.088	0.11
	STP14	1±0.0	1±0.0	1±0.3	1-3	0.088	0.11
	CRK13	1±0.0	1±0.0	1±0.3	1-2	0.116	0.02
	CRK14	1±0.0	1±0.0	1±0.4	1-3	0.116	0.02

^a FHB=FHB severity (% infected kernels), DON=deoxynivalenol (in ppm), HD=heading date (days from sowing to when 50% of spikes in a row emerged from the boot), HT=plant height (in cm), SD=spike density (# of nodes per cm rachis), SA=spike angle (1=completely erect spike, 3=nodding spike more than 90° from the vertical)

^b NJG=Nanjing, China; STP=St. Paul, MN; CRK=Crookston, MN

^c Due to the difficulty in growing wild barley in the field at the Minnesota locations, data for W-365 were obtained from a limited number of samples; ND = no data

^d No Standard Deviation available because there was only one sample

Table 2.6. Parental and population means plus standard deviations and population range for Fusarium head blight (FHB) severity, DON concentration, and related agronomic and morphological traits in the Quest/Kutahya advanced backcross population (six-rowed subset only).

Trait ^a	Environment ^b	Kutahya	Quest	Population Mean	Population Range	H ²	P-value
FHB	BRD12	1.1±0.4	2.8±0.8	3.4±1.2	1-5	0.077	0.08
	BRD13	2.2±0.6	3.3±0.9	4.0±1.0	1-5	0.077	0.08
	NJG13	11.7±3.5	18.7±9.6	14.4±7.4	0.3-40.6	ND	ND
	STP13	5.1±2.9	4.7±3.9	6.3±3.7	1.3-19.1	0.119	0.14
	STP14	12.2±6.5	5.6±2.6	7.5±3.5	1.0-25.3	0.119	0.14
	CRK13	6.4±3.5	6.4±3.1	9.4±4.8	0.3-27.8	0.107	0.04
	CRK14	13.5±3.1	14.1±5.3	18.7±5.8	5.1-48.1	0.109	0.03
	DON	BRD12	0.5±0.2	2.9±1.2	4.0±3.0	0.2-27.4	0
	BRD13	27.3±15.9	27.6±12.3	32.8±15.6	6.5-104.2	0	0.54
	STP13	2.4±1.3	3.3±2.4	4.4±2.9	0.1-19.0	0.030	0.10
	STP14	22.3±6.5	13.0±2.6	3.5±5.8	0.0-35.9	0.030	0.10
	CRK13	6.5±5.3	13.7±6.8	16.6±7.8	1.2-42.9	0.180	0.002
	CRK14	21.4±16.2	39.4±30.1	36.3±15.2	12.8-104.4	0.118	0.02
HD	BRD12	50±1.1	52±2.8	52±2.7	48-60	0.337	<0.0001
	BRD13	45±4.1	48±1.7	49±2.6	40-59	0.337	<0.0001
	STP13	52±1.4	54±2.1	54±2.6	48-59	0.565	<0.0001
	STP14	50±2.1	50±1.8	55±3.3	46-68	0.565	<0.0001
	CRK13	59±1.7	61±6.9	62±2.3	52-69	0.680	<0.0001
	CRK14	49±1.6	50±1.9	53±2.4	46-60	0.692	<0.0001
	HT	BRD12	80.6±3.9	72.0±3.6	74.9±5.8	55-95	0.349
	BRD13	116.8±7.5	99.6±2.1	104.2±6.7	86-127	0.349	<0.0001
	STP13	89.6±8.4	83.3±6.3	84.3±8.1	60-104	0.301	<0.0001
	STP14	78.0±4.3	70.0±4.4	69.1±8.6	39-94	0.301	<0.0001
	CRK13	89.7±10.1	84.4±10.2	85.9±6.6	49.5-101.5	0.237	<0.0001
	CRK14	103.7±11.5	86.2±7.4	93.5±6.4	71.5-108.3	0.274	<0.0001
SD	STP13	1.3±0.1	1.4±0.1	1.4±0.2	1.1-2.1	0.204	0.0006
	STP14	1.3±0.1	1.3±0.1	1.3±0.1	1.0-2.0	0.204	0.0006
	CRK13	1.3±0.2	1.3±0.1	1.4±0.1	1.1-1.7	0.104	0.03
	CRK14	1.4±0.1	1.4±0.1	1.3±0.1	1.0-1.7	0.201	0.0003
SA	STP13	3±0.0	1±0.0	1±0.3	1-3	0.242	<0.0001
	STP14	2±0.0	1±0.0	1±0.4	1-3	0.242	<0.0001
	CRK13	2±0.0	1±0.0	1±0.3	1-3	0.871	<0.0001
	CRK14	2±0.0	1±0.0	1±0.4	1-3	0.251	<0.0001

^a FHB=FHB severity (% of infected kernels except BRD which was scored on a 1-5 scale), DON=deoxynivalenol (in ppm), HD=heading date (days from sowing to when 50% of spikes in a row emerged from the boot), HT=plant height (in cm), SD=spike density (# of nodes per cm rachis), SA=spike angle (1=completely erect spike, 3=nodding spike more than 90° from the vertical)

^b BRD=Brandon, MB, Canada; NJG=Nanjing, China; STP=St. Paul, MN; CRK=Crookston, MN

Table 2.7. Summary of quantitative trait loci (QTL) identified for FHB severity, DON accumulation, and related agronomic traits in the Rasmusson/PI 466423 advanced backcross population determined using the composite interval mapping feature of QGene (six-rowed subset only).

QTL	Trait ^a	Chrom. ^b	Peak significant marker	Bin ^c	α	R ²	LOD	Sig. ^d
FHB-qtl-2H-4	FHB STP 10	2H	SCRI_RS_182408	4	-3.1	0.11	5.5	0.01
FHB-qtl-2H-4	FHB STP 11	2H	SCRI_RS_182408	4	3.6	0.16	7.8	0.05
FHB-qtl-2H-4	FHB STP 12	2H	SCRI_RS_182408	4	4.9	0.15	7.1	0.01
FHB-qtl-2H-4	FHB CRK 10	2H	SCRI_RS_182408	4	-9.3	0.38	21.4	0.01
FHB-qtl-2H-4	FHB CRK 11	2H	BOPA2_12_10219	4	-4.3	0.09	4.4	0.01
FHB-qtl-5H-9	FHB CRK 12	5H	SCRI_RS_240059	9	-1.8	0.10	4.7	0.01
DON-qtl-2H-4	DON STP 10	2H	SCRI_RS_182408	4	4.3	0.15	7.3	0.01
DON-qtl-2H-4	DON STP 11	2H	SCRI_RS_182408	4	3.0	0.16	7.6	0.01
DON-qtl-2H-4	DON STP 12	2H	SCRI_RS_182408	4	ND	ND	ND	
DON-qtl-2H-4	DON CRK 10	2H	BOPA2_10715	4	3.9	0.16	7.6	0.01
DON-qtl-2H-4	DON CRK 11	2H	SCRI_RS_182408	4	9.6	0.26	13.5	0.01
HT-qtl-2H-4	HT STP 10	2H	SCRI_RS_182408	4	5.0	0.16	7.8	0.01
HT-qtl-2H-4	HT STP 11	2H	SCRI_RS_182408	4	3.3	0.13	6.4	0.01
HT-qtl-2H-4	HT STP 12	2H	SCRI_RS_182408	4	-	-	-	
HT-qtl-2H-4	HT CRK 10	2H	SCRI_RS_182408	4	6.6	0.37	21.0	0.01
HT-qtl-2H-4	HT CRK 11	2H	SCRI_RS_182408	4	6.6	0.38	21.7	0.01
HT-qtl-2H-4	HT CRK 12	2H	SCRI_RS_182408	4	7.5	0.32	17.5	0.01
HD-qtl-2H-4	HD STP 10	2H	SCRI_RS_182408	4	3.4	0.47	28.5	0.01
HD-qtl-2H-4	HD STP 11	2H	SCRI_RS_182408	4	4.0	0.64	46.6	0.01
HD-qtl-2H-4	HD STP 12	2H	SCRI_RS_182408	4	5.3	0.41	23.8	0.01
HD-qtl-2H-4	HD CRK 10	2H	SCRI_RS_182408	4	1.6	0.39	22.0	0.01
HD-qtl-2H-4	HD CRK 11	2H	SCRI_RS_182408	4	3.5	0.64	46.3	0.01
HD-qtl-2H-4	HD CRK 12	2H	SCRI_RS_182408	4	4.2	0.50	31.0	0.01
HD-qtl-7H-4	HD CRK 12	7H	SCRI_RS_194080	4	2.1	0.13	6.1	0.01

^a FHB=FHB severity, DON=deoxynivalenol, HT=plant height, HD=heading date; HZO=Hangzhou (China), STP=St. Paul, MN, CRK=Crookston, MN

^b Chrom=chromosome

^c Bin position is based on Kleinhofs et al. (2005) and Ahmad Sallam (*unpublished*)

^d Significance level was determined using permutation tests.

Table 2.8. Summary of quantitative trait loci (QTL) identified for FHB severity, DON accumulation, and related agronomic traits in the Quest/W-365 advanced backcross population determined using the composite interval mapping feature of QGene (six-rowed subset only).

QTL	Trait ^a	Chrom. ^b	Peak significant marker	Bin ^c	a	R ²	LOD	Sig. ^d
FHB-qtl-4H-3	FHB NJG13	4H	SCRI_RS_157832	3	-5.9	0.08	6.0	0.01
FHB-qtl-2H-2	FHB STP 13	2H	SCRI_RS_207144	2	-1.9	0.08	5.8	0.01
FHB-qtl-2H-4	FHB STP 14	2H	SCRI_RS_207399	4	-4.0	0.21	16.5	0.01
FHB-qtl-2H-4	FHB CRK 13	2H	BOPA2_12_30145	4	-10.4	0.28	22.8	0.01
FHB-qtl-2H-4	FHB CRK 14	2H	SCRI_RS_207399	4	-9.1	0.20	15.9	0.01
DON-qtl-2H-4	DON STP 13	2H	BOPA1_2477-377	4	-2.6	0.07	4.9	0.01
DON-qtl-2H-4	DON STP 14	2H	BOPA2_12_30145	4	-10.5	0.62	68.3	0.01
DON-qtl-2H-4	DON CRK 14	2H	BOPA2_12_30145	4	7.9	0.06	4.0	0.05
HT-qtl-2H-4	HT STP 13	2H	BOPA2_12_30145	4	5.4	0.09	6.5	0.01
HT-qtl-2H-4	HT STP14	2H	BOPA2_12_30145	4	7.7	0.18	13.7	0.01
HT-qtl-2H-4	HT CRK 13	2H	BOPA2_12_30145	4	11.4	0.24	19.1	0.01
HT-qtl-2H-4	HT CRK 14	2H	BOPA2_12_30145	4	12.5	0.34	29.8	0.01
HD-qtl-2H-4	HD STP 13	2H	BOPA2_12_30145	4	4.2	0.54	54.0	0.01
HD-qtl-2H-4	HD STP 14	2H	BOPA2_12_30145	4	4.7	0.56	57.6	0.01
HD-qtl-2H-4	HD CRK 13	2H	BOPA2_12_30145	4	4.1	0.66	75.7	0.01
HD-qtl-2H-4	HD CRK 14	2H	BOPA2_12_30145	4	4.5	0.62	68.6	0.01

^a FHB=FHB severity, DON=deoxynivalenol, HT=plant height, HD=heading date; NJG=Nanjing (China), STP=St. Paul, MN, CRK=Crookston, MN

^b Chrom=chromosome

^c Bin position is based on Kleinhofs et al. (2005) and Ahmad Sallam (*unpublished*)

^d Significance level was determined using permutation tests.

ND=no data

Table 2.9. Summary of quantitative trait loci (QTL) identified for FHB severity, DON accumulation, and related agronomic traits in the Quest/Kutahya advanced backcross population determined using the composite interval mapping feature of QGene (six-rowed subset only).

QTL	Trait	Chrom. ^b	Peak significant marker)	Bin ^c	α	R ²	LOD	Sig. ^d
FHB-qtl-6H-10	FHB BRD 12	6H	SCRI_RS_239959	10	-0.4	0.09	6.0	0.01
FHB-qtl-1H-4	FHB BRD 13	1H	BOPA2_12_30948	4	-0.8	0.09	6.2	0.05
FHB-qtl-4H-5	FHB BRD 13	4H	BOPA2_12_30328	5	-0.8	0.08	5.8	0.05
DON-qtl-2H-3	DON BRD 13	2H	SCRI_RS_166237	3	-7.2	0.07	4.7	0.05
HT-qtl-3H-5	HT BRD 12	3H	BOPA1_12_30732	5	-1.9	0.05	3.7	0.01
HT-qtl-6H-6	HT BRD 12	6H	SCRI_RS_208339	6	-2.4	0.11	7.6	0.01
HT-qtl-3H-9	HT BRD 13	3H	BOPA1_11657-398	9	-3.9	0.17	12.4	0.01
HT-qtl-6H-6	HT BRD 13	6H	SCRI_RS_208339	6	-2.7	0.12	8.6	0.01
HT-qtl-3H-6	HT STP 13	3H	SCRI_RS_175220	6	-2.7	0.06	4.0	0.05
HT-qtl-6H-6	HT STP 13	6H	SCRI_RS_208399	6	-2.0	0.05	3.6	0.05
HT-qtl-3H-9	HT STP 14	3H	BOPA1_11657-398	9	-2.8	0.06	4.1	0.01
HT-qtl-6H-6	HT STP 14	6H	SCRI_RS_208399	6	-2.4	0.06	4.3	0.01
HT-qtl-3H-9	HT CRK 14	3H	BOPA1_11657-398	9	-2.3	0.06	4.4	0.01
HT-qtl-6H-6	HT CRK14	6H	SCRI_RS_208399	6	-2.8	0.13	9.4	0.01
HD-qtl-2H-7	HD STP 14	2H	SCRI_RS_191136	7	-2.2	0.08	5.6	0.01
HD-qtl-7H-4	HD STP 14	7H	SCRI_RS_193044	4	3.1	0.25	18.9	0.01
HD-qtl-2H-7	HD CRK 13	2H	SCRI_RS_191136	7	-2.0	0.12	8.5	0.01
HD-qtl-7H-4	HD CRK 13	7H	SCRI_RS_193044	4	2.4	0.32	25.3	0.01
HD-qtl-2H-7	HD CRK 14	2H	SCRI_RS_191136	7	-2.2	0.13	8.9	0.01
HD-qtl-7H-4	HD CRK 14	7H	SCRI_RS_193044	4	2.0	0.22	16.4	0.01

^a FHB=FHB severity, DON=deoxynivalenol, HT=plant height, HD=heading date, BRD=Brandon, MB, Canada; CHN=Nanjing (China); STP=St. Paul, MN; CRK=Crookston, MN

^b Chrom=Chromosome

^c Bin position is based on Kleinhofs et al. (2005) and Ahmad Sallam (*unpublished*)

^d Significance level was determined using permutation tests

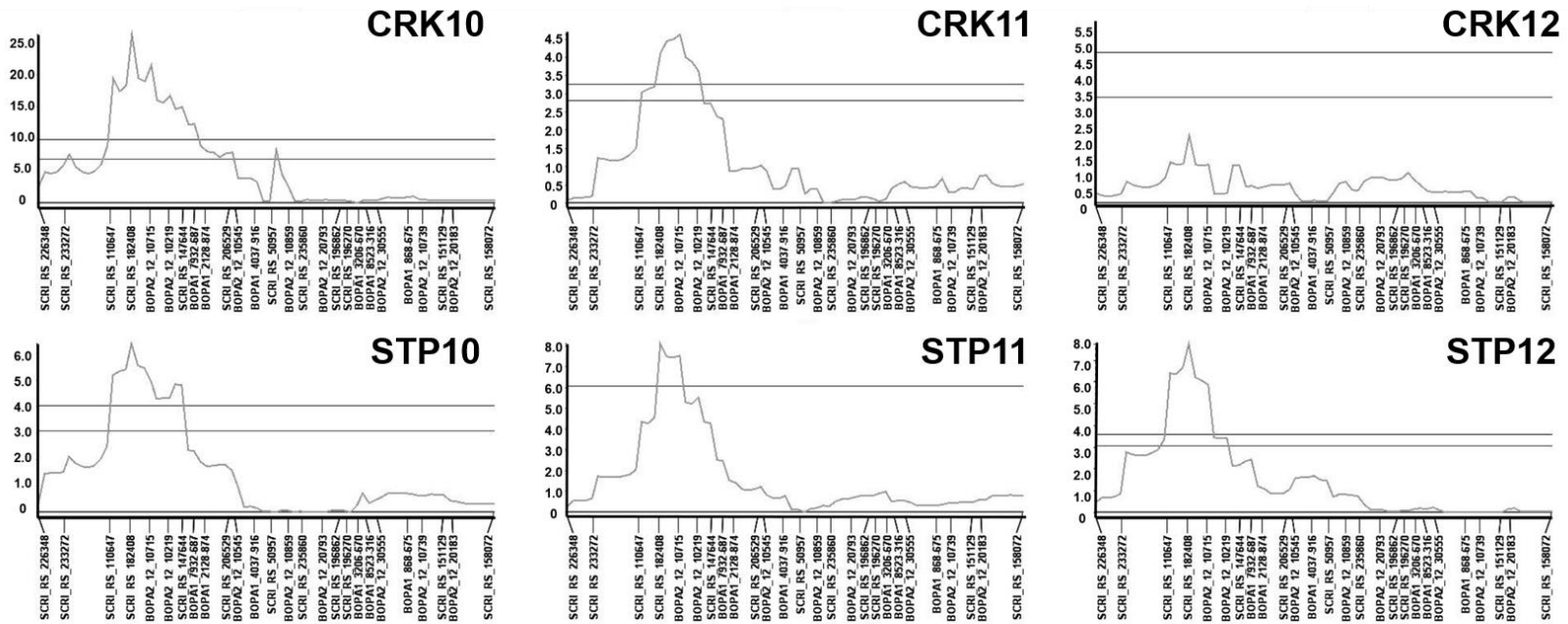


Figure 2.1.1. Quantitative trait loci trace of chromosome 2H for *Fusarium* head blight severity in the Rasmusson/PI 466423 advanced backcross population using the composite interval mapping maximum likelihood estimator (CIM MLE) function of QGene (Joehanes and Nelson 2008) for the Crookston (CRK) and Saint Paul (STP), MN nurseries from 2010-2012. Not pictured: Hangzhou 2011 where no significant QTL were detected.

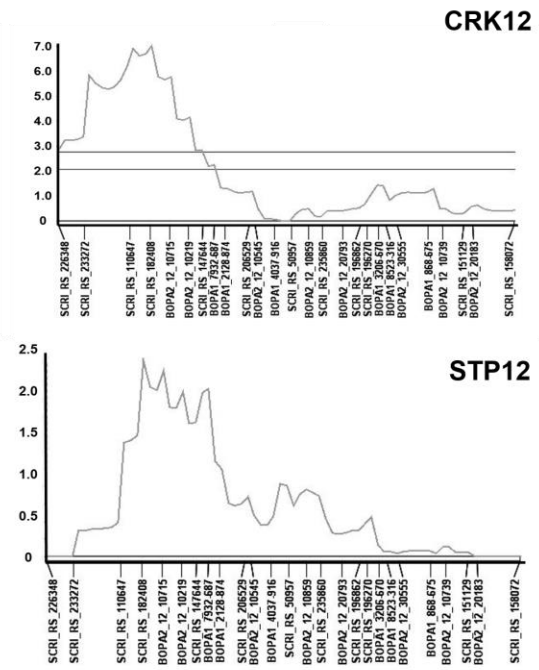
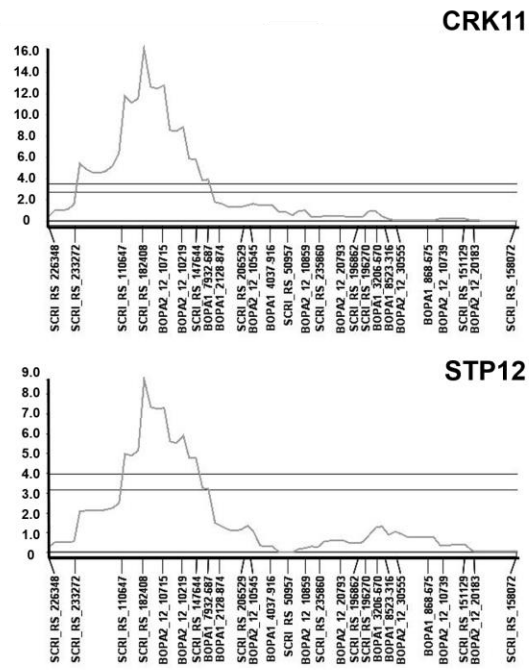
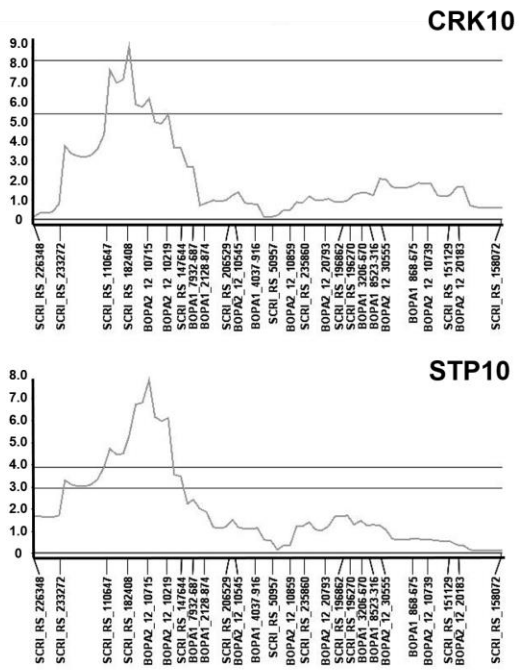


Figure 2.1.2. Quantitative trait loci trace of chromosome 2H for deoxynivalenol concentration in the Rasmusson/PI 466423 advanced backcross population using the composite interval mapping maximum likelihood estimator (CIM MLE) function of QGene (Joehanes and Nelson 2008) for the Crookston (CRK) and Saint Paul (STP), MN nurseries from 2010-2012.

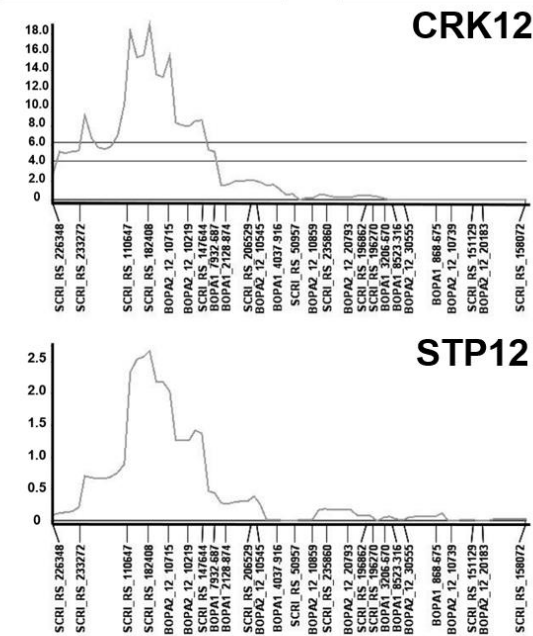
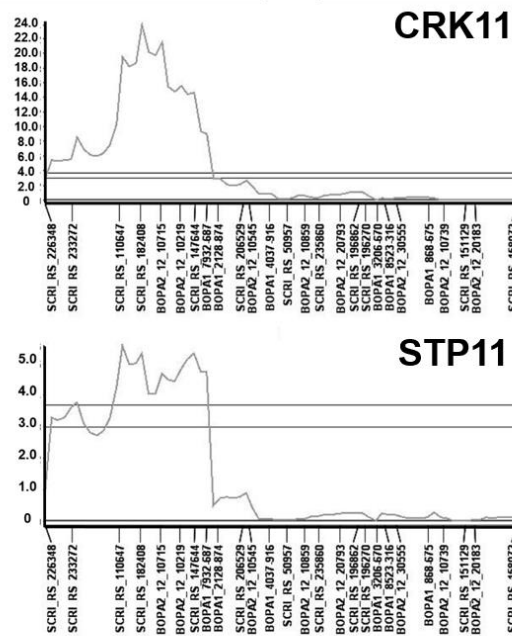
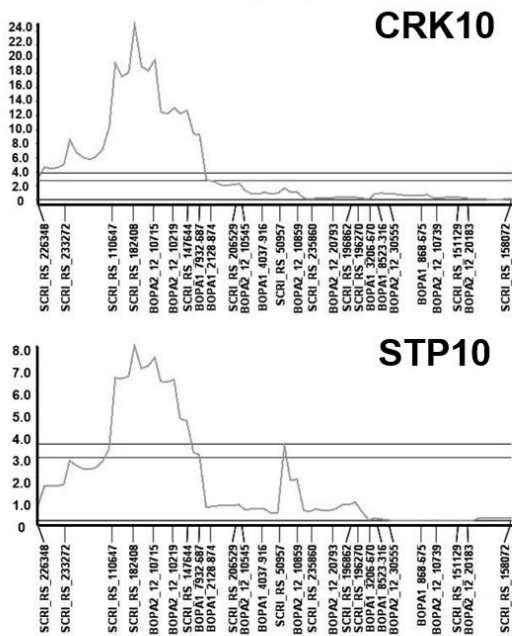


Figure 2.1.3. Quantitative trait loci trace of chromosome 2H for plant height in the Rasmusson/PI 466423 advanced backcross population using the composite interval mapping maximum likelihood estimator (CIM MLE) function of QGene (Joehanes and Nelson 2008) for the Crookston (CRK) and Saint Paul (STP), MN nurseries from 2010-2012.

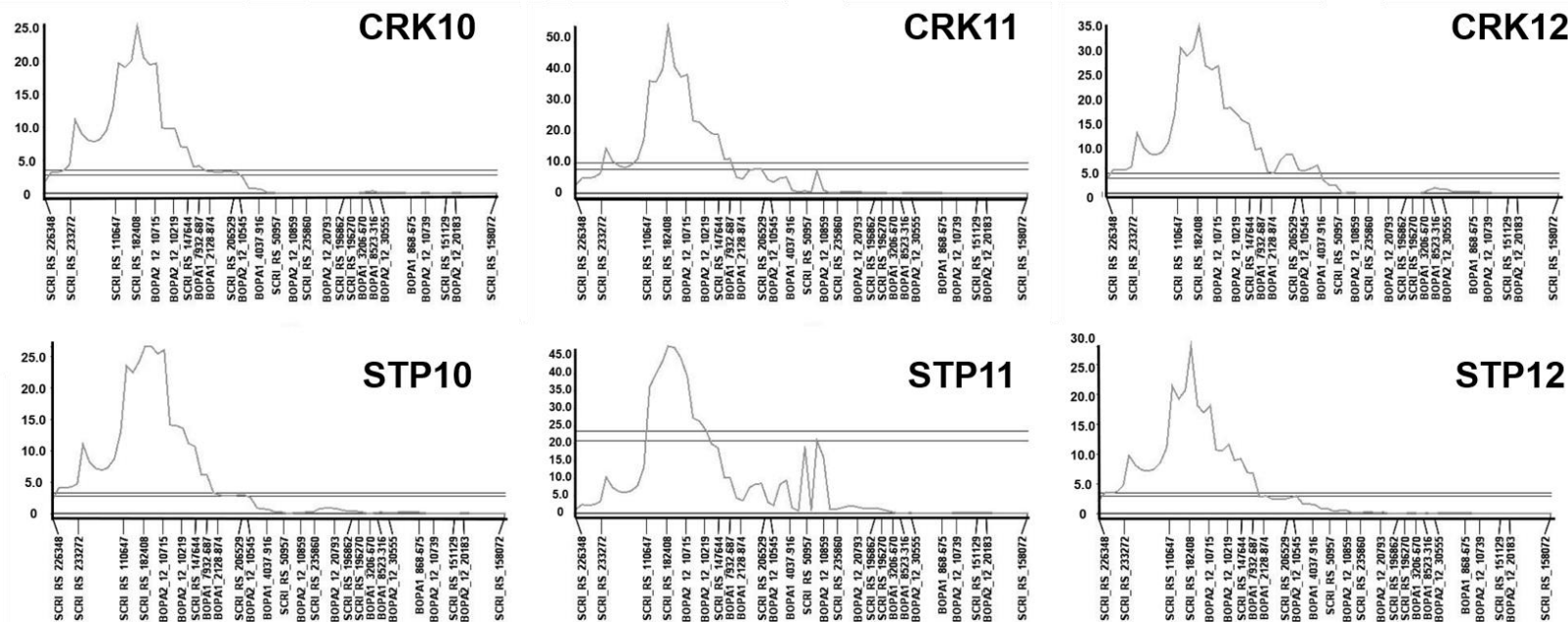


Figure 2.1.4. Quantitative trait loci trace of chromosome 2H for heading date in the Rasmusson/PI 466423 advanced backcross population using the composite interval mapping maximum likelihood estimator (CIM MLE) function of QGene (Joehanes and Nelson 2008) for the Crookston (CRK) and Saint Paul (STP), MN nurseries from 2010-2012.

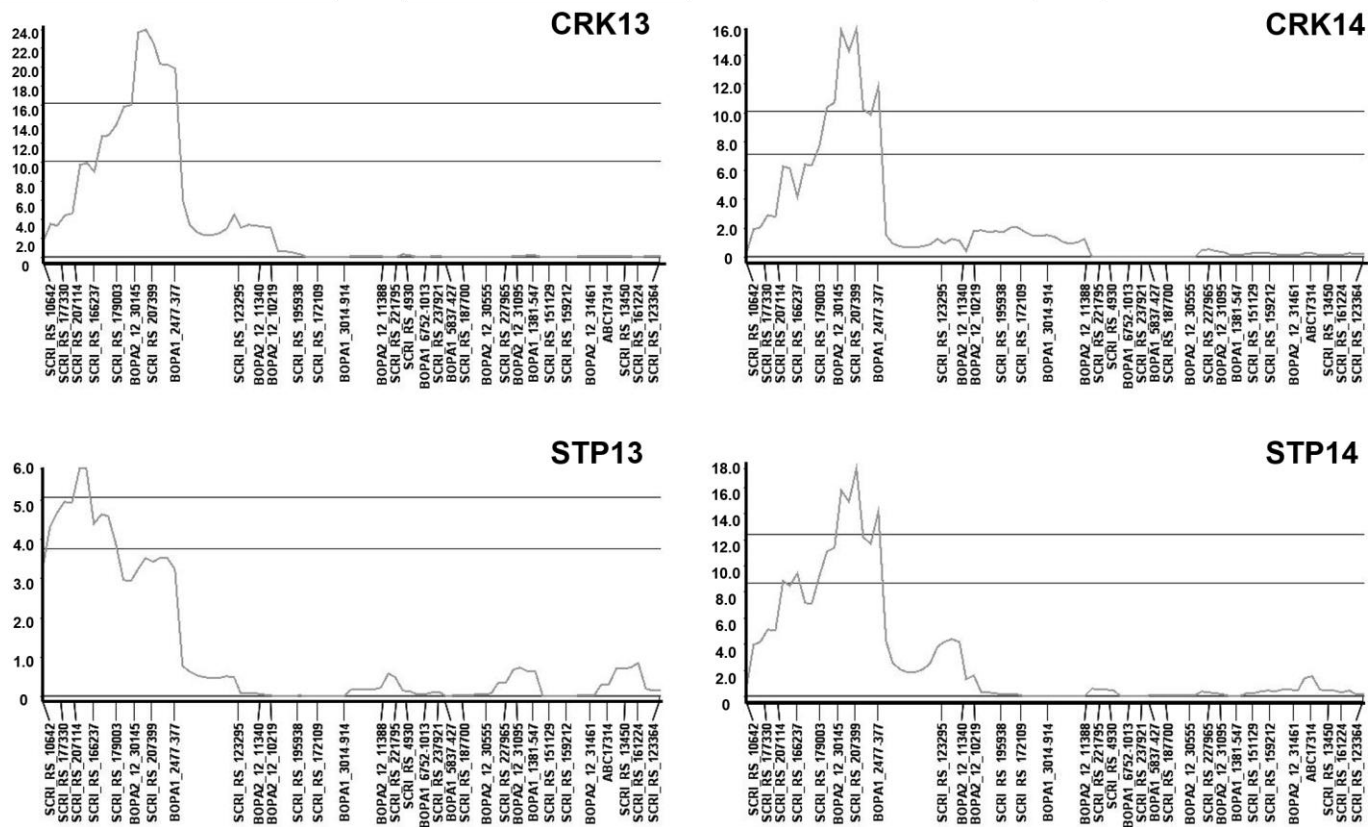


Figure 2.2.1. Quantitative trait loci trace of chromosome 2H for Fusarium head blight severity in the Quest/W-365 advanced backcross population using the composite interval mapping maximum likelihood estimator (CIM MLE) function of QGene (Joehanes and Nelson 2008) for the Crookston (CRK) and Saint Paul (STP), MN nurseries from 2013-2014.

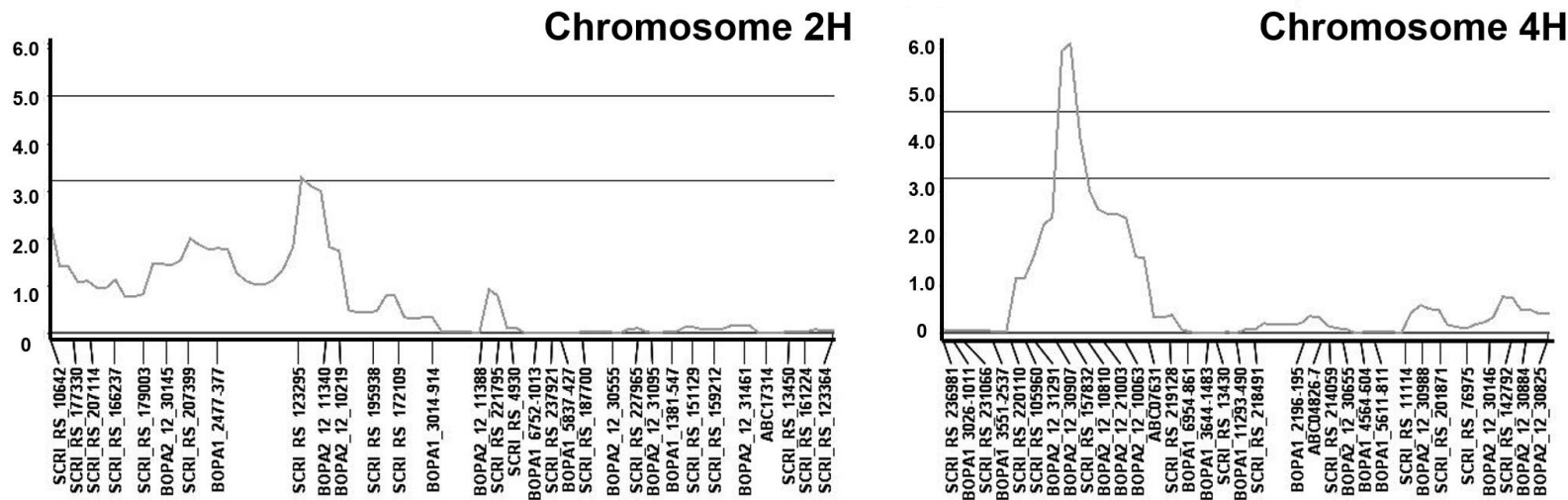
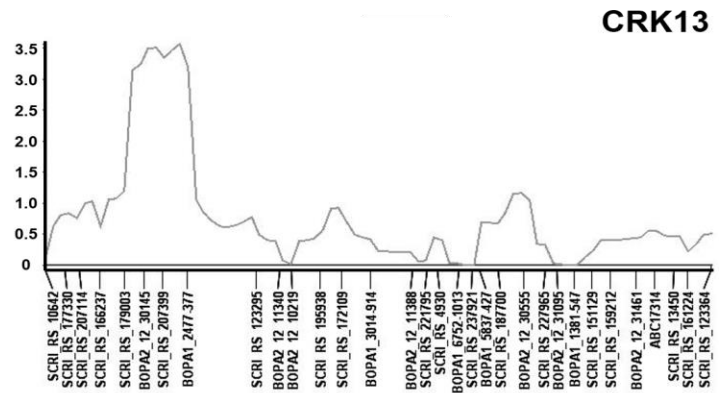
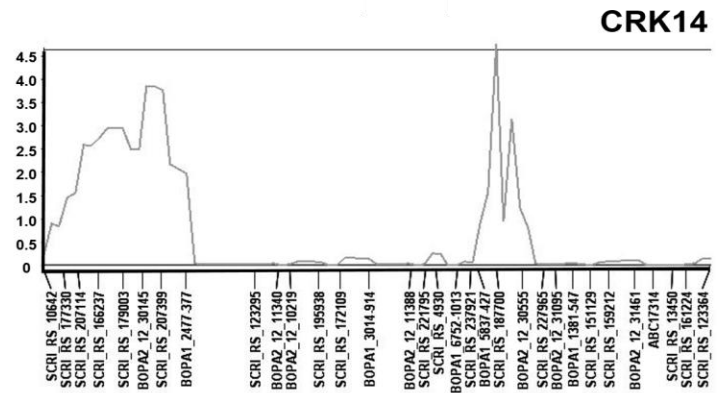


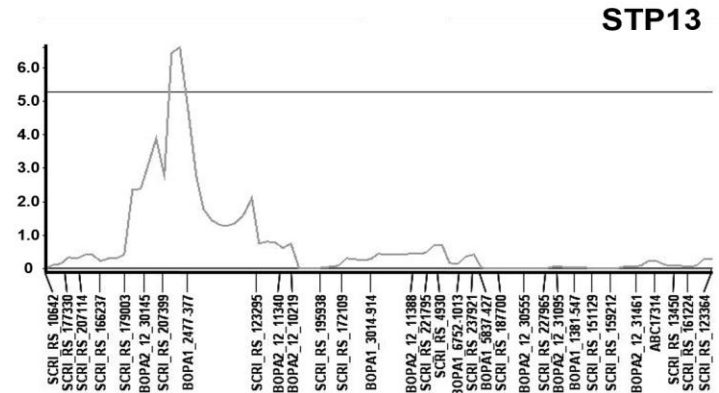
Figure 2.2.2. Quantitative trait loci trace for Fusarium head blight severity in the Quest/W-365 advanced backcross population in Nanjing, China (2013) using the composite interval mapping maximum likelihood estimator (CIM MLE) function of QGene (Joehanes and Nelson 2008) for chromosome 2H (left) and chromosome 4H (right).



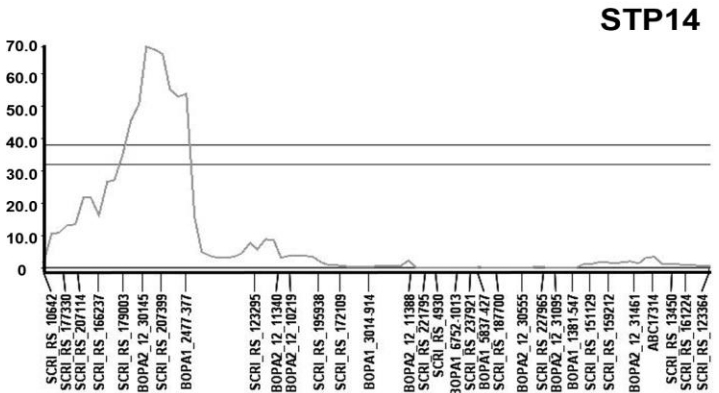
CRK13



CRK14



STP13



STP14

Figure 2.2.3. Quantitative trait loci trace on chromosome 2H for deoxynivalenol concentration in the Quest/W-365 advanced backcross population using the composite interval mapping maximum likelihood estimator (CIM MLE) function of QGene (Joehanes and Nelson 2008) for the Crookston (CRK) and Saint Paul (STP),MN nurseries from 2013-2014.

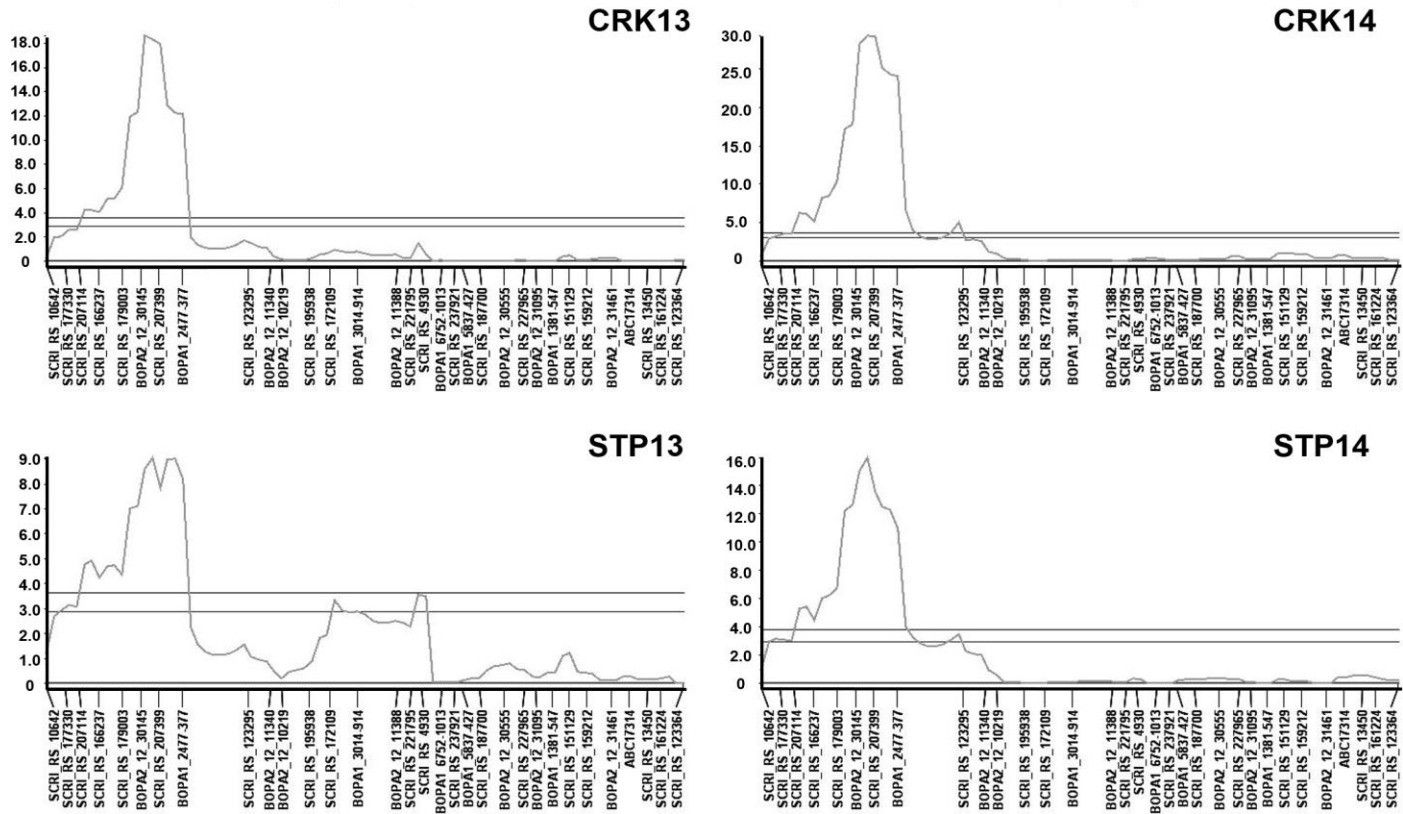


Figure 2.2.4. Quantitative trait loci trace on chromosome 2H for plant height in the Quest/W-365 advanced backcross population using the composite interval mapping maximum likelihood estimator (CIM MLE) function of QGene (Joehanes and Nelson 2008) for the Crookston (CRK) and Saint Paul (STP), MN nurseries from 2013-2014.

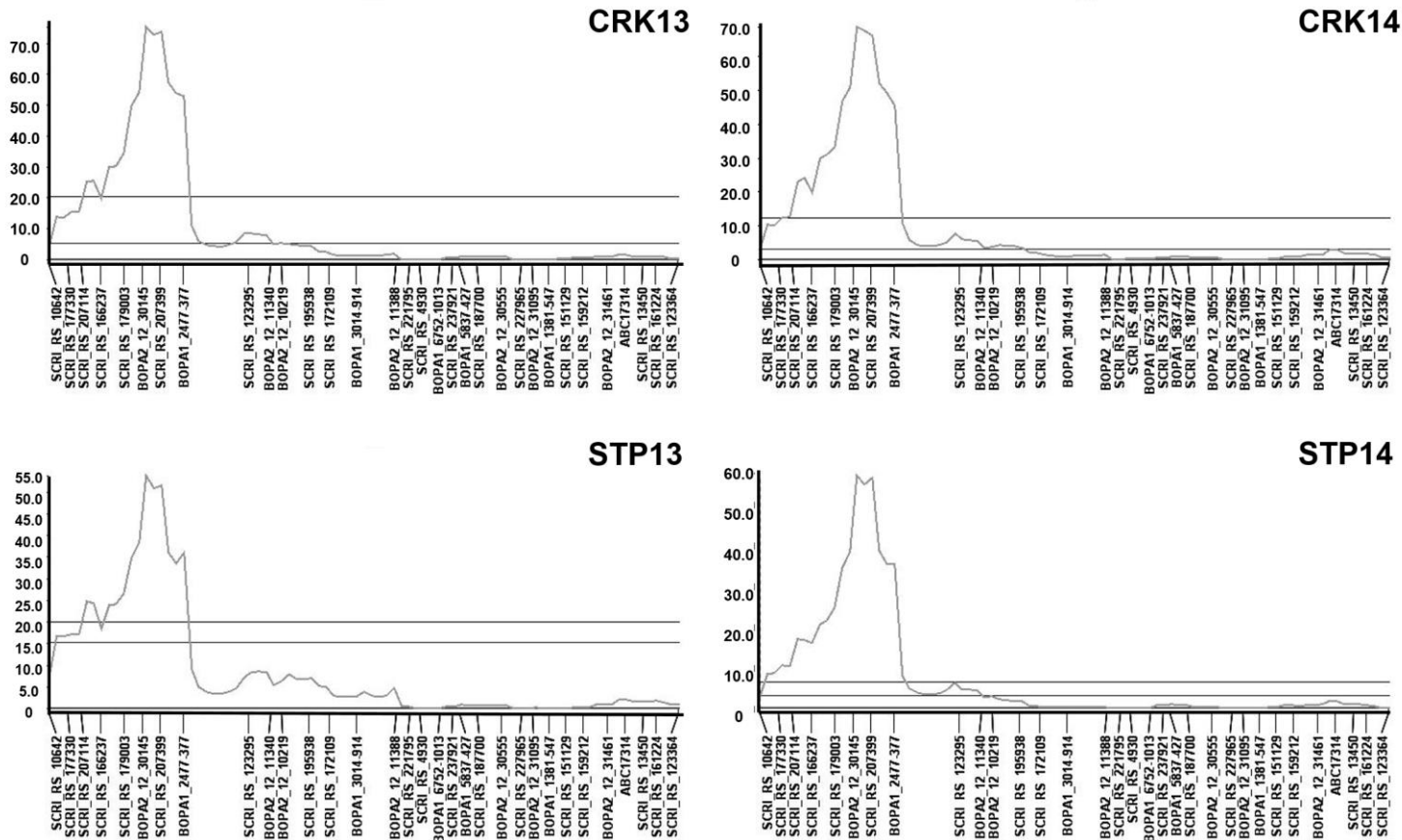


Figure 2.2.5. Quantitative trait loci trace on chromosome 2H for heading date in the Quest/W-365 advanced backcross population using the composite interval mapping maximum likelihood estimator (CIM MLE) function of QGene (Joehanes and Nelson 2008) for the Crookston (CRK) and Saint Paul (STP), MN nurseries from 2013-2014.

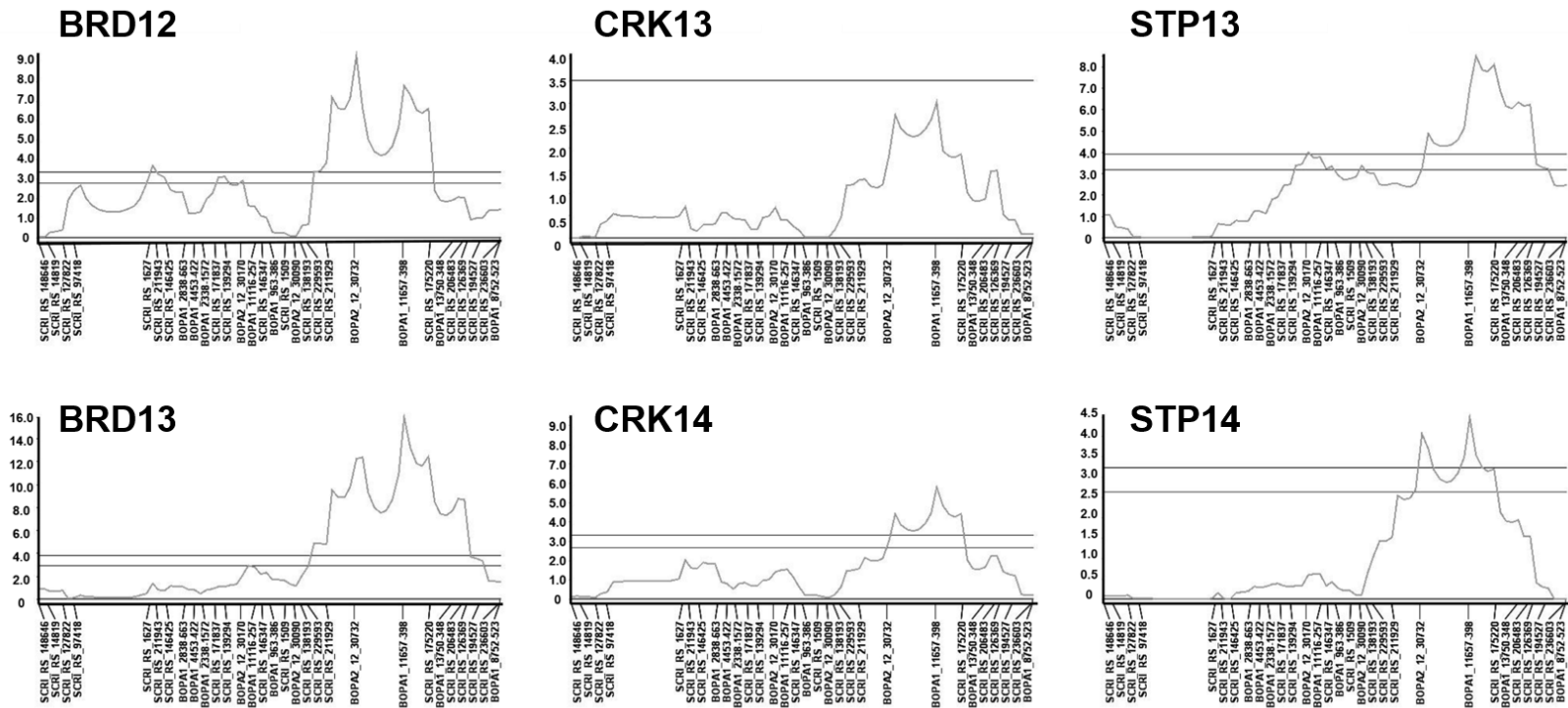


Figure 2.3.1. Quantitative trait loci trace on chromosome 3H for plant height in the Quest/Kutahya advanced backcross population using the composite interval mapping maximum likelihood estimator (CIM MLE) function of QGene (Joehanes and Nelson 2008) for Brandon (BRD), MB FHB nurseries from 2012-2013 and the Crookston (CRK) and Saint Paul (STP), MN FHB nurseries from 2013-2014.

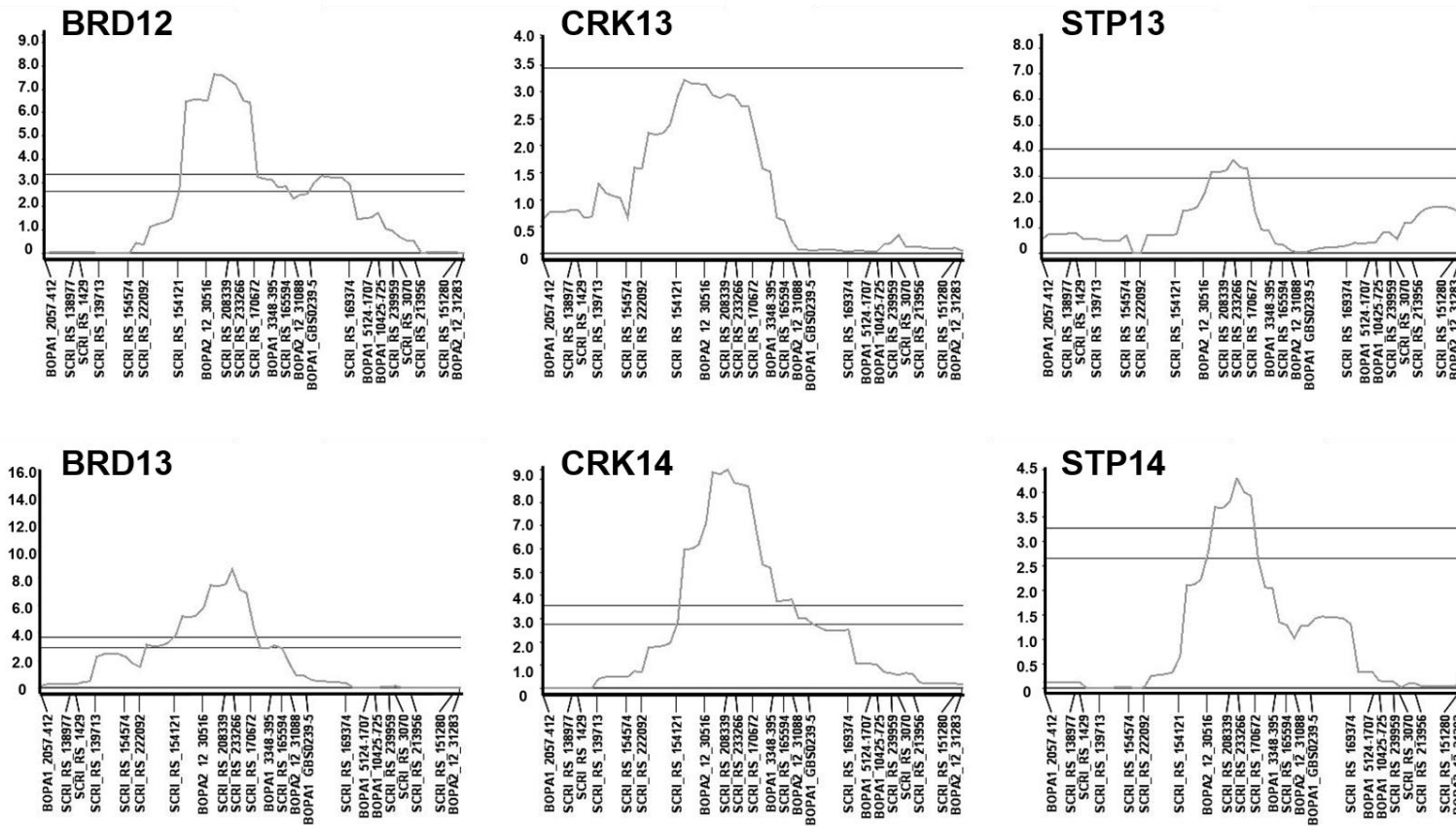


Figure 2.3.2. Quantitative trait loci trace on chromosome 6H for plant height in the Quest/Kutahya advanced backcross population using the composite interval mapping maximum likelihood estimator (CIM MLE) function of QGene (Joehanes and Nelson) for Brandon (BRD), MB FHB nurseries from 2012-2013 and the Crookston (CRK) and Saint Paul (STP), MN nurseries from 2013-2014.

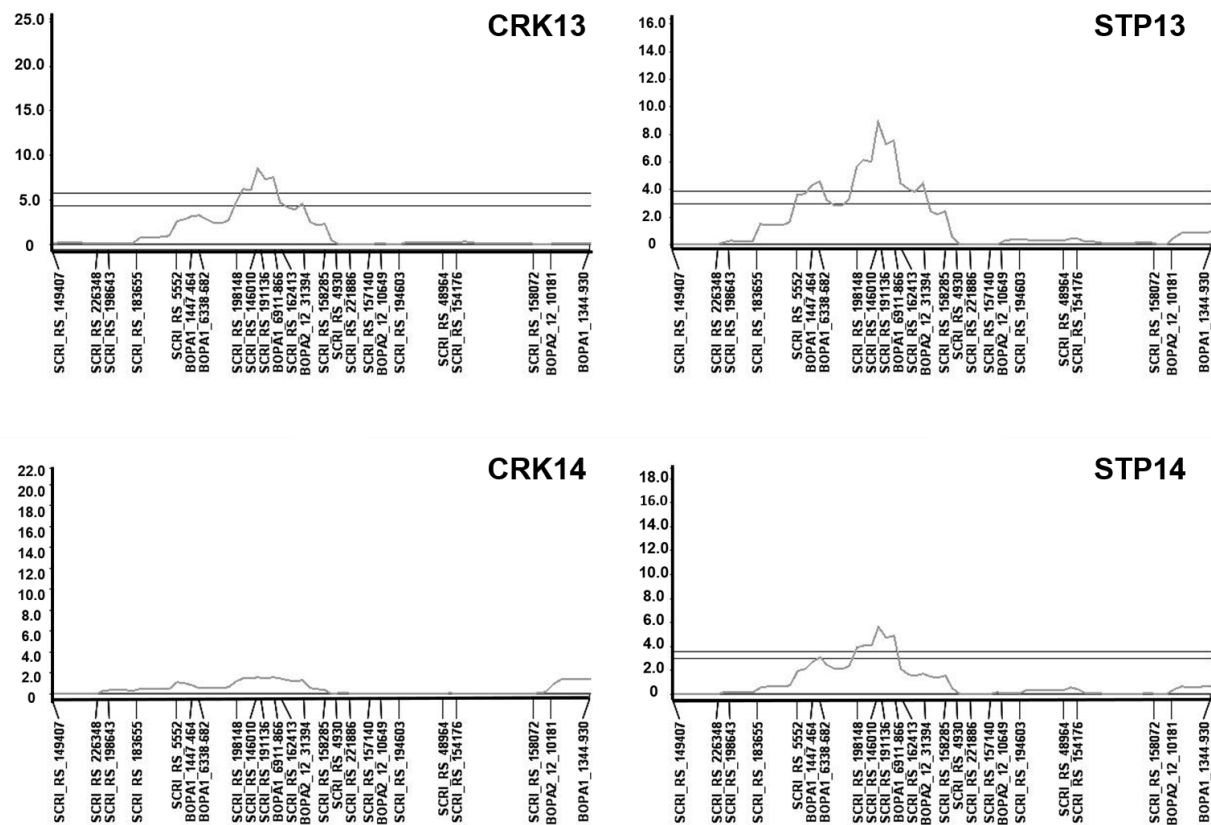


Figure 2.3.3. Quantitative trait loci trace on chromosome 2H for heading date in the Quest/Kutahya advanced backcross population using the composite interval mapping maximum likelihood estimator (CIM MLE) function of QGene (Joehanes and Nelson 2008) for the Crookston (CRK) and Saint Paul (STP), MN FHB nurseries from 2013-2014. Not pictured: Brandon 2012 and 2013 (where no significant QTL were detected).

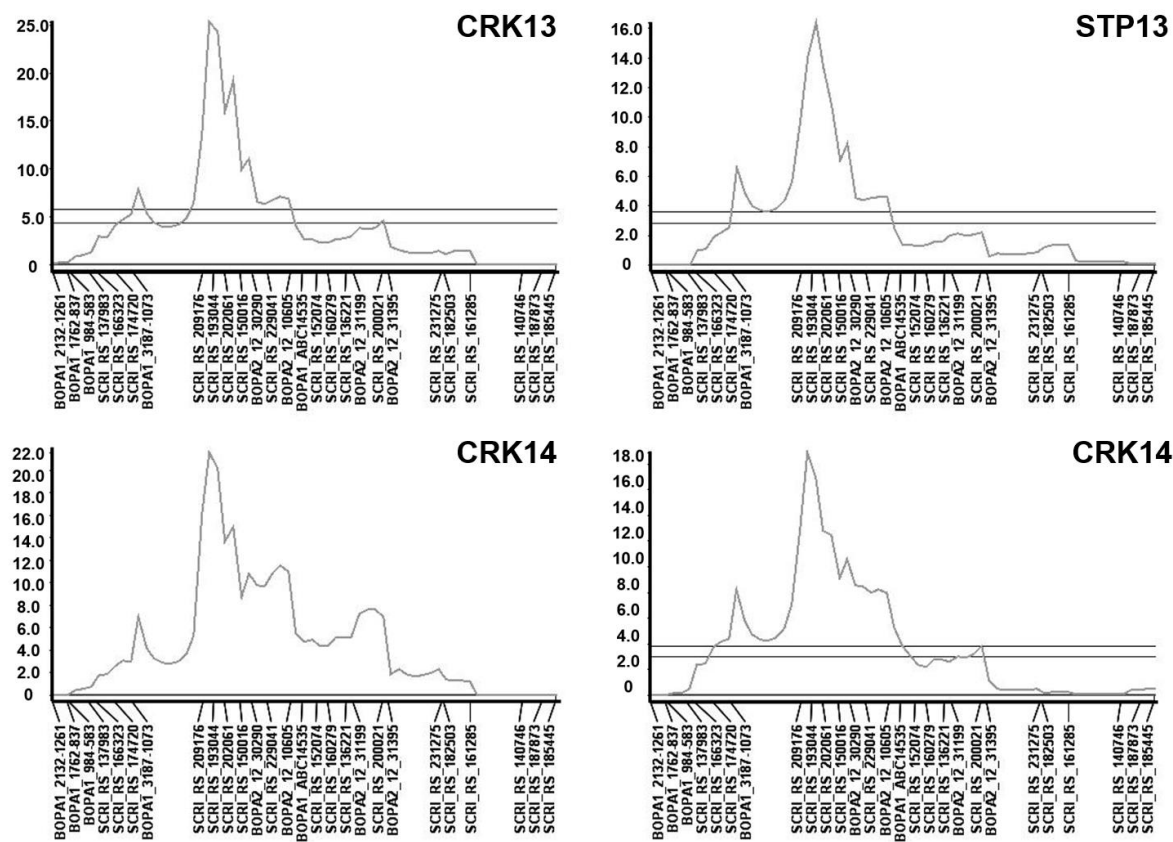


Figure 2.3.4. Quantitative trait loci trace on chromosome 7H for heading date in the Quest/Kutahya advanced backcross population using the composite interval mapping maximum likelihood estimator (CIM MLE) function of QGene (Joehanes and Nelson 2008) for the Crookston (CRK) and Saint Paul (STP), MN nurseries from 2013-2014. Not pictured: Brandon 2012 and 2013 (where no significant QTL were detected).

Chapter 3

Mapping QTL Conferring Resistance to a Widely Virulent Isolate of *Cochliobolus sativus* in Wild Barley Accession PI 466423

Introduction

Spot blotch is caused by the fungal pathogen *Cochliobolus sativus* (Ito & Kurib.) Drechs. ex Dastur (anamorph: *Bipolaris sorokiniana* (Sacc.) Shoem.) and historically has been an important disease of barley (*Hordeum vulgare* L.) in the Upper Midwest region of the United States. The disease is capable of causing yield losses up to 36% in susceptible cultivars and also reducing malting quality (Clark 1979). Deployment of resistant cultivars is the best strategy for controlling the disease. NDB112 (CIho 11531) is a six-rowed breeding line derived from the cross CIho 7117-77/Kindred (Wilcoxson et al. 1990) that possesses a high level of resistance to many pathotypes of *C. sativus* (Valjavec-Gratian and Steffenson 1997). It was used extensively in the Minnesota and North Dakota barley breeding programs to develop cultivars with spot blotch resistance. These breeding efforts were highly successful as no outbreaks of the disease have occurred in six-rowed cultivars with the NDB112 resistance for 50 years, an extraordinary example of durable resistance. In a recent study by Zhou and Steffenson (2013), the genetics of this durable resistance was characterized in a genome-wide association study (GWAS) of a very large collection of US barley breeding germplasm (N = 3,072). They identified three quantitative trait loci (QTL) contributing to the Midwest Six-rowed Durable Resistance Haplotype (MSDRH): *Rcs-qt1-1H-11_10764* on chromosome 1H, *Rcs-qt1-3H-11_10565* on chromosome 3H and *Rcs-qt1-7H-11_20162* on chromosome 7H.

The same high degree of resistance and broad protection against a diversity of *C. sativus* pathotypes bred into six-rowed cultivars has not been achieved in two-

rowed cultivars (Valjavec-Gratian and Steffenson 1997). Bowman (PI 483237), a two-rowed feed cultivar bred for the western Dakotas (Franckowiak et al. 1985), has NDB112 in its pedigree and apparently all three resistance QTL required for the MSDRH (Zhou and Steffenson 2013). Despite possessing the favorable allele configuration for the MSDRH, Bowman does not possess the same level of resistance as six-rowed types and succumbed to a new pathotype of *C. sativus* (isolate ND90Pr or pathotype 2) to which the six-rowed types are resistant (Fetch and Steffenson 1994; Zhou and Steffenson 2013). Bilgic et al. (2005) studied spot blotch resistance in four populations of barley derived from parents differing in row type (e.g. two- or six-rowed) and end-use (e.g. malting or feed). A notable finding from their study was the detection of a major effect QTL in chromosome 1H that was detected in the Steptoe/Morex (six- x six-rowed) population, but not any of the other three populations that included both two- x six- and six- x six-rowed crosses. Two of those three populations involved Morex (which donated the resistance allele in the Steptoe/Morex population), so it was particularly surprising that the 1H QTL was not identified. Additional analyses to resolve this result revealed minimal variation due to epistasis (4.4%) and no QTL x environment interactions. Therefore, Bilgic et al. (2005) postulated that spot blotch resistance may be differentially expressed in different genetic backgrounds. Zhou and Steffenson (2013) later identified the 1H QTL as one of the three comprising the MSDRH. Moreover, based on the SNP genotyping of parental lines, they suggested that the failure to detect this QTL in the latter

populations may be due to the fixation of the resistance locus in the biparental populations studied by Bilgic et al. (2005). However, in the Steptoe/Morex population, both parents carry the same SNP allele for marker 11_10764 in chromosome 1H (coincident with the resistance QTL in that population), but still segregate for reaction to spot blotch at that locus, suggesting that a recombination event occurred between marker 11_10764 and the resistance QTL.

Most of the research done on screening barley for breeding purposes and QTL mapping for spot blotch resistance in the United States has focused on isolate ND85F (pathotype 1) and to a lesser extent isolate ND90Pr (pathotype 2). However, there is strong evidence that NDB112-virulent isolates exist in the pathogen population. Ghazvini and Tekauz (2007) examined 127 *C. sativus* isolates from Canada and other parts of the world for their reaction on 12 differential barley lines and found that isolates varied from avirulent to highly virulent, but generally lacked classic gene-for-gene interactions. Significantly, several isolates were found with higher virulence (infection response of 6 or higher on a 1-9 scale) on NDB112. In another study, Zhong et al. (2015) described an isolate (ND4008) with virulence on NDB112 from North Dakota. Isolate ND4008 was originally isolated from the roots of barley (Gyawali 2010), and while no outbreaks of spot blotch have been recorded on cultivars with the NDB112 resistance in the field, this finding raises serious concerns regarding the vulnerability of barley in the region.

Isolate ND4008 is widely virulent on accessions in the genus *Hordeum*. From the evaluation of the core collection of cultivated barley (N = 2,062) from United States Department of Agriculture-Agricultural Research Service National Small Grains Collection (USDA-ARS NSGC), only 44 accessions were identified with a high level of seedling resistance to isolate ND4008 (Y. Leng *personal communication*). GWAS of 1,480 two- and six-rowed accessions from the NSGC core collection identified two QTL for resistance to isolate ND4008: one in chromosome 1H (*Rcs-qt1-1H-P3*) and the second in chromosome 6H (*Rcs-qt1-6H-P3*) (R. Wang and S. Zhong *personal communication*). Other QTL for resistance to isolate ND4008 were identified in chromosomes 2H (*Rcs-qt1-2H-P3*) and 3H (*Rcs-qt1-3H-P3*), but only when the six- or two-rowed subsets were used, respectively. Similar to the screening of the NSGC cultivated barley core collection, a relatively low frequency (11% of N = 318) of wild barley (*Hordeum vulgare* ssp. *spontaneum*) accessions was found resistant to isolate ND4008 from seedling evaluations of the Wild Barley Diversity Collection (WBDC) (S. Zhong, *personal communication*). Wild barley is a well-established repository of genetic diversity for disease resistance (Ames et al. 2015, Fetch et al. 2003, Steffenson et al. 2007, Yun et al. 2005). During routine screening of mapping population parents developed in our project at the University of Minnesota, we identified a wild barley parent (PI 466423) with a high level of seedling resistance to isolate ND4008. PI 466423 was originally collected near the village of Mehola by the Jordan River in Israel and was used as a source of Fusarium head blight

(FHB) resistance for barley (Dahl et al. 2009, Huang et al. 2013). Given the potential losses that spot blotch can cause on barley in the region and the wide virulence of isolate ND4008, it is important to fully characterize the few sources of resistance available in *Hordeum*. Thus, the objective of this study was to characterize the number, chromosomal position and effect of loci conferring resistance to isolate ND4008 in an advanced backcross population involving PI 466423.

Materials and Methods

Population Development

An advanced backcross population (Tanksley and Nelson 1996) involving the wild barley resistance source PI 466423 and the susceptible cultivar Rasmusson (Smith et al. 2010) was used in this investigation. Rasmusson is a six-rowed malting barley cultivar developed at the University of Minnesota and was used as the recurrent parent. Methodology for population development was according to Matus et al. (2003). All crosses and single-seed descent increases were made in a greenhouse (18-25°C with a 16-hr photoperiod supplemented by sodium vapor lights emitting $>300 \mu\text{mol photons s}^{-1} \text{ m}^{-2}$) at the Plant Growth Facility on the Saint Paul campus of the University of Minnesota from 2005-2010. Plants were grown in square plastic pots (13.3 x 13.3 x 10.2 cm, l x w x h) filled with a 50:50 mixture of steam-sterilized field soil and Sunshine MVP potting mix (Sun Gro Horticulture, Quincy, MI). At planting, all pots were fertilized with Osmocote

slow-release fertilizer (14-14-14 N-P-K) as well as water-soluble Peters Dark Weather fertilizer (15-0-15) (Scott's Company, Marysville, OH). After these initial fertilization treatments, plants were fertilized every two weeks with another water-soluble fertilizer (20-20-20) (J.R. Peters Inc., Allentown, PA). At physiological maturity, spikes were harvested, dried in a forced air dryer for two days at 35°C and subsequently threshed and cleaned for the next generation advance and testing. In total, 244 BC₂F₄ progeny derived from 68 BC₁ individuals were used for genotyping and phenotyping.

Genotyping

To obtain DNA for genotyping, lyophilized leaf tissue was first ground to a fine powder using the Qiagen TissueLyser II (Qiagen, Valencia, CA) and 3 mm tungsten carbide beads (one bead per microtube). Then, genomic DNA was extracted using the Qiagen BioSprint 96 workstation and the BioSprint 96 DNA Plant Kit (Qiagen). Genotyping was done using the Illumina Infinium assay according to the manufacturer's instructions at the USDA-ARS Biosciences Research Laboratory in Fargo, North Dakota. The Illumina genotyping array is capable of interrogating 7,842 single nucleotide polymorphism (SNP) markers within the barley genome simultaneously, although not with equal efficiency. SNPs for the Illumina genotyping array were developed from expressed sequence tags (ESTs) and next-generation sequencing (NGS) as described by Close et al. (2009) and Comadran et al. (2012), respectively. Of these SNPs,

2,832 were EST-derived and 5,010 came from NGS data. Further information regarding these SNP markers is available online at:

bioinf.hutton.ac.uk/iselect/app/. Genotype calls for all SNP markers were visually inspected and validated using Illumina's GenomeStudio Genotyping Module v.2011. Of the 7,842 potential SNPs, 2,554 were polymorphic between PI 466423 and Rasmusson.

Genetic Map Construction

JoinMap 4.1[®] was used to create genetic linkage maps for QTL analysis. All 2,554 polymorphic loci were considered for map construction. Polymorphic markers were sorted into groups based on recombination frequency beginning with a frequency of 0.250 and ending with 0.050 in increments of -0.05. The maximum likelihood algorithm was used for ordering marker loci and calculating genetic distances for each linkage group. Markers that were uninformative or flagged in suspect linkages (defined as having a recombination frequency with another marker greater than 0.5) were removed from consideration. Barley chromosome designations (i.e. 1H to 7H) were assigned based on markers in common with the consensus map of Muñoz-Amatriaín et al. (2014).

Plant Growth Conditions for Disease Phenotyping

For the disease phenotyping tests, seeds of the parents, progeny population and controls were sown in cone-tainers (5 cm diameter x 18 cm depth, Stuewe and

Sons, Inc., Tangent, OR) filled with a 50:50 mixture of steam-sterilized field soil and Sunshine MVP potting mix. The cone-tainers were held in racks at 50 per rack. After sowing, plants were placed in a cold room (4°C) for 4-5 days to break any residual dormancy, thereby ensuring even germination and plant development. Then, the racks containing the cone-tainers were set into shallow plastic pans partially filled with potting mix to facilitate even moisture conditions for plants during the course of the experiment. Cone-tainers were spaced out after inoculation to a density of 25 per rack to avoid plant to plant shading. Greenhouse conditions during the pre- and post-inoculation periods were as described above.

Preparation of Pathogen Inoculum and Inoculation

Isolate ND4008 of *C. sativus* was originally derived from a single spore and maintained as conidia stored on silica gel crystals in glass vials at 4°C. It was provided by Shaobin Zhong at North Dakota State University (Fargo, ND). Protocols for inoculum production were described in Fetch and Steffenson (1999). When the second leaves of plants were fully expanded (14 days after planting), seedlings were inoculated with a conidial suspension (4,000 conidia/ml) of isolate ND4008 using an artist's airbrush (Model H, Paasche Airbrush Company, Chicago, IL) pressurized by a CO₂ tank (207 kPa). To facilitate even distribution and adherence of conidia on the barley leaves, a surfactant (Tween[®] 20: polyoxyethylene-20-sorbitan monolaurate) was added

(100 µl/L) to the conidial suspension. The volume of inoculum suspension applied to each plant was approximately 0.3 ml. After inoculation, plants were placed in mist chambers where ultrasonic humidifiers were turned on for 45 minutes to create a fine layer of free moisture on the leaf surfaces. At the end of the initial misting period, humidifiers were programmed to come on for 2 minutes every hour overnight (~16 h) to maintain leaf wetness. During this time, the plants were kept at 18–22°C and near 100% RH in the dark. In the morning, the mist chamber doors were opened and the humidifiers turned off in order to allow the plants to dry slowly (for about 4 h) before moving them back into the greenhouse.

Disease phenotyping

For the seedling tests, infection responses (IRs) were recorded 11–12 days post-inoculation on the second leaves according to the 1-9 rating scale of Fetch and Steffenson (1999). The experiment was conducted in a completely randomized design and was repeated three sequential times. If any of the progeny lines exhibited variable responses across experiments or failed to germinate, additional evaluations were made to obtain a clear consensus reading. Progeny lines that consistently had both resistant (IR=1-5) and susceptible (IR=6-9) plants in near equal frequencies were considered heterozygous and removed from the QTL analysis. Mendelian ratios based on the classification of progeny into resistant (infection responses of 1-5) or susceptible (infection responses of 6-9) classes were not apparent. In addition to the seedling assays with isolate

ND4008, the Rasmusson/PI 466423 population was also evaluated against the commonly used isolate ND85F (pathotype 1) at both the seedling and adult stages. These experiments were done to determine the pathotype specificity of QTL conferring seedling resistance to isolate ND4008 and also to validate the MSDRH phenotype contributed by Rasmusson. Conditions and experimental design for seedling tests to isolate ND85F were the same as described above. Adult plant evaluations to isolate ND85F were conducted in the field on the Saint Paul campus of the University of Minnesota in 2015. For this experiment, seeds of the parents, progeny, and controls were sown in paired hill plots (15-25 seeds/plot) spaced 0.3 m apart with a four-row planter. On the sides adjacent to the hill plots, continuous spreader rows of the susceptible line ND5883 (PI 643237) were sown to facilitate development of a spot blotch epidemic. Inoculations were performed by placing about 40 g of barley straw infected with isolate ND85F at the base of the spreader row plants when they reached the mid-tillering stage of development (Fetch et al. 2008). Adult plant resistance was assessed at the mid-dough stage of development based on standard disease diagrams (James 1971) that describe the amount of leaf area affected by disease as well as one of four qualitative IR classes: resistant (R), moderately resistant (MR), moderately susceptible (MS), or susceptible (S) according to Fetch and Steffenson (1999). If more than one IR type was present on a progeny line, they were recorded in order of their relative frequency. The field experiment with isolate ND85F was conducted once in 2015 on a subset of the population as a

completely randomized design with two replicates (N = 145). Due to the spatial variation present in the spot blotch nursery, adult data were corrected with a Best Linear Unbiased Estimator (BLUE) using the PROC MIXED procedure in SAS (v.9.3, SAS Institute 2011).

QTL analysis

QGene was used in QTL analysis because it is capable of handling advanced backcross populations, which have an unbalanced population structure (Joehanes and Nelson 2008). The composite interval mapping (CIM) maximum likelihood estimator algorithm, developed by Lander and Botstein (1989), was used for all QTL analyses on all mapped markers. However, QTL traces in the figures include only a subset of markers so marker names would be legible. The markers included in these figures were selected to ensure full coverage across the chromosomes and be evenly spaced. Identified QTL were named according to the following convention: *R* Reaction to *C* Cochliobolus *s* sativus; *qtl* for quantitative trait locus; chromosome number; and the SNP marker most significantly associated with the trait.

Results

Genetic map construction

The final genetic map used for QTL analysis contains 1,083 polymorphic SNP markers, spanning a genetic distance of 1,147 cM (Appendix B). Overall, the

map is well-saturated with markers every 1-3 cM. However, there are one to several large gaps in each of the seven chromosomes as follows: 9 and 15 cM on chromosome 1H; 8, 14 and 15 cM on chromosome 2H; 7 and 19 cM on chromosome 3H, 7 cM on chromosome 4H; 8, 10 and 20 cM on chromosome 5H; 6 cM on chromosome 6H and 7 cM on chromosome 7H.

Disease phenotyping

Isolate ND4008. The controls reacted as expected to isolate ND4008 based on previous experiments: NDB112 exhibited an IR mode of 6-7 with a range of 5 to 8; Bowman an IR mode of 3-4 with a range of 2 to 5; and ND5883 an IR mode of 6-7 with a range of 5 to 8. The parents PI 466423 and Rasmusson differed markedly in their reactions giving IR mode 2 (range of 1 to 3) and 7 (range of 6 to 8), respectively (Fig. 3.1). Reactions of individual progeny from the Rasmusson/PI 466423 population ranged from 2 to 7, and the overall population mean was 4.60 with a standard deviation of 1.46 (Fig. 3.2). The frequency distribution for spot blotch reaction in the population was skewed toward those with lower IRs, suggesting that both parents were contributing alleles for resistance.

Isolate ND85F. The controls of NDB112, Bowman and ND5883 reacted as expected based on many previous experiments. For NDB112, this is an IR of 2-3;

Bowman an IR of 4-5 and ND5883 an IR of 7-8 (Fetch and Steffenson 1994, Valjavec-Gratian and Steffenson 1997). Both PI 466423 and Rasmusson were resistant to isolate ND85F at the seedling stage, exhibiting IRs of 3 (range of 2 to 4) and 3 (range of 3 to 4), respectively (Appendix E). Reactions of individual progeny from the Rasmusson/PI 466423 population ranged from 2 to 7, and the overall population mean was 3.79 with a standard deviation of 1.12 (Fig. 3.3). At the adult plant stage in the field, PI 466423 had an average severity of 63% (range: 50-90%, excluding a few outliers in a low disease section of the nursery) with mostly S-MS IRs. In contrast, Rasmusson exhibited a low average severity of 11.1% (range: 5-30%) with mostly R or R-MR IRs. The overall population mean severity was 17.3% with a standard deviation of 11.4 (Fig. 3.4). Distribution of disease severity of the progeny was skewed strongly toward lower severity levels (Fig. 3.4).

QTL analysis

Isolate ND4008. QTL for seedling resistance to isolate ND4008 were identified on chromosomes 1H (designated *Rcs-qt1-1H-12_30404*), 2H (*Rcs-qt1-2H-SCRI_RS_233272*), 4H (*Rcs-qt1-4H-SCRI_RS_168399*) and 5H (*Rcs-qt1-5H-SCRI_RS_138933*) (Fig. 3.5), explaining 10.3%, 7.4%, 6.4% and 8.4% of the phenotypic variation, respectively (Table 3.1). The resistance alleles for *Rcs-qt1-1H-12_30404*, *Rcs-qt1-4H-SCRI_RS_168399* and *Rcs-qt1-5H-SCRI_RS_138933*

were contributed by PI 466423, whereas the one for *Rcs-qt1-2H-SCRI_RS_233272* was contributed by Rasmusson (Table 3.1).

Isolate ND85F. Seedling resistance to isolate ND85F was controlled by a single QTL on chromosome 3H (*Rcs-qt1-3H-1630-1150*), explaining 9.5% of the variation (Table 3.1, Fig. 3.6). At the adult plant stage, one QTL on chromosome 2H (*Rcs-qt1-2H-12_10715*) was identified for resistance explaining 28.7% of the variation (Table 3.1, Fig. 3.7). The favorable alleles for both seedling and adult plant resistance QTL were contributed by Rasmusson.

Haplotype analysis

Information regarding the complete set of resistance QTL in select germplasm is of critical importance to breeders. Zhou and Steffenson (2013) showed that to achieve the highest (and presumably most durable) level of resistance possible in a collection of barley breeding germplasm, all three resistance QTL of the MSDRH must be present. Therefore, we conducted haplotype analysis to determine the effect of single and also various combinations of multiple resistance QTL in this population. Individuals were classified into groups according to their genotypes. For example, individuals lacking any resistance QTL had a haplotype of “AA” at *Rcs-qt1-1H-12_30404*, “BB” at *Rcs-qt1-2H-SCRI_RS_233272*, “AA” at *Rcs-qt1-4H-SCRI_RS_168399* and “AA” at *Rcs-qt1-5H-SCRI_RS_138933* on chromosomes 1H, 2H, 4H and 5H, respectively. In

contrast, individuals with all four resistance QTL had a haplotype of “BB” at *Rcs-qt1-1H-12_30404*, “AA” at *Rcs-qt1-2H-SCRI_RS_233272*, “BB” at *Rcs-qt1-4H-SCRI_RS_168399* and “BB” at *Rcs-qt1-5H-SCRI_RS_138933*. Individuals with one to three QTL had various combinations of the respective resistance alleles of “BB”, “AA”, “BB” and “BB.” The mean IR for progeny without any of the resistance QTL to isolate ND4008 was 5.6 compared with a mean IR of 2.3 (58.9% reduction) for those progeny with all four QTL (Table 3.2). Progeny with a single QTL exhibited a 10.7 to 14.3% reduction in the mean IR, whereas those with the 1H QTL exhibited a 25% higher mean IR. Progeny with two QTL exhibited a 32.4 to 46.4% reduction in the mean IR, and those with three QTL exhibited a 37.5 to 50.0% reduction (Table 3.2). The mean IR values for some of these haplotypes must be interpreted with caution because they were based on only a few progeny.

Data availability

Given that the primary source of resistance alleles in this population is a wild barley accession, there is potential for deleterious traits to be carried along with the target gene(s) through linkage drag. Therefore, data for disease phenotypes and several key agronomic traits of progeny from the Rasmusson/PI 466423 population are publicly available through the Triticeae Coordinated Agricultural Project (TCAP) at <http://triticeaetoolbox.org/barley/>. Seeds from this population have been deposited in the USDA-ARS NSGC in Aberdeen, ID and are assigned

Genetic Stock Hordeum (GSHO) numbers GSHO 13101-GSHO 13339 in the Germplasm Resources Information Network (GRIN).

Discussion

The durability of spot blotch resistance derived from line NDB112 has been remarkable in six-rowed malting barley cultivars. However, recent reports of *C. sativus* isolates with virulence for NDB112 (Ghazvini and Tekauz 2007, Gyawali 2010) highlight the vulnerability of current six-rowed cultivars to epidemics and underscores the urgency for identifying new sources of resistance. Extensive screening of *Hordeum* germplasm to isolate ND4008 revealed very few sources of resistance. These efforts led to the identification of 44 resistant accessions from the evaluation of 2,062 accessions in the NSGC barley core collection, 30 of which also had adult plant resistance (Y. Leng and S. Zhong *personal communication*). GWAS of this core collection identified resistance QTL in chromosomes 1H and 6H (*Rcs-qt1-1H-P3* and *Rcs-qt1-6H-P3*) in the whole panel and ones in chromosomes 3H (*Rcs-qt1-3H-P3*) and 2H (*Rcs-qt1-2H-P3*) when the two- and six-rowed subsets, respectively, were considered separately (R. Wang and S. Zhong *personal communication*). An independent screening effort in our laboratory identified wild barley accession PI 466423 as a source of resistance to isolate ND4008. This wild barley accession was previously used as a source of *Fusarium* head blight resistance in the advanced backcross population of Rasmusson/PI 466423 (Dahl et al. 2009).

In this study, we utilized the Rasmusson/PI 466423 population to characterize the genetic architecture of resistance to the virulent *C. sativus* isolate ND4008. We detected three resistance QTL contributed by PI 466423 residing in chromosomes 1H, 4H and 5H, and one additional one contributed by Rasmusson residing in chromosome 2H (Fig. 3.5 and Table 3.1). The four combined alleles of these resistance QTL contributed to a 58.9% reduction in the seedling IR compared to progeny carrying no resistance QTL (Table 3.2). Of the four QTL for resistance to isolate ND4008 identified in this study, only one resides in a chromosomal region that is likely coincident with an allele of the MSDRH. *Rcs-qtl-1H_12_30404* maps to 42.2 cM in chromosome 1H, a position very close to QTL *Rcs-qtl-1H-11_10764* at 41.0 cM reported by Zhou and Steffenson (2013). Although marker 11_10764 maps to 33.6 cM in the Rasmusson/PI 466423 population, it still falls within the confidence interval for QTL *Rcs-qtl-1H_12_30404* when the CIM algorithm is applied (Table 3.1). QTL *Rcs-qtl-1H-11_10764* explains the highest percentage of variation for seedling resistance to isolate ND85F of the MSDRH (Zhou and Steffenson 2013). The genotypes at SNP marker 11_10764 differ between PI 466423 and Rasmusson. Despite segregation for 11_10764 in the Rasmusson/PI 466423 population, this marker was not found significantly associated with resistance to ND85F—the identical isolate used previously by Zhou and Steffenson (2013). One possible reason for the lack of detection of QTL *Rcs-qtl-1H-11_10764* is that both parents may carry an effective resistance allele at that locus or that a recombination occurred

between the resistance QTL and 11_10764, resulting in the observed segregation for the marker. With its coincident mapping position to *Rcs-qtl-1H-11_10764* in chromosome 1H, *Rcs-qtl-1H_12_30404* may represent a novel allele at this previously identified locus of the MSDRH or a closely linked locus. Additional genetic studies will be needed to resolve this question. A QTL conferring resistance to isolate ND4008 was identified in chromosome 1H in NSGC germplasm through GWAS, but it maps to the long arm of chromosome 1H and is therefore unique from the one identified in this study (R. Wang and S. Zhong *personal communication*). In this study, the PI 466423 allele of QTL *Rcs-qtl-4H-SCRI_RS_168399* conferring resistance to isolate ND4008 was identified at 137.7 cM in chromosome 4H (Table 3.1, Fig. 3.5C). A minor effect QTL (*Rcs-qtl-4H-10-11*) conferring seedling resistance to isolate ND85F was reported by Bilgic et al. (2006) in this same interval in the Calicuchima-sib/Bowman-BC population; thus, *Rcs-qtl-4H-SCRI_RS_168399* may be detecting this same resistance locus. The resistance QTL *Rcs-qtl-5H-SCRI_RS_138933*, also identified in this study, maps to 147 cM in chromosome 5H (Table 3.1, Fig. 3.5D). In a previous investigation, Bilgic et al. (2005) identified a spot blotch resistance QTL (*Rcs-qtl-5H-10-11*) around 150 cM in the Harrington/TR306 population; thus, it is possible that *Rcs-qtl-5H-SCRI_RS_138933* is coincident with this QTL in chromosome 5H. Two other resistance QTL were found by Roy et al. (2010) in bins 6-7 and 14 of chromosome 5H through GWAS of the of the Wild Barley

Diversity Collection (WBDC), but neither is coincident with *Rcs-qt1-5H-SCRI_RS_138933*.

The resistance QTL *Rcs-qt1-2H-SCRI_RS_233272* mapped to 8.5 cM in chromosome 2H and was contributed by Rasmusson (Table 3.1, Fig. 3.5B). Roy et al. (2010) found SNP marker 12_10970 (*Rcs-qt1-2H-12_10970*) significantly associated with spot blotch resistance in a GWAS of the WBDC. That resistance QTL maps close to *Rcs-qt1-2H-SCRI_RS_233272* at 7.8 cM in chromosome 2H, but is not part of the MSDRH. Thus, the coincident mapping locations suggest that QTL *Rcs-qt1-2H-SCRI_RS_233272* may not be a novel spot blotch resistance locus. Another resistance QTL effective against isolate ND4008 was identified in the long arm of chromosome 2H by GWAS in the six-rowed panel of the NSGC (R. Wang and S. Zhong *personal communication*). The QTL *Rcs-qt1-2H-SCRI_RS_233272* identified in this study maps to the short arm of chromosome 2H and is therefore a distinct locus for resistance to isolate ND4008.

Two distinct QTL for resistance to isolate ND85F were detected in the Rasmusson/PI 466423 population: one in chromosome 3H at the seedling stage (Fig. 3.6) and one in chromosome 2H at the adult plant stage (Fig. 3.7). The seedling resistance QTL in chromosome 3H, *Rcs-qt1-3H-11_1630-1150*, is located near the reported position of the 3H QTL (*Rcs-qt1-3H-11_10565*)

comprising part of the MSDRH (Zhou and Steffenson 2013). The adult plant resistance QTL in chromosome 2H, *Rcs-qtl-2H-12_10715*, was mapped to 42.3 cM in chromosome 2H in the study presented here (Table 3.1, Fig. 3.7) and is likely coincident with one (*Rcs-qtl-2H-3-5*) contributed by Morex at this position in the Steptoe/Morex population (Bilgic et al. 2005). However, the chromosome 2H QTL *Rcs-qtl-2H-3-5* found by Bilgic et al. (2005) is not part of the MSDRH. Based on haplotype analysis by Zhou and Steffenson (2013), Rasmusson should carry the complete MSDRH like all other six-rowed malting cultivars from the Midwest. However, in this study, the other two QTL of the MSDRH (i.e. *Rcs-qtl-1H-11_10764* and *Rcs-qtl-7H-11_20162* in chromosomes 1H and 7H, respectively) were not identified in either the seedling or adult plant evaluations to ND85F, the standard *C. sativus* isolate used in nearly all previous genetic studies. One possible explanation for this is that PI 466423 may carry the same resistance alleles as Rasmusson at these loci, and therefore no segregation occurred in the population. Another possibility is that QTL x genotype interactions masked the presence of *Rcs-qtl-1H-11_10764* and *Rcs-qtl-7H-12_20162*.

Haplotype analysis provided some insight into the effects of the four QTL identified for resistance to isolate ND4008 in this study, albeit with different levels of robustness given the low number of progeny samples in some cases. Progeny with a single QTL exhibited a 10.7 to 14.3% reduction in the mean IR, but the 1H QTL surprisingly exhibited a 25% higher mean IR (Table 3.2). This unexpected

value may be due to the low number of samples (two) upon which the mean was based, but could also indicate that the presence of other QTL are required for effective resistance. Progeny with two QTL exhibited a 32.4 to 46.4% reduction in the mean IR, those with three QTL exhibited a 37.5 to 50.0% reduction, and those with all four QTL exhibited a 58.9% reduction (Table 3.2). Thus, to achieve the highest level of resistance possible to isolate ND4008, it is recommended that all four QTL be combined. Since it appears that three of the four QTL for resistance to isolate ND4008 are independent of the MSDRH, it should be possible to combine all of the resistance alleles in a broadly effective haplotype. An advantage of the advanced backcross population structure used in this study is that it yielded several agronomically advanced lines that are resistant to both isolates ND85F and ND4008, which can be used directly as parents in a breeding program for development of broadly resistant cultivars.

Surprisingly, the QTL contributing resistance to isolates ND4008 and ND85F were all of minor effect, each explaining less than 11% of the total phenotypic variation (Table 3.1). A possible explanation for this result is that the population is derived from just 68 BC₁ individuals, which limits effective recombination and means that many of the 244 BC₂F₄ individuals are more closely related than if they had been derived from 244 BC₁ individuals. The close relatedness of some progeny can result in an overestimation of allelic effects. Thus, the actual allelic effect of *Rcs-qt1-1H-12_30404*, *Rcs-qt1-4H-SCRI_RS_168399* and *Rcs-qt1-5H-*

SCRI_RS_138933 (all derived from PI 466423) could be greater, while *Rcs-qtI-2H-SCRI_RS_233272* and *Rcs-qtI-3H-1630-1150* (both derived from Rasmusson) may be lower than these estimates. The phenotypic variation for spot blotch resistance found in previous biparental populations was typically much higher, although this was not true for all identified QTL as some values were on the same order of magnitude as those found in this study (Alsop 2009, Bilgic et al. 2005, Steffenson et al. 1996, Yun et al. 2005). The missing variance may also be attributed to the effect of other resistance loci not detected here due to fixation of resistance alleles in the parents or hidden heritability as suggested by Zhou and Steffenson (2013). To dissect the effect of these resistance QTL with greater genetic resolution, the Rasmusson/PI 466423 population should be expanded to include more individuals derived from independent BC₁ plants. This approach should result in greater marker density, since there would be additional recombinations across the genome, closing some of the large gaps observed in the current population. Alternatively, QTL validation populations could be developed with progeny from this population.

In addition to being used as the base population for identifying QTL for resistance to isolate ND4008, the Rasmusson/PI 466423 population can serve as a source of resistant parents for the next breeding cycle and also as a training population for genomic selection. Some of the most resistant progeny from Rasmusson/PI 466423 population have been used as parents in the Minnesota barley breeding

program. Preliminary screening tests of the F₃ generation have identified lines with resistance to isolate ND4008, which could be used for validation studies in the future. Genomic selection should hasten the recovery of advanced germplasm lines that combine the MSDRH with a high level of resistance to isolate ND4008.

In the Midwest production region, spot blotch infection usually increases markedly after anthesis; thus, adult plant or all-stage resistance is key in protecting against losses. In this study, the phenotyping tests conducted in response to isolate ND4008 were done only at the seedling stage in the greenhouse because of the threat this isolate poses to barley production in the field. A preliminary test for adult plant resistance was conducted on six progeny showing seedling resistance in the greenhouse. Several, but not all of these progeny exhibited MR to R reactions at the adult plant stage. These results suggest that the seedling resistance identified in our study can be effective at the adult plant stage in some genotypes, although not to the same high degree as the resistance derived from NDB112 to isolate ND85F. Additional phenotyping tests should be done on adult plants in the greenhouse to identify the most resistant progeny for use in breeding programs. The information and germplasm generated from this study should facilitate the development of advanced germplasm with more broad-based spot blotch resistance before virulent isolates such as ND4008 become widespread.

Table 3.1. The chromosomal position, allelic effect (α), phenotypic variance (R^2), and LOD scores of quantitative trait loci (QTL) for resistance to *Cochliobolus sativus* isolates ND4008 (N = 244) and ND85F at the seedling (N = 244) and/or adult plant stages (N=145) in the Rasmusson/PI 466423 advanced backcross population.

QTL	Chromosome	Position (cM)	Confidence Interval	Allelic Effect (α)	R^2	LOD
Isolate ND4008 (N=244)						
<i>Rcs-qt1-1H-12_30404</i>	1H	42.2	SCRI_RS_4891 - SCRI_RS_132028	0.58	0.103	5.76
<i>Rcs-qt1-2H-SCRI_RS_233272</i>	2H	8.5	SCRI_RS_233272 ^c	-0.55	0.074	4.08
<i>Rcs-qt1-4H-SCRI_RS_168399</i>	4H	137.5	BOPA2_12_30655 - BOPA1_5611-811	0.57	0.064	3.53
<i>Rcs-qt1-5H-SCRI_RS_138933</i>	5H	147.0	SCRI_RS_168141 - SCRI_RS_13320	0.61	0.084	4.66
Isolate ND85F seedling (N=244)						
<i>Rcs-qt1-3H-11_1630-1150</i>	3H	6.9	BOPA1_1630-1150 – BOPA1_6573-369	-0.30	0.095	5.31
Isolate ND85F adult (N=145)						
<i>Rcs-qt1-2H-12_10715</i>	2H	36.4	BOPA2_12_10715 – BOPA2_10219	-1.32	0.287	17.96

^a Composite interval mapping maximum likelihood estimator (QGene CIM MLE) was applied to seedling infection response. Values for α are in reference to the Rasmusson allele: positive values indicate that PI 466423 contributed the resistance allele, while negative values indicate that Rasmusson contributed the resistance allele.

^b Significance thresholds varied between permutation tests, but usually fell between 3.5 and 3.6 for significance at 0.01.

^c Confidence interval for *Rcs-qt1-2H-SCRI_RS_233272* is narrow.

Table 3.2. Number of progeny of the Rasmusson/PI 466423 advanced backcross population with resistance allele(s) at each QTL, the associated infection response (IR), range of IR for each class and the % reduction in IR compared to those progeny that carry none of the resistance allele(s)^a.

QTL combination	No. of Individuals	Average IR	IR Range	% Reduction
No resistance QTL				
0 QTL	29	5.6	3 - 8	-
Any single identified QTL				
1H only	2	7.0	7 - 7	+25.0 ^b
2H only	133	4.9	3 - 7	12.5
4H only	4	4.8	3 - 6	14.3
5H only	2	5.0	4 - 6	10.7
Any pair of identified QTL				
1H + 2H	36	3.7	2 - 7	33.9
1H + 4H	0	ND	ND	ND
1H + 5H	0	ND	ND	ND
2H + 4H	11	3.6	3 - 6	35.7
2H + 5H	16	3.8	3 - 5	32.4
4H + 5H	1	3.0	3	46.4
Any triplet of identified QTL				
1H + 2H + 4H	2	3.5	3 - 4	37.5
1H + 2H + 5H	4	2.8	2 - 4	50.0
1H + 4H + 5H	1	3.0	3	46.4
2H + 4H + 5H	0	ND	ND	ND
All identified QTL				
4 QTL	3	2.3	2 - 3	58.9

^a Data is presented first without regard to specific QTL, followed sequentially by all possible combinations of one, two, and three QTL in order to assess the relative benefit of each QTL.

^b This represents an increase in disease severity.

ND = No data

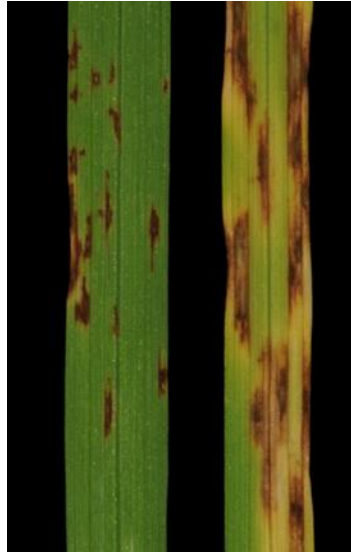


Figure 3.1. The seedling infection response of PI 466423 (left) and Rasmusson (right) infected with *Cochliobolus sativus* isolate ND4008 in the greenhouse.

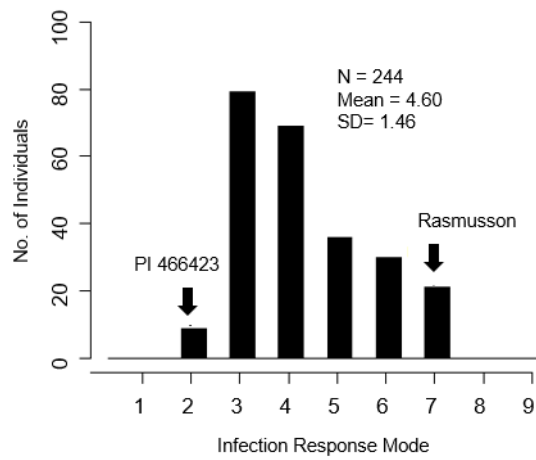


Figure 3.2. Frequency distribution of the seedling infection response mode of progeny from the Rasmusson/PI 466423 advanced backcross population to *Cochliobolus sativus* isolate ND4008 in the greenhouse.

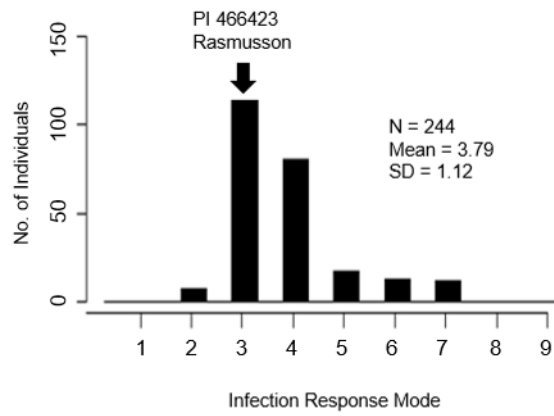


Figure 3.3. Frequency distribution of the seedling infection response mode of progeny from the Rasmusson/PI 466423 advanced backcross population to *Cochliobolus sativus* isolate ND85F in the greenhouse.

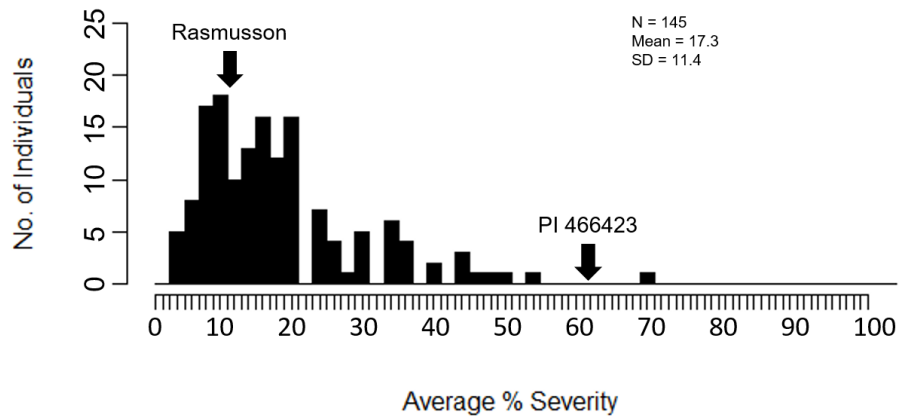


Figure 3.4. Frequency distribution of the mean adult plant spot blotch severity of progeny from the Rasmusson/PI 466423 advanced backcross population to *Cochliobolus sativus* isolate ND85F in the field at Saint Paul, Minnesota.

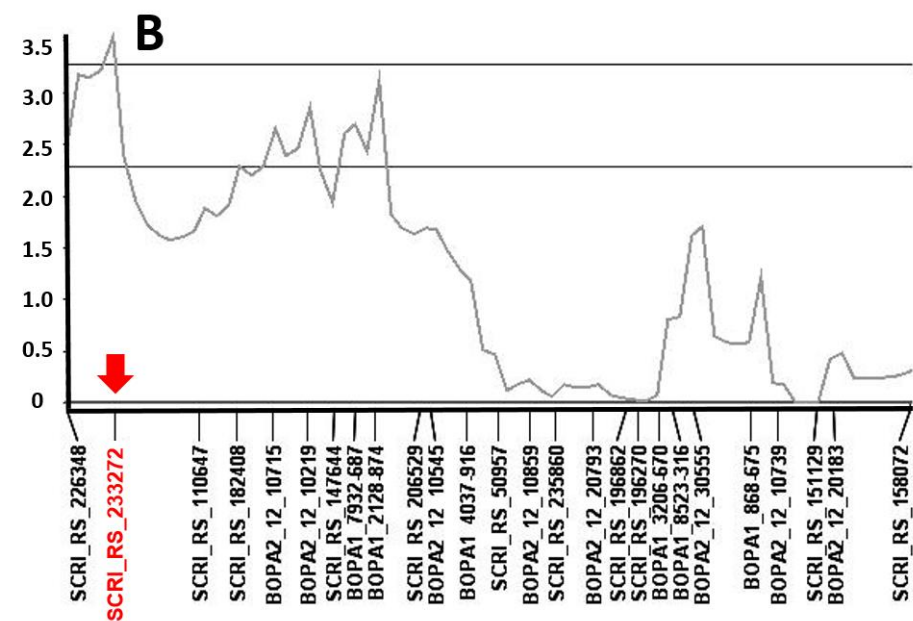
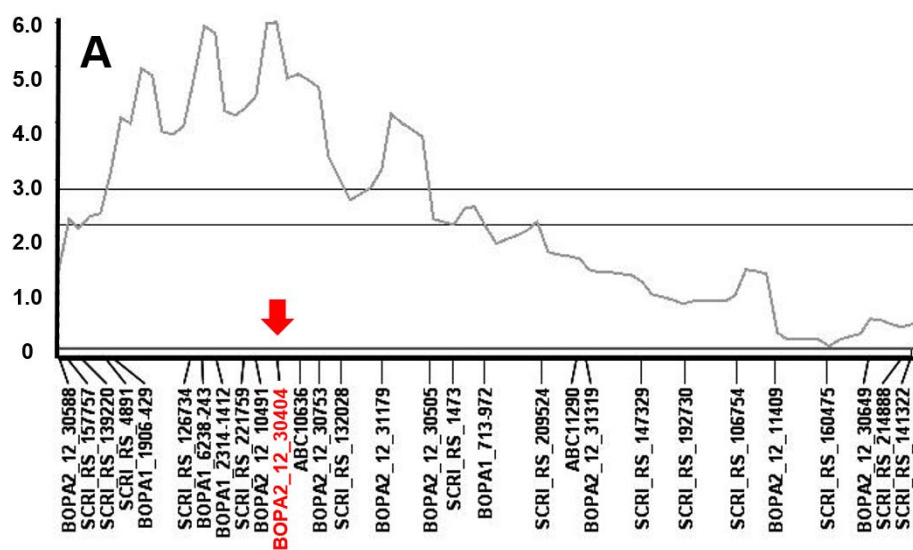


Figure 3.5. Quantitative trait loci traces for seedling resistance to *Cochliobolus sativus* isolate ND4008 in the Rasmusson/PI 466423 advanced backcross population (N = 244) using the composite interval mapping maximum likelihood estimator (CIM MLE) function of QGene. (A) Chromosome 1H, (B) Chromosome 2H, (C) Chromosome 4H, and (D) Chromosome 5H.

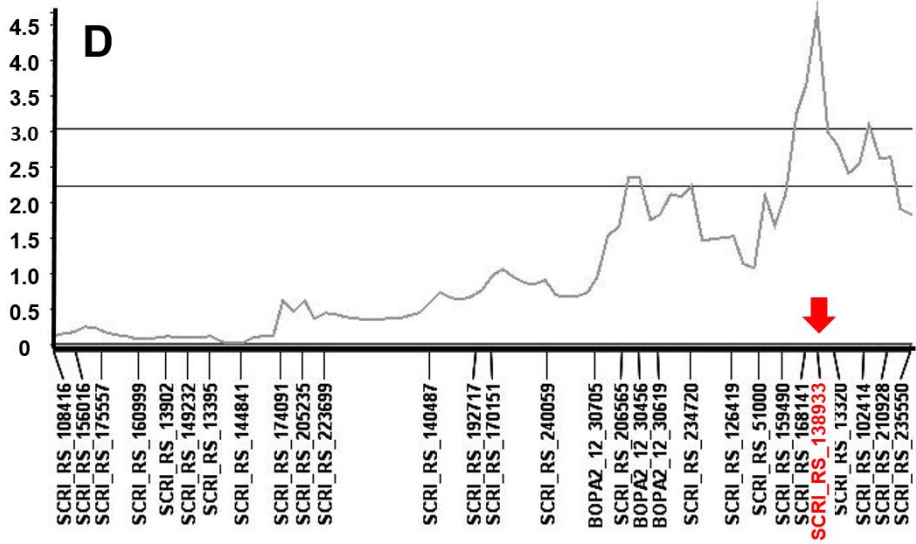
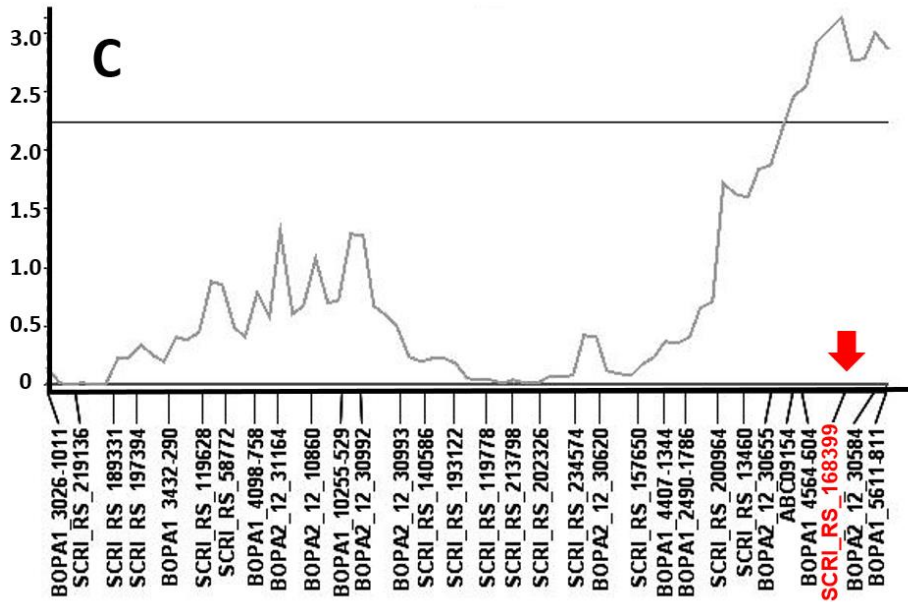


Figure 3.5. Continued.

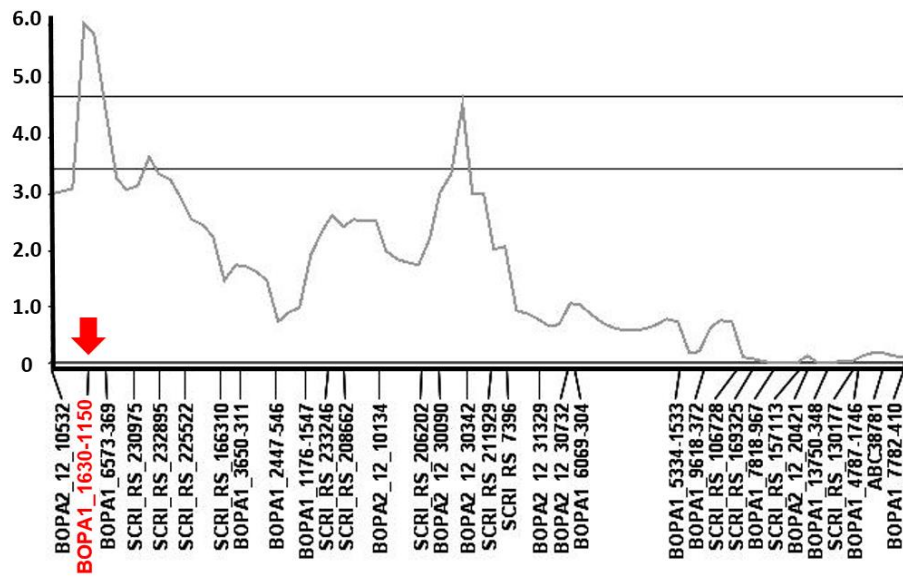


Figure 3.6. Quantitative trait loci trace on chromosome 3H for seedling resistance to *Cochliobolus sativus* isolate ND85F in the Rasmusson/PI 466423 advanced backcross population (N = 244) using the composite interval mapping maximum likelihood estimator (CIM MLE) function of QGene.

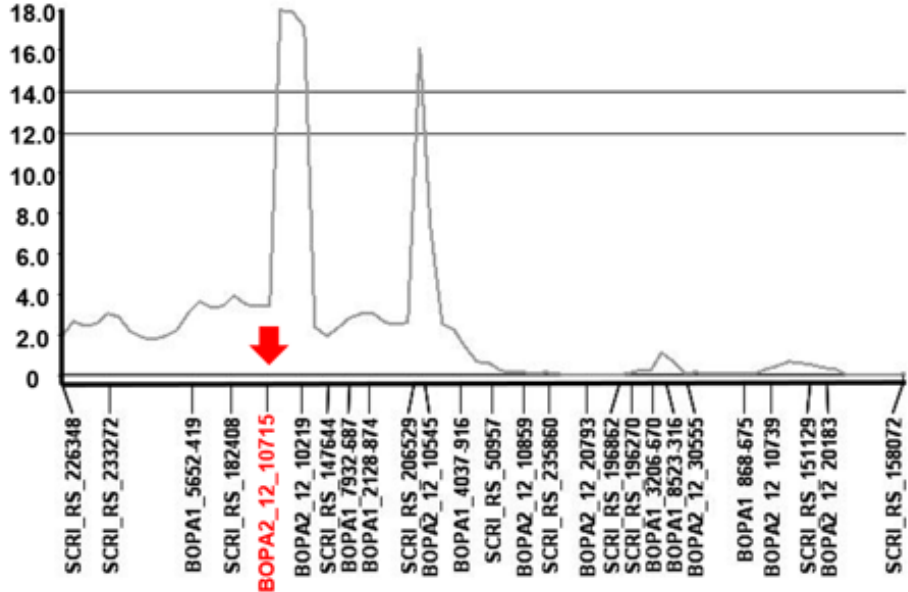


Figure 3.7. Quantitative trait loci trace on chromosome 2H for adult plant resistance to *Cochliobolus sativus* isolate ND85F in the Rasmusson/PI 466423 advanced backcross population (N = 145) using the composite interval mapping maximum likelihood estimator (CIM MLE) function of QGene.

Chapter 4

A Description of the Spot Blotch Susceptible Morex Mutant 14-40

Introduction

'Morex' is a six-rowed Midwestern malting barley cultivar (Rasmusson and Wilcoxson 1979) that carries durable resistance to two devastating diseases in barley: stem rust and spot blotch caused by *Puccinia graminis* f. sp. *tritici* and *Cochliobolus sativus*, respectively. The resistance to stem rust is conferred by the resistance gene *Rpg1* (Brueggeman et al, 2002), and the resistance to spot blotch is conferred by the Midwest Six-rowed Durable Resistance Haplotype (MSDRH) (Zhou and Steffenson 2013). As part of a broad approach to investigate the molecular basis of the resistance pathways in barley, we identified and characterized mutants with compromised resistance reactions to the two diseases. We screened gamma irradiated seed of 'Morex' in a stem rust/spot blotch disease nursery in St. Paul in 2008. From 3,000 M₂ families, we found seven and nine barley lines with possible compromised resistance reactions to stem rust and spot blotch, respectively. Here, we describe a Morex mutant (Mx14-40) that is highly susceptible to spot blotch at the seedling stage (Fig. 4.1). Additionally, in the absence of the pathogen, this mutant exhibits extremely large necrotic lesions that mimic those of highly susceptible barley lines infected with the disease at the adult plant stage (Fig. 4.2). The susceptibility of Mx14-40 to spot blotch at the adult stage is confounded with the adult onset necrosis.

Many disease lesion mimic mutants have been reported in different plants (Johal et al. 1995, Rostoks et al. 2003, Rostoks et al. 2006). These mutants were demonstrated to be defective in a variety of proteins involved in the regulation of

cell death by lowering the activation threshold or the inability to arrest the cell death cascade once it has begun (Johal et al. 1995). The phenotypes of other disease lesion mimic mutants are due to auto-active R genes (Wang et al. 2015). Clearly, defects in the complex resistance pathways can have diverse and profound effects on the regulation of cell death.

The genetic architecture of durable spot blotch resistance in Midwestern six-rowed barley has been well-characterized (Zhou and Steffenson 2013), and is associated with three quantitative trait loci (QTL) located in chromosomes 1H, 3H, and 7H that, in combination, reduce the seedling infection response by 47% and adult plant disease severity by 83% (Zhou and Steffenson 2013). However, less is known about the specific mechanism underlying the resistance. To further elucidate the resistance response, we created a segregating population from true breeding (homozygous) individuals of the spot blotch susceptible mutant Mx14-40 and the resistant wild type Morex (Morex WT). Progeny were phenotyped in the F₃ generation to recover homozygous resistant and homozygous susceptible families. Homozygous susceptible families were recovered from the F₃ generation and verified in the F₄ and F₅ generations. Homozygous resistant families were identified in the F₃ generation, but none of them exhibited the distinctly low infection responses typical of the cultivars carrying the MSDRH. This result suggests that there are multiple mutations in Mx14-40 that affect the reaction to spot blotch or that some component of the MSDRH has been lost in

the progeny of the Mx4-40/Morex WT population. Phenotyping of the Mx14-40/Morex WT population at the seedling stage showed that susceptibility to spot blotch is inherited recessively, albeit not in a Mendelian fashion among F₂ progeny, which segregated 61:11 for resistant : susceptible ($X^2=3.2$, $p=0.0736$) and 27:30:15 (resistant : segregating : susceptible) among the F₃ families tested ($X^2=6.0$, $p=0.0498$), highlighting the complexity of the molecular mechanisms underlying this trait. Other crosses created with Mx14-40 as the susceptible parent support this mode of inheritance (J. Franckowiak, *personal communication*). Mutant Mx14-40 and the Morex WT were also used in an RNAseq experiment that is described in Chapter 5 of this thesis. This study was undertaken to elucidate the molecular basis of the contrasting disease phenotypes, thereby providing a greater understanding of the spot blotch resistance pathways in durably resistant cultivars like Morex. This report describes the phenotype of mutant Mx14-40 in detail. ‘

Materials and Methods

Identification of Mutant Mx14-40

A bulk collection of ‘Morex’ seed was irradiated with a dose of 15 kR (kilo roentgen) gamma radiation (15,000 R = 150 Gy) by A. Kleinhofs at Washington State University (Pullman, WA, USA) in 2005. The M₁ generation was grown in Pullman, and the resulting M₂ generation was sown at the Minnesota Agricultural Experiment Station on the Saint Paul campus of the University of Minnesota

(Saint Paul, MN, USA) in 2008. Mutant Mx14-40 was a single plant selection from an M₂ family that was segregating for spot blotch severity and infection response (Fetch and Steffenson 1999). The Morex genetic background of Mx14-40 was confirmed with cultivar-specific probes and also SNP genotyping (Close et al. 2009, Comadran et al. 2012) in comparison to Morex WT (*data not shown*).

Disease phenotyping experiments in the greenhouse and growth chamber

Mutant Mx14-40, Morex WT, and the derived segregating population were tested for reaction to *C. sativus* isolate ND85F in the greenhouse and growth chamber. Seeds were sown in cone-tainers (5 cm diameter x 18 cm depth, Stuewe and Sons, Inc., Tangent, OR) filled with a 50:50 mixture of steam-sterilized soil and Sunshine MVP potting mix (Sun Gro Horticulture, Quincy, MI). The cone-tainers were held in racks at 50 per rack and spaced out to 25 per rack after inoculation. After the spot blotch assays, all viable progeny from the F₂ generation were transplanted to obtain F₃ lines. In subsequent generations, only progeny lines showing clear resistant and susceptible reactions were advanced.

Plants for observation of lesion development were sown in square plastic pots (13.3 x 13.3 x 10.2 cm, l x w x h) filled with the same mixture specified above. At planting, all pots were fertilized with Osmocote slow-release fertilizer (14-14-14 N-P-K) as well as water-soluble Peters Dark Weather fertilizer (15-0-15) (Scott's Company, Marysville, OH). After these initial fertilization treatments, plants were

fertilized every two weeks with a 20-20-20 N-P-K fertilizer (J.R. Peters Inc., Allentown, PA). In the greenhouse, plants were subjected to 16 hours light/8 hours darkness with a temperature range of 20-25°C. Supplemental greenhouse light was provided by sodium vapor lights emitting $>300 \mu\text{mol photons s}^{-1} \text{ m}^{-2}$. In the growth chamber experiment, the photoperiod and temperature were the same, but light was provided by florescent lights emitting $1400 \mu\text{mol photons s}^{-1} \text{ m}^{-2}$.

Results

Mutant Mx14-40 closely resembles the Morex WT for all gross morphological traits and also development. Both genotypes germinate and emerge from the soil at the same time after planting with subsequent development continuing at the same rate for several weeks after planting. Under the described growth conditions, Mx14-40 begins to exhibit spontaneous necrotic lesions about 4 to 5 weeks (± 3 days) after planting, whereas the Morex WT remains completely free of such symptoms. The lesions forming on Mx14-40 are very large (3-4 cm) and closely resemble those exhibited by susceptible barley genotypes infected with *C. sativus*. Despite the difficulty in precisely predicting when these necrotic lesions will appear, they always manifest themselves on the oldest leaves first.

The lesions start as very small, round spots (1-2 mm) and then grow increasingly larger (3-4 cm) during the weeks that follow. Lesions are initially light brown and diffuse when viewed from the adaxial leaf surface, but are a much darker shade

of brown when viewed from the abaxial side of the leaf. Size expansion of existing lesions proceeds at a slow rate following the initial appearance of symptoms. The same is true for the number of new lesions forming on leaves. Once tillering ceases and the flag leaves fully emerge, the size of existing lesions and the number of new lesions forming increase markedly (Fig. 4.3-4.5). Once the plant has fully matured, necrotic lesions are present on every leaf of the mutant plant. At this stage, lesions often coalesce with one another, resulting in large sections of the leaves being necrotic (Figure 4.2).

Natural greenhouse infection of adult plants of mutant Mx14-40 by powdery mildew (*Blumeria graminis* f. sp. *hordei*) resulted in the premature formation of necrotic lesions around the powdery mildew infection sites, suggesting that microbial infection is capable of inducing lesion formation. One hypothesis to explain the appearance of these lesions is that they are caused by hypersensitivity to ambient non-pathogens (i.e. *Alternaria* or *Trichoderma*), meaning that mutant Mx14-40 is an activation mutant as proposed by Johal et al. (1995). An independent observation on plants free of any possible powdery mildew infection revealed that, from a whole-plant perspective, appearance of the necrotic lesions is non-random. The location of necrotic lesions forming on any given leaf of a plant is seemingly unpredictable; however, the progression of necrotic lesion development follows a predictable pattern. Lesions invariably first appear on the oldest leaves. Following initial appearance of the necrotic lesions

on basal leaves, new lesions begin to appear on younger leaves of the same affected tillers. If *Alternaria*- or *Trichoderma*-induction of a hypersensitive reaction was responsible for the appearance of these lesions, we would expect a random pattern of the lesions on the barley plant. Since the appearance and rate of development of these lesions depends on the presence of lesions on more mature leaves on the same tiller, we can infer that fungus-induced hypersensitivity is probably not involved. To provide further evidence that this hypersensitivity is not induced by a fungal microbe, the Mx14-40 and Morex WT would need to be grown to full maturity in a completely sterile environment (i.e., planting disinfected seeds in sterile environment, grown in a hydroponic solution inside a chamber with filtered air). Such an experiment may not even be possible. The observed pattern of lesion development suggests there is a cell death-promoting signal that originates from initial lesions of older basal leaves that spreads to distal regions of younger leaves in affected tillers through the vascular system.

Discussion

Mutant Mx14-40 provides a unique opportunity to study regulation of the cell death pathway in barley, in addition to spot blotch susceptibility. The genomic locus (or loci) of the mutation(s) in Mx14-40 remain unknown; however, gamma radiation is capable of causing a wide range of genomic alterations, ranging from small point mutations to rearrangements of entire chromosomal segments

throughout the genome (Sachs et al. 2000). The RNAseq experiment described in Chapter 5 of this thesis did not identify informative deletions in candidate genes. Moreover, due to the close genetic similarity of parents in the Mx14-40/Morex WT population, QTL mapping of the causal locus was not possible because the available SNP markers were monomorphic. We cannot exclude the possibility that a mutation in a promoter or enhancer is responsible for the altered gene expression rather than a deletion or mutation in an exonic region.

Techniques such as florescent in-situ hybridization (FISH) could enable detection of chromosomal regions that are altered in the mutant compared with the wild type. In addition, a segregating population should be made between mutant Mx14-40 and an accession genetically dissimilar to Morex to enable the genetic mapping of seedling spot blotch susceptibility as well as adult-onset necrosis since the two traits co-segregate.

The available observation data suggests that Mx14-40 is a propagation mutant (Johal et al. 1995), meaning that it is unable to arrest the pro-cell death signal. Chapter 5 of this thesis will propose a potential candidate for that cell death signal. Mutant Mx14-40 is unique because it forms exceptionally large necrotic lesions that closely resemble those induced by *C. sativus* infection of a susceptible barley genotype at the adult plant stage. As such, Mx14-40 should be of interest to the barley genetics community as well as other researchers interested in disease resistance and cell death regulatory pathways.

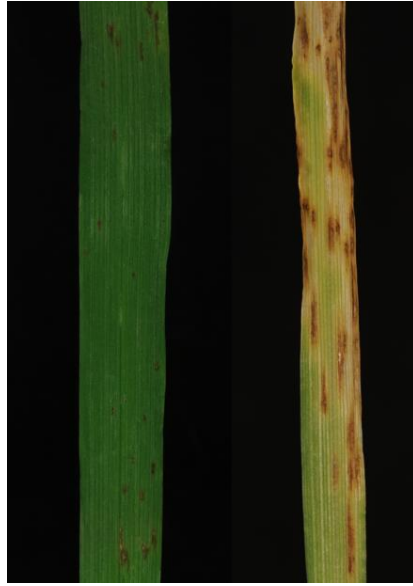


Figure 4.1. Seedling response to infection by *Cochliobolus sativus* isolate ND85F for Morex wild-type (left) and mutant Mx14-40 (right).

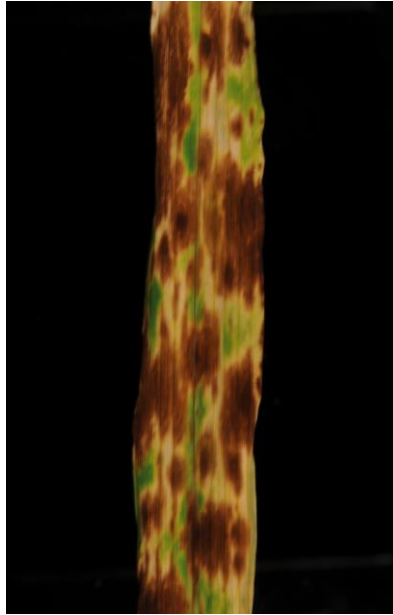


Figure 4.2. Adult-onset necrosis symptoms of mutant Mx14-40.

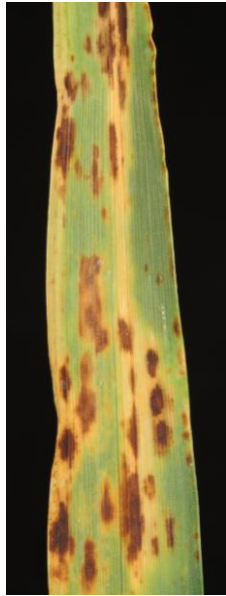


Figure 4.3. Adult onset necrosis of Mutant Mx14-40 on the flag-3 leaf after anthesis.



Figure 4.4. Adult onset necrosis of Mutant Mx14-40 on the flag-2 leaf after anthesis.



Figure 4.5. Adult onset necrosis of Mutant Mx14-40 on the flag-1 leaf after anthesis.

Chapter 5

RNAseq Reveals a Role for Lipid Metabolism in the Resistance Response of Barley to Infection by the Spot Blotch Pathogen *Cochliobolus sativus*

Introduction

Spot blotch, caused by *Cochliobolus sativus* (Ito & Kurib.) Drechs ex. Dastur (anamorph: *Bipolaris sorokiniana* (Sacc.) Shoem.), is an important disease of barley (*Hordeum vulgare* L.) that can cause up to 30% yield loss in susceptible cultivars (Mathre et al. 1997). Over the past 50 years, the disease has been of little consequence in the Upper Midwest region because the widely grown six-rowed cultivars carry durable resistance (Johnson 1984), contributed by the breeding line NDB112. Quantitative trait loci (QTL) mapping and genome-wide association studies (GWAS) have revealed that the NDB112-derived resistance is conferred by three QTL on chromosomes 1H, 3H, and 7H, referred to as the Midwest Six-rowed Durable Resistance Haplotype (MSDRH). (Steffenson et al. 1996, Bilgic et al. 2005, Roy et al. 2010, Zhou and Steffenson 2013). Despite the wealth of knowledge about the genetic architecture of durable spot blotch resistance, little is known about the molecular basis for resistance.

Elucidation of the molecular pathways underlying resistance may be useful for identifying breeding targets that contribute to durable resistance. In one investigation, Drader et al. (2010) attempted to clone the spot blotch resistance gene *Rcs5*, which confers seedling resistance to *C. sativus* isolate ND85F (Steffenson et al. 1996) and is also coincident with the chromosome 7H QTL (*Rcs-qtl-7H-11_20162*) of the MSDRH. That study narrowed the *Rcs5*-containing region to a small window containing two wall-associated kinases, suggesting a

putative function of *Rcs5*. In another study using a microarray, Millett et al. (2009) investigated the transcriptomic response of barley to *C. sativus*; however, the main focus of that study was to contrast the defense response of barley to *C. sativus* (a hemi-biotroph) and the leaf rust pathogen *Puccinia hordei* (a biotroph). The primary conclusion of that study was that genes related to oxidative stress are important for conferring the resistance of barley to *C. sativus* and that the pathogen switches its lifestyle from biotrophic to necrotrophic around 24 hours after inoculation. In particular, the precise timing of the accumulation and removal of reactive oxygen species (ROS) determines the effectiveness of the defense response (Millett et al. 2009).

Mutagenesis is a common technique for inducing variation in plants (Sikora et al. 2011). Generally, mutations are inherited in a recessive manner since they lead to a loss of function in the genes of the wild-type (Koornneef 2002). In barley, mutations result in a wide variety of morphological alterations, including chlorina, albino, brachytic (dwarf), eceriferum (glossy), and others (Lundqvist 2005). One particularly common mutant class is the disease lesion mimic, so named for their close resemblance to disease-induced lesions caused by necrotrophic organisms (Johal et al. 1995). To explore the spot blotch resistance pathways in a cultivar carrying the MSDRH (Zhou and Steffenson 2013), we treated seed of cultivar Morex (a six-rowed malting barley cultivar from Minnesota) (Rasmusson and Wilcoxson 1979) with gamma-irradiation and screened M₂ families for

segregation for spot blotch reaction. One putative mutant identified in the field (Mx14-40) proved to be highly susceptible to spot blotch at the seedling stage (Fig. 5.1). Further observations of this mutant revealed that it also exhibits very large disease mimic lesions at the adult plant stage (Fig. 5.2). The mutant may also be susceptible to spot blotch at the adult stage, but the similarity between the disease mimic lesions and those actually caused by *C. sativus* made it difficult to discern the relative contribution of each factor. The genomic position of the mutated gene(s) contributing to the disease mimic lesions in Mx14-40 are unknown at this time.

The regulation of cell death is important for the plant defense response to pathogens. In plants, the rapid induction of cell death is an important defense response against biotrophic organisms (i.e. rust and mildew pathogens) because damage can be confined to a small number of cells (Glazebrook 2005).

Necrotrophic organisms feed on dead plant tissue or actively kill living plant tissue through the use of toxins, so a successful plant defense response would be to keep infected cells alive (Glazebrook 2005). A third parasitic lifestyle is hemi-biotrophy, where organisms begin infection as biotrophs before transitioning into necrotrophs at a later stage of development. The spot blotch pathogen is considered a hemi-biotroph (Kumar et al. 2002).

Much of the research done to characterize the gene products (discussed below) associated with these lesion mimic phenotypes involved the model plant species *Arabidopsis*. However, several studies have been carried out on cereals. Within the cereal crops, ion channel proteins (Rostoks et al. 2003, 2006), a membrane-anchored protein (Jørgensen 1992), a uroporphyrinogen decarboxylase (Hu et al. 1998), and a heat transcription factor (Yamanouchi et al. 2002) have been implicated in the phenotype of disease lesion mimics. A list of well-characterized lesion mimic mutants is given in Table 5.1. The diverse functions of these genes suggest that the molecular pathways controlling cell death are complex. Further, many of these genes' involvement in the defense response may simply be only one of their native functions. The observed necrotic lesion may be a pleiotropic effect, resulting from a perturbation of normal plant physiology. Previous transcriptomic studies in the barley-*C. sativus* pathosystem have relied on microarrays which, while useful, were hindered by being reliant on existing gene sequences, which were limited to ~3,000. The emergence and application of next-generation sequencing (NGS) to the sequencing of RNA has provided unparalleled access to the transcriptome (Wang et al. 2009). Compared with microarrays, the digital nature of RNAseq allows for detection of more genes and a more accurate measurement across a greater range of gene expression levels.

RNAseq has been used to study plant-microbe interactions in a variety of pathosystems including: potato-*Phytophthora infestans* (Gao et al. 2013),

soybean-*Xanthomonas axonopodis* (Kim et al. 2011), cotton-*Verticillium dahliae* (Xu et al. 2011), creeping bentgrass-*Sclerotinia homoeocarpa* (Orshinsky et al. 2012), and others. In this study, we used RNAseq to study the transcriptional response of barley to *C. sativus*. Through the mapping of short sequence reads generated in this experiment to the barley reference assembly (Morex WGS assembly version 3) and subsequent differential expression analysis, we identified key genes involved in the barley defense response to *C. sativus*.

The primary objectives of this experiment are to: 1) use an RNAseq approach to study the defense response of a susceptibility mutant in barley to *C. sativus* at the seedling stage, 2) identify putative gene knock-outs, and 3) relate the information obtained from the seedling defense response to adult onset disease mimic lesions.

Materials and Methods

Plant material

The spot blotch resistant barley genotype used in this experiment is the six-rowed malting cultivar 'Morex', which derives its resistance from the breeding line NDB112 (Clho 11531). NDB112 originated from the cross Clho 7117-77/Kindred (Wilcoxson et al. 1990) and is highly resistant to most pathotypes of *C. sativus* (Valjavec-Gratian and Steffenson 1997). The wild type (WT) Morex used in this experiment was from the original 'Morex' seed source used to create the Steptoe/Morex doubled haploid (DH) population (Kleinhofs et al. 1993). To

generate mutants susceptible to spot blotch, WT Morex seed was irradiated with gamma radiation. Approximately 3,000 seeds of Morex were subjected to 15 kilo roentgen (=150 gray) gamma radiation by A. Kleinhofs at Washington State University in 2005, and then the M₁ generation was increased in the field at Pullman, WA during the same year. The resulting M₂ families were then sown at the Minnesota Agricultural Experiment Station (MAES) on the St. Paul campus of the University of Minnesota in 2006. The M₂ nursery was inoculated with the spot blotch pathogen according to established protocols (Steffenson et al. 1996) to identify families segregating for reaction to the disease. Several families segregating for spot blotch severity and infection response (IR) were identified in the nursery. Cleaved Amplified Polymorphic Sequences (CAPS) markers specific for Morex were used to verify that putative susceptibility mutants were indeed of this genetic constitution and not due to a seed admixture. The genomic locus/loci of the mutation(s) rendering Mx14-40 susceptible is unknown at this time.

Ionizing radiation such as gamma rays produce mutations (i.e., point mutations, indels) in the genome with a high frequency, although the size of the deletions are smaller than those produced with fast neutrons (Koornneef 2002). Large-scale deletions, such as entire chromosome arms, are possible; however, these are generally not preserved in the M₂ or later generations due to deleterious effects on plant fitness (Koornneef 2002). Initially, we sought to minimize in Mx14-40 the effect of background mutations on the expression of spot blotch

susceptibility by crossing it with WT Morex and then selecting progeny with the most extreme phenotype. Since evaluations of the Mx14-40/WT population did not yield true breeding progeny with the same extreme susceptible and resistant phenotypes as the parents, we used Mx14-40 and WT Morex in the RNAseq experiment.

Inoculum preparation

Inoculum was prepared according to the methods of Fetch and Steffenson (1999). Briefly, conidia of *C. sativus* isolate ND85F, retrieved from silica gel crystals stored at 4°C, were plated out onto minimal media agar plates 10 days before inoculation. To harvest conidia for inoculation, water was poured onto the surface of the *C. sativus* cultures and then gently rubbed with a rubber spatula to dislodge the conidia. The conidia-water suspension was then passed through four layers of cheesecloth, and then the concentration of spores was estimated using a hemacytometer. The suspension was diluted to 6,000 conidia/ml, and a surfactant (Tween-20: polyoxyethylene-20-sorbitan monolaurate) was added at a rate of 10 µl per 100 ml of the suspension to increase the distribution and adhesion of conidia to the leaf surfaces. Approximately 0.3 ml of the conidial suspension was applied to each inoculated leaf.

Experimental design

One experimental unit consisted of fifty individual plants sown in cone-tainers (5 cm diameter x 18 cm depth, Stuewe and Sons, Inc., Tangent, OR) filled with a 50:50 mixture of steam-sterilized field soil and Sunshine MVP potting mix. The cone-tainers were held in racks at 50 per rack. Forty of these individuals were one of the two different genotypes: WT Morex (WT) or mutant Mx14-40 (MUT), which were subjected to RNAseq. The controls of NDB112 (Clho 11531), Bowman (PI 483237), and ND5883 (PI 6434237) (Fetch and Steffenson 1999) as well as extra WT and MUT individuals were also included to verify the disease reaction of the genotypes to isolate ND85F, but were not subjected to RNAseq. The barley plants were organized in a cone rack in randomized complete block design (RCBD). Plants were grown for 14 d until the second leaf of each seedling was fully expanded. Then, they were inoculated or mock (water) inoculated. After inoculation, the plants were moved to a mist chamber kept near 100% relative humidity by ultrasonic humidifiers running for 2 minutes of continuous misting every 60 minutes. During this infection period, the plants were kept in the dark overnight. The first tissue samples were harvested 12 hours after inoculation (hai) when the pathogen was beginning to ramify beyond the initial penetrated cells (Millet et al. 2009). At this time point, pathogenesis-related (PR) proteins begin to accumulate (Kumar et al. 2002). Tissue samples (5-6 second leaves per sample) were collected by cutting the leaf sheaths with a scissors and placing them into 50 mL tubes. Then, the samples were immediately frozen in liquid nitrogen before moving them into storage in a -80°C ultra-low freezer. In total, 36

independent samples were collected from three biological replicates of the two treatments (*C. sativus*-inoculated and water-inoculated), two plant genotypes (WT and MUT), and three time points (12, 24, and 36 hai). Time points were selected based on a previous study that indicated *C. sativus* switches its lifestyle from biotroph to necrotroph around 24 hours after initial infection (Millet et al. 2009). The 12 and 36 hour time points were selected to capture potentially interesting defense responses at even intervals before and after the 24 hour time point.

RNA extraction

RNA was extracted using the Trizol method. Briefly, tissue samples were ground into a fine powder with a mortar and pestle in liquid nitrogen. This powder was then transferred to a 50 ml tube with 10 ml Trizol reagent (Ambion, Foster City, CA). Samples were kept on ice until all of them were processed. Next, the samples were incubated for 15 min in a pre-heated water bath at 60°C. The tubes were then spun for 10 min at 4,000 rpm, and the supernatant transferred to an RNase-free 50 ml tube to which 2 ml chloroform was added. Tubes were then inverted 5-10 times. The tubes were again centrifuged for 15 min at 4,000 rpm, and the supernatant was transferred to a new RNase-free 50 ml tube. One half of one volume of isopropanol and Na-citrate/NaCl solutions were added per 1 ml aqueous solution followed by incubation at -20°C for 20 min. Following the incubation period, samples were centrifuged for 10 min at 4,000 rpm, rinsed with

10 ml of 70% EtOH, and centrifuged for another 5 min at 4,000 rpm. The supernatant was then discarded, and the pellet was allowed to air dry for at least 10 min while inverted on a laboratory tissue. Finally, the pellet was dissolved in 300 µl DEPC-treated (RNase-free) water and frozen at -20°C for storage until all samples were ready to be submitted to sequencing. Upon thawing, 2 µl RNasin (Promega, Madison, WI) was added to each sample. RNA was purified using an RNeasy Cleanup Kit (Qiagen, Foster City, CA).

RNA samples were checked for quality and quantity on a Nanodrop 1000 machine (Thermo Fisher Scientific Inc., Wilmington, DE) before taking samples to the University of Minnesota Genomics Center (UMGC) for RNAseq library preparation with the TruSeq RNA v2 library kit (Illumina Inc., San Diego, CA). The UMGc also checked the quality and integrity of the RNA samples with an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). Libraries were barcoded and multiplexed into three pools before being subjected to 100 bp paired-end sequencing using the Illumina Hi-Seq 2000 machine (Illumina). One exception to this protocol was with sample *R3_INOC_MUT_24*, which was run later using the Illumina Hi-Seq 2500 1T instrument (Illumina). The reason for this was that this sample was inadvertently omitted by the Genomics Center in the first run of the experiment and instead sample *R3_INOC_WT_12* was sequenced twice.

Sequencing of MLOC_17240

We determined computationally that MLOC_17240 was possibly deleted in mutant Mx14-40 with command-line arguments that would return gene identifiers that had mapped reads for WT Morex samples, but not the mutant samples, allowing for up to 5 reads in mutant samples to account for barcoding errors. In order to assess whether this gene was truly deleted rather than simply not expressed due to a deletion elsewhere in the genome affecting its expression, we designed 10 primers covering most of approximately 8,000 bp of the gene using the complete genomic sequence downloaded from the European Nucleotide Archive via the Barlex Genome Explorer website (Colmsee et al. 2015). Primers were designed using the design tool from the National Center for Biotechnology Information (NCBI) (Ye et al. 2012). Genomic DNA was extracted from WT Morex and Mx14-40 using a modified CTAB method (Guotai Yu, *personal communication*) (Appendix F). PCR was performed by Functional Biosciences (Madison, WI, USA). Once it was confirmed that the PCR products generated from WT Morex DNA were from MLOC_17240, DNA from mutant Mx14-40 were sent to Functional Biosciences to determine if the same primers could amplify a product.

Validation of results by qPCR (SYBR® Green)

First-strand cDNA synthesis was generated from 1 µg of total RNA extracted from the second leaves of both inoculated and mock-inoculated WT Morex and

mutant Mx14-40 plants with the Superscript first-strand synthesis system (Invitrogen, Foster City, CA). Guidelines from the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (Bustin et al. 2009) were used to design the quantitative real-time PCR (qPCR) experiment. Primers for qPCR were designed using the NCBI Primer design tool (Ye et al. 2012) and synthesized commercially (Integrated DNA Technologies, Coralville, IA USA). For qPCR, 20 µl qPCRs were run in three technical replicates on the Roche 480 Light Cycler (Applied Biosystems) using 5 µl of first-strand cDNAs and SYBR® Green PCR Master Mix (Applied Biosystems). PCR cycles were as follows: one cycle of 1 min at 94°C, followed by 45 cycles at 94°C for 15 s and 58°C for 45 s. Following amplification, all products were subjected to melt curve analysis. A negative control without a cDNA template was run with each analysis to evaluate the overall specificity. SYBR® Green binds with grooves of double-stranded DNA, so the signal is non-specific. The barley GAPDH gene (Horvath et al. 2002) was used as the reference gene to normalize the total amount of cDNA in each reaction. Relative fold changes were calculated with Microsoft Excel® using C_T values extracted from the Roche 480 Real Time PCR System (Applied Biosystems) according to the comparative C_T method of Schmittgen and Livak. (2008).

Validation of results by qPCR (Probe-based)

In order to verify the RNAseq results as well as the qPCR results determined using the SYBR® Green system, we also submitted cDNA generated with the same protocol as described above to the UMGC for qPCR with analysis by the Roche Universal Probe Library (UPL) system, which is functionally similar to the TAQMAN® assay. The Roche UPL system is a more accurate method of quantifying transcripts because each probe (8-9 nt) consists of two parts: a reporter (fluorescein) at the 5' end and a quencher dye at the 3' end of the probe. Upon integration of the probe into the transcript during qPCR, the quencher is cleaved as a result of integration into a double-stranded DNA molecule. The intensity of the signal is therefore related to the number of integrated probes. Three primers were designed for each transcript of interest in order to maximize the likelihood that one would be efficient enough to proceed to the qPCR step. Efficiency is calculated by performing serial dilutions with the most efficient primer pair being one that approximately doubles each cycle of PCR. Efficiencies greater than 100% indicate non-specific binding, while efficiencies under 90% indicate poor primer design or sub-optimal PCR conditions. Analyses were performed on an ABI 7900HT machine (Applied Biosystems).

Results

WT Morex × Mutant Mx14-40 population

The F₁ individual from the cross WT Morex × Mx14-40 was moderately resistant to *C. sativus* isolate ND85F exhibiting an IR of 5. Segregation ratios in the F₂ and

F₃ generations indicated that the mutation is not simply inherited, although resistance was inherited in a dominant manner ($X^2=3.2$, $p=0.0736$). Other crosses made with mutant Mx14-40 showed similar patterns of non-Mendelian inheritance (J. Franckowiak, *personal communication*). Due to the close genetic similarity of the parents of the WT Morex × Mx14-40 population, all tested SNP markers from BOPA1 and BOPA2 were monomorphic; therefore, it was not possible to map the locus/loci conferring the susceptibility phenotype. Mapping the mutated locus/loci was further complicated by the presence of QTL for spot blotch resistance that are fixed in most Midwest germplasm lines. One possible solution would be to use a more distantly-related (e.g. non-Midwestern) parent to map QTL for the adult onset necrosis as a proxy for seedling disease response.

Spot blotch symptoms

The disease phenotypes of resistant (Bowman and NDB112) and susceptible (ND5883) controls as well as the WT Morex and Morex mutant to isolate ND85F were assessed 11 d after inoculation. NDB112 and Bowman exhibited the expected resistant response (IR = 2-3) of small distinct necrotic spots and no associated chlorosis, indicative of a hypersensitive response. In contrast, the susceptible control ND5883 had large diffuse areas of necrosis with a high degree of surrounding chlorosis, indicative of a compatible interaction. WT Morex gave a resistant reaction (IR = 2-3), while Mx14-40 gave a susceptible reaction (IR = 6-7) (Fig. 5.1).

RNAseq analysis

Changes in transcript levels between the resistant WT Morex and susceptible mutant Mx14-40 were assessed at 12, 24, and 36 hai by RNAseq. A total of 1.6 billion reads were generated by 100 bp paired-end sequencing from 36 cDNA libraries. Half of these libraries (18) came from three independent replicates of mock (water) inoculated plants, harvested at 12, 24, and 36 hai from both WT Morex and mutant 14-40 seedlings, and the other half came from three independent replicates of *C. sativus* inoculated plants of the same barley genotypes harvested at the same time intervals. Approximately 88% of the sequenced reads (1.4 billion mapped reads) were successfully aligned to the barley genome reference assembly (Morex WGS assembly version 3) using TopHat2 software (Table 5.2) (Trapnell et al. 2009). Of the 26,159 predicted high-confidence genes in the barley genome, 24,244 high-confidence genes were mapped using these sequence data, and expression levels were measured based on read count using HTseq (Anders et al. 2015). DEseq (Anders and Huber 2010) was used to calculate differential expression. Differential expression was calculated by contrasting the effect of genotype (WT Morex vs. mutant Mx14-40), while controlling for the effect of treatment (inoculation and mock inoculation). Principle Component Analysis (PCA) of the expression profiles for the 36 samples show that the first principle component (PC1) explains 74% of the variance between *C. sativus*-inoculated and mock-inoculated plants. The

second principle component (PC2) explains 11% of the variance and separates samples of both genotypes at the 12 hai time point from the 24 and 36 hai time points (Figure 5.3).

Transcriptome analysis in response to C. sativus infection

The number of differentially expressed genes in WT Morex were compared with Mx14-40 at each time point [$P < 0.001$ and \log_2 (fold change) > 2.0 or < -2.0]. A total of 45 unique genes were up- or down-regulated in WT Morex across all inoculated time points. Of these, 18 and 27 were up- and down-regulated, respectively, at 12, 24, and/or 36 hai (Fig. 5.4). Compared with Mx14-40, 9 genes were up-regulated and 8 genes were down-regulated at 12 hai, 11 were up-regulated and 19 were down-regulated at 24 hai, and 13 were up-regulated and 11 down-regulated at 36 hai. Some of these genes were either up-regulated (8) or down-regulated (8) at multiple time points in WT Morex compared with Mx14-40. Of the 45 genes that were differentially expressed, only 7 of these were up-regulated and 3 were down-regulated at all three time points (Tables 5.3-5.4).

Genes that were upregulated in the resistant WT Morex compared to the susceptible mutant Mx14-40 include a glycine rich protein (MLOC_69866), a Sec14p-like phosphatidylinositol transfer family protein (MLOC_34706), a long-chain fatty acid CoA ligase (MLOC_17240), an elongation factor (MLOC_56627), an RNA-binding protein Nova-1 (MLOC_18824), and an

esterase/lipase/thioesterase-like protein (MLOC_37652) at 12 hai. The same genes plus a disease resistance protein (powdery mildew resistance protein pm3 variant) (MLOC_68128), and genes of unknown function were upregulated at 24 hai. In addition, all of the genes common to 12 and 24 hai as well as an F-box domain containing protein (MLOC_55208), a response regulator (MLOC_11341), an NBS-LRR disease resistance protein (MLOC_64708) and some genes of unknown function at 36 hai (Table 5.3). Several genes were also downregulated in WT Morex compared with mutant Mx14-40. Like those that were upregulated, many downregulated genes encode genes for unknown proteins. The only gene identified at 12 hai with an annotation is a diacylglycerol kinase (MLOC_63451).

At 24 hai, the diacylglycerol kinase was also differentially expressed, but so were other genes such as those encoding a methyl esterase (MLOC_56070), a chlorophyll a-b binding protein (MLOC_41838), a cytochrome P450 family protein (MLOC_64131), a 3C-like chlorophyll a-b binding protein (MLOC_9033), and a glutathione S-transferase (MLOC_54448). At 36 hai, a methyl esterase (MLOC_56070), a NADH dehydrogenase (MLOC_51239), and an enolase (MLOC_37167) gene were identified, among other unknown proteins (Table 5.4).

In order to ascertain the function of these unknown proteins, sequences of each were downloaded from the European Nucleotide Archive via the Barley Genome Explorer (Colmsee et al. 2015) and used in a NCBI Basic Local Alignment

Search Tool (BLAST) search for homology. This resulted in the putative assignment of some, but not all of the unknown proteins. Genes down-regulated in WT Morex compared with mutant Mx14-40 include: a retrotransposon (MLOC_2328), a drug resistance protein (MLOC_25487), a cytochrome P450 (MLOC_33504), a DNA-binding protein (MLOC_4070), a coiled-coil-NBS-LRR disease resistance protein (MLOC_54513), an ethylene-responsive transcription factor (MLOC_71255), and a chlorophyll a-b binding protein (MLOC_76504). Most genes that could not be assigned a functional annotation mapped to contigs that were very short (around 200 bp), which may explain why no functional assignment could be made. Some genes (MLOC_10417, MLOC_20028, MLOC_21608, MLOC_64780) had high identities (97-100%) with a wide variety of genes and many hits with the non-specific class “predicted coding sequence” so they were not assigned a putative function here. As the assembly of the barley genome improves, so too should the functional annotation of these genes.

Detection of genomic mutation

Prior to conducting the RNAseq experiment, the location(s) of the mutant locus/loci in the genome causing the susceptible seedling response as well as the large necrotic lesions at the adult plant stage were unknown. Therefore, a simple code was written to filter genes based on read counts to see if there were any genes which had reads for the wild type samples but no reads for the mutant sample. Some leniency was allowed to account for possible errors in barcoding

samples. This process yielded two candidates: MLOC_69866 (a glycine-rich repeat protein) and MLOC_17240 (a long-chain fatty acid acyl CoA ligase). The glycine-rich protein was expressed at a higher level (in WT Morex) than the long-chain fatty acid acyl CoA ligase, but the annotation indicated that the protein encoded by the gene could not be called with high confidence. Since biological significance would be difficult to infer from the general term *glycine-rich repeat*, only MLOC_17240 was selected for further investigation. The NCBI primer design tool (Ye et al. 2012) was used to design primers that had good coverage of the ~8,000 bp length of the genomic sequence, except for a small region (68 bp) at the 5' end of the gene.

Validation of RNAseq based gene expression by qPCR

To validate the RNAseq-based gene expression levels that correlated with the number of tags obtained, qPCR was performed on selected genes with different expression levels and functional assignments. The RNA used for qPCR validation was extracted from tissue harvested from independent biological replicates. Results for five of the genes tested for validation are presented in Fig. 5.6. Two genes tested for validation (MLOC_34706 and MLOC_68128) could not be validated. The likely reasons for this are homology of MLOC_34706 with other similar proteins (Sec14p-like phosphatidylinositol transfer family) that would have overestimated its relative expression in the validation samples. MLOC_68128 is a disease resistance protein and is expressed at low levels. Aside from these two

genes, the overall qPCR results agree with RNAseq data. Changes in relative expression for down-regulated (MLOC_63451, MLOC_51239, MLOC_54448, and MLOC_25487) and up-regulated (MLOC_37652) are consistent across expression assays (RNAseq, SYBR green, and probe-based). The differences in magnitude of relative expression could be caused by the fact that DESeq, used for differential expression analysis, takes the expression of mock-inoculated samples into consideration whereas qPCR only considered the inoculated samples.

Discussion

The durability of NDB112-derived spot blotch resistance in Midwest six-rowed barley germplasm is remarkable and the genetic basis is well-characterized (Zhou and Steffenson 2013). The purpose of this study was to elucidate the molecular basis of compatible and incompatible interactions between barley and *C. sativus* at the seedling stage through the use of the susceptible Morex mutant Mx14-40. The differential expression results from the RNAseq experiment presented here suggest that lipid signaling (i.e., MLOC_17240, MLOC_34706), leading to activation of the jasmonic acid pathway, plays a pivotal role in the defense response of barley to *C. sativus* and that failure to appropriately regulate the jasmonic acid-induced cell death is responsible for the susceptible phenotype.

Association with known spot blotch resistance loci

Some of the genes identified by RNAseq are coincident with previously identified major genes or quantitative trait loci (QTL) for spot blotch resistance. Tables 5.3 and 5.4 provide chromosome and position (cM) mapping information obtained through Barlex: The Barley Genome Explorer (Colmsee et al. 2015). Starting with disease resistance (NBS-LRR) genes, one identified by RNAseq (MLOC_68128) maps to a position on the short arm of chromosome 1H where *Reaction to C. sativus 6 (Rcs6)* was mapped in the Bowman-BC/Calicuchima-sib population for resistance to isolate ND90Pr (pathotype 2) (Bilgic et al. 2006). Resistance to spot blotch in barley is not strictly controlled by major effect resistance genes. Minor effect QTL also play an important role in defense. With that frame of reference, QTL with modifying effects may have a variety of functions. Bilgic et al. (2005) identified QTL for spot blotch resistance in chromosomes 3H and 7H at the seedling stage and resistance QTL in chromosomes 1H, 2H, 3H, 5H, and 7H at the adult plant stage. Of the genes identified in this study with a known genomic position (Tables 5.3 and 5.4), three (MLOC_11341, MLOC_37652, MLOC_55208) of the up-regulated and six (MLOC_25487, MLOC_4070, MLOC_54513, MLOC_56070, MLOC_71255, MLOC_78528) of the down-regulated genes map to regions previously found to harbor QTL for spot blotch resistance. Coincident positions were determined by comparing the known map position (in cM) with the bin positions reported for those QTL based on the bin map of Kleinhofs et al. (2005). Half of these genes have a known function, but

the results of this comparison should be interpreted with caution. For example, both a response regulator (MLOC_11341) and an esterase/lipase/thioesterase-like protein (MLOC_37652) map to the chromosome 7H bin 2-4 region that was identified by Bilgic et al. (2005) and is part of the MSDRH (Zhou and Steffenson 2013). Drader et al. (2010) showed that the major gene/large-effect QTL for spot blotch resistance in this region (*Rcs5*) is a wall-associated kinase, but this gene was not differentially expressed in this experiment. These bins cover large regions of the genome and the comparative position of the genes identified through RNAseq with previously identified QTL should not be misinterpreted as the only, or even the best, candidate genes at known resistance loci.

Lipid metabolism

Phosphatidic acid (PA) is well-known as a signaling molecule in plants under stress (Ryu and Wang 1996 & 1998, Young et al. 1996, Lee et al. 1997, Frank et al. 2000, Munnik et al. 2000, van der Luit et al. 2000, Welti et al. 2002). PA may be synthesized from smaller constituent molecules, but hydrolysis of glycerophospholipids from the plant cell membranes provide an alternate route towards accumulation of PA by the phospholipases C and D (PLC and PLD) (Saucedo-García et al. 2015). Following the PLC pathway, phosphatidylinositol 4,5-bisphosphate is hydrolyzed to form inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG). IP₃ leads to increased levels of cytosolic Ca²⁺, while DAG is immediately converted into PA by diacylglycerol kinase (DGK) (Laxalt and

Munnik 2002). DGK must therefore be constitutively expressed at a level sufficient enough to maintain its activity at biologically meaningful levels. Andersson et al. (2006) studied the accumulation of PA in *Arabidopsis* in response to two transgenically-expressed effectors (AvrRpm1 and AvrRpt2) from *Pseudomonas syringae*. The authors demonstrated that PA is induced in two waves. Early induction of the PLC pathway induces a small amount of PA, followed by an increase in cytosolic Ca²⁺ levels. A second, larger wave of PA produced from the PLD pathway, ultimately results in an oxidative burst and the hypersensitive response. The PLD pathway has been associated with activation of the jasmonic acid pathway (Wang et al. 2000).

Since DGK is down-regulated in WT Morex compared with mutant Mx14-40 (Table 5.2, Figure 5.5), we should expect PA to accumulate at higher levels in the mutant and presumably lead to resistance if PA is in fact signaling the presence of an invading pathogen. The reason for this discrepancy may be due to the inability of mutant Mx14-40 to stop the production of PA, resulting in runaway cell death. The over-production of PA also provides a potential mechanism to explain the adult-onset necrosis. Two basic types of adult-onset necrosis exist: one type is caused by over-sensitivity of the cell death initiation pathway and the other is caused by the inability of the cell to stop the cell death signals and return the plant to a basal state (Johal et al. 1995). A potential hypothesis is that the PLC and PLD pathways play a role in the spot blotch

resistance pathway and that the adult-onset necrosis is triggered by the same mechanism. If this hypothesis is correct, then mutant Mx14-40 is deficient in the ability to arrest propagation of the cell death signal.

Potential roles of other differentially expressed genes

F-box containing proteins are members of a large superfamily of proteins that tag proteins for degradation with ubiquitin as part of the Skp, Cullin, F-box (SCF) complex (Patton et al. 1998, Maniatis 1999). They are commonly identified in plant-pathogen interactions (Yan et al. 2009). The *Arabidopsis* mutant *coronatine insensitive 1-1 (coi1-1)* has enhanced resistance to coronatine-producing strains of *Pseudomonas syringae* (Feys et al. 1994). Yan et al. (2009) demonstrated that this resistance is caused by the inability of coronatine to interact with CORONATINE INSENSITIVE 1 (COI1), an F-box containing protein that also binds jasmonoyl-isoleucine, an oxylipin and an active form of jasmonic acid. These results demonstrate precedence for the native role of F-box proteins in the jasmonic acid pathway and provide an example that pathogens may co-opt the defense network through mimicking plant hormones to aid in pathogenesis.

Jasmonates have previously been shown to negatively regulate chloroplastic proteins in barley, including subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco), chlorophyll a/b binding proteins (CAB), and others in order to recycle the constituent amino acids of those proteins to

synthesize proteins induced by jasmonates in the cytosol (Reinbothe et al. 1994). Jasmonate-induced proteins (JIPs) are a broad group of proteins that are induced by JA including JIP60, which maps to the long arm of chromosome 4H in the same region as QTL for spot blotch and powdery mildew resistance (Rustgi et al. 2014). JIP60 has both a ribosome-inactivating domain and a translation-initiating domain, indicating that it has a dual function in translational reprogramming, determined by cleavage of the JIP60 protein. The exact timing of this processing step is unknown (Rustgi et al. 2014). In the context of the barley-*C. sativus* results presented here, this mechanism could explain the up-regulation of proteins in the jasmonic acid pathway and the F-box protein, as well as the down-regulation of CAB proteins in WT Morex compared with mutant Mx14-40.

Other roles for lipids in disease resistance

The Sec14p-like phosphatidylinositol transfer family protein likely resides in the endoplasmic reticulum and moves substrates to the golgi apparatus (Mousley et al. 2007, Bankaitis et al. 2009). An *Arabidopsis* mutant *defective in induced resistance 1-1* (*dir1-1*) is defective in its ability to initiate systemic acquired resistance (SAR) and develops spontaneous necrotic lesions as a pleiotropic effect of the mutation. The protein encoded by the mutated gene, DIR1, encodes a putative apoplastic lipid transfer protein (Maldonado et al. 2002), providing

corroborative evidence that lipid transfer proteins are involved with the plant defense response and the development of spontaneous lesion mimics.

Esterase/lipase/thioesterase-like proteins have also previously been implicated in plant defense. Two of the best-characterized genes required for SAR are *Enhanced Disease Susceptibility 1 (EDS1)* and *Phytoalexin Deficient 4 (PAD4)*, both of which encode lipases (Falk et al. 1999, Jirage et al. 1999). In addition, an *Arabidopsis* mutant *glp1* that is defective in a gene encoding a GDSL LIPASE 1 (GLP1) was rendered susceptible to the necrotrophic pathogen *Alternaria brassicicola*, although the mutant was still resistant to *Pseudomonas syringae* pv. *tomato* DC3000. Collectively, these data suggest that lipases play a role in plant defense, but do not affect salicylic acid-induced cell death (Oh et al. 2005).

Oxidative stress

The downregulation of enolase, which catalyzes the penultimate reaction in glycolysis, can be interpreted in a number of different ways. In one scenario, enolase is expressed at higher levels in mutant Mx14-40 in order to maintain ATP production during the stress response. Other differentially expressed genes in mutant Mx14-40 directly (chlorophyll a-b binding proteins, glutathione-S-transferase) or indirectly (diacylglycerol kinase) suggest an oxygen-rich environment caused by ROS of the oxidative burst. Glycolysis is important for maintaining ATP production under anaerobic stress, so it is somewhat

paradoxical that enolase was identified in an oxygen-rich environment. Tissue staining techniques using DAB to visualize ROS levels were not done, so the presence of ROS is being inferred through the differential expression results and the observation of large and widespread necrosis expected to result from ROS-induced cell death. Under a second scenario, enolase is down-regulated rapidly in the WT Morex and not in mutant Mx14-40. Down-regulation of glycolysis-related genes, including enolase, have been observed in the incompatible (resistant) responses in other pathosystems (Xie et al. 2013).

Two chlorophyll A/B binding (CAB) genes were down-regulated in the WT Morex compared with mutant Mx14-40, not including the CAB gene identified in the BLAST search. These were likely induced by the salicylic acid pathway initiated by the phosphatidic acid signalling pathway. Schenk et al. (2001) previously demonstrated that this cross-talk is possible.

In plants, cytochrome P450s play roles in conjugation of fatty acids (Bleé 2002), hormone synthesis (Booker et al. 2005), synthesis of defense compounds (Qi et al. 2006), and more. From the available data, it is not known what the exact role of the cytochrome P450 expressed in Mx14-40 may be, but its known functions fit into the general model of plant defense.

Other aspects of the defense response

The glycine-rich repeat protein up-regulated in the WT Morex may be associated with the cell wall like other reported glycine-rich proteins (Showalter 1993); however, association with the cell wall is only one possible function of glycine-rich proteins. The *Arabidopsis* genome, for example, has eight glycine-rich proteins that are associated with RNA binding (Lorković 2009). This alternative function, along with the RNA-binding NOVA1 protein that was differentially expressed at 12 hai, could indicate that post-transcriptional regulation of an undetermined gene or genes is important for the defense response. Without further data, it will not be possible to conclude anything specific about the role these genes play in the defense response.

The (pseudo) response regulator 9 (PRR9) is likely involved with circadian rhythm as its native function (Salomé and McClung 2005). Pseudo response regulators including PRR9 are redundant since perturbations of one regulator can be compensated by the activity of other response regulators. Knock-downs of PRR9, for example, result in a moderate increase in the length of an oscillation of the *Arabidopsis* circadian rhythm; however, a double knockout of PRR9 and PRR7 has a more dramatic effect on the circadian rhythm (Salomé and McClung 2005). Another protein that is in the family of pseudo response regulators is *Timing of CAB Expression 1 (TOC1)* (Matsushika et al. 2000). TOC1 is a transcription factor that accumulates in the evening before being targeted for degradation by the SCF complex mediated by the F-box protein ZEITLUPE

(Salomé and McClung 2005). This is a single feedback loop since the genes upregulated by TOC1 (CIRCADIAN CLOCK ASSOCIATED 1 [CCA1] and LATE AND ELONGATED HYPOCOTYL [LHY]) suppress the expression of TOC1 and are attenuated by some unknown mechanism (Salomé and McClung 2005). The detection of circadian rhythm genes could mean that there is overlap between the circadian clock and pathogen response. It is not likely that these genes were detected due to the time of harvest because the differential expression comparisons were made between the two different tissues (WT and MUT) at the same time point and should cancel out.

Conclusion

The results of this study suggest that lipid signaling is an important part of the defense response of barley to infection by *C. sativus*. The differential expression of genes encoding diacylglycerol kinase and oxidative-stress proteins supports the hypothesis that over-accumulation of phosphatidic acid in mutant Mx14-40 is responsible for the compatible (susceptible) interaction between barley and *C. sativus* as well as the adult-onset necrosis as a pleiotropic effect of disruption in the phospholipid signaling pathway. In order to reach a definitive conclusion, future work should be done to monitor the activities of these proteins including labeling of candidate phospholipids to monitor sub-cellular localization, assays to measure protein-protein interactions, and independent mutations of candidate genes to independently re-create the phenotypes of mutant Mx14-40. Genetic

mapping of these phenotypes in a segregating population should narrow the list of candidate genes for targeted mutagenesis.

Table 5.1. A sample of mutated genes and their putative products that have been implicated in disease lesion mimic mutants in a variety of plants.

Mutant Name	Species	Putative Product	Reference
<i>Accelerated cell death 5 (acd5)</i>	<i>Arabidopsis</i>	Ceramide kinase	Berkey et al. (2012)
<i>Accelerated cell death 11 (acd11)</i>	<i>Arabidopsis</i>	Sphingolipid transporter	Berkey et al. (2012)
<i>Constitutive expresser of PR genes 22 (cpr22)</i>	<i>Arabidopsis</i>	Unknown	Yoshioka et al. (2001)
<i>Copine 1 (Cpn1)</i>	<i>Arabidopsis</i>	Copine calcium-dependent phospholipid binding protein	Jambunathan et al. (2001)
<i>Defective in induced resistance 1-1 (dir1-1)</i>	<i>Arabidopsis</i>	Lipid transfer protein	Maldonado et al. (2002)
<i>Defense no death 1 (Dnd1)</i>	<i>Arabidopsis</i>	Cyclic nucleotide-gated ion channel (CNGC2)	Clough et al. (2000)
<i>Hypersensitive-induced reaction (HIR)</i>	Barley	Ion channel	Rostoks et al. (2003)
<i>HR-like lesion mimic (Hlm1)</i>	<i>Arabidopsis</i>	Cyclic nucleotide-gated ion channel (CNGC4)	Balague et al. (2003)
<i>Lesion mimic (Les22)</i>	Maize	Uroporphyrinogen decarboxylase	Hu et al. (1998)
<i>Mildew locus O (mlo)</i>	Barley	Membrane-anchored protein	Jørgensen (1992)
<i>Necrotic locus (Nec1)</i>	Barley	Barley homolog of <i>Arabidopsis</i> CNGC4	Rostoks et al. (2006)
<i>Spotted leaf 7 (Spl7)</i>	Rice	Heat transcription factor	Yamanouchi et al. (2002)

Table 5.2. Mapping statistics from TopHat2.

Treatment	Sample	Left reads mapped	Right reads mapped	Properly paired reads (%)	
Inoculated	Rep 1 MUT 12 hai	42,352,931	42,299,431	90	
	Rep 2 MUT 12 hai	38,576,504	38,508,617	89	
	Rep 3 MUT 12 hai	39,409,842	39,355,855	87	
	Rep 1 MUT 24 hai	38,673,328	38,621,148	90	
	Rep 2 MUT 24 hai	31,285,572	31,234,535	90	
	Rep 3 MUT 24 hai	143,086,312	142,413,634	88	
	Rep 1 MUT 36 hai	36,976,776	36,928,982	86	
	Rep 2 MUT 36 hai	29,523,502	29,494,033	89	
	Rep 3 MUT 36 hai	34,879,870	34,835,877	88	
	Rep 1 WT 12 hai	35,413,107	35,371,088	90	
	Rep 2 WT 12 hai	41,688,309	41,612,118	90	
	Rep 3 WT 12 hai	42,727,925	42,668,903	88	
	Rep 1 WT 24 hai	35,331,627	35,290,071	90	
	Rep 2 WT 24 hai	35,831,008	35,767,094	90	
	Rep 3 WT 24 hai	37,295,016	37,249,103	89	
	Rep 1 WT 36 hai	31,920,551	31,878,580	90	
	Rep 2 WT 36 hai	34,549,599	34,502,358	88	
	Rep 3 WT 36 hai	33,219,199	33,185,645	87	
	Mock (water)	Rep 1 MUT 12 hai	44,841,693	44,748,719	84
		Rep 2 MUT 12 hai	39,705,195	39,633,968	89
		Rep 3 MUT 12 hai	45,233,346	45,143,421	86
Rep 1 MUT 24 hai		40,151,087	40,086,464	90	
Rep 2 MUT 24 hai		37,172,200	37,086,135	88	
Rep 3 MUT 24 hai		38,836,586	38,772,035	88	
Rep 1 MUT 36 hai		40,480,947	40,398,594	88	
Rep 2 MUT 36 hai		36,946,885	36,888,056	88	
Rep 3 MUT 36 hai		35,387,613	35,334,497	88	
Rep 1 WT 12 hai		39,274,314	39,208,695	89	
Rep 2 WT 12 hai		34,115,257	34,038,736	89	
Rep 3 WT 12 hai		41,933,099	41,873,255	85	
Rep 1 WT 24 hai		42,437,286	42,381,045	89	
Rep 2 WT 24 hai		39,909,208	39,831,748	89	
Rep 3 WT 24 hai		40,836,557	40,779,431	87	
Rep 1 WT 36 hai		39,532,750	39,471,064	90	
Rep 2 WT 36 hai		41,709,129	41,653,733	89	
Rep 3 WT 36 hai		42,235,553	42,190,467	85	

Table 5.3. Differential expression results. Genes upregulated in the resistant wild type Morex compared to the susceptible Morex mutant 14-40. Genes are ordered by value of gene ID.

Gene ID	Annotation	Chromosome	Position (cM)	Time Point
MLOC_10417	Unknown protein	NA	NA	12, 24, 36
MLOC_11341	Response regulator 9	7H	48.3	36
MLOC_17240	Long-chain-fatty-acid CoA ligase, putative	NA	NA	12, 24, 36
MLOC_18224	RNA-binding protein Nova-1	6H	28.5	12
MLOC_20028	Unknown protein	NA	NA	24
MLOC_21608	Unknown protein	1H	122.1	12, 24, 36
MLOC_34706	Sec14p-like phosphatidylinositol transfer family protein	NA	NA	12, 24, 36
MLOC_37652	Esterase/lipase/thioesterase-like protein	7H	21.9	12, 36
MLOC_55208	F-box domain containing protein, expressed	3H	132.9	36
MLOC_56627	Elongation factor 2	1H	11.8	12, 24, 36
MLOC_57548	Unknown protein	NA	NA	36
MLOC_59569	Unknown protein	7H	109.8	24
MLOC_64708	NBS-LRR disease resistance protein, putative	5H	166.3	36
MLOC_64780	Unknown protein	5H	98.6	12, 24, 36
MLOC_65892	Unknown protein	NA	NA	36
MLOC_68128	Disease resistance/powdery mildew resistance protein pm3 variant	1H	3.2	24
MLOC_69866	Glycine rich protein	NA	NA	12, 24, 36
MLOC_71422	Unknown protein	NA	NA	24

Table 5.4. Differential expression results. Genes downregulated in the resistant wild type Morex compared to the susceptible Morex mutant 14-40. Genes are ordered by value of gene ID.

Gene ID	Annotation	Chromosome	Position (cM)	Time Point
MLOC_2328	Unknown protein	4H	51.4	24
MLOC_25487	Unknown protein	2H	57.1	12, 24, 36
MLOC_25995	Unknown protein	NA	NA	12, 24, 36
MLOC_31070	Unknown protein	NA	NA	36
MLOC_33504	Unknown protein	NA	NA	24, 36
MLOC_33716	Unknown protein	NA	NA	12
MLOC_33745	Unknown protein	NA	NA	12
MLOC_37167	Enolase	NA	NA	36
MLOC_4070	Unknown protein	3H	51.0	24, 36
MLOC_41838	Chlorophyll a-b binding protein 2	NA	NA	24
MLOC_42641	Unknown protein	NA	NA	12
MLOC_47203	Unknown protein	NA	NA	24
MLOC_4849	Unknown protein	1H	97.5	24
MLOC_51239	NADH dehydrogenase	NA	NA	36
MLOC_54448	Glutathione S-transferase	7H	1.9	24
MLOC_54513	Unknown protein	2H	58.9	12, 24, 36
MLOC_56070	Methyl esterase 1	3H	128.0	24, 36
MLOC_63451	Diacylglycerol kinase 2	NA	NA	12, 24
MLOC_63777	Unknown protein	NA	NA	36
MLOC_64131	Cytochrome P450 family protein	5H	44.1	24
MLOC_67332	Unknown protein	NA	NA	24, 36
MLOC_71255	Unknown protein	1H	48.1	24
MLOC_73205	Unknown protein	NA	NA	24
MLOC_76504	Unknown protein	6H	79.6	24
MLOC_78528	Unknown protein	5H	143.6	24
MLOC_9033	Chlorophyll a-b binding protein 3C-like	NA	NA	24
MLOC_9436	Unknown protein	NA	NA	12

Table 5.5: Primers used for SYBR green real-time PCR

Primer Name	Primer Sequence	Product Length (bp)
Long-chain fatty acid CoA ligase (MLOC_17240)	5'-CTGTTCTTGAAGGGCGTGC-3' 5'-ACGACCGTATCTTTGGCTCC-3'	239
Sec14p-like phosphatidylinositol transfer family protein (MLOC_34706)	5'-TGGAGAAAGGAGTTCGGCAC-3' 5'-TTTGGTCAATGTGGCGCTTG-3'	274
Elongation factor (MLOC_56627)	5'-TCCAAGCAAATGCACTGGC-3' 5'-TGGCGATCGATCAAGTGGAG-3'	202
NADH dehydrogenase (MLOC_51239)	5'-GTTCACTCCTCTGTTGCCGA-3' 5'-AGCATTGGTCCCATCACCTG-3'	181
Glutathione-S-transferase (MLOC_54448)	5'-ATCTCCAGAGCAAGAGCGAC-3' 5'-CTCGCGCACTTTTGTCCAG-3'	214
Cytochrome P450 (MLOC_64131)	5'-TTCGCCTTCGACACCATCTG-3' 5'-GACTTCCCCTCCGTCAAAGC-3'	86
Disease resistance protein (MLOC_68128)	5'-AGAGATGTCGTGGCTCTCCT-3' 5'-TGTGTGCTGGCAAATCGTTG-3'	168
Unknown protein (MLOC_25487)	5'-TAGGCGACACAAGAAGGAGG-3' 5'-CCCAGCCAAACTCATTCCAGA-3'	143
Diacylglycerol kinase (MLOC_63451)	5'-AGGTAACCTCATTGGGCACG-3' 5'-GCCCGACATCGTCAGTTACA-3'	174
GAPDH	5'-CGTTCATCACCACCGACTAC-3' 5'-CAGCCTTGTCCTGTCAAGT-3'	N/A

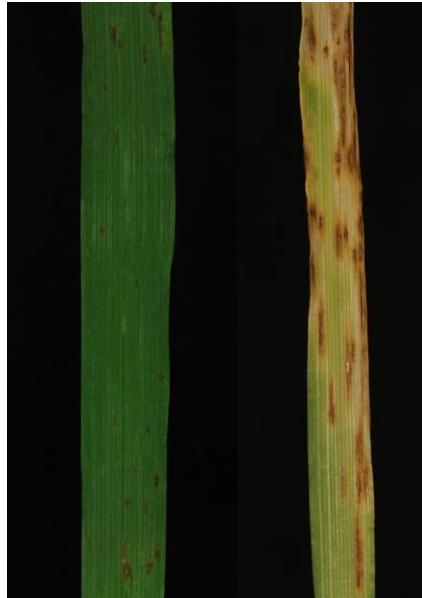


Figure 5.1. Seedling response to infection by *Cochliobolus sativus* isolate ND85F for Morex wild-type (left) and Morex mutant 14-40 (right).

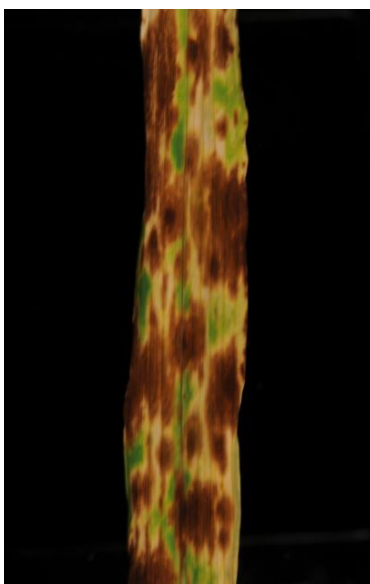


Figure 5.2. Adult-onset necrosis symptoms of mutant Mx14-40.

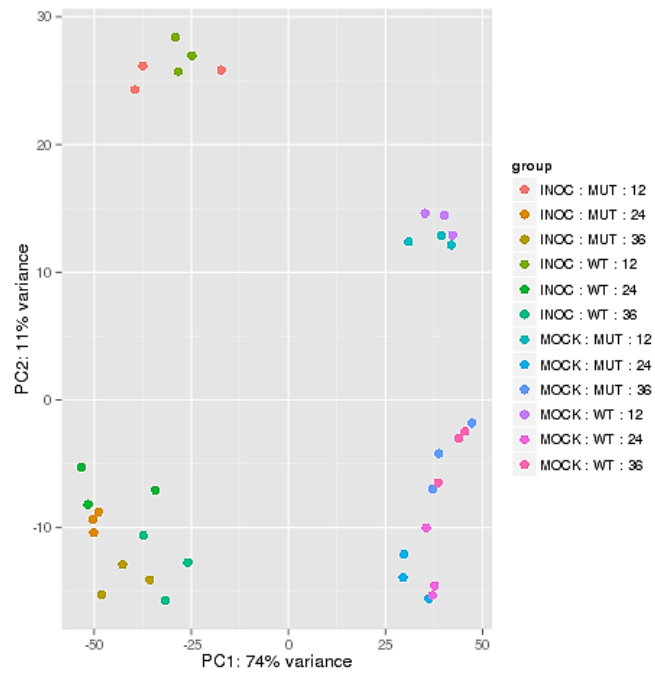


Figure 5.3. Principle Component Analysis of RNA-seq data.

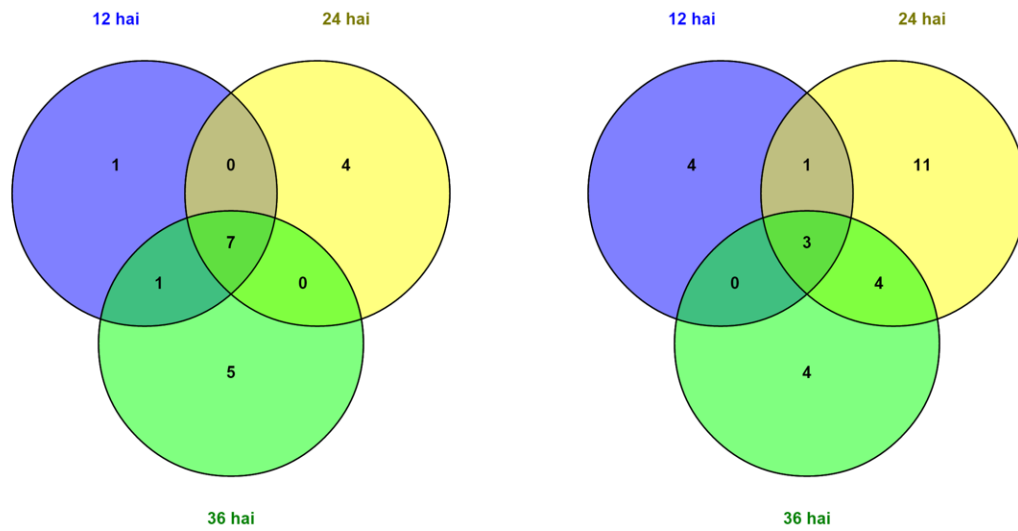


Figure 5.4. Number of gene transcripts that were up- or down-regulated (left and right, respectively) in the Morex wild-type compared with Morex mutant 14-40 [$P < 0.001$ and \log_2 fold change > 2.0 or < -2.0].

Gene ID	Inoculated						Gene ID	Mock-inoculated					
	Morex WT			Morex Mutant 14-40				Morex WT			Morex Mutant 14-40		
	12	24	36	12	24	36		12	24	36	12	24	36
MLOC_11341	3.470328	5.34229	6.273649	0.590078	1.866513	2.146325	MLOC_11341	6.925479	7.670321	8.31473	4.007297	4.138086	5.063603
MLOC_17240	6.686266	7.064552	8.062456	0	0	0	MLOC_17240	7.144462	7.056144	7.597248	0	0	0
MLOC_18224	7.090449	8.01759	7.764633	4.432159	5.516478	5.675897	MLOC_18224	7.761259	8.125707	7.555035	5.666454	5.847335	5.591651
MLOC_23395	5.106381	6.019677	5.444035	1.567169	2.058286	1.875301	MLOC_23395	2.974125	5.397685	4.30666	1.102602	1.592454	2.311906
MLOC_31603	1.35752	3.893891	3.270668	0	0	0	MLOC_31603	2.484326	5.119653	3.763673	0	1.141437	0.985387
MLOC_34706	6.377996	6.98481	6.493825	1.651373	2.681425	2.514952	MLOC_34706	4.954991	5.230096	5.257223	1.463128	0.051529	0.575583
MLOC_35121	4.081329	4.543724	3.382452	6.99686	6.981066	6.30266	MLOC_35121	4.081329	3.361444	3.582538	6.99686	6.283727	6.257664
MLOC_37652	8.342321	7.881705	7.865128	5.219653	5.768135	4.183551	MLOC_37652	10.09712	8.824263	9.212616	6.706747	5.822961	6.356827
MLOC_4070	0.30314	0.188065	0.478387	5.036609	6.013353	6.59483	MLOC_4070	0	0.737173	0	5.793743	4.77323	5.109788
MLOC_46970	2.172345	3.256209	3.864331	0	0	0	MLOC_46970	3.04719	4.977117	4.967366	0	0	0
MLOC_55208	4.766624	6.249875	6.730219	0	0	0	MLOC_55208	4.926837	5.653762	6.912628	0	0	0
MLOC_55612	3.746808	4.858909	3.97559	1.923436	1.619085	2.180219	MLOC_55612	2.934752	4.888187	4.91723	1.358734	2.120779	2.004104
MLOC_55886	8.261849	11.39775	11.32676	5.637783	11.26636	10.95611	MLOC_55886	8.965031	8.791218	8.518823	5.665208	6.334608	6.043079
MLOC_56627	7.82024	8.919735	8.795018	4.865298	5.977065	6.082739	MLOC_56627	8.540314	9.132431	9.07868	5.194921	6.101199	6.404472
MLOC_59648	4.105418	3.131454	3.461012	7.524471	5.740295	6.468445	MLOC_59648	5.094529	3.971209	4.094567	7.90513	6.874574	7.309178
MLOC_63451	3.610754	3.4533	3.56993	6.871447	7.218453	6.040551	MLOC_63451	3.610754	4.174816	3.815569	6.871447	6.998506	6.418662
MLOC_63777	0.053859	1.82977	0	4.157063	4.006215	6.31047	MLOC_63777	0	0	0	4.866188	6.661122	6.102982
MLOC_64708	5.597003	5.405621	5.351045	0.232426	0.840471	0.409823	MLOC_64708	5.234748	5.568215	5.269038	0.481085	0.048412	0.839611
MLOC_64780	8.403289	8.44528	8.23492	4.459657	3.90871	4.507332	MLOC_64780	7.857939	7.87859	7.942434	4.035313	3.955458	4.456556
MLOC_65020	9.035443	9.253605	8.456125	7.062088	6.778951	5.884055	MLOC_65020	11.25274	10.52534	10.38989	8.766155	8.258785	8.423284
MLOC_66104	3.424493	4.056904	2.833517	0	0	0	MLOC_66104	2.532281	4.115888	3.045808	0	0	0
MLOC_69617	7.525505	7.343184	6.993684	5.56233	4.94145	4.568669	MLOC_69617	6.52812	7.491702	6.731004	4.163348	4.955589	4.234898
MLOC_69866	11.90178	11.98252	11.51826	2.63206	0.772854	1.715947	MLOC_69866	12.9067	11.80751	11.57032	1.95726	2.290083	2.57802
MLOC_7242	5.5543	2.40956	5.105533	9.105812	6.386817	7.941084	MLOC_7242	8.182735	3.925942	7.021614	11.54572	7.910169	11.05028

Figure 5.5. Heat map of differentially expressed gene transcripts in Morex WT and Morex mutant 14-40. Values represent gene counts normalized using a negative binomial distribution.

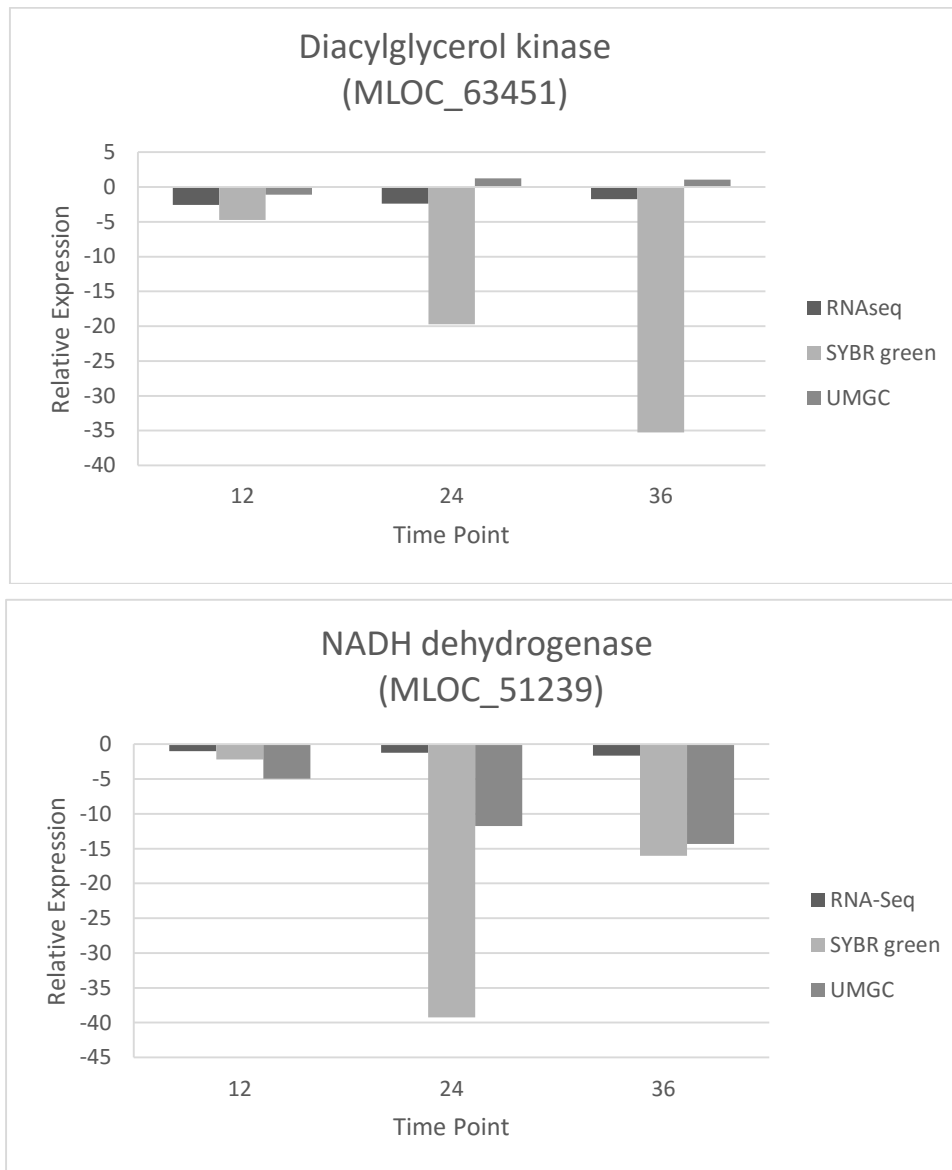


Figure 5.6. Validation of RNAseq data with quantitative real-time PCR (qPCR).

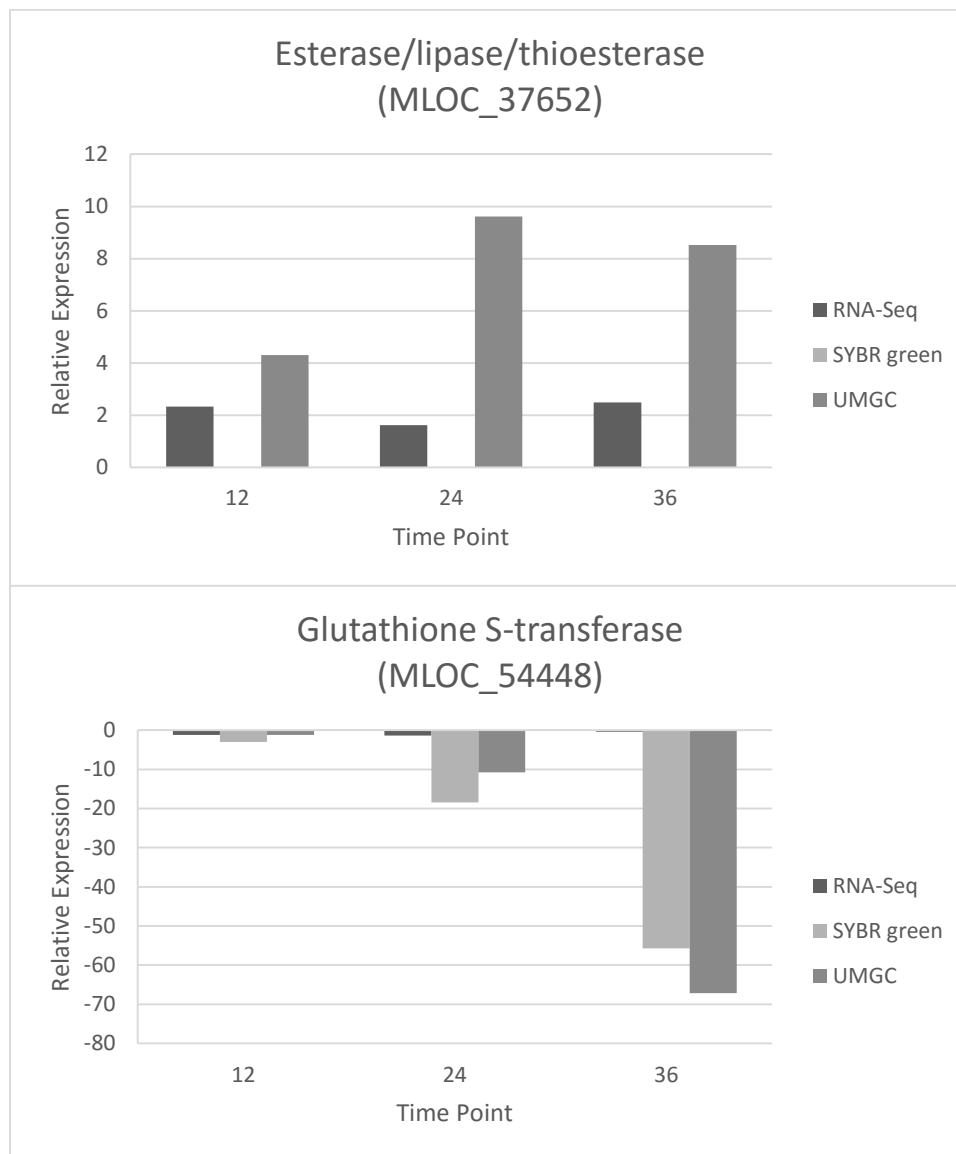


Figure 5.6. Continued

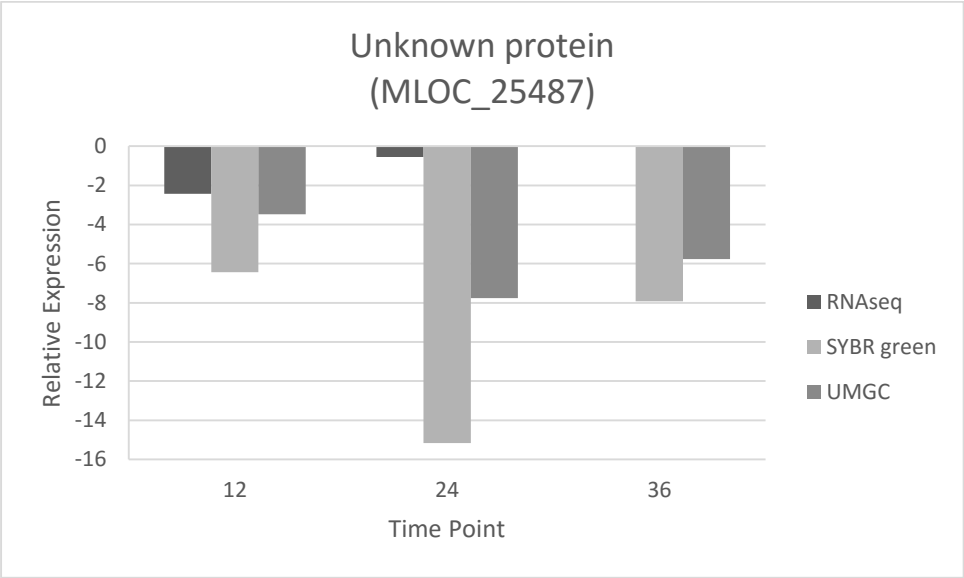


Figure 5.6. Continued

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Appendices

Appendix A1. Information for isolates of *Fusarium graminearum* used in the St. Paul 2010 Fusarium head blight nursery.

#	Accession Number		Collection Information					Previous Crop	Species	Storage Information		
	SGP ID No:	Field ID No:	Other ID No:	Host Crop	Cultivar	Year	Location County, State			Town / additional info.	Soil	Silica Gel
	1	10113001	W-3-A-13	-	Wheat	-	2013			Roseau, MN	Roseau Wheat trial/ Roseau, MN	-
2	10113002	B-4-A-13	-	Barley	-	2013	Roseau, MN	Roseau Wheat trial/ Roseau, MN	-	GZ	Oct-13	Oct-13
3	10113003	B-4-B-13	-	Barley	-	2013	Roseau, MN	Roseau Wheat trial/ Roseau, MN	-	GZ	Oct-13	Oct-13
4	10113004	W-5-A-13	-	Wheat	-	2013	-	-	-	GZ	Oct-13	Oct-13
5	10113005	W-11-A-13	E1	Wheat	Rollag	2013	Kittson, MN	Jupiter/ on Hwy 7	Soybeans	GZ	Oct-13	Oct-13
6	10113006	W-12-A-13	C4	Wheat	Prosper	2013	Norman, MN	Lee	Soybeans	GZ	Oct-13	Oct-13
7	10113007	W-12-B-13	C4	Wheat	Prosper	2013	Norman, MN	Lee	Soybeans	GZ	Oct-13	Oct-13
8	10113008	W-13-A-13	C3	Wheat	Soren	2013	Otter Tail, Mn	Carlisle	Soybeans	GZ	Oct-13	Oct-13
9	10113010	W-17-A-13	D3	Wheat	Prosper	2013	Pennington, MN	Bray	Soybeans	GZ	Oct-13	Oct-13
10	10113011	W-17-B-13	D3	Wheat	Prosper	2013	Pennington, MN	Bray	Soybeans	GZ	Oct-13	Oct-13
11	10113012	W-18-A-13	D1	Wheat	Prosper	2013	Red Lake, MN	Lake Pleasant/ 110th Ave SW and CR114	Soybeans	GZ	Oct-13	Oct-13
12	10113013	W-19-A-13	F3	Wheat	Prosper	2013	Clay, MN	on US75; N side is adjacent to housing development	Soybeans	GZ	Oct-13	Oct-13
13	10113014	W-20-A-13	F2	Wheat	Prosper	2013	Clay, MN	Moland/house on NW 1/4	Soybeans	GZ	Oct-13	Oct-13
14	10113015	W-20-B-13	F2	Wheat	Prosper	2013	Clay, MN	Moland/house on NW 1/4	Soybeans	GZ	Oct-13	Oct-13
15	10113016	W-23-A-13	E4	Wheat	Norden	2013	Marshall, MN	Sinnott/entered at SW of 1/4	Soybeans	GZ	Oct-13	Oct-13
16	10113017	W-23-B-13	E4	Wheat	Norden	2013	Marshall, MN	Sinnott/entered at SW of 1/4	Soybeans	GZ	Oct-13	Oct-13
17	10113018	W-23-C-13	E4	Wheat	Norden	2013	Marshall, MN	Sinnott/entered at SW of 1/4	Soybeans	GZ	Oct-13	Oct-13
18	10113019	W-25-A-13	D4	Wheat	Jenna	2013	Marshall, MN	Excel	Alfalfa	GZ	Oct-13	Oct-13
19	10113020	W-25-B-13	D4	Wheat	Jenna	2013	Marshall, MN	Excel	Alfalfa	GZ	Oct-13	Oct-13
20	10113021	B-29-A-13	-	Barley	-	2013	Wilkin, MN	Foxhome	-	GZ	Oct-13	Oct-13
21	10113022	W-30-A-13	B2	Wheat	-	2013	Norman, MN	-	-	GZ	Oct-13	Oct-13
22	10113023	W-31-A-13	B3	Wheat	Brennan	2013	Clay, MN	Highland Grove, entered through farm on hwy 32	Soybeans	GZ	Oct-13	Oct-13
23	10112001	W-2-A-12	-	Wheat	Albany	2012	Polk, MN	Vineland	-	Gz	Sep-12	Sep-12
24	10112002	W-2-B-12	-	Wheat	Albany	2012	Polk, MN	Vineland	-	Gz	Sep-12	Sep-12
25	10112003	W-3-B-12	-	Wheat	-	2012	Polk, MN	Vineland	-	Gz	Sep-12	Sep-12
26	10112004	W-7-A-12	-	Wheat	-	2012	Marshall, MN	Warrenton	-	Gz	Sep-12	Sep-12
27	10112005	W-9-A-12	-	Wheat	-	2012	Marshall, MN	Tamarac	-	Gz	Sep-12	Sep-12

Appendix A1 (Continued). Information for isolates of *Fusarium graminearum* used in the St. Paul 2010 Fusarium head blight nursery.

#	Accession Number			Collection Information					Previous Crop	Species	Storage Information	
	SGP	Field	Other	Host	Cultivar	Year	Location				Soil	Silica
	ID No:	ID No:	ID No:	Crop			County, State	Town / additional info.				
28	10112006	W-9-B-12	-	Wheat	-	2012	Marshall, MN	Tamarac	-	Gz	Sep-12	Sep-12
29	10112007	W-13-A-12	-	Wheat	-	2012	Polk, MN	Higdem	-	Gz	Sep-12	Sep-12
30	10112008	W-13-C-12	-	Wheat	-	2012	Polk, MN	Higdem	-	Gz	Sep-12	Sep-12
31	10112009	W-14-A-12	-	Wheat	-	2012	-	-	-	Gz	Sep-12	Sep-12
32	10112010	W-14-B-12	-	Wheat	-	2012	-	-	-	Gz	Sep-12	Sep-12
33	10112011	W-16-A-12	-	Wheat	-	2012	-	-	-	Gz	Sep-12	Sep-12
34	10112012	W-16-B-12	-	Wheat	-	2012	-	-	-	Gz	Sep-12	Sep-12
35	10111001	W-1-A-11	-	Wheat	-	2011	-	-	-	Gz	Aug-11	Aug-11
36	10111005	W-3-B-11	-	Wheat	-	2011	-	-	-	Gz	Aug-11	Aug-11
37	10111007	W-4-A-11	-	Wheat	-	2011	-	-	-	Gz	Aug-11	Aug-11
38	10110004	W-3-A-10	-	Wheat	-	2010	Marshall, MN	S of Argyle, Hwy 75	-	Gz	Oct-10	Oct-10
39	10110007	W-6-B-10	-	Wheat	-	2010	Polk, MN	Hwy 9 & County Rd 41	-	Gz	Oct-10	Oct-10

Appendix A2. Information for isolates of *Fusarium graminearum* used in the St. Paul 2011 Fusarium head blight nursery.

Accession Number		Collection Information							Species	Storage Information	
SGP	Field	Other	Host		Year	Location		Previous		Soil	Silica
ID No:	ID No:	ID No:	Crop	Cultivar		County, State	Town / additional info.	Crop			Gel
10110001	W-1-A-10	-	Wheat	-	2010	Marshall, MN	Stephen	-	Gz	Oct-10	Oct-10
10110002	W-1-B-10	-	Wheat	-	2010	Marshall, MN	Stephen	-	Gz	Oct-10	Oct-10
10110003	B--2-A-10	-	Barley	-	2010	Marshall, MN	Stephen	-	Gz	Oct-10	Oct-10
10110004	W-3-A-10	-	Wheat	-	2010	Marshall, MN	S of Argyle, Hwy 75	-	Gz	Oct-10	Oct-10
10110005	W-3-B-10	-	Wheat	-	2010	Marshall, MN	S of Argyle, Hwy 75	-	Gz	Oct-10	Oct-10
10110006	W-5-B-10	-	Wheat	-	2010	Polk, MN	S of Euclid, Hwy 75	-	Gz	Oct-10	Oct-10
10110007	W-6-B-10	-	Wheat	-	2010	Polk, MN	Hwy 9 & County Rd 41	-	Gz	Oct-10	Oct-10
10110008	W-8-A-10	-	Wheat	-	2010	Polk, MN	S of Beltrami, Hwy 9	-	Gz	Oct-10	Oct-10
10110009	W-8-B-10	-	Wheat	-	2010	Polk, MN	S of Beltrami, Hwy 9	-	Gz	Oct-10	Oct-10
10110010	W-10-A-10	-	Wheat	-	2010	Polk, MN	Between Beltrami & Borup, Hwy 9	-	Gz	Oct-10	Oct-10
10110011	W-11-B-10	-	Wheat	-	2010	Norman, MN	S of Borup, Hwy 9	-	Gz	Oct-10	Oct-10
10109001	W-5-09	-	Wheat	-	2009	Norman, MN	Hwy 9, S of Borup	-	Gz	Sep-09	Sep-09
10109007	B-17-09	-	Barley	-	2009	Clay, MN	Hwy 9 & 80th Ave	-	Gz	Sep-09	Sep-09
10109008	W-18-09	-	Wheat	-	2009	Polk, MN	Hwy 2 & Hwy 102	-	Gz	Sep-09	Sep-09
10109009	W-19-09	-	Wheat	-	2009	Ottertail, MN	County Rd 82	-	Gz	Sep-09	Sep-09
10109010	W-20-09	-	Wheat	-	2009	Norman, MN	County Rd 123 & 250th Ave	-	Gz	Sep-09	Sep-09
10109011	W-21-09	-	Wheat	-	2009	Norman, MN	Hwy 9 & Wild Rice River	-	Gz	Sep-09	Sep-09
10109012	W-23-09	-	Wheat	-	2009	Clay, MN	Barnsville	-	Gz	Sep-09	Sep-09
10109013	W-25-09	-	Wheat	-	2009	Norman, MN	Hwy 9	-	Gz	Sep-09	Sep-09
10108004	W-4-08	-	Wheat	-	2008	Polk, MN	N of Euclid, Hwy 75	-	Gz	Sep-08	-
10108005	B-8-08	-	Barley	-	2008	Marshall, MN	N of Alvarado	-	Gz	Sep-08	Sep-08
10108006	W-10-08	-	Wheat	-	2008	Marshall, MN	N of Alvarado	-	Gz	Sep-08	Sep-08
10108007	W-11-08	-	Wheat	-	2008	Kittson, MN	E of Donaldson, Hwy 11	-	Gz	Sep-08	Sep-08
10108008	W-14-08	-	Wheat	-	2008	Kittson, MN	S of Karlstad	-	Gz	Sep-08	Sep-08
10108009	O-19-08	-	Oat	-	2008	Polk, MN	Crookston, Hwy 2	-	Gz	Sep-08	Sep-08
10108011	W-23-08	-	Wheat	-	2008	Norman, MN	S of Beltrami, Hwy 9	-	Gz	Sep-08	Sep-08
10108012	W-25-08	-	Wheat	-	2008	Norman, MN	S of Beltrami, Hwy 9	-	Gz	Sep-08	Sep-08

Appendix A2 (Continued). Information for isolates of *Fusarium graminearum* used in the St. Paul 2011 Fusarium head blight nursery.

Accession Number			Collection Information						Species	Storage Information	
SGP	Field	Other	Host		Year	Location		Previous		Soil	Silica
ID No:	ID No:	ID No:	Crop	Cultivar		County, State	Town / additional info.	Crop			Gel
10108013	W-28-08	-	Wheat	-	2008	Norman, MN	Borup	-	Gz	Sep-08	Sep-08
10108016	W-32-08	-	Wheat	-	2008	Polk, MN	Erskine, Hwy 59	-	Gz	Sep-08	Sep-08
10108018	W-35-08	-	Wheat	-	2008	Red Lake, MN	Extension Trial in Oklee	-	Gz	Sep-08	Sep-08
10108019	W-38-08	-	Wheat	Kuntz	2008	Polk, MN	240th St S & 310th Ave SW, by Fisher	-	Gz	Sep-08	Sep-08
10108022	W-45-08	-	Wheat	Samson	2008	Polk, MN	Euclid, 240th & 310th Ave	-	Gz	Sep-08	Sep-08
10107001	W-5-07	-	Wheat	-	2007	Norman, MN	W of Ada, Hwy 200	-	Gz	Feb-10	Oct-07
10107002	W-7-07	-	Wheat	-	2007	Clark, MN	Neillsville	-	Gz	Feb-10	Oct-07
10107003	W-8-07	-	Wheat	-	2007	Polk, MN	N of Angus	-	Gz	Feb-10	Oct-07
10107004	W-9-07	-	Wheat	-	2007	Marshall, MN	N of Warren	-	Gz	Feb-10	Oct-07
10107005	W-10c-07	-	Wheat	-	2007	Marshall, MN	Stephen Airport	-	Gz	Feb-10	Oct-07
10107006	W-13-07	-	Wheat	-	2007	Marshall, MN	Eagle Point	-	Gz	Feb-10	Oct-07
10107007	W-16-07	-	Wheat	-	2007	Polk, MN	Sandsville	-	Gz	Feb-10	Oct-07
10107008	W-23-07	-	Wheat	-	2007	Norman, MN	Ada	-	Gz	Feb-10	Oct-07
10107009	W-26-07	-	Wheat	Granger	2007	Norman, MN	Borup, Hwy 9	-	Gz	Feb-10	Oct-07
10106001	W-10-06	-	Wheat	Norpro	2006	Norman, MN	Borup	-	Gz	Apr-07	Apr-07
10106002	N-C	-	Corn	-	2006	Dakota, MN	Rosemount	-	Gz	May-07	May-07
10105057	B-2-1	-	Barley	-	2005	Polk, MN	Fertile	-	Gz	Apr-08	Jun-07
10105064	B-3-5	-	Barley	-	2005	Mahnomen, MN	Mahnomen, Hwy 200	-	Gz	Apr-08	Jun-07
10105068	B-4-3	-	Barley	Stander	2005	Dakota, MN	Rosemount	-	Gz	Feb-09	Jun-07
10105008	W-2-4	-	Wheat	Oklee	2005	Norman, MN	Borup, Agripro Site	-	Gz	May-07	May-07
10105014	W-3-5	-	Wheat	-	2005	Polk, MN	Angus	-	Gz	May-07	May-07
10105015	W-4-1	-	Wheat	-	2005	Polk, MN	Crookston by Northland Inn	-	Gz	May-07	May-07
10105037	W-9-1	-	Wheat	Hanna	2005	Norman, MN	Borup, Agripro Site	-	Gz	Jun-07	Jun-07

Appendix A3. Information for isolates of *Fusarium graminearum* used in the St. Paul 2012 Fusarium head blight nursery.

Accession Number			Collection Information					Species	Storage Information		
SGP ID No:	Field ID No:	Other ID No:	Host Crop	Cultivar	Year	Location County, State	Town / additional info.	Previous Crop	Soil	Silica Gel	
10111001	W-1-A-11	-	Wheat	-	2011	-	-	-	Gz	Aug-11	Aug-11
10111002	W-1-B-11	-	Wheat	-	2011	-	-	-	Gz	Aug-11	Aug-11
10111003	W-2-B-11	-	Wheat	-	2011	-	-	-	Gz	Aug-11	Aug-11
10111004	W-3-A-11	-	Wheat	-	2011	-	-	-	Gz	Aug-11	Aug-11
10111005	W-3-B-11	-	Wheat	-	2011	-	-	-	Gz	Aug-11	Aug-11
10111006	W-3-D-11	-	Wheat	-	2011	-	-	-	Gz	Aug-11	Aug-11
10111007	W-4-A-11	-	Wheat	-	2011	-	-	-	Gz	Aug-11	Aug-11
10111008	W-4-B-11	-	Wheat	-	2011	-	-	-	Gz	Aug-11	Aug-11
10111009	W-4-D-11	-	Wheat	-	2011	-	-	-	Gz	Aug-11	Aug-11
10110001	W-1-A-10	-	Wheat	-	2010	Marshall, MN	Stephen	-	Gz	Oct-10	Oct-10
10110002	W-1-B-10	-	Wheat	-	2010	Marshall, MN	Stephen	-	Gz	Oct-10	Oct-10
10110003	B-2-A-10	-	Barley	-	2010	Marshall, MN	Stephen	-	Gz	Oct-10	Oct-10
10110004	W-3-A-10	-	Wheat	-	2010	Marshall, MN	S of Argyle, Hwy 75	-	Gz	Oct-10	Oct-10
10110005	W-3-B-10	-	Wheat	-	2010	Marshall, MN	S of Argyle, Hwy 75	-	Gz	Oct-10	Oct-10
10110006	W-5-B-10	-	Wheat	-	2010	Polk, MN	S of Euclid, Hwy 75	-	Gz	Oct-10	Oct-10
10110007	W-6-B-10	-	Wheat	-	2010	Polk, MN	Hwy 9 & County Rd 41	-	Gz	Oct-10	Oct-10
10110008	W-8-A-10	-	Wheat	-	2010	Polk, MN	S of Beltrami, Hwy 9	-	Gz	Oct-10	Oct-10
10110010	W-10-A-10	-	Wheat	-	2010	Polk, MN	Between Beltrami & Borup, Hwy 9	-	Gz	Oct-10	Oct-10
10110011	W-11-B-10	-	Wheat	-	2010	Norman, MN	S of Borup, Hwy 9	-	Gz	Oct-10	Oct-10
10109001	W-5-09	-	Wheat	-	2009	Norman, MN	Hwy 9, S of Borup	-	Gz	Sep-09	Sep-09
10109003	W-12-09	-	Wheat	-	2009	Polk, MN	Hwy 9, S of Hwy 10	-	Gz	Sep-09	Sep-09
10109004	W-13-09	-	Wheat	-	2009	Polk, MN	S of Crookston, Hwy 9	-	Gz	Sep-09	Sep-09
10109006	W-16-09	-	Wheat	-	2009	Norman, MN	S of Beltrami on Hwy 9	-	Gz	Sep-09	Sep-09
10109008	W-18-09	-	Wheat	-	2009	Polk, MN	Hwy 2 & Hwy 102	-	Gz	Sep-09	Sep-09
10109009	W-19-09	-	Wheat	-	2009	Ottertail, MN	County Rd 82	-	Gz	Sep-09	Sep-09
10109010	W-20-09	-	Wheat	-	2009	Norman, MN	County Rd 123 & 250th Ave	-	Gz	Sep-09	Sep-09
10109011	W-21-09	-	Wheat	-	2009	Norman, MN	Hwy 9 & Wild Rice River	-	Gz	Sep-09	Sep-09
10109012	W-23-09	-	Wheat	-	2009	Clay, MN	Barnsville	-	Gz	Sep-09	Sep-09
10109013	W-25-09	-	Wheat	-	2009	Norman, MN	Hwy 9	-	Gz	Sep-09	Sep-09
10107009	W-26-07	-	Wheat	Granger	2007	Norman, MN	Borup, Hwy 9	-	Gz	Feb-10	Oct-07

Appendix A4. Information for isolates of *Fusarium graminearum* used in the St. Paul 2013 Fusarium head blight nursery.

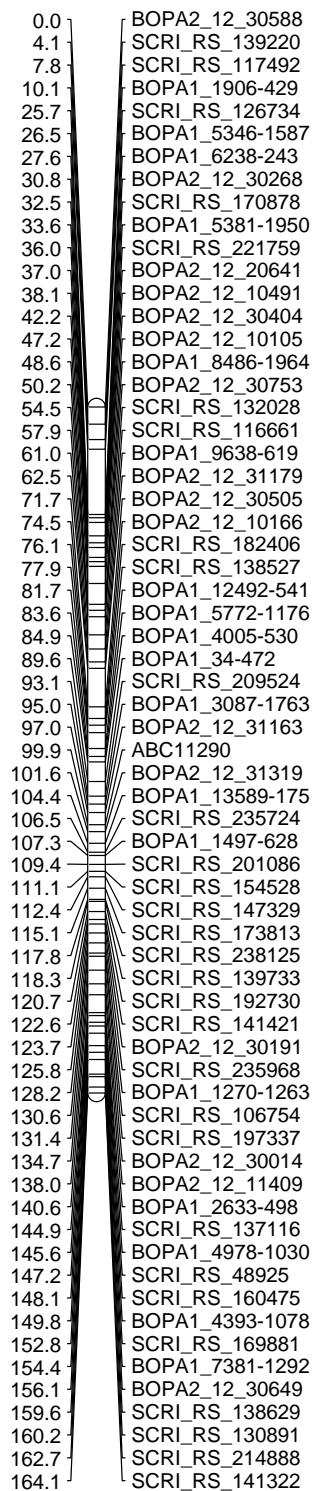
Accession Number		Collection Information						Species	Storage Information		
SGP ID No:	Field ID No:	Other ID No:	Host Crop	Cultivar	Year	Location County, State Town / additional info.		Previous Crop	Soil	Silica Gel	
10112001	W-2-A-12	-	Wheat	Albany	2012	Polk, MN	Vineland	-	Gz	Sep-12	Sep-12
10112002	W-2-B-12	-	Wheat	Albany	2012	Polk, MN	Vineland	-	Gz	Sep-12	Sep-12
10112003	W-3-B-12	-	Wheat	-	2012	Polk, MN	Vineland	-	Gz	Sep-12	Sep-12
10112004	W-7-A-12	-	Wheat	-	2012	Marshall, MN	Warrenton	-	Gz	Sep-12	Sep-12
10112005	W-9-A-12	-	Wheat	-	2012	Marshall, MN	Tamarac	-	Gz	Sep-12	Sep-12
10112006	W-9-B-12	-	Wheat	-	2012	Marshall, MN	Tamarac	-	Gz	Sep-12	Sep-12
10112007	W-13-A-12	-	Wheat	-	2012	Polk, MN	Higdem	-	Gz	Sep-12	Sep-12
10112008	W-13-C-12	-	Wheat	-	2012	Polk, MN	Higdem	-	Gz	Sep-12	Sep-12
10112009	W-14-A-12	-	Wheat	-	2012	-	-	-	Gz	Sep-12	Sep-12
10112010	W-14-B-12	-	Wheat	-	2012	-	-	-	Gz	Sep-12	Sep-12
10112011	W-16-A-12	-	Wheat	-	2012	-	-	-	Gz	Sep-12	Sep-12
10112012	W-16-B-12	-	Wheat	-	2012	-	-	-	Gz	Sep-12	Sep-12
10111001	W-1-A-11	-	Wheat	-	2011	-	-	-	Gz	Aug-11	Aug-11
10111003	W-2-B-11	-	Wheat	-	2011	-	-	-	Gz	Aug-11	Aug-11
10111004	W-3-A-11	-	Wheat	-	2011	-	-	-	Gz	Aug-11	Aug-11
10111005	W-3-B-11	-	Wheat	-	2011	-	-	-	Gz	Aug-11	Aug-11
10111006	W-3-D-11	-	Wheat	-	2011	-	-	-	Gz	Aug-11	Aug-11
10111007	W-4-A-11	-	Wheat	-	2011	-	-	-	Gz	Aug-11	Aug-11
10111009	W-4-D-11	-	Wheat	-	2011	-	-	-	Gz	Aug-11	Aug-11
10110001	W-1-A-10	-	Wheat	-	2010	Marshall, MN	Stephen	-	Gz	Oct-10	Oct-10
10110002	W-1-B-10	-	Wheat	-	2010	Marshall, MN	Stephen	-	Gz	Oct-10	Oct-10
10110003	B-2-A-10	-	Barley	-	2010	Marshall, MN	Stephen	-	Gz	Oct-10	Oct-10
10110004	W-3-A-10	-	Wheat	-	2010	Marshall, MN	S of Argyle, Hwy 75	-	Gz	Oct-10	Oct-10
10110005	W-3-B-10	-	Wheat	-	2010	Marshall, MN	S of Argyle, Hwy 75	-	Gz	Oct-10	Oct-10
10110006	W-5-B-10	-	Wheat	-	2010	Polk, MN	S of Euclid, Hwy 75	-	Gz	Oct-10	Oct-10
10110007	W-6-B-10	-	Wheat	-	2010	Polk, MN	Hwy 9 & County Rd 41	-	Gz	Oct-10	Oct-10
10110008	W-8-A-10	-	Wheat	-	2010	Polk, MN	S of Beltrami, Hwy 9	-	Gz	Oct-10	Oct-10
10109008	W-18-09	-	Wheat	-	2009	Polk, MN	Hwy 2 & Hwy 102	-	Gz	Sep-09	Sep-09
10109009	W-19-09	-	Wheat	-	2009	Ottertail, MN	County Rd 82	-	Gz	Sep-09	Sep-09
10109012	W-23-09	-	Wheat	-	2009	Clay, MN	Barnsville	-	Gz	Sep-09	Sep-09

Appendix A5. Information for isolates of *Fusarium graminearum* used in the St. Paul 2014 Fusarium head blight nursery.

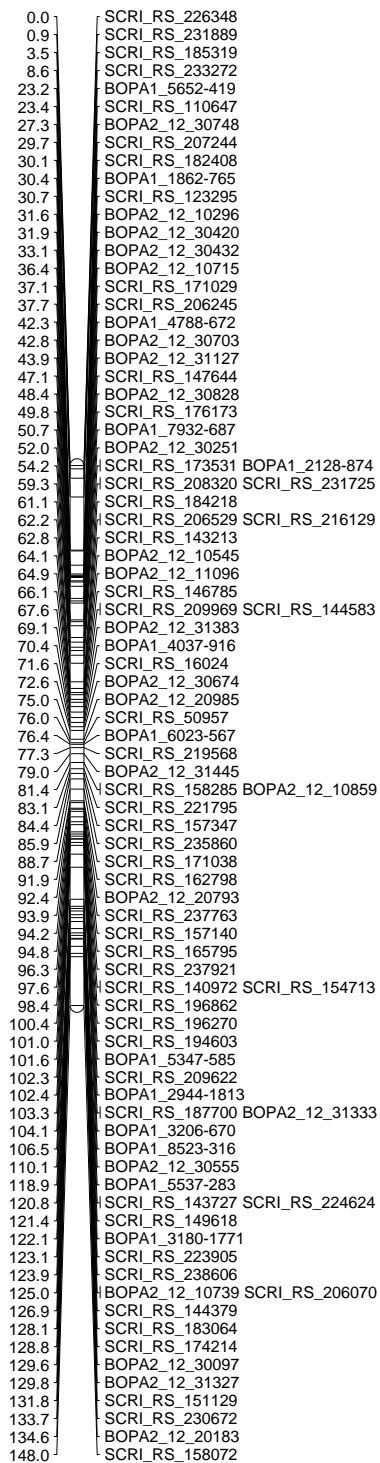
Accession Number		Collection Information						Previous Crop	Species	Storage Information	
SGP ID No:	Field ID No:	Other ID No:	Host Crop	Cultivar	Year	Location County, State	Town / additional info.			Soil	Silica Gel
10113001	W-3-A-13	-	Wheat	-	2013	Roseau, MN	Roseau Wheat trial/ Roseau, MN	-	GZ	Oct-13	Oct-13
10113002	B-4-A-13	-	Barley	-	2013	Roseau, MN	Roseau Wheat trial/ Roseau, MN	-	GZ	Oct-13	Oct-13
10113003	B-4-B-13	-	Barley	-	2013	Roseau, MN	Roseau Wheat trial/ Roseau, MN	-	GZ	Oct-13	Oct-13
10113004	W-5-A-13	-	Wheat	-	2013	-	-	-	GZ	Oct-13	Oct-13
10113005	W-11-A-13	E1	Wheat	Rollag	2013	Kittson, MN	Jupiter/ on Hwy 7	Soybeans	GZ	Oct-13	Oct-13
10113006	W-12-A-13	C4	Wheat	Prosper	2013	Norman, MN	Lee	Soybeans	GZ	Oct-13	Oct-13
10113007	W-12-B-13	C4	Wheat	Prosper	2013	Norman, MN	Lee	Soybeans	GZ	Oct-13	Oct-13
10113008	W-13-A-13	C3	Wheat	Soren	2013	Otter Tail, Mn	Carlisle	Soybeans	GZ	Oct-13	Oct-13
10113010	W-17-A-13	D3	Wheat	Prosper	2013	Pennington, MN	Bray	Soybeans	GZ	Oct-13	Oct-13
10113011	W-17-B-13	D3	Wheat	Prosper	2013	Pennington, MN	Bray	Soybeans	GZ	Oct-13	Oct-13
10113012	W-18-A-13	D1	Wheat	Prosper	2013	Red Lake, MN	Lake Pleasant/ 110th Ave SW and CR114	Soybeans	GZ	Oct-13	Oct-13
10113013	W-19-A-13	F3	Wheat	Prosper	2013	Clay, MN	on US75; N side is adjacent to housing development	Soybeans	GZ	Oct-13	Oct-13
10113014	W-20-A-13	F2	Wheat	Prosper	2013	Clay, MN	Moland/house on NW 1/4	Soybeans	GZ	Oct-13	Oct-13
10113015	W-20-B-13	F2	Wheat	Prosper	2013	Clay, MN	Moland/house on NW 1/4	Soybeans	GZ	Oct-13	Oct-13
10113016	W-23-A-13	E4	Wheat	Norden	2013	Marshall, MN	Sinnott/entered at SW of 1/4	Soybeans	GZ	Oct-13	Oct-13
10113017	W-23-B-13	E4	Wheat	Norden	2013	Marshall, MN	Sinnott/entered at SW of 1/4	Soybeans	GZ	Oct-13	Oct-13
10113018	W-23-C-13	E4	Wheat	Norden	2013	Marshall, MN	Sinnott/entered at SW of 1/4	Soybeans	GZ	Oct-13	Oct-13
10113019	W-25-A-13	D4	Wheat	Jenna	2013	Marshall, MN	Excel	Alfalfa	GZ	Oct-13	Oct-13
10113020	W-25-B-13	D4	Wheat	Jenna	2013	Marshall, MN	Excel	Alfalfa	GZ	Oct-13	Oct-13
10113021	B-29-A-13	-	Barley	-	2013	Wilkin, MN	Foxhome	-	GZ	Oct-13	Oct-13
10113022	W-30-A-13	B2	Wheat	-	2013	Norman, MN	-	-	GZ	Oct-13	Oct-13
10113023	W-31-A-13	B3	Wheat	Brennan	2013	Clay, MN	Highland Grove, entered through farm on hwy 32	Soybeans	GZ	Oct-13	Oct-13
10112001	W-2-A-12	-	Wheat	Albany	2012	Polk, MN	Vineland	-	Gz	Sep-12	Sep-12
10112002	W-2-B-12	-	Wheat	Albany	2012	Polk, MN	Vineland	-	Gz	Sep-12	Sep-12
10112003	W-3-B-12	-	Wheat	-	2012	Polk, MN	Vineland	-	Gz	Sep-12	Sep-12
10112004	W-7-A-12	-	Wheat	-	2012	Marshall, MN	Warrenton	-	Gz	Sep-12	Sep-12
10112005	W-9-A-12	-	Wheat	-	2012	Marshall, MN	Tamarac	-	Gz	Sep-12	Sep-12

Appendix A5 (Continued). Information for isolates of *Fusarium graminearum* used in the St. Paul 2014 Fusarium head blight nursery.

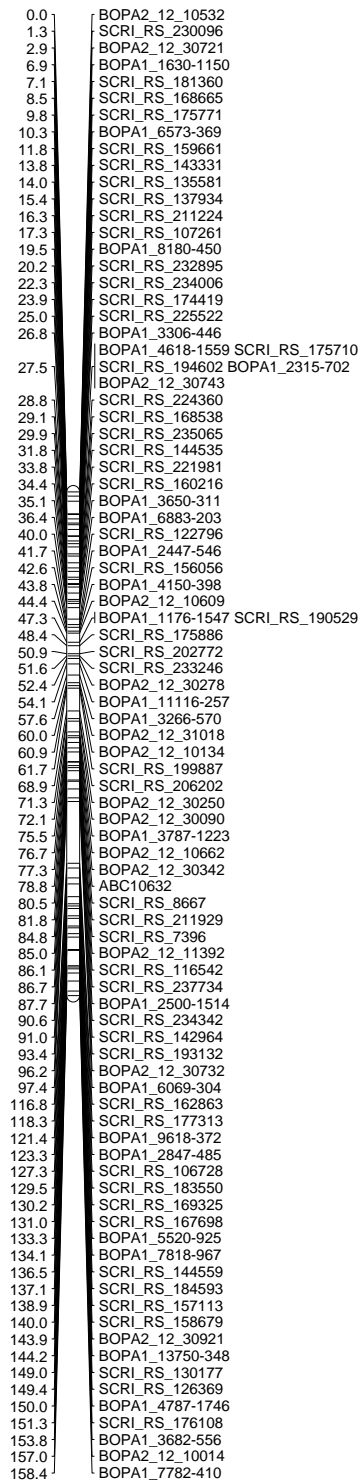
Accession Number		Collection Information						Species	Storage Information		
SGP ID No:	Field ID No:	Other ID No:	Host Crop	Cultivar	Year	Location County, State	Town / additional info.	Previous Crop	Soil	Silica Gel	
10112006	W-9-B-12	-	Wheat	-	2012	Marshall, MN	Tamarac	-	Gz	Sep-12	Sep-12
10112007	W-13-A-12	-	Wheat	-	2012	Polk, MN	Higdem	-	Gz	Sep-12	Sep-12
10112008	W-13-C-12	-	Wheat	-	2012	Polk, MN	Higdem	-	Gz	Sep-12	Sep-12
10112009	W-14-A-12	-	Wheat	-	2012	-	-	-	Gz	Sep-12	Sep-12
10112010	W-14-B-12	-	Wheat	-	2012	-	-	-	Gz	Sep-12	Sep-12
10112011	W-16-A-12	-	Wheat	-	2012	-	-	-	Gz	Sep-12	Sep-12
10112012	W-16-B-12	-	Wheat	-	2012	-	-	-	Gz	Sep-12	Sep-12
10111001	W-1-A-11	-	Wheat	-	2011	-	-	-	Gz	Aug-11	Aug-11
10111005	W-3-B-11	-	Wheat	-	2011	-	-	-	Gz	Aug-11	Aug-11
10111007	W-4-A-11	-	Wheat	-	2011	-	-	-	Gz	Aug-11	Aug-11
10110004	W-3-A-10	-	Wheat	-	2010	Marshall, MN	S of Argyle, Hwy 75	-	Gz	Oct-10	Oct-10
10110007	W-6-B-10	-	Wheat	-	2010	Polk, MN	Hwy 9 & County Rd 41	-	Gz	Oct-10	Oct-10



Appendix B1. A graphical representation of chromosome 1H from the Rasmuson/PI 466423 population.



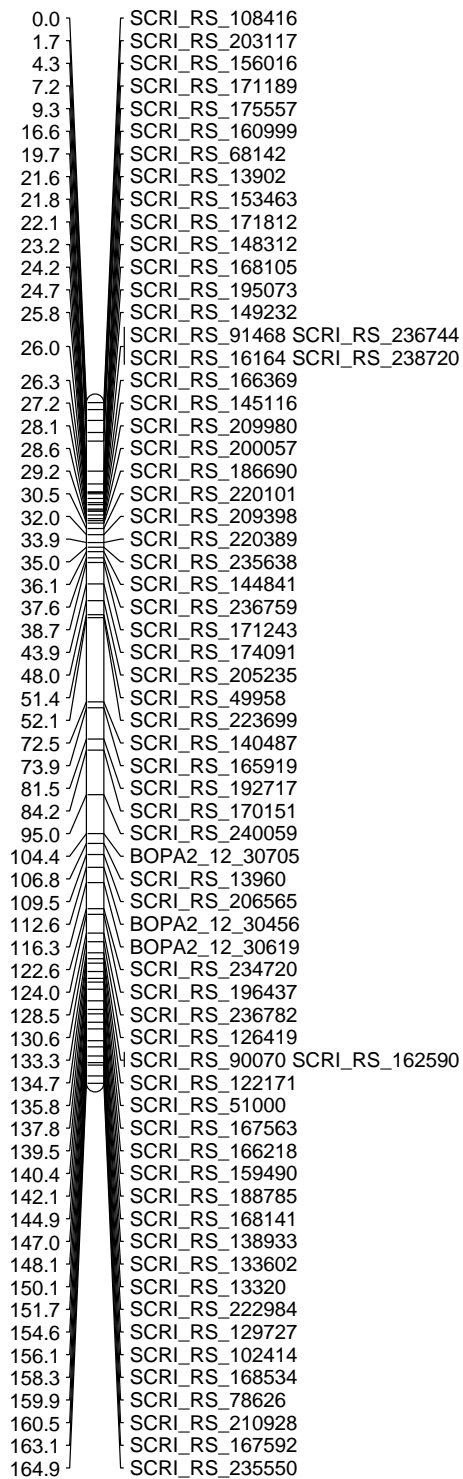
Appendix B2. A graphical representation of chromosome 2H from the Rasmuson/PI 466423 population.



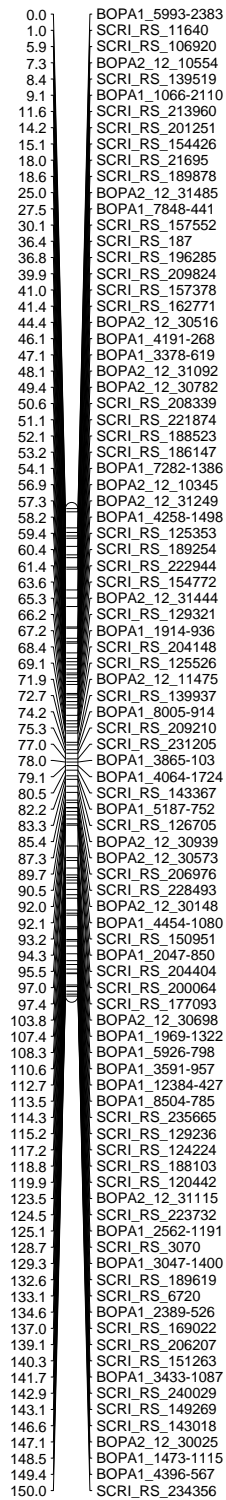
Appendix B3. A graphical representation of chromosome 3H from the Rasmuson/PI 466423 population.

0.0	BOPA1_3026-1011
1.3	SCRI_RS_206744
5.0	SCRI_RS_219136
7.2	BOPA1_3551-2537
11.5	SCRI_RS_189331
13.5	BOPA2_12_11300
15.6	SCRI_RS_197394
21.0	BOPA1_3432-290
24.6	SCRI_RS_154178
26.7	SCRI_RS_119628
29.9	BOPA1_9027-454
30.7	SCRI_RS_58772
31.1	BOPA1_1593-1597
32.8	SCRI_RS_125487
35.9	BOPA1_4098-758
36.2	BOPA1_2832-377
38.7	SCRI_RS_143144
40.0	BOPA2_12_31164
41.9	BOPA2_12_30865
43.5	BOPA2_12_31313
45.7	BOPA2_12_10860
48.0	SCRI_RS_161528
49.0	SCRI_RS_75805
51.1	BOPA1_10255-529
52.7	BOPA1_14765-388
53.1	BOPA2_12_30328
54.1	BOPA2_12_30992
61.0	BOPA2_12_30993
63.3	SCRI_RS_164612
64.2	SCRI_RS_192458
65.2	SCRI_RS_140586
66.7	BOPA2_12_30597
67.3	BOPA2_12_30564
68.1	BOPA1_5475-1355
69.2	SCRI_RS_133407
70.3	SCRI_RS_193122
72.8	BOPA2_12_30331
73.3	BOPA2_12_30777
74.3	SCRI_RS_143191
75.8	SCRI_RS_119778
76.2	BOPA1_3489-854
77.3	BOPA2_12_30427
78.1	BOPA2_12_30625
79.5	BOPA2_12_21137
80.1	SCRI_RS_213798
82.7	SCRI_RS_196044
83.7	SCRI_RS_109600
84.3	BOPA1_4276-1082
85.0	SCRI_RS_202326
86.3	SCRI_RS_136645
88.1	SCRI_RS_170560
89.4	SCRI_RS_163440
91.2	SCRI_RS_234574
93.5	SCRI_RS_229658
94.9	SCRI_RS_151213
95.2	BOPA2_12_30620
95.8	SCRI_RS_194525
96.6	BOPA1_10321-364
97.9	BOPA1_11293-490
101.9	SCRI_RS_179444
104.8	SCRI_RS_160624
106.5	BOPA1_4407-1344
109.2	SCRI_RS_175327
110.2	BOPA1_2490-1786
111.4	BOPA2_12_31362
112.9	SCRI_RS_179438
113.3	SCRI_RS_165833
115.7	SCRI_RS_200964
116.8	SCRI_RS_200957
119.6	BOPA1_1523-1136
120.2	SCRI_RS_13460
121.7	BOPA2_12_31246
122.8	BOPA1_3272-1578
123.3	SCRI_RS_125524
124.4	BOPA1_4986-1214
128.7	ABC09154
129.5	BOPA1_5245-304
130.4	BOPA1_4564-604
133.4	SCRI_RS_233444
137.5	SCRI_RS_168399
138.2	BOPA1_4361-1867
139.6	BOPA1_2574-410
142.5	BOPA2_12_30584
143.9	SCRI_RS_129218
144.6	BOPA1_5611-811

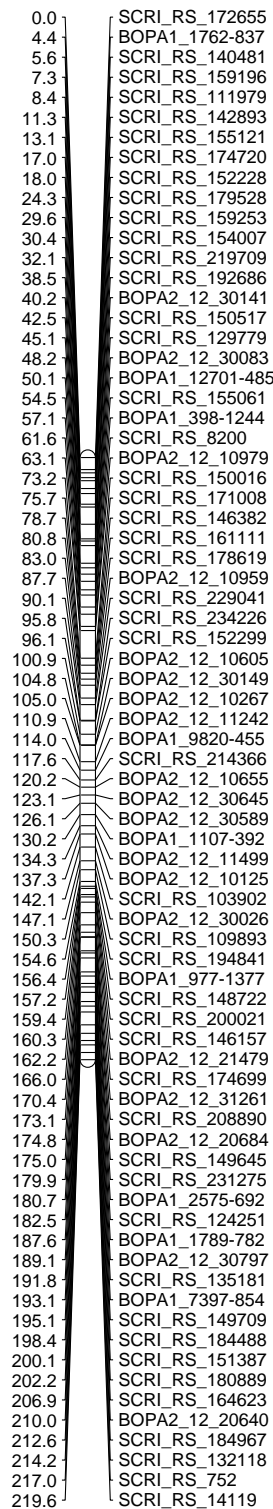
Appendix B4. A graphical representation of chromosome 4H from the Rasmuson/PI 466423 population.



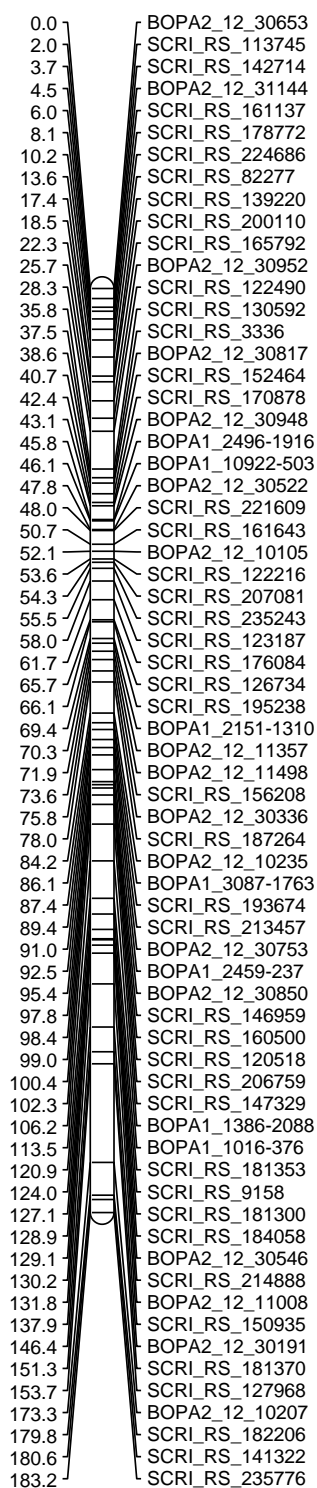
Appendix B5. A graphical representation of chromosome 5H from the Rasmuson/PI 466423 population.



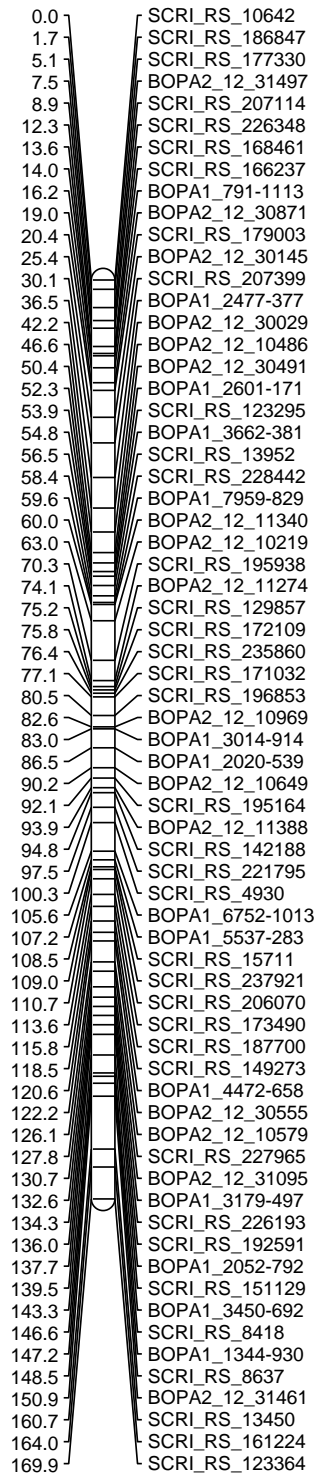
Appendix B6. A graphical representation of chromosome 6H from the Rasmuson/PI 466423 population.



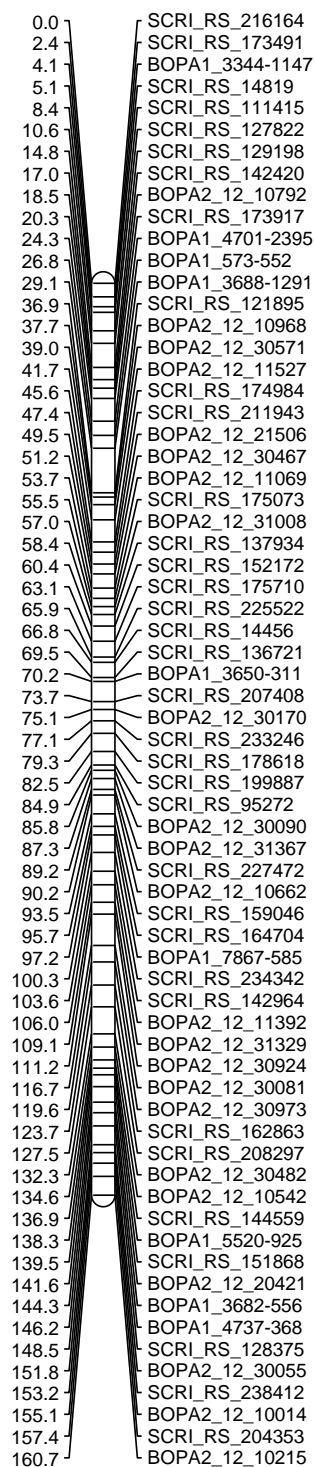
Appendix B7. A graphical representation of chromosome 7H from the Rasmuson/PI 466423 population.



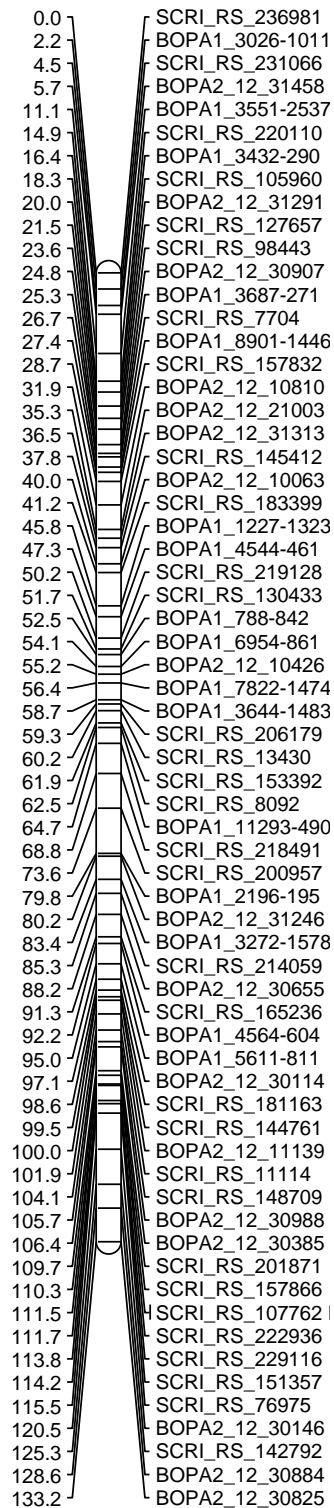
Appendix C1. A graphical representation of chromosome 1H from the Quest/W-365 population.



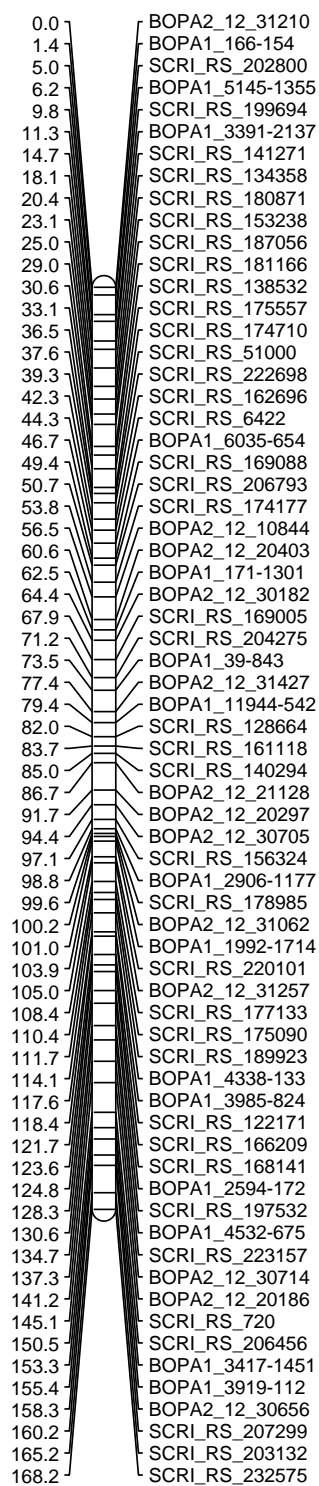
Appendix C2. A graphical representation of chromosome 2H from the Quest/W-365 population.



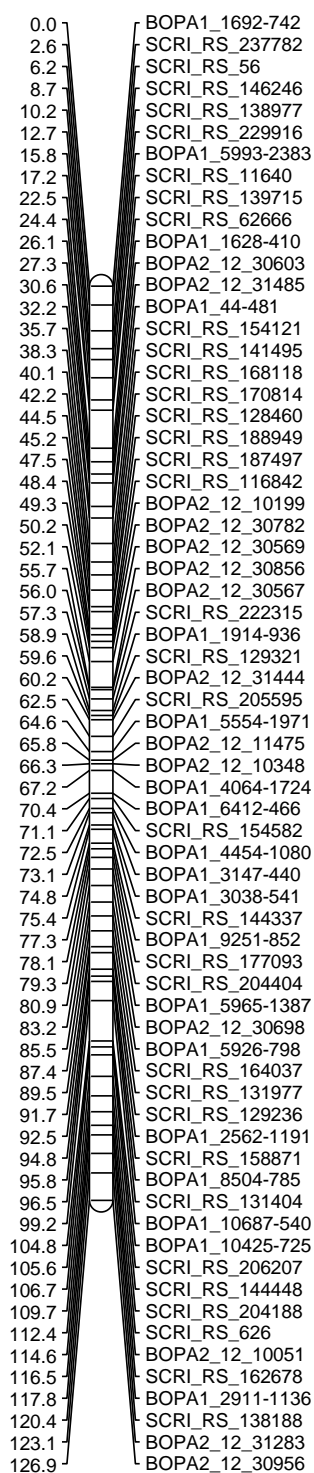
Appendix C3. A graphical representation of chromosome 3H from the Quest/W-365 population.



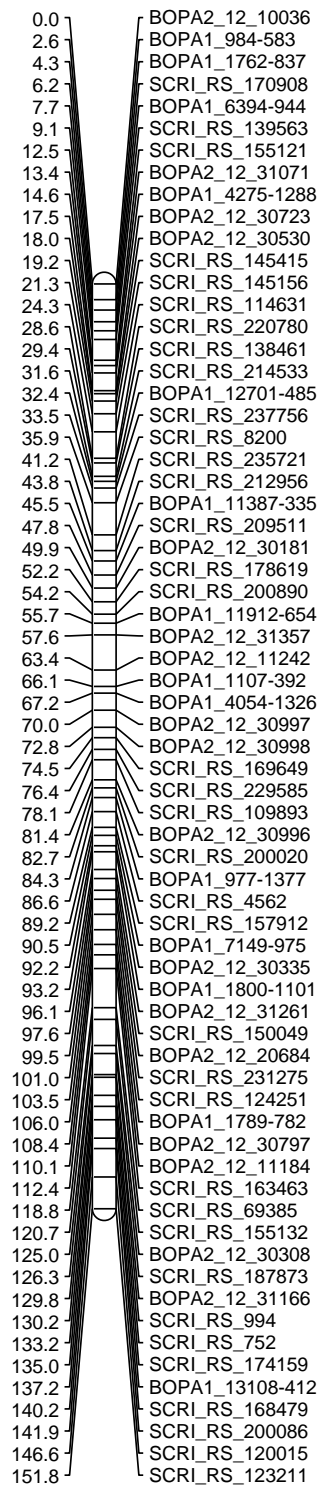
Appendix C4. A graphical representation of chromosome 4H from the Quest/W-365 population.



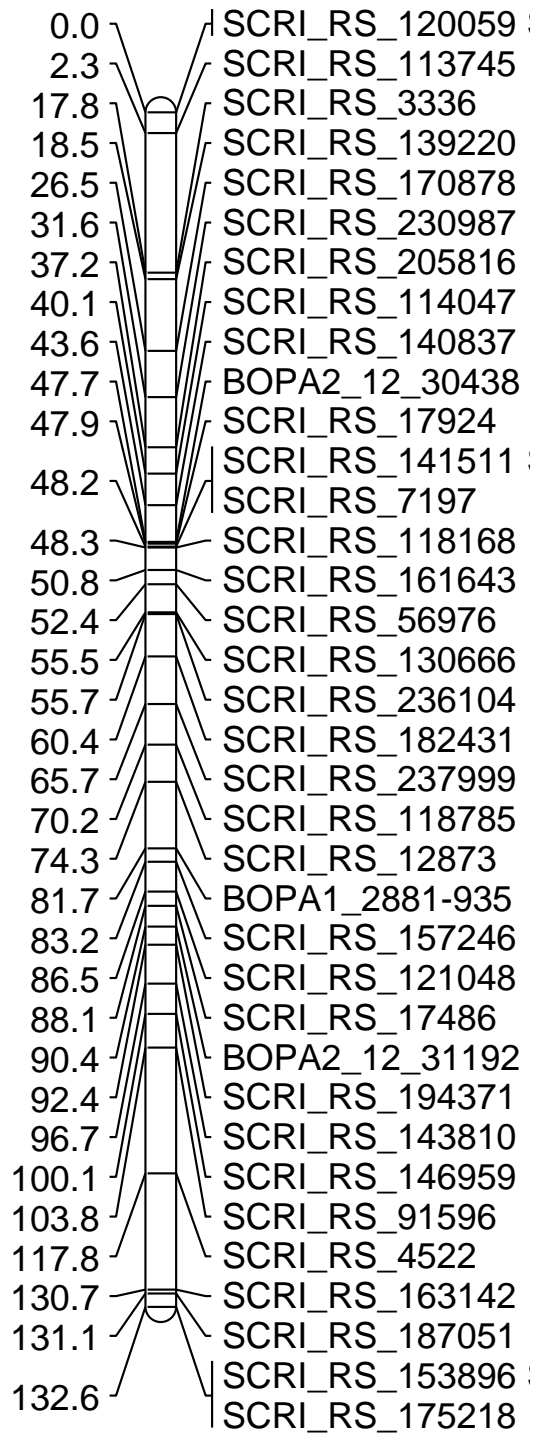
Appendix C5. A graphical representation of chromosome 5H from the Quest/W-365 population.



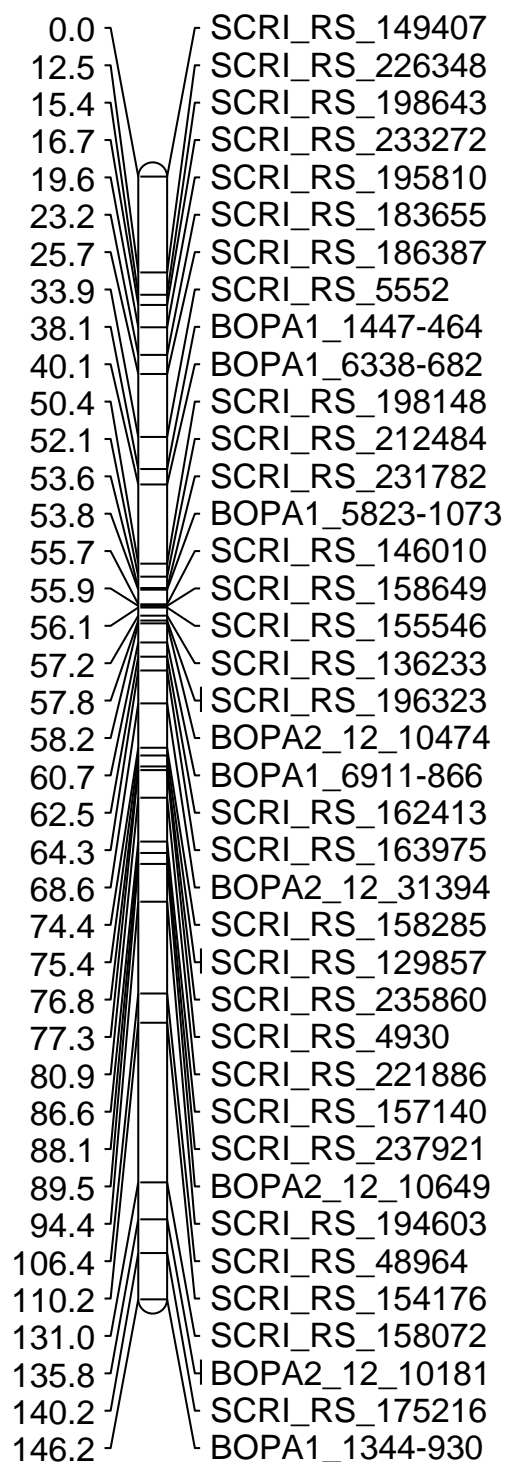
Appendix C6. A graphical representation of chromosome 6H from the Quest/W-365 population.



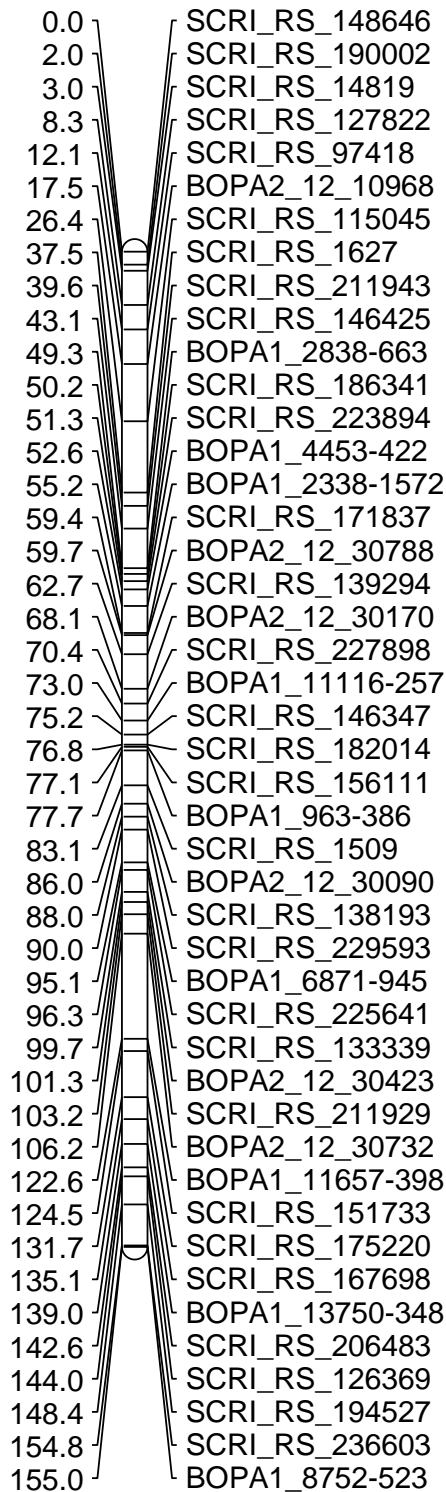
Appendix C7. A graphical representation of chromosome 7H from the Quest/W-365 population.



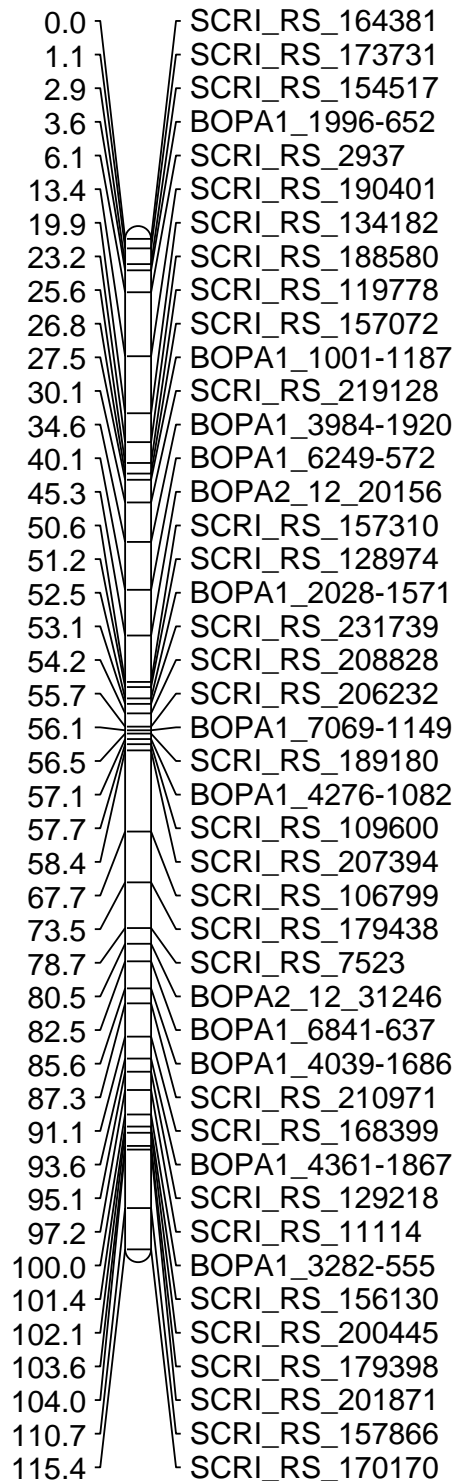
Appendix D1. A graphical representation of chromosome 1H from the Quest/Kutahya population.



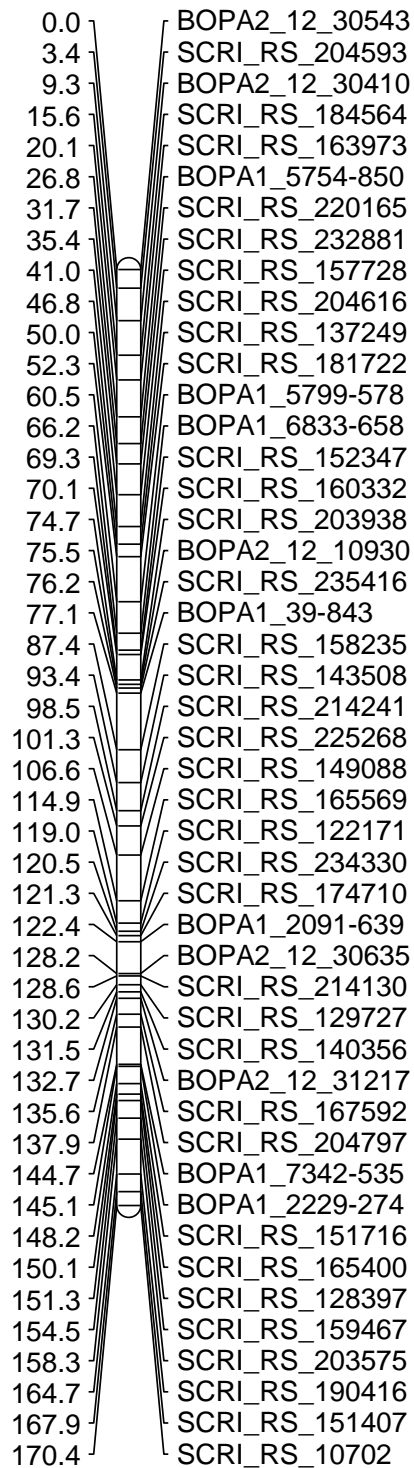
Appendix D2. A graphical representation of chromosome 2H from the Quest/Kutahya population.



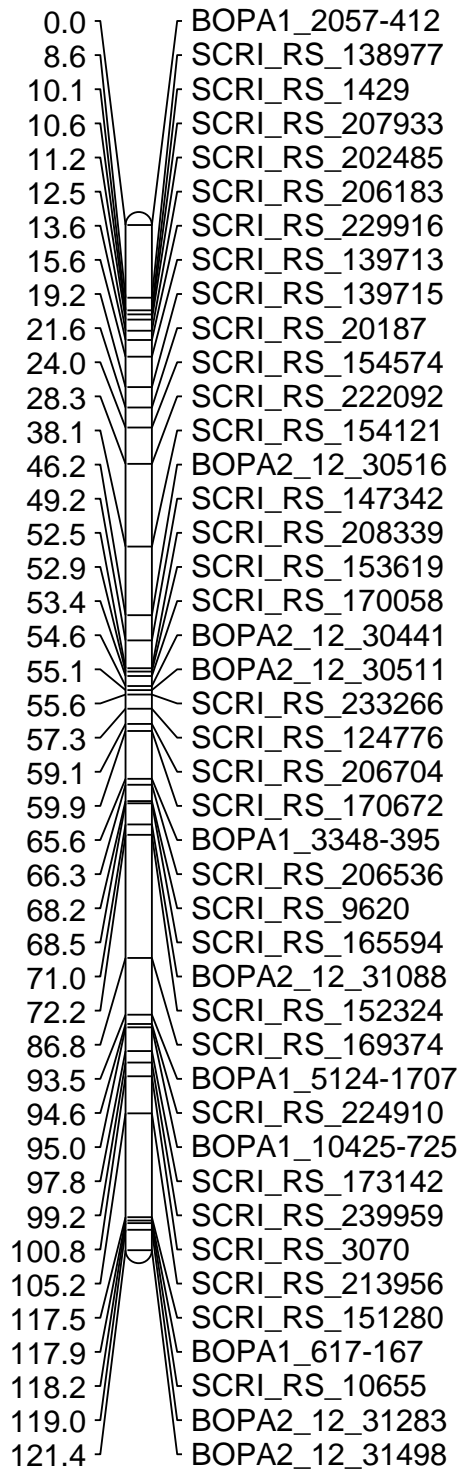
Appendix D3. A graphical representation of chromosome 3H from the Quest/Kutahya population.



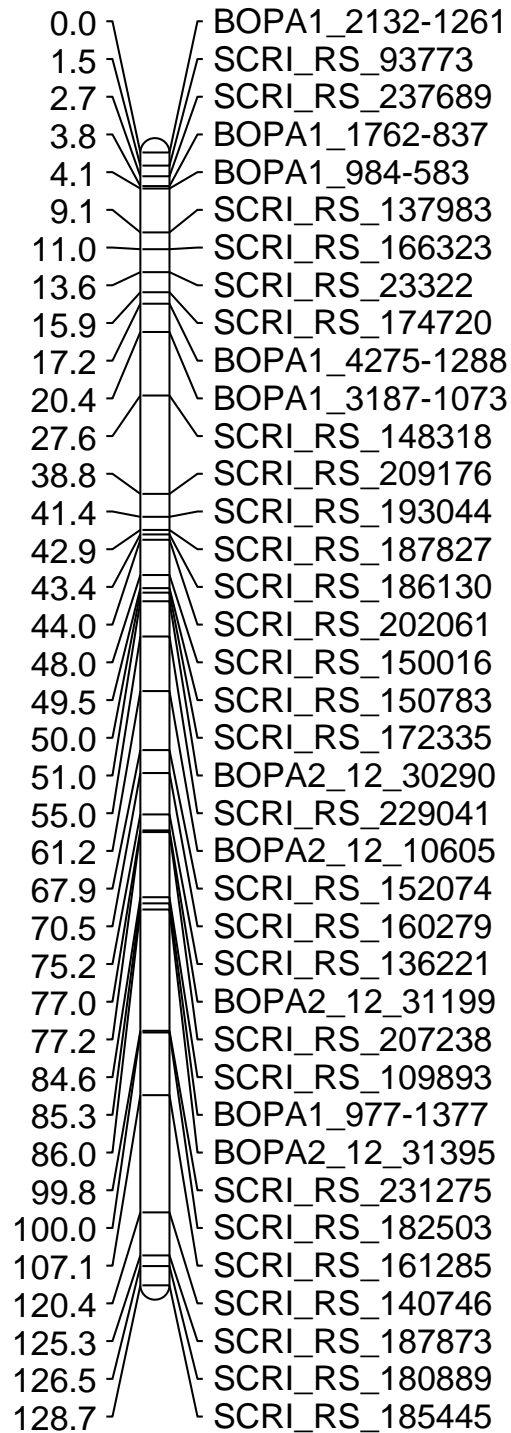
Appendix D4. A graphical representation of chromosome 4H from the Quest/Kutahya population.



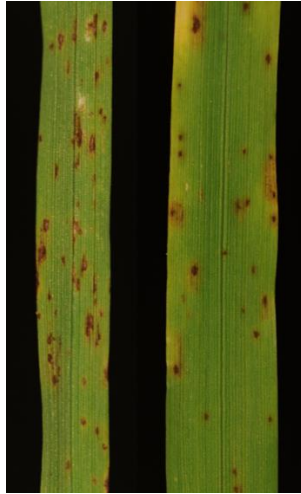
Appendix D5. A graphical representation of chromosome 5H from the Quest/Kutahya population.



Appendix D6. A graphical representation of chromosome 6H from the Quest/Kutahya population.



Appendix D7. A graphical representation of chromosome 7H from the Quest/Kutahya population.



Appendix E. The seedling infection response of PI 466423 (left) and Rasmusson (right) infected with *Cochliobolus sativus* isolate ND4008 in the greenhouse.

Appendix F

CTAB DNA Extraction

DNA extractions were carried out in 2 ml tubes. Tissue was harvested from the second and third leaves of seedlings of each sample and immediately frozen in liquid nitrogen. Samples were ground using beads and a shaker at a speed of 28 l/s for 2 min in one direction and another 2 min in the opposite direction. The resulting powder was spun down at 1000 rpm. Next, 800 µl of extraction buffer (at 65°C) was added to the samples, which were then mixed at a speed of 20 l/s and spun down at 1000 rpm. Next, the samples were placed in a water bath at 65°C for 30 min and gently inverted every 10 min. After the water bath treatment, the tubes were spun at 1000 rpm for 2 min, and 800 µl of a mixture of chloroform: isoamyl alcohol (24:1) was added to each sample. The sample was again gently inverted 60 times to ensure mixture with the chloroform treatment. Next, the samples were centrifuged for 15 min at 12,000 rpm at 20°C to separate the phases. Using 1000 µl tubes cut in the middle of point portion, 525 µl of the top layer was transferred to a new tube to ensure that the chloroform layer was not disturbed. Then, 1000 µl of 100% ethanol was added to each sample to wash down the walls of each tube. After placing the caps back on, the tubes were gently rocked for 1 min before being spun down at 12,000 rpm for 2 min. The solution was then carefully decanted. Next, 300 µl TE+RNase was added to the tube, which was inverted to dissolve the DNA pellet until it was no longer visible. The samples were then incubated at 37°C to allow the RNA to be digested for 1 hr. Next, 600 µl of 100% ethanol was used to wash the sides of each tube, which were then gently inverted for 2 min. The tubes were spun down at 12,000 rpm for 2 min and the solution decanted. Next, 1 ml of 70% ethanol was added to the tubes and allowed to sit for 1 hr, before being spun down at 12,000 rpm for 2 min and the solution decanted. DNA was allowed to air dry for 60 min before 100 µl 1X TE buffer was added.