Using Near Isogenic Barley Lines to Validate Deoxynivalenol (DON) QTL Previously Identified Through Association Analysis

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Stephanie Lynn Navara

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Abstract

Fusarium head blight (FHB) is a serious disease of cereal grains caused by the fungal pathogen *Fusarium graminearum*. Deoxynivalenol (or DON), the associated trichothecene mycotoxin is of special concern to barley producers and consumers. A recent association mapping (AM) study of U.S. six-row spring barley identified several modest effect quantitative trait loci (QTL) for DON and FHB. To date, few studies have attempted to verify the results of association analyses, particularly for complex traits such as FHB and DON resistance in barley. Despite control measures used to mitigate the effects of population structure and multiple testing in AM, false positives may still occur. To verify previously reported associations we evaluated the effects of nine DON QTL using near isogenic lines (NILs) for each QTL region. Families of contrasting homozygous haplotypes for each region were derived from lines in the original AM populations that were heterozygous for DON QTL. Seventeen NIL families were evaluated for FHB and DON in three field experiments. Significant differences between contrasting NIL haplotypes were detected for three QTL across environments and/or genetic backgrounds, thereby confirming QTL from the original AM study. Several explanations for those QTL that were not confirmed are discussed, including the effect of genetic background and incomplete sampling of relevant haplotypes.

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Chapter 1 Introduction

The field of plant breeding has benefited from technological advancements made over past decades. High throughput genotyping platforms make complex trait dissection a feasible and gradually more cost effective endeavor through readily available molecular marker resources. Robust statistical procedures coupled with advanced computing technology allow breeders to identify and dissect genomic regions of interest, drawing information from broader resources than those used in the past. A multitude of techniques for complex trait dissection are being successfully exploited toward this end, including both linkage and association mapping. As breeders begin to translate these genetic discoveries into breeding applications an emphasis must be placed on validating both results and methodologies. No approach is immune to error; validation is an integral part of the discovery process. Confidence based on robust analyses will in turn lead to confidence in discoveries, which can then be implemented in crop improvement. This research examines the application of association mapping of Fusarium head blight resistance by using near-isogenic barley lines for quantitative trait loci (QTL) associated with the disease.

Fusarium Head Blight

Fusarium head blight (FHB) or "scab" is a disease of small grains caused primarily in the United States by the fungal pathogen *Fusarium graminearum* Schwabe [teleomorph: *Gibberella zeae* (Schwein)]. *F. graminearum* infects cereal grains and can cause stem and root rot in maize. Epidemics occur periodically in grain producing regions of the Midwest varying in degree of severity. Most recently in the summer of 1993, closely related barley cultivars grown in the Midwest succumbed to disease pressure from a perfect storm of favorable weather conditions, large amounts of inoculum in crop

debris, and lack of host resistance. Contaminated grain resulting from infection led to immense profit losses for growers. Between 1998 and 2000, roughly 2.7 billion dollars in losses were reported as a result of widespread infection in both wheat and barley throughout the Northern Great Plains and Central United States (Nganje et al. 2004). The increased risk of this disease to barley has contributed to a reduction in national acreage from almost 7.8 million acres in 1993 to less than 2.6 million acres in 2011 (National Agricultural Statistics Service 2012, http://www.nass.usda.gov/index.asp). As a result higher value crops such as corn and soybeans are actively displacing barley due in part to the increased risk to farmers in terms of disease, and the benefits of economic support through insurance and other subsidies.

Kernels infected by the fungus may eventually display brown, water-stained lesions indicating infection and cell death. Kernel discoloration is often but not always an indicator for the presence of deoxynivalenol (DON, or vomitoxin), a trichothecene compound produced by *F. graminearum* and other *Fusarium* species, including *F. culmorum*. Vomitoxin, as its common name suggests, can cause emesis, feed refusal in non-ruminant animals such as pigs (the most sensitive animal), and immune suppression. Exposure can also lead to adverse health effects and toxicosis with differential sensitivity depending on length and amount of exposure (Pestka 2007). Common exposure reactions include symptoms of diarrhea and vomiting, but in sufficiently high quantities can lead to tissue damage or mortality (≥27 mg/kg of body weight in experimental animals (Pestka 2007)).

Acceptable contamination levels of DON in wheat are well established. Generally less than 1 parts per million (ppm) are recommended for finished products and between 5 to 10 ppm in grain destined for end use as livestock feed (http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/default.htm; United States Food and Drug Administration 2010). No such FDA rules currently dictate

acceptability for barley. Instead, the malting and brewing industries have generally imposed a threshold of less than 0.5 ppm due in part to the "gushing" associated with toxin contaminated malt (Schwarz 1996) and public concern over safety. Harvested grain is immediately assayed for mycotoxin contamination post arrival at grain elevators. If above acceptable levels, entire harvests can be sold at reduced prices or rejected entirely.

Life Cycle

Like many fungi, this monocyclic fungus thrives under warm, moist conditions. The life cycle of *F. graminearum* begins on the residue of plants grown the previous season. Sources of residue have been shown to greatly influence disease pressure the following season. In wheat, FHB incidence and severity were highest when planted after corn, and lowest after soybeans (Dill-Macky and Jones 2000). Warm spring weather facilitates perithecial development on crop debris that coincides with barley head maturation. Ascospores released from the residue-borne perithecia are wind dispersed onto the head, where they germinate within hours and gain access to the kernel. The protective barley lemma and palea are highly lignified structures with tough adaxial and abaxial walls which themselves resist infection; the fungus circumvents these and other plant defenses to gain entry into the kernels. Stomata, anther exposure during pollination, gaps between the lemma and palea, and wounds have been postulated as potential entry points though the precise mechanism and host-pathogen interactions remain uncertain (Bushnell et al. 2003).

Barley displays type II disease resistance to FHB which hinders the spread of the disease post infection (Zhu et al. 1999). Infection is often limited to the central and lateral spikelets at a particular node. Once inside the plant, the fungus exhibits a short biotrophic period without significant harm to the host. Approximately two days later, a

switch from a biotroph to a necrotrophic lifestyle occurs as a significant amount of host protein degradation occurs. A transcript analysis by Boddu et al. (2006) of the susceptible cultivar Morex indicated three stages of fungal infection corresponding to different intervals of fungal development; an early stage (0-48 hours after infection (hai)), an intermediate stage (48-96 hai), and a late (48-144 hai) stage with active infection observed 72 hai, eventually leading to plant tissue necrosis.

Management of the disease is possible through cultural practices and chemical control. Current conservation tillage strategies are beneficial for maintaining soil quality and decreasing erosion and runoff, however such practices allow the persistence of the pathogen on crop debris over the winter. These practices along with rotation schemes alternating between small grains and corn have further perpetuated the problem (McMullen et al. 1997). Fungicides alone are often ineffective at lowering severity to an acceptable level, and the window of effective application timing is short. Additionally when considering chemical control, many factors must be weighed including inoculum source, level of cultivar resistance, climatic factors, crop sensitivity, yield potential, fungicide capabilities, frequency of crop inspection, and management inputs (Wale 1994). Currently several fungicides are registered for the use in wheat and barley and have the potential to reduce FHB and DON with well-timed applications (Jones 2000), though disease reduction is often not low enough to achieve industry standards. Genetically resistant cultivars remain the most cost effective and environmentally sound option for control (Steffenson 1998).

Genetics of Resistance

Resistance to FHB and accumulation of DON is both complex and quantitative; many loci of small effect condition a phenotype that can be greatly influenced by the environment. No sources of complete immunity have been identified in barley, though

sufficient genetic variation exists to identify some sources of resistance (Rudd et al. 2001). Several bi-parental mapping studies conducted between 1999 and 2008 have identified QTL for resistance to FHB (Dahleen et al. 2003; Mesfin et al. 2003; De la Pena et al. 1999; Zhu et al. 1999; Ma et al. 2000; Horsley et al. 2006). These regions span all seven chromosomes of barley and were detected across a number of environments using parental resistance sources Chevron (6-row), Frederickson (2-row), CIho 4196 (2-row), and Zhedar 2 (2-row). These QTL have been summarized by Massman et al. (2011) and generally explain a small amount of variation, only a few of which have been detected in multiple trials (most notably on chromosomes 2H, 4H, and 6H). FHB and DON analyses often produce conflicting results in terms of effect differences across locations, further stressing the important role of environment on DON accumulation and FHB development. Despite the difficulty of breeding for resistance in barley, moderate heritability estimates show that genetic gain can be achieved when selecting for resistance in breeding programs (Capettini et al. 2003).

While it is clear many regions of the barley genome affect the disease phenotype, several have been associated with undesirable morphologic or agronomic traits (late flowering, tall plant height, or high grain protein concentration) reducing the utility of these regions in marker assisted selection (MAS). Associations between FHB and DON with plant height and heading are well documented (Canci et al. 2004; Hori et al. 2006; Mesfin et al. 2003; Nduulu et al. 2007; Zhu et al. 1999). QTL for these traits have been mapped, however low mapping resolution in some studies generally does not allow for discrimination between pleiotropic effects and tightly linked loci. Gain achieved in terms of FHB resistance and DON accumulation may come at the sacrifice of other agronomically important traits, as negative associations seem to exist (Gervais et al. 2003). Late maturing, tall plants are undesirable for barley producers, and tall plants predispose plants to lodging, interfering with harvest.

Association Mapping

Bi-parental vs. Association Mapping

A common approach toward genetic dissection of complex traits is through family-based linkage mapping (bi-parental mapping). Contrasting parents are crossed to create populations of segregating progeny used to associate traits with genetic markers. Two important advantages of these family-based populations are the creation of linkage blocks of markers in close proximity to QTL and predictable allele frequencies based on the family structure of the population. This allows for detection of QTL using sparsely spaced markers.

Though successful at identifying major effect QTL, linkage-based studies also suffer from several limitations in terms of resolution and diversity. First, QTL detection is based on segregation of only two alleles per locus and conducted in a narrow genetic context. As a result, QTL can be population specific and therefore of limited application. Vales et al. (2005) reported that in a study of stripe rust resistance in barley, only large effect QTL were detected in small populations (n = 94) but small effect QTL were detected only by increasing the population to over 400 lines. Because of the resources required to create and evaluate mapping populations, their sizes have typically been small (100 – 200 individuals) resulting in overestimation of effect sizes and limited power to detect small effect QTL (Beavis 1998). Finally, mapping resolution is often low (10-20 cM) due to few generations of recombination among individuals in these populations (Holland 2007).

Association Mapping

Association mapping (AM) is a promising technique for identifying loci involved in the inheritance of complex traits, such as FHB resistance and DON accumulation (Risch and Merikangas 1996). Also known as linkage-disequilibrium (LD) mapping or genome-wide association mapping (GWAS), AM takes advantage of historical recombination events among populations and existing LD information to infer relationships between quantitative traits and causative loci. Analyses based on LD are able to detect and locate QTL based on the strength of the correlation between a trait and marker. This strategy, unlike other methods, identifies associations between markers and QTL without relying on bi-parental mapping populations (Zhu et al. 2008). Instead panels are designed for a specific purpose, for example to represent the diversity in a germplasm collection, geographic region, or breeding program. Because no new populations need to be developed, lines from a broader context can be utilized and AM can survey the genetic diversity of the population. In some cases, it is possible to assemble mapping panels that have already been phenotyped so that only genotype data is needed to carry out AM.

The effectiveness of this approach has transcended the human-based studies from which it originated to find application in plant species, including *Arabidopsis* (Aranzana et al. 2005; Zhao et al. 2007), maize (Yu and Buckler 2006), soybean (Fasoula et al. 2004), and wheat (Breseghello and Sorrells 2006). In barley, AM studies have generally been successful in identifying associations, however results are often hard to reproduce, effect sizes are small, and the QTL detected only account for a fraction of explainable variation (Cockram et al. 2008; Cockram et al. 2010; Kraakman et al. 2006; Massman et al. 2011; Rostoks et al. 2006; Roy et al. 2010; Stracke et al. 2009).

LD and Other Factors Affecting AM Detection

As the name implies, LD-mapping relies on the strength of association of a genetic marker with phenotype-affecting variant. It is affected by many factors involving population dynamics, including mating tendencies, recombination, selection, and genetic bottlenecks, among others and has been reviewed (Flint-Garcia et al. 2003; Gaut and Long 2003). LD based studies rely on the recombination histories of a species to make marker-trait inferences. Each subsequent generation of crossing decays LD until only loci in close proximity are inherited together. Intuitively inbreeding species like barley contain more extensive LD (Caldwell et al. 2006) than those with out-crossing tendencies (in maize for example, LD decays rapidly over 1 kbp (Remington et al. 2001)). This generalization does not always hold true, as in the case of autogamous wild barley in which the LD observed at a majority of loci is more consistent with an outcrossing species (Morrell et al. 2005). The extent of LD varies not only between but also within species, and with "islands" of high LD surrounded by regions of high recombination. In cultivated barley, gene-rich regions potentially represent up to 60% of the physical length of barley extended across "genetic centromeres" (Comadran et al. 2011).

The extent of LD has important implications for AM. Populations with extensive LD will require fewer markers and result in lower resolution than populations in which LD decays rapidly. In *ad-hoc* or natural populations, the extent of LD is generally low, reflecting the recombination histories of unrelated individuals. In contrast, bi-parental populations provide a population in which LD is maximized in the F2 generation, a result of the recent recombination of chromosomes from divergent parents. Therefore, fewer markers are needed to detect functional polymorphisms.

Other factors affecting QTL detection include: heritability of the trait, size of the population, frequency of marker alleles, and the effect of environment. Studies more successfully detect associations when heritability is moderate (Yu and Buckler 2006). In

addition, a large population provides a greater range of backgrounds in which to test for associations. Effect sizes can be overestimated if populations are small, as described by Beavis (1994) in which a small number of individuals in the population (n) leads to fewer detected QTL and an upward bias in the estimated effects of those few QTL. Increasing n in the mapping population can mitigate this effect. In an AM study of spring barley breeding lines evaluating a highly heritable trait (heading date), a minimum of 384 lines were required to detect consistent effects across simulations of balanced and unbalanced data (Wang et al. 2012). In addition QTL are more likely to be detected if the frequency matches that of the marker allele frequency (Mackay and Powell 2007). Finally, rare variants of large effect can be missed due to weak association signals among those more frequently represented.

Limitations of AM Studies

One area of concern regarding AM is the potential for the generation of false positive associations as a result of differential relatedness among lines. Complex breeding histories lead to varying degrees of population structure that must be accounted for in any AM analysis. Spurious associations can potentially result, and occur when lines are more related to each other than the population as a whole and lead to misleading conclusions. Extensive population structure has been observed in cultivated barley world-wide, with major subdivisions including two or six-row head types and spring or winter growth habit (Malysheva-Otto et al. 2006). A study of 10 North American breeding programs using 1,536 single nucleotide polymorphism (SNP) markers and 1816 barley breeding lines identified extensive population structure not only between row type and growth habit subdivisions, but also between breeding programs (Hamblin et al. 2010).

Accounting for population structure is a necessary component of analysis and can be approached in several ways. Genomic control using random markers to adjust inflation of the test statistic is one potential option (Devlin and Roeder 1999). The transmission disequilibrium test (TDT; Spielman et al. 1993) will detect linkage in the presence of disequilibrium but is most useful for qualitative traits. Others involve structured association using random markers to estimate the population structure to be incorporated into statistical analysis. An estimate of population structure (Q) can be obtained using population membership estimates obtained through model-based approaches, such as those obtained through STRUCTURE (Pritchard et al. 2000) or principal component analysis (Price et al. 2006). These estimates can be used as a covariate in analysis as a probability of membership in each determined subgroup. A unified mixed model approach suggested by Yu et al. (2005) successfully reduced type I and type II errors in association analysis in maize by incorporating both a population structure Q-matrix (fixed effects) and a relative kinship K-matrix (random effects) calculated using pedigree information or random genome-wide markers. This approach when used in *Arabidopsis* successfully decreased the false-positive error rate and maintained statistical power when compared to other population structure control methods (Zhao et al. 2007). The mixed linear model accounts for both coarse and fine degrees of similarity with the model $y = X\beta + Z\mu + e$, where y is the vector of observations, β represents the fixed effects in the model and includes markers and populations structure Q, and μ represents random additive genetic effects from multiple background QTL for individuals or lines.

Another concern in population-based associations is the issue of multiple testing. When many hypotheses are tested simultaneously, an expected number of type I errors will occur as an artifact of the number of tests performed. An arbitrary significance threshold is set to a level allowing for an acceptable level of type I error detection, while attempting to maintain a high level of power for detecting true

associations. False positive results are mitigated by decreasing the number of significant associations tested. Among these adjustments include the most stringent Bonferroni correction which minimizes the family-wise error rate by adjusting the threshold for detection based on the number of independent tests conducted. Other approaches include adjusting for the false discovery rate by accounting for the proportion of errors committed by falsely rejecting null hypotheses (Benjamini and Hochberg 1995). Despite measures to control for false positives and due to the nature of complex traits, errors can still occur and significant associations can still be missed. Therefore the results of association studies should be validated to confirm their usefulness in future crop improvement endeavors with confidence.

QTL Validation

Mapping studies have reported thousands of potentially beneficial QTL, unfortunately many of those identified in genetic studies remain unutilized (Bernardo 2008). Post identification, validation of putative QTL regions is crucial, but rarely occurs. If unconfirmed, breeding efforts can potentially be wasted on spurious associations. QTL mapping and QTL validation are often separate endeavors and the additional time and cost investments necessary for testing make the additional step undesirable. Despite the costs, an investment in sound results before MAS in breeding or initiating a gene-cloning project is worth the upfront investment. Several methods toward QTL identification include, but are not limited to independent testing of QTL in different backgrounds and environments, selective genotyping, and testing using near isogenic lines.

By creating new populations using an early season cold-tolerant sorghum line parent, Knoll and Ejeta (2008) validated three QTL previously identified with a cross using the same parent. In order to validate barley disease QTL from a cross between wild and

two-row barley, Yun et al. (2006) developed a population using the original OUH602 donor parent crossed to two-row recurrent malting parent Harrington. The QTL analysis of this population resulted in detection of the same associated regions as the mapping study, and detected an additional QTL for adult plant spot blotch resistance. Validation using this approach is robust, however development of the validation population requires an additional investment in time and resources. Validation has also been successful via the analysis of lines from the mapping population that were not used in the QTL study. Using doubled haploid (DH) lines from the Steptoe/Morex cross not used for mapping, Romagosa et al. (1999) validated four yield QTL by comparing the effects of contrasting alleles in three environments; two QTL effects were confirmed, the others were very highly influenced by the environment resulting in inconsistent detection across trials. Selection of both the beneficial QTL alleles in DH lines resulted in significantly higher yield responses. Selective genotyping of extreme phenotypes has also been successful in the validation of two sunflower stalk rot resistance QTL from a previous study (Micic et al. 2005).

Near Isogenic Lines

Near isogenic lines (NILs) have also been used for validation, and have shown utility for a number of breeding purposes including the integration of molecular and genetic marker maps (Muehlbauer et al. 1988) and genetic mapping (Kaeppler et al. 1993). NILs are attractive because the QTL under investigation is not affected by segregation of other genomic regions. Direct comparisons between contrasting allele classes in a genetically identical background enable verification that a phenotype is associated with a specific locus.

Marker assisted-recurrent selection of a donor parent allele into a recurrent parent is a strategy that depends on marker information to transfer beneficial alleles from un-

adapted lines creating NILs. This approach, while successful, requires several generations of backcrossing and marker assisted selection. The linkage drag introduced during introgression may also affect results, particularly if the source of the favorable allele is exotic or not adapted to the target breeding region (Stam and Zeven 1981). An alternative involves deriving lines from existing breeding populations not completely homozygous at all loci. One generation of selfing and selection will generate segregating heterogeneous inbred families (HIFs) of NILs with contrasting alleles at target loci (Tuinstra et al. 1997). This approach has been tested in several crop species, including sorghum where the procedure was used to characterize seed weight (Tuinstra et al. 1997) and drought resistance QTL (Tuinstra et al. 1998). HIFs have also been used successfully to validate and characterize QTL regions associated with determining varietal plant type during rice development (Kobayashi et al. 2006).

Often NILs are generated from a single mapping population and therefore conclusions drawn may only be relevant to the population from which they were derived. (Pumphrey et al. 2007) circumvented this issue by developing NILs from several existing wheat breeding populations segregating for *Fhb1*, a major Fusarium head blight resistance QTL. By developing NILs derived from thirteen different populations, they showed that lines homozygous for the resistance allele significantly decreased disease severity and infected kernels in both greenhouse and field trials. *Fhb1* has been widely used resistance source derived from the Chinese cultivar Sumai 3 conferring a large effect on disease. The *Fhb1* gene in wheat has been mapped repeatedly and accounts for 20-60% of the variation observed in bi-parental mapping populations (Anderson et al. 2001; Buerstmayr et al. 2002; Buerstmayr et al. 2003; Waldron et al. 1999; Zhou et al. 2002). Though a major FHB resistance resource, NIL analysis only detected differences in nine of nineteen NIL pairs in greenhouse inoculations where the effect is most robust. In barley, it is unlikely that effects this

large are available to FHB, further underscoring the importance of validation, especially for QTL of small effect.

Advantages of NILs include the ease of line development from pre-existing populations in which only one generation of selfing of a heterozygous parent results in contrasting homozygous progeny. If derived from elite breeding material, a majority of genomic loci are already fixed and one round of selfing and selection enables relatively straightforward comparison between contrasting homozygous classes. An additional advantage, especially in the context of AM validation is the balanced evaluation of allele frequencies. The trade-off with AM studies is the gain in terms of allelic diversity at a locus, although there will be a loss of power to identify rare variants of large effect. NIL evaluation does however have the potential to resolve questions of effect size in addition to validation.

The disadvantage of NILs relates to the nature of the lines; a single locus is tested for an effect in a fixed background, but recreating that exact background is nearly impossible because of the segregation at other loci. One caveat of a NIL approach is the assumption of a fixed genetic background. In reality other small effect QTL contributing to a phenotype can be scattered across the genome and heterozygous, producing NIL that segregate for more than one QTL and thereby confounding results. Monitoring segregation at sites known to influence a trait would resolve this possibility. Recreating identical NILs is difficult, but is increasingly less so if more advanced generations are used for development. Additionally, power to detect an effect can be low if only a few lines representing each allele are in each family. This issue can be more or less circumvented through many different HIFs to test the effect of a QTL.

In the present study, NILs were selected from three, six-row barley breeding programs in the Upper Mid-West United States including the University of Minnesota, North Dakota State University, and Busch Agriculture Resources Inc. as assembled by the Barley Coordinated Agriculture Project (Barley CAP). These lines were used to generate HIFs for comparison of DON QTL that were identified in a previous AM study. AM has proven to be a powerful tool for identifying QTL involved in FHB resistance and DON accumulation. If beneficial QTL are successfully validated, they can be deployed into a breeding program with greater confidence.

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Chapter 2 Using Near Isogenic Barley Lines to Validate Deoxynivalenol (DON) QTL Previously Identified Through Association Analysis

Introduction

Genetic studies have identified many useful loci contributing to complex traits; unfortunately, many of them remain unutilized in plant breeding (Bernardo 2008). Validation is an important and often overlooked step between quantitative trait loci (QTL) identification and subsequent research in QTL cloning, genomics studies, or marker assisted breeding. Efforts to conduct validation are often avoided due to the large numbers of QTL identified in mapping studies and the substantial amount of time and resources required to generate independent and appropriate testing populations. Despite these deterrents, validation studies have been conducted in many crops, including maize (Austin and Lee 1996; Landi et al. 2005), soybean (Fasoula et al. 2004), sunflower (Micic et al. 2005), and tomato (Foolad et al. 2001). Successful validation studies in barley have confirmed QTL with effects on agronomic, disease resistance, and quality-related traits, among others (Ahmad Naz et al. 2012; Canci et al. 2004; Spaner et al. 1999; Mundt et al. 2003; Muñoz-Amatriaín et al. 2008; Romagosa et al. 1999; Yun et al. 2006). However these validation efforts represent only a small fraction of the published QTL.

Association mapping (AM) is a powerful tool for utilizing genotype and phenotype data from diverse germplasm to detect marker-trait associations and has its own challenges in terms of validation (Zhu et al. 2008). In barley, AM studies have generally been successful in identifying causative regions, however results are difficult to reproduce across genetic backgrounds and experiments, and effect sizes are often small (Cockram et al. 2010; Kraakman et al. 2006; Massman et al. 2011; Rostoks et al. 2006; Roy et al. 2010; Stracke et al. 2009). Despite the expanded opportunities to discover QTL

through AM, few of these QTL have been confirmed by independent validation studies to enable subsequent genetic research or application in marker-based breeding. Verification of AM results is especially important due to the complex population structure that often occurs in AM panels. Varying degrees of relatedness within a population can result in spurious associations if not properly accounted for (Lander and Schork 1994). The diverse nature of AM panels and the potential for multiple alleles segregating at a QTL make it particularly important to validate QTL effects in relevant germplasm. Furthermore, the power to detect QTL is contingent upon many factors that vary among studies, including population size, marker density, linkage disequilibrium (LD) pattern, and effect size (Long and Langley 1999). Finally, the number of marker tests conducted in analyses along with phenotyping and/or genotyping errors can increase the potential for false positive associations.

Methods to validate QTL include, but are not limited to, testing of QTL with additional progeny from the original or independent mapping populations, confirming effects via marker assisted selection (MAS), and comparison of contrasting alleles using near isogenic lines (NILs). NILs in particular have been shown to be useful for a number of purposes aside from validation, including integrating molecular and genetic marker maps (Muehlbauer et al. 1988), identifying QTL (Kaeppler et al. 1993), and fine mapping (Brouwer and St. Clair 2004). When QTL are initially identified in wide crosses using exotic parents, NILs are attractive to breeders as they allow confirmation of QTL and quantification of allelic effects. Such application has supported QTL results for salt tolerance in soybeans (Hamwieh et al. 2011), Fusarium head blight (FHB) resistance and grain protein content in wheat (Pumphrey et al. 2007; Prasad et al. 2003; Singh et al. 2001), and disease resistance QTL in barley (Kongprakhon et al. 2009; Smith et al. 2004).

Past studies using recurrent selection strategies to introgress donor QTL alleles into recipient lines thereby constructing NILs have been successful in confirming QTL effects. However, these populations require additional time and resources to develop. In contrast, heterogeneous inbred families (HIFs) can be relatively easily developed from partially inbred lines after one generation of selfing and selection (Tuinstra et al. 1997). This approach has been tested in several crop species, including sorghum to characterize seed weight (Tuinstra et al. 1997) and drought resistance QTL (Tuinstra et al. 1998). These advanced inbred lines not completely homozygous at all loci provide a resource for simultaneous breeding and testing of effects in relevant backgrounds (Pumphrey et al. 2007).

Fusarium head blight, or "scab", is a disease of small grains caused primarily in the United States by the fungal pathogen *Fusarium graminearum* Schwabe [teleomorph: *Gibberella zeae* (Schwein)] and has been a major target for small grains breeding programs in the U.S. (Bai and Shaner 2004; Buerstmayr et al. 2009; Steffenson and Smith 2006). Resistance to FHB and the accumulation of the mycotoxin produced by the pathogen (deoxynivalenol (or DON)) is both complex and quantitative, greatly affected by environmental factors, and therefore an appropriate candidate for MAS. No current barley varieties are immune however some resistance sources exist. Several bi-parental mapping studies have identified QTL for FHB resistance distributed across the barley genome (Dahleen et al. 2003; Mesfin et al. 2003; De la Pena et al. 1999; Zhu et al. 1999; Ma et al. 2000; Horsley et al. 2006). These studies have limited resolution (less than 10-20 cM; Holland 2007) and are often based on wide crosses using exotic sources of resistance. As a result numerous other traits can potentially co-segregate with resistance making interpretation of disease resistance difficult.

A previous AM study by Massman et al. (2011) identified QTL for FHB and DON accumulation, respectively, using elite germplasm from four Midwest U.S. barley

breeding programs. In this study, we present a rare validation of QTL identified by association mapping using near isogenic lines. Our specific objectives were to 1) validate DON QTL detected in the original AM study 2) compare allelic effects from the AM study to those from the NIL study 3) investigate haplotype diversity at DON QTL within the original AM panel.

Materials and Methods

To validate DON QTL previously identified through association mapping, we developed sets of NILs for QTL regions using selected SNP markers informative for the regions under investigation. These markers were used to genotype progeny from the single plants genotyped for the prior association mapping study to identify near-isogenic homozygotes at the QTL regions of interest.

Marker Selection

A total of 28 candidate QTL were identified in the six-row mapping panels from the original AM study (Appendix A). We inspected SNP genotypes at those QTL in 463 six-row lines from CAPI and CAPII AM panels and selected those that identified heterozygotes. All lines were previously genotyped by two sets of SNP markers referred to as BOPA1 and BOPA2 as part of the barley Coordinated Agricultural Project (barley CAP; www.barleycap.org; Close et al. 2009). The SNP data for these lines are available in The Hordeum Toolbox data repository (http://thehordeumtoolbox.org; Blake et al. 2011). In addition to identifying markers that were heterozygous, we also considered the level of significance (p-value) from the Massman et al. 2011 AM studies and distribution across the QTL region. Since the original mapping study, data for an additional 1,536 SNP markers (BOPA2) became available. Heterozygous markers within this pool were used to supplement the BOPA1 markers to span a QTL region. Based on

these criteria, a total of forty-eight markers were selected to generate the NIL families for fifteen QTL regions with from one to ten markers for each QTL (Table 1).

NIL Development and Genotyping

Markers and CAP lines to serve as parents for NIL development were selected simultaneously, considering both the number of heterozygous loci per line and the significant, segregating markers spanning a QTL. Most parent lines were chosen so that they were heterozygous at only one DON QTL region. However, we initially selected lines that were heterozygous at multiple DON QTL regions. Twenty-four lines were identified to develop 24 heterogeneous inbred families (HIFs; (Tuinistra et al. 1997) hereafter referred to as NIL families. Of those lines, 11 were developed from lines originating from the University of Minnesota (MN), 12 from North Dakota State University (ND), and 1 from Busch Agriculture Resources, Inc. (BA) (Table 2).

NIL families were generated by planting fifteen seeds from each of the twenty-four selected CAP lines in separate pots (one seed/pot) in a greenhouse. The seed source was self-pollinated seed from the plant that was genotyped with BOPA markers as part of the original AM study, thus we expected a 1:2:1 segregation ratio in the progeny from the parent line that was heterozygous at the QTL. Tissue was harvested at the two-leaf stage from each plant and freeze dried for storage until genotyping. DNA extraction from the leaf tissue was carried out at the Fargo USDA-ARS genotyping lab using a modified wheat and barley extraction protocol (Pallotta et al. 2003). All lines were genotyped using the forty-eight SNP markers with a chip purchased from Illumina as part of a custom Veracode Genotyping Assay System and run using Ilumina's Bead Express Technology. The Illumina Genome Studio software was used to score the marker genotypes. These data were imported into Microsoft Excel and used to assign lines into NIL classes of contrasting haplotypes. Six families were excluded from further

analysis because there was segregation at more than two QTL, or all lines of the NIL family remained heterozygous for at least one marker, or the genotypic class assignment was unclear based on Genome Studio clustering results. A total of 92 NILs were developed from 18 families that segregated for nine DON QTL regions (Table 3). The segregation of the 48 SNP markers for the 18 parents of the NIL families is shown in Appendix B. The genotypes of the NILs are shown in Figure 1, Figure 2, and Appendix C for families evaluating the effects of significantly different one-QTL families, two-QTL families, and the remaining families, respectively.

Phenotypic Evaluation of NILs

The 92 selected NILs, eighteen NIL parent lines and parents of the NIL parents were planted in the summer of 2010 in Stephen MN to increase seed used to plant disease trials in the summer of 2011 at three locations: St. Paul MN, Crookston MN, and Osnabrock ND. All three locations were planted in a randomized complete block design, treating each NIL family as a separate experiment; NILs, NIL parent, and parents of the NIL parent randomized within families, and families randomized across five blocks. For each entry 4 g of seed were planted in a 1.5-m single row spaced 0.3 m apart in St Paul (planted April 25) and Crookston (planted May 18). The Osnabrock disease nursery was planted on June 14th with approximately 15 kernels in 0.3 m single row plots.

The St. Paul location was inoculated with a mixture of 50 *F. graminearum* isolates collected between 2005 and 2010 from Minnesota wheat and barley fields (Appendix D). Plots were inoculated twice with micro-conidia using CO_2-powered backpack sprayers; once at heading when greater than 90% of the spikes per row had emerged from the boot and again approximately four days later (Steffenson et al. 2003). Due to the differential flowering times observed among families over half the field (439 plots,

including: all five reps of NIL families FEG149-18, ND25665, M04-29, ND25694, FEG126-12, FEG148-40, ND25661, FEG132-63, ND25657, and ND25684; one rep of M04-45; two reps of ND25681; four reps of ND25732) was inoculated first on June 28 and again on July 1, while the second half (296 plots, including remaining NIL families and replications) was inoculated on July 1 and again July 5. All entries within a replication within a family were inoculated at the same time. Inoculum at Crookston and Osnabrock was applied as a *Fusarium*-colonized grain spawn at approximately 56 kg/ha at two weeks and one week before flowering (Horsley et al. 2006). All fields received mist-irrigation after inoculum was applied to facilitate disease development.

Heading date was assessed as the number of days after planting in which 50% of the heads in a plot had emerged half way or more from the boot. All entries for a block within a NIL family experiment were rated on the same day. At the St. Paul and Crookston locations, ten arbitrarily selected spikes within each row were scored using the following scale corresponding to the percent of infected kernels on a spike: 0, 1, 3, 5, 10, 15, 25, 35, 50, 75, 100%. FHB severity and HD data from Osnabrock were not taken.

Plots were harvested in St. Paul and Crookston using a hand sickle and threshed on site with a custom Vogel thresher. Samples from Osnabrock were hand harvested by sickle, placed in paper bags, and later threshed in St. Paul. All grain samples were cleaned to remove excess chaff using a belt thresher. After cleaning, the grain was hand mixed and sub-sampled before grinding for toxin analysis. Approximately 20 grams of seed from each plot were ground using a Cyclotec sample mill with a 1 mm mesh sieve and analyzed for DON using gas chromatography and mass spectrometry (Tacke and Casper 1996).

Data Analysis

NIL Mean Comparison Tests

Minnesota line M04-29 was excluded from analysis based on segregation at multiple QTL which would have confounded results. Other observations from the phenotypic data sets were excluded from analysis if the values exceeded three standard deviations from the NIL family means. Data for each family were checked for departure from normality and homogeneity of variance by plotting quantiles as a QQ-plot and performing the Bartlett test, respectively. Phenotypic data for all locations were analyzed using the "Proc MIXED" procedure implemented in the computing software, SAS v 9.2 (SAS Institute, 2008). Analysis of variance was conducted for both individual and combined locations on a family basis. For a single location, the mixed model for all three traits included the fixed effects of haplotype and lines nested within haplotype, with the random effect of replication. Across locations, a similar model was used including the effects of haplotype and lines nested within haplotype as fixed effects in conjunction with location and replication nested within location as random effects. A QTL was considered validated if significant differences between haplotypes were observed below the $\alpha = 0.05$ threshold.

A QTL haplotype was considered as the genotype of an individual defined by the set of markers selected to represent the QTL region. Haplotype effects were calculated on a NIL family bases, comparing the difference in class performance as a percentage of the family mean. Effect size and direction were calculated by subtracting the numerically lower haplotype from the higher haplotype (i.e. haplotype 2 minus haplotype 1, or haplotype 4 minus haplotype 3). Haplotype numbers were arbitrarily assigned.

AM Study Haplotype Effects

To assess the haplotype performance from the original mapping data sets, we grouped CAPI and CAPII lines based on marker haplotype for the set of markers used to define each QTL region. The two haplotypes defined by each NIL family represents a subset of the haplotypes that exist in the association panel. Least square means were calculated with data from four trials described in the Massman et al. (2011) study. Groups representing the same haplotypes as in the NIL study were used to calculate effects. Effect sizes for DON, FHB, and HD were calculated based on the average performance of lines comprising a QTL haplotype as a percentage of the population mean. These calculations did not account for population structure and were based solely on class phenotype observed in 2006 and 2007. A two-sided unpaired t-test was then used to determine whether the same haplotype comparisons made in NIL trials were significantly different than the AM study. Finally, a Tukey's honest significance difference (HSD) means separation procedure was used to identify differences in mean performance among all the haplotypes observed in the AM panel.

Results

Family Phenotypic Evaluation

Disease pressure was sufficient at all three disease nurseries to assess phenotypic variation with average DON levels of 10.6, 11.5, and 32.7 ppm for Osnabrock, St. Paul, and Crookston, respectively (Appendix E, entire field). FHB severity was on average 5% in St. Paul and 31% in Crookston, and HD was on average about 10 days earlier in Crookston (Appendix F, entire field). Error variances were homogenous for all traits allowing for combined environment analysis. All three traits were significantly different among checks at all environments ($p < 0.0002$). At all locations, ND23899 and ND25657 consistently resulted in the highest DON concentration while ND25694 was among the lowest.

NIL Phenotypic Evaluation

Seventeen NIL families were used to evaluate nine DON QTL on the basis of forty informative SNP markers across three environments. Of the seventeen, fourteen isolated genomic differences to a single QTL region while three families segregated at two QTL. NIL family performance for DON are presented in Appendix G; FHB and HD in Appendix H. NIL heading date means differed most often from check means, exceeding check ranges in over half of comparisons (boxed cells). Consistent with performance of the NIL parents, NIL family ND23899 and ND25657 resulted in the highest DON concentration across locations while ND25694 and FEG148040 were among the least.

Single QTL NIL Families

Fourteen NIL families segregating at a single QTL region were used to evaluate eight DON QTL across three locations (Appendix I). In three instances, a single family was used to evaluate a region; in four cases two families were used, and in one case three families were used. Two haplotypes were defined by each NIL family and each haplotype within a family was represented by between one and seven lines. A QTL effect was considered validated if a difference was detected between haplotypes in the combined location analysis at a significance level of $\alpha = 0.05$ (Table 4, NIL haplotype study). Effects for the single QTL NIL families for all locations are presented in Appendix J. Five of the fourteen NIL families segregating at a single QTL had significant differences between haplotypes for DON. Five NIL families were significant for HD, of which two were in common with families significant for DON. Six NIL families were not associated with any of the three traits. FHB severity was not significant between haplotypes in any family.

The effects of haplotypes 2 and 4 of DON.10 decreased DON relative to haplotypes 1 and 3 by 10% and 15%, respectively (Table 4). These two haplotypes share common alleles for three adjacent markers that span 1.6 cM within the five marker region which spans 6.3 cM (Figure 1a). This QTL was validated with NILs in both the University of Minnesota and North Dakota State University backgrounds and was also identified by several previous bi-parental mapping studies. In the original AM study, DON.10 was significant in the CAPI (384 lines contributed by the University of Minnesota (MN), two-row (N2) North Dakota State University six-row (N6), and Busch Agricultural Resources Inc. (BA) breeding programs in 2006) and CAPI six-row (six-row lines from the MN, N6, and BA breeding programs) panels, and was among the most significant of the QTL detected in CAPII six-row (six-row lines from the same six-row breeding programs in 2007).

At DON.13, haplotypes 2 and 4 conferred a decrease in DON concentration relative to haplotype 3 by 14% and 23% respectively (Table 4). HD was also significant in two of the NIL families with haplotype 2 conferring earlier flowering and lower DON. Haplotypes 2 and 4 share common alleles for four adjacent markers that span 4.1 cM within the six marker region that spans 13.0 cM (Figure 1b). This QTL was significant in several Massman mapping panels, including the CAP I, CAP II, CAP I six-row, and CAP II six-row populations.

HD differences between haplotypes were also detected in both DON.17 and DON.18 families (Table 4). In DON.17, HD was marginally significant between haplotypes 1 and 2, resulting in a decrease of 1.1 days. Only two of the six markers were polymorphic between the classes, narrowing the causal region from 11.8 cM to 1.74 cM (Figure 1c). Differences at DON.18 were statistically but not necessarily biologically significant resulting in a 0.5-day difference in HD.

Finally, DON.33 was evaluated using a single marker and tested in two families. The effect of haplotype 2 both significantly decreased DON by 9% and decreased heading date by 1.6 days relative to haplotype 1 in the ND25728 NIL family (Table 4).

Two QTL NIL Families

NIL family FEG126-12 segregated for DON.17 and DON.18 and was significant for DON and FHB (Table 5). In this family haplotype 2 in DON.17 is identical to haplotype 2 of single NIL family FEG168-09 which had no effect on DON (Figure 2A). The other haplotype, 3, is distinct from those in FEG168-09. The two haplotypes of DON.18 (haplotypes 5 and 6) are distinct from the other four haplotypes identified in single NIL families FEG148-09 and ND23899. Haplotype 3 at DON.17 and haplotype 5 at DON.18 occur together in two of the NILs and haplotype 2 at DON.17 and 6 at DON.18 occur together in the other NIL. The 3 and 5 haplotype combination is lower for DON and FHB compared to the 2 and 6 haplotype combination. When these QTL were assessed individually in NILs there was no effect on disease suggesting that the unique haplotypes segregating in the FEG126-12 family are responsible for the effect on disease.

NIL family ND25665 was highly significant for heading date among the three haplotypes based on the two QTL regions DON.10 and DON.20 (Table 5). The two haplotypes for DON.10 were the same as haplotypes 1 and 4 in the single QTL NILs (Figure 2B). The NIL containing haplotype 4 for DON.10 and 1 for DON.20 had earlier heading by 3 days relative to the combination of haplotype 1 (DON.10) and 2 (DON.20), and by 1.6 days relative to the combination of haplotypes 4 (DON.10) and 2 (DON.20). The DON.10 region was not associated with HD with the four haplotypes assessed in the single QTL NILs suggesting that DON.20 is affecting HD.

ND25694 segregated at DON.10 and DON.31 and was significant for HD (Figure 2C). Four haplotypes are defined by these two QTL. These four haplotypes are the result of the combinations of DON.10 haplotypes 1 and 3 and DON.31 haplotypes 1 and 2. In single QTL analysis, haplotypes 1 and 3 at DON.10 were not associated with HD and were both higher in DON compared to haplotypes 2 and 4. In the single QTL NILs, DON.31 was not associated with any of the traits. It is not clear why the two QTL NIL family shows an effect on HD while the single QTL families do not. One possibility is that each QTL has a small effect and only the combination of both is sufficient to detect in this experiment.

Overall, three QTL were validated for an effect on DON based on NIL haplotype comparisons and five were found to have no effect. Four of the eight DON regions were found to have an effect on HD. NIL families segregating at multiple QTL suggest that haplotypes that were not segregating in the single QTL analysis could be responsible for the effect of those regions on DON or HD.

Haplotype Effects in the Association Panel

There were additional QTL haplotypes in the AM panel (referred to as AM haplotypes) than those represented in the NILs (Table 6). Additional AM haplotypes with greater than 1% frequency within the population were identified in six of the QTL. The haplotypes evaluated with NILs were generally the most frequent haplotypes observed in the AM panel, with the exception of DON.13 haplotype 1, present in only one line and DON.18 haplotype 4, unique to the NIL family testing it.

AM haplotype effects were significant in nine and seven of fourteen families for DON and FHB, respectively (Table 4, AM haplotype study). DON effects ranged from 11% to 44% and FHB from 16% to 72%. HD effects were small (less than 3%) and significant in

six families. HD and DON effects were in the same direction in four families, and opposite in one family. When a DON effect was detected in the NIL study there was a similar effect in the AM study with one exception. In family ND25728, the direction of the effect was negative the NIL study and positive in the AM study. In terms of the DON effect size the two studies generally agreed though AM haplotype effects were generally higher except for ND25661. As mentioned earlier no FHB effects were detected in the NIL study. Heading date effects were inconsistent across the studies except for ND25728.

Discussion

Validating QTL prior to further genetic investigation or implementing MAS in breeding is a prudent step to insure effective use of resources. This is particularly true for QTL discovered by AM since there is an increased risk of false positive discoveries due to the complex population structure that typically exists in AM panels. We were able to use NILs with contrasting marker haplotypes at QTL for DON detected by AM to validate and directly estimate haplotype effects. We validated both QTL that were consistently detected in the original AM study (DON.10 and DON.13) as well as one that was detected with less confidence (DON.33).

The FHB related QTL we studied typically explained only 1-5% of the observed variation in the AM study (Massman et al. 2011). In contrast, the *Fhb1* gene in wheat has been mapped repeatedly and accounts for 20-60% of the variation observed in bi-parental mapping populations (Anderson et al. 2001; Buerstmayr et al. 2002; Buerstmayr et al. 2003; Waldron et al. 1999; Zhou et al. 2002). *Fhb1* NILs containing the resistant allele averaged a 23% and 27% decrease in disease severity and infected kernels, respectively across several populations evaluated in the field (Pumphrey et al. 2007). Interestingly, only half of the NIL pairs for *Fhb1* studied showed a significant

effect for disease spread as measured in a greenhouse assay; the phenotype that *Fhb1* directly impacts. In another study, Häberle et al. (2007) validated the effect size and direction of two FHB resistance related QTL, which individually decreased severity by 27% relative to the susceptible allele in a backcross population of winter wheat. Despite the fact that the QTL we studied explained a very small proportion of the genetic variation in the AM panel, the allelic effects of validated QTL ranged from a 9% to 23% reduction in DON. This suggests validation efforts may be warranted even for associations that appear to have small effects when identified by AM.

Our primary objective was to validate DON QTL, but we also observed associations between DON and heading date that have been previously noted (de la Pena et al. 1999; Ma et al. 2000). In the AM study, three of the five DON QTL investigated (DON.10, DON.13, and DON.29) were associated with heading date (Massman et al. 2011). It is often speculated that later heading results in lower disease as a result of disease avoidance rather than disease resistance *per se*, however there is some evidence supporting tight linkage of HD and resistance genes (Massman et al. 2011; Nduulu et al. 2007). From our study of DON.13 the differential association between DON and HD among three NIL families indicates that linkage between traits has been broken. Breeding strategies using the allele corresponding to haplotype 4 from ND25661 should result in lower DON without altering HD.

In addition to our study, an independent AM analysis of four years of CAP data across the MN and ND six-row breeding lines resulted in the identification of a HD QTL on chromosome 4H detected in the same region as DON.18 in the Massman study (Vikram et al., unpublished). Interestingly, this QTL was detected when AM was conducted with MN breeding lines, and the combined analysis using MN and ND breeding lines, but not when using the ND breeding lines alone. Our investigation of this region using NILs derived from both ND and MN backgrounds detected a heading

date difference in the MN derived FEG168-09 NILs, but not in those from ND. This indicates that detection of the HD QTL in the AM study was likely the result of segregation within a subpopulation of the AM panel.

Failure to Validate QTL

NILs provided a relatively simple means to validate QTL. No difference among NILs would suggest the QTL was a false positive. Not surprisingly and consistent with past studies of FHB and DON, not all regions were confirmed in our study; however several potential explanations could explain the failure to validate a QTL.

First the effect of genetic background may have played a role in detecting differences among NILs. Evidence for a background effects is given by the two DON.33 NIL families that were both from the ND breeding program but have different pedigrees. Both tested the effects of identical haplotypes in the region using a single marker, however, only one family was significant for DON. Pumphrey et al. (2007) found evidence for a background effect in the validation of *Fhb1*. The authors hypothesized that higher levels of background resistance in some NIL families might make it more difficult to see a difference between NILs compared to NILs from families with lower base levels of resistance. Greater background resistance may also explain what we observed at DON.13. The two ND NIL families generally resulted in higher overall DON levels than the MN family; however the lower DON in the MN line M04-45 may be due to the fact that it is hulless and lower DON may result from the loss of the hull during harvest (Legzdina and Buerstmayr 2004; Clear et al. 1997).

Another explanation for lack of validation is that the haplotypes conferring an effect in the AM panel were not those that were contrasted in the NILs that we developed. This is another potential explanation for why at DON.13 only two of the three NIL families

showed a difference in DON. A total of four haplotypes were evaluated, but only those families with NILs that contrasted haplotype 3 were significantly different for DON. Haplotype effect estimates from AM population indicated that haplotype 3 conferred the least resistance which is consistent with the effect directions observed in the NIL study.

The complications of validation using NILs when multiple haplotypes for a QTL are segregating is apparent when we look at the large number of haplotypes present at some QTL regions (Table 6). Since any NIL family will compare only two haplotypes, it is possible that our sample may miss the most important haplotypes. This may indeed have been the case for DON.07 where haplotypes 1 and 2 were tested as NILs and did not differ in DON as would have been predicted by the haplotype analysis of the AM data (Table 6). However, the AM results indicate that haplotype 3 would have shown a significant effect on DON if contrasted with either haplotypes 1 or 2. We also note there are cases where a difference in performance predicted by the AM study was not observed in the NILs as occurred with comparisons of haplotypes 1 and 2 at DON.18 (Tables 4 and 6). Consideration of multiple haplotypes in the context of NIL validation also suggests that conducting AM by haplotype rather than by SNP could increase power to detect associations (Hamblin and Jannink 2011; Lorenz et al. 2010).

Finally, if a QTL was not validated it is possible that even among near-isogenic lines there could be other loci for disease resistance still segregating, masking the effect for which the NILs were designed. We attempted to account for this by genotyping all NILs with a set of 48 markers that mapped to known QTL. In fact, several NIL parents were segregating at more than two DON QTL and were excluded from our study because of the anticipated complexity in interpreting those results. Given the large number of loci that likely contribute to DON and the level of residual heterozygosity present in the

CAP line parents, SNPs associated with DON that were not included in our 48 SNP screening panel could still be segregating among NILs.

Advantages of Using NIL Validation

The choice of QTL validation method is a function of the trait of interest and the organism under study. The vast majority of AM studies have been conducted in humans for disease related traits (http://www.genome.gov/gwastudies) and approaches for validation are limited to conducting subsequent association studies in panels that are distinct from the original discovery panel. Therefore, the factors that limit power and increase bias in the original study are still relevant in the validation study (i.e. rare variants and population structure).

Despite some of the caveats mentioned above, the ability to easily generate NILs in plant systems offers substantial advantages with regard to validation and characterization of QTL. Measuring allelic effects in near isogenic backgrounds eliminates the factors present in AM studies that can limit detection, such as population structure, varying allele frequency, and extent of LD. Using NILs each allele or haplotype is evaluated at a designed frequency determined by the number of NILs generated, creating a situation with optimum power to detect an association. This reduces the confounding relationships of both differential allele frequencies and background effects. NIL-based analysis is not subject to bias caused by population structure because QTL are tested in a fixed genetic background. However, analysis of identical NIL haplotypes developed across different populations provides us an opportunity to investigate background effects on isolated QTL (see failure to validate QTL section above).

In addition, the construction of NILs offered the possibility to refine the QTL interval and increase the map resolution of the QTL. At DON.10 and DON.13, we were able to significantly reduce the size of the QTL region defined in the original study (Figure 1A and 1B). We developed NILs by screening 15 progeny from each heterozygous parent and selecting the contrasting homozygotes. To further increase mapping resolution one could easily select the heterozygotes, allow them to self-pollinate to generate large numbers of progeny, screen them with appropriate flanking markers, and obtain more recombinant NILs.

Finally, because NILs were derived from current breeding material instead of mapping populations, the simultaneous benefits of validation and germplasm improvement can be accomplished (Pumphrey et al. 2007). Breeding for FHB resistance and lower DON has been difficult due to the complex nature of the trait and unfavorable linkages of other traits with resistance (Mesfin et al. 2003, Nduulu et al. 2007). Thus, the major resistance QTL identified in bi-parental mapping populations have been linked to tall plant height and late heading which are both undesirable from a breeding perspective. AM and subsequent validation in elite breeding material has identified QTL that can reduce DON by measurable amounts without negatively effecting other traits. We were able to validate QTL with larger effects that could be exploited by traditional MAS approaches. However, it is likely that much of the variation for FHB resistance and lower DON is explained by loci with relatively small effects. Recently, genomic selection approaches have been shown to be effective in predicting DON with a level of accuracy that should accelerate gain from selection (Lorenz et al. 2012). Ultimately, the cost and format of the available marker genotyping technology will determine which approach is most promising. Taken as a whole, our results suggest a combination of MAS for QTL regions such as DON.10 and DON.13 and genomic selection may best serve breeding objectives for the reduction of DON in barley.

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Table 1. Forty-eight markers and criteria used to select these as informative markers associated with deoxynivalenol (DON) for near isogenic line (NIL) identification

				Position		Association Study[b]		Allele Class[d]		
Marker	BOPA[a]	SNP	DON QTL	Chr	cM	CAP I	CAP II	AA	BB	AB
12_31394	2	[C/G]	DON.07	2H	74.37	NA[c]	NA	0.83	0.16	0.004
11_20960	1	[T/C]	DON.07	2H	74.37	1.75E-07	-	0.83	0.17	0.004
11_20734	1	[T/C]	DON.07	2H	75.18	-	-	0.71	0.28	0.004
11_10446	1	[T/C]	DON.10	2H	125.46	2.31E-08	4.67E-06	0.32	0.67	0.006
11_21440	1	[A/G]	DON.10	2H	126.03	2.31E-08	1.18E-05	0.67	0.33	0.006
11_21459	1	[A/G]	DON.10	2H	127.06	4.40E-08	6.55E-06	0.33	0.66	0.006
11_10065	1	[A/G]	DON.10	2H	130.01	4.96E-04	-	0.33	0.67	0.006
11_20895	1	[A/G]	DON.10	2H	131.77	5.64E-05	0.0695	0.32	0.67	0.009
11_21129	1	[T/C]	DON.13	3H	52.5	1.16E-09	3.56E-04	0.76	0.23	0.011
11_11086	1	[T/C]	DON.13	3H	53.27	6.16E-10	3.56E-04	0.76	0.23	0.011
12_31372	2	[T/C]	DON.13	3H	54.4	NA	NA	0.08	0.91	0.004
11_20995	1	[T/C]	DON.13	3H	58.64	5.31E-10	0.0393	0.73	0.25	0.011
11_21120	1	[T/C]	DON.13	3H	64.19	1.70E-04	-	0.31	0.68	0.009
11_11391	1	[A/C]	DON.13	3H	65.52	7.19E-05	0.0207	0.69	0.31	0.004
12_30736	2	[T/C]	DON.15	3H	168.4	NA	NA	0.17	0.82	0.004
11_20422	1	[C/G]	DON.17	4H	24.59	0.0041	6.21E-07	0.13	0.86	0.006
11_20302	1	[A/G]	DON.17	4H	26.19	0.0041	3.17E-05	0.13	0.86	0.006
11_20777	1	[T/C]	DON.17	4H	26.66	0.0041	4.23E-11	0.84	0.15	0.009
11_21374	1	[A/G]	DON.17	4H	28.4	-	-	0.98	0.02	0.002
11_21122	1	[A/G]	DON.17	4H	33.38	0.0012	2.50E-07	0.10	0.90	0.002
12_10860	2	[G/C]	DON.17	4H	36.37	NA	NA	0.89	0.10	0.002
12_30328	2	[T/C]	DON.18	4H	40.96	NA	NA	0.13	0.86	0.004
11_21073	1	[T/C]	DON.18	4H	48.5	2.53E-04	0.0212	0.64	0.35	0.011
11_10756	1	[T/G]	DON.18	4H	48.5	2.53E-04	0.0212	0.35	0.64	0.011
11_20289	1	[A/G]	DON.18	4H	50.4	-	0.0118	0.67	0.32	0.009
11_11114	1	[T/C]	DON.18	4H	54.25	0.0146	-	0.67	0.32	0.009
11_20361	1	[A/G]	DON.18	4H	59.37	-	0.01	0.58	0.41	0.011
11_21191	1	[T/C]	DON.18	4H	61.04	0.0075	-	0.64	0.35	0.011
11_20838	1	[T/C]	DON.20	4H	96.59	3.71E-05	-	0.68	0.32	0.009
11_10869	1	[G/C]	DON.26	5H	173.08	3.23E-04	-	0.57	0.43	0.004
11_10401	1	[T/A]	DON.27	5H	191.97	-	1.73E-05	0.28	0.71	0.009
12_30360	2	[A/T]	DON.27	5H	191.97	NA	NA	0.09	0.90	0.002
11_10129	1	[A/G]	DON.29	6H	42.36	-	-	0.51	0.48	0.009
11_21281	1	[A/G]	DON.29	6H	43.83	-	-	0.74	0.26	0.006
11_10817	1	[A/C]	DON.29	6H	45.44	2.00E-04	-	0.41	0.59	0.006
12_30569	2	[T/G]	DON.29	6H	51.41	NA	NA	0.80	0.19	0.006
11_21158	1	[T/C]	DON.29	6H	53.95	-	2.01E-04	0.15	0.85	0.004
11_20600	1	[C/G]	DON.29	6H	55	-	7.94E-05	0.86	0.13	0.004
12_10758	2	[A/G]	DON.29	6H	60.23	0.0308	7.39E-04	0.17	0.82	0.002
11_21069	1	[A/G]	DON.29	6H	63.95	0.0308	7.39E-04	0.81	0.18	0.006
11_20904	1	[A/G]	DON.29	6H	64.36	-	-	0.16	0.84	0.006
11_20714	1	[T/C]	DON.29	6H	67.04	-	8.22E-05	0.83	0.17	0.004
11_11349	1	[T/G]	DON.30	6H	71.08	-	7.74E-08	0.15	0.84	0.004
11_20868	1	[C/G]	DON.31	6H	124.85	-	-	0.46	0.52	0.011
11_21437	1	[T/C]	DON.33	7H	17.2	2.99E-04	-	0.61	0.38	0.009
11_11014	1	[A/G]	DON.35	7H	60.69	0.0017	-	0.86	0.13	0.009
11_10534	1	[A/G]	DON.36	7H	80.94	2.34E-05	-	0.19	0.81	0.002
11_10055	1	[G/C]	DON.36	7H	79.6	-	-	0.11	0.88	0.006

a Single nucleotice polymorphism (SNP) marker platform BOPA1 or BOPA2

b p-values of significant markers in either CAPI (2006) or CAPII (2007) association mapping panels from Massman et al. (2011)

c NA = BOPA2 markers not tested in association analyses

d Marker frequency of genotype AA, BB, or AB in combined CAPI and II population on the basis of 463 lines

Table 2. Twenty-four barley CAP lines from three six-row breeding programs selected as parents for NIL development

CAP Line Name	Program[a]	CAP year[b]	DON QTL[c]
6B01-2221	BA	2006	DON.18
FEG126-12	MN	2006	DON.17, DON.18
FEG132-63	MN	2006	DON.29
FEG148-40	MN	2006	DON.18
FEG149-18	MN	2006	DON.10
FEG167-10	MN	2007	DON.17
FEG168-09	MN	2007	DON.17
M03-66	MN	2006	DON.27
M03-82	MN	2006	DON.10
M04-29	MN	2007	DON.10, DON.29, DON.33
M04-31	MN	2007	DON.07, DON.10, DON.26, DON.29, DON.35
M04-45	MN	2007	DON.13
ND23899	N6	2006	DON.18
ND25657	N6	2007	DON 0.31
ND25660	N6	2007	DON 0.36
ND25661	N6	2007	DON.13, DON.18
ND25665	N6	2007	DON.10, DON.20
ND25681	N6	2007	DON.07
ND25684	N6	2007	DON.33
ND25691	N6	2007	DON.13
ND25694	N6	2007	DON.10, DON 0.31
ND25697	N6	2007	DON.10, DON.29, DON 0.30
ND25728	N6	2007	DON.33
ND25732	N6	2007	DON.29

a BA = Busch Agricultural Resources, Inc; MN = University of Minnesota; ND = North Dakota State University

b CAPI = 2006 lines; CAPII = 2007 lines

c DON QTL regions in which at least one marker is heteroygous

Table 3. Eighteen CAP lines selected for near isogenic line (NIL) haplotype comparisons in field trials

CAP Line	Parents of CAP line	Program[a]	CAP year[b]	DON QTL[c]
FEG126-12	FEG66-31/M120	MN	2006	DON.17, DON.18
FEG132-63	FEG80-74/FEG67-12	MN	2006	DON.29
FEG148-40	FEG96-22/Rassmusson	MN	2006	DON.18
FEG149-18	ND20407/M118	MN	2006	DON.10
FEG168-09	Comp351/Rassmusson/M98-102	MN	2007	DON.17
M04-29	M01-63/M120	MN	2007	DON.10, DON.29, DON.33
M04-45	M001-71/M01-87	MN	2007	DON.13
ND23899	Drummond/ND17643	N6	2006	DON.18
ND25657	Stellar/ND20481	N6	2007	DON 0.31
ND25661	Stellar/ND20481	N6	2007	DON.13, DON.18*
ND25665	Stellar/ND20481	N6	2007	DON.10, DON.20
ND25681	Stellar/ND20481	N6	2007	DON.07
ND25684	Stellar/ND20481	N6	2007	DON.33
ND25691	Stellar/ND20481	N6	2007	DON.13
ND25694	Stellar/ND20481	N6	2007	DON.10, DON 0.31
ND25697	Stellar/ND20603	N6	2007	DON.10, DON.29, DON 0.30*
ND25728	ND19474/ND20477	N6	2007	DON.33
ND25732	ND19474/ND20477	N6	2007	DON.29

a BA = Busch Agricultural Resources, Inc; MN = University of Minnesota; ND = North Dakota State University

b CAPI = 2006 lines; CAPII = 2007 lines

c DON QTL regions in which at least one marker is heteroygous

* CAP parent heterozygous at multiple QTL, though progeny (NILs) segregated for only one

Table 4. Effects of significant[a] haplotype differences from the near isogenic line (NIL) and association mapping (AM) studies for deoxynivalenol (DON), Fusarium head blight (FHB), and heading date (HD)

						DON QTL region								Haplotype Effects[c]					
		# lines per haplotype				DON.07	DON.10	DON.13	DON.17	DON.18	DON.20	DON.29	DON.31 DON.33	NIL haplotype study[d]			AM haplotype study[e]		
NIL family	# markers[b]	1	2	3	4									DON	FHB	HD	DON	FHB	HD
ND25681	3	2	1			X								-	-	-	-11%**	-	-0.7%**
FEG149-18	5(4)	4	3				X							-10%*	-	-	-20%***	-	-0.8%**
ND25697	5(3)			1	1		X							-15%**	-	-	-29%***	-	-
M04-45	6(3)	1	2					X						-	-	-1.8%*	-	-	-
ND25691	6(5)		2	4				X						14%***	-	2.4%***	17%***	17%**	-
ND25661	6(3)			1	1			X						-23%*	-	-	-17%**	-29%**	-0.8%*
FEG168-09	6(3)	3	4						X					-	-	1.9%*	27%**		-
FEG148-40	7(6)	7	2							X				-	-	-1.1%**	44%***	72%**	1.1%*
ND23899	7(2)			2	4					X				-	-	-	-	-	-
FEG132-63	10(1)	1	1									X		-	-	-	-	29%*	-2%***
ND25732	10(2)	4		2								X		-	-	-		-	-
ND25657	1	1	2										X	-	-	-	-	-16%***	-
ND25684	1	5	4										X	-	-	-	17.1%***	21%***	-
ND25728	1	1	1										X	-9%**	-	-1.6%**	17.1%***	21%***	-0.4%*

a *,**,*** = significant difference between haplotypes at p-value < 0.05, 0.01, and 0.001, respectively

b Number of markers per haplotype, () indicate the number polymorphic between haplotypes

c Effects presented only if effect >10%, or significant in either NIL or AM analysis

d Effect of haplotype based on performance of lines from NIL study

e Effect of haplotype based on performance of lines from AM study

Table 5. Significant p-value differences between near isogenic line (NIL) family haplotypes isolating the effect of two QTL across three locations for Fusarium head blight (FHB), deoxynivalenol (DON) concentration, and heading date (HD)

		DON QTL haplotypes evaluated[b]										Significant p-values								
		DON.10			DON.17		DON.18		DON.20		DON.31										
		Hap 1	Hap 3	Hap 4	Hap 2	Hap 3	Hap 5	Hap 6	Hap 1	Hap 2	Hap 1	Hap 2	St. Paul, MN			Crookston, MN			Osnabrock, ND	Combined Locations		
NIL family	Class[a]												DON	FHB	HD	DON	FHB	HD	DON	DON	FHB	HD
FEG126-12	1					X	X						0.012	0.003	0.0186	-	-	-	0.0273	0.002	0.014	-
	2				X			X														
ND25665	1			X					X				-	-	0.0003	-	-	-	-	-	-	0.004
	2	X								X												
	3			X						X												
ND25694	1	X									X		-	-	0.0083	-	-	-	-	-	-	0.017
	2		X									X										
	3		X								X											
	4	X										X										

a Haplotype classes in multiple QTL comparisons based on the combination of NIL haplotypes across two QTL

b DON QTL, as defined by Massman et al. (2011) and associated NIL haplotypes

Table 6. Haplotype diversity within the CAPI and CAPII association panels for six deoxynivalenol (DON) QTL and average associated trait values

		haplotype		Trait[b]		
Study[a]	QTL	number	n	DON (ppm)[c]	FHB severity (%)	HD (DAP)[d]
NIL	DON 0.07	1	74	25.3	10.2	52.7
NIL	DON 0.07	2	327	22.7	11.0	52.3
AM	DON 0.07	3	54	20.9	11.6	52.6
total haplotypes identified		**5**				
NIL	DON 0.10	1	154	22.9	9.9	52.7
NIL	DON 0.10	2	118	18.7	9.8	52.3
NIL	DON 0.10	3	119	26.4	12.0	52.3
NIL	DON 0.10	4	21	19.0	10.2	51.8
NIL*	DON 0.10	5	27	26.0	13.8	52.4
AM	DON 0.10	6	8	26.1	15.2	52.5
total haplotypes identified		**11**				
NIL	DON 0.13	1	1	11.7	13.9	50.8
NIL	DON 0.13	2	289	21.7	10.4	52.5
NIL	DON 0.13	3	76	25.6	12.2	52.4
NIL	DON 0.13	4	20	21.4	9.0	52.0
AM	DON 0.13	5	14	22.7	12.0	51.7
total haplotypes identified		**15**				
NIL	DON 0.17	1	7	17.4	8.6	52.1
NIL	DON 0.17	2	379	23.9	11.4	52.4
AM	DON 0.17	3	37	17.0	7.7	52.2
AM	DON 0.17	4	22	19.2	7.8	52.2
total haplotypes identified		**11**				
NIL	DON 0.18	1	13	16.9	7.9	52.1
NIL	DON 0.18	2	9	26.0	16.1	52.6
NIL	DON 0.18	3	105	25.6	11.4	52.5
NIL	DON 0.18	4	-	-	-	-
NIL*	DON 0.18	5	247	22.1	10.5	52.4
NIL*	DON 0.18	6	17	25.0	12.2	52.2
AM	DON 0.18	7	8	11.4	5.5	50.5
AM	DON 0.18	8	14	27.3	18.0	52.4
AM	DON 0.18	9	8	18.2	8.8	52.4
AM	DON 0.18	10	7	23.6	8.6	52.6
total haplotypes identified		**23**				
NIL	DON 0.29	1	20	21.3	9.0	52.8
NIL	DON 0.29	2	38	20.2	12.1	51.9
NIL	DON 0.29	3	85	23.6	9.8	52.7
AM	DON 0.29	4	69	23.5	12.9	52.1
AM	DON 0.29	5	24	30.2	16.8	52.2
AM	DON 0.29	6	25	26.8	11.9	52.2
AM	DON 0.29	7	12	20.9	9.5	52.8
AM	DON 0.29	8	16	25.4	12.1	52.5
AM	DON 0.29	9	20	22.1	8.4	53.2
AM	DON 0.29	10	11	22.4	7.9	52.3
AM	DON 0.29	11	12	19.5	9.5	52.5
AM	DON 0.29	12	17	22.0	8.8	52.3
AM	DON 0.29	13	14	22.4	8.5	53.0
AM	DON 0.29	14	6	22.0	7.8	52.6
AM	DON 0.29	15	9	14.9	7.6	51.0
AM	DON 0.29	16	5	19.1	13.0	52.1
AM	DON 0.29	17	5	19.8	53.5	16.3
total haplotypes identified		**60**				

a NIL = haplotypes in near isogenic line study; AM = additional haplotypes in association panel with > 0.01 frequency

b Average of all lines of the haplotype

c DON concentration (in parts per million)

d Heading date in days after planting

* NILs from analysis of two QTL NIL families

A

	Position		CAP Parent	FEG149-18							CAP Parent	ND25697	
marker	cM	Chr		A	F	K	O	B	E	J		J	K
11_10446	125.46	2	AB	BB	BB	BB	BB	AA	AA	AA	AB	BB	AA
11_21440	126.03	2	AB	AA	AA	AA	AA	BB	BB	BB	AB	AA	BB
11_21459	127.06	2	AB	BB	BB	BB	BB	AA	AA	AA	AB	BB	AA
11_10065	130.01	2	AB	BB	BB	BB	BB	AA	AA	AA	BB	BB	BB
11_20895	131.77	2	BB	BB	BB	BB	BB	BB	BB	BB	AA	AA	AA
haplotype				**1**	**1**	**1**	**1**	**2**	**2**	**2**		**3**	**4**

B

	Position		CAP Parent	M04-45			CAP Parent	ND25691								CAP Parent	ND25661	
marker	cM	Chr		C	G	L		B	H	O	C	G	K	L	M		F	K
11_21129	52.5	3	AB	BB	AA	AA	AB	AA	AA	AA	BB	BB	BB	BB	BB	AB	BB	AA
11_11086	53.27	3	AB	BB	AA	AA	AB	AA	AA	AA	BB	BB	BB	BB	BB	AB	BB	AA
12_31372	54.4	3	AB	AA	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
11_20995	58.64	3	AA	AA	AA	AA	AB	AA	AA	AA	BB	BB	BB	BB	BB	AB	BB	AA
11_21120	64.19	3	BB	BB	BB	BB	AB	BB	BB	BB	AA	AA	AA	AA	AA	AA	AA	AA
11_11391	65.52	3	AA	AA	AA	AA	AB	AA	AA	AA	BB	BB	BB	BB	BB	BB	BB	BB
haplotype:				**1**	**2**	**2**		**2**	**2**	**2**	**3**	**3**	**3**	**3**	**3**		**3**	**4**

C

	Position		CAP Parent	FEG168-09						
marker	cM	Chr		A	D	F	B	C	H	I
11_20422	24.59	4	BB	BB	BB	BB	BB	BB	BB	BB
11_20302	26.19	4	BB	BB	BB	BB	BB	BB	BB	BB
11_20777	26.66	4	AB	BB	BB	BB	AA	AA	AA	AA
11_21374	28.4	4	AB	BB	BB	BB	AA	AA	AA	AA
11_21122	33.38	4	BB	BB	BB	BB	BB	BB	BB	BB
12_10860	36.37	4	AA	AA	AA	AA	AA	AA	AA	AA
haplotype				**1**	**1**	**1**	**2**	**2**	**2**	**2**

Figure 1. The segregation of significant and interesting NIL families for deoxynivalenol (DON) accumulation QTL derived from CAP parent lines for the QTL under investigation. A) NIL families FEG149-18 and ND25697 testing DON.10 B) NIL families M04-45, ND25691, and ND25661 testing DON.13 C) NIL family FEG169-09 testing DON.17. NIL family names are listed above letters A-O representing individual NILs. The genotypes AA and BB represent homozygous marker alleles, AB represents those that are heterozygous.

A

marker	Position Chr	cM	DON	CAP Parent	NIL family FEG126-12 A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
11_20422	4	24.59	DON.17	AB	AA	AA	AB	AB	AB	AB	AB	BB	AB	AB	BB	AA	AA	AB	BB
11_20302	4	26.19	DON.17	AB	AA	AA	AB	AB	AB	AB	AB	BB	AB	AB	BB	AA	AA	AB	BB
11_20777	4	26.66	DON.17	AB	BB	BB	AB	AB	AB	AB	AB	AA	AB	AB	AA	BB	BB	AB	AA
11_21374	4	28.4	DON.17	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
11_21122	4	33.38	DON.17	AB	AA	AA	BB	AB	AB	AB	BB	BB	AB	AB	BB	AA	AA	AB	BB
12_10860	4	36.37	DON.17	AB	BB	AB	AA	AB	AB	AB	AA	AA	AB	AB	AA	BB	BB	AB	AA
			haplotype		**3**							**2**			**2**	**3**	**3**		**2**
12_30328	4	40.96	DON.18	AB	BB	AB	AA	AB	AB	AB	AA	AA	AB	AB	AA	BB	AB	AB	AA
11_10756	4	48.5	DON.18	AB	BB	AB	AA	AB	AB	AB	AA	AA	AB	AB	AA	BB	AB	AB	AA
11_21073	4	48.5	DON.18	AB	AA	AB	BB	AB	AB	AB	BB	BB	AB	AB	BB	AA	AB	AB	BB
11_20289	4	50.4	DON.18	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
11_11114	4	54.25	DON.18	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
11_20361	4	59.37	DON.18	AB	AA	AB	BB	AB	AB	AB	BB	BB	--	AA	BB	AA	AB	AB	BB
11_21191	4	61.04	DON.18	AB	AA	AB	BB	--	AB	AB	BB	BB	AB	AA	BB	AA	AB	AB	BB
			haplotype	**P**	**5**		**6**				**6**	**6**			**6**	**5**			**6**

B

marker	Position Chr	cM	DON	CAP Parent	NIL family ND25665 A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
11_10446	2	125.46	DON.10	AB	AA	AA	AB	AA	BB	AA	AB	AB	AB	AB	AB	AB	AB	AA	BB
11_21440	2	126.03	DON.10	AB	BB	BB	AB	BB	AA	BB	AB	AB	AB	AB	AB	AB	AB	BB	AA
11_21459	2	127.06	DON.10	AB	AA	AA	--	AA	BB	AA	--	--	--	--	--	--	--	AA	BB
11_10065	2	130.01	DON.10	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
11_20895	2	131.77	DON.10	AB	AA	AA	AB	AA	BB	AA	BB	BB	AB	AB	AB	AB	BB	AA	BB
			haplotype		**4**	**4**		**4**	**1**	**4**								**4**	**1**
11_20838	4	96.59	DON.20	AB	AB	AB	BB	BB	AA	AB	AB	AB	AB	AB	AA	AB	BB	AA	AB
			haplotype				**1**	**1**	**2**						**2**			**2**	

C

marker	Position Chr	cM	DON	CAP Parent	NIL family ND25694 A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
11_10446	2	125.46	DON.10	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
11_21440	2	126.03	DON.10	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
11_21459	2	127.06	DON.10	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
11_10065	2	130.01	DON.10	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
11_20895	2	131.77	DON.10	AB	BB	AA	AB	AA	AA	BB	AB	BB	AA	AB	AB	AB	AA	AA	AB
			haplotype		**1**	**3**		**3**	**3**	**1**		**1**	**3**				**3**	**3**	
11_20868	6	124.85	DON.31	AB	BB	AB	BB	AA	BB	AA	AB	AA	AB	BB	AA	AA	AA	AB	BB
			haplotype		**1**		**1**	**2**	**1**	**2**		**2**		**1**	**2**	**2**	**2**		**1**

Figure 2: Two-QTL NIL families and associated haplotypes within each QTL region. A) NIL family FEG126-12 at DON.17 and DON.18 B) NIL family ND25665 at DON.10 and DON.20 C) NIL family ND25694 at DON.10 and DON.31. Boxes indicate NIL lines classes compossed of the combined haplotypes of two QTL. NILs are denoted by letters (A-O). The genotypes AA and BB represent homozygous markers, AB represents heterozygotes

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Appendix A. Pool of candidate QTL detected in the original six-row mapping populations

		Position		CAP I six-row		CAP II six-row	
QTL	QTL name[a]	CH	cM	p[a]	$r^{2,b}$	p	r^2
DON.03		1H	99	6.52E-06	0.016		
DON.05		2H	28-34	6.32E-11	0.033		
DON.06		2H	50			1.33E-05	0.017
DON.07		2H	74-78	2.75E-11	0.034		
DON.08		2H	86	1.49E-05	0.015		
DON.10	DON2H.125-132	2H	125-132	9.97E-10	0.029	3.73E-07	0.024
DON.13	DON3H.52-65	3H	52-65	5.00E-15	0.046	4.01E-05	0.016
DON.15		3H	168-170			5.67E-05	0.015
DON.16	DON4H.03	4H	3			1.04E-05	0.018
DON.17	DON4H.21-36	4H	21-36	9.40E-08	0.022	2.21E-10	0.035
DON.18	DON4H.40-61	4H	40-61	5.71E-11	0.033		
DON.19		4H	65-78	3.95E-06	0.017		
DON.20		4H	86-98	9.26E-09	0.025		
DON.21		5H	7	1.06E-05	0.015		
DON.22		5H	57			1.09E-04	0.014
DON.23		5H	75-80			5.37E-05	0.015
DON.25		5H	151-159	9.67E-05	0.012		
DON.26		5H	173	1.13E-04	0.012		
DON.27	DON5H.190-192	5H	190-192			1.00E-04	0.014
DON.29	DON6H.42-67	6H	42-67	9.96E-09	0.025	1.81E-06	0.021
DON.30		6H	70-77			2.03E-07	0.025
DON.31		6H	124			3.47E-06	0.02
DON.33		7H	17-22	9.92E-09	0.025		
DON.35		7H	60-71	3.12E-05	0.013		
DON.36		7H	79-88	2.70E-06	0.017		
DON.37		7H	98	9.09E-05	0.012		
DON.38		7H	110	2.44E-06	0.017		
DON.39		7H	130-145	4.75E-07	0.02		

a QTL identified in at least two mapping subsets across environments by Massman et al. (2011)

b p-values of most significant marker associated with deoxynivalenol (DON)

c r^2 = the amount of explainable variation due to a QTL

Appendix B. Segregation[a] of eighteen CAP parent lines across ten QTL associated with deoxynivalenol (DON) concentration

DON QTL	marker	Position chr	Position cM	ND23899	ND25657	ND25661	ND25665	ND25681	ND25684	ND25691	ND25694	ND25697	ND25728	ND25732	FEG148-40	FEG149-18	FEG126-12	FEG132-63	FEG168-09	M04-45	M04-29
DON.07 Chr 2H (74-78)	11_20960	2	74.4	AA	BB	BB	BB	AB	AA	BB	BB	AA	BB	BB	AA	AA	AA	AA	AA	AA	AA
	12_31394	2	74.4	AA	BB	BB	BB	AB	AA	BB	BB	AA	BB	BB	AA	AA	AA	AA	AA	AA	AA
	11_20734	2	75.2	BB	BB	BB	BB	AB	AA	BB	BB	AA	AA	BB	AA	AA	BB	AA	AA	AA	AA
DON.10 Chr 2H (125-132 cM)	11_10446	2	125	BB	BB	AA	AB	AA	BB	BB	BB	AB	BB	BB	BB	AB	AA	AA	BB	AA	BB
	11_21440	2	126	AA	AA	BB	AB	BB	AA	AA	AA	AB	AA	AA	AA	AB	BB	BB	AA	BB	AA
	11_21459	2	127	BB	BB	AA	AB	AA	BB	BB	BB	AB	BB	BB	BB	AB	AA	AA	BB	AA	BB
	11_10065	2	130	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	AA	AB	AA	AA	AA	AA	BB
	11_20895	2	132	AA	AA	AA	AB	BB	BB	BB	AB	AA	BB	BB	BB	BB	BB	BB	BB	BB	AB
DON.13 Chr 3H (52-65 cM)	11_21129	3	52.5	BB	BB	AB	BB	BB	AA	AB	AA	AA	AA	AA	AA	AA	AA	AA	AA	AB	AA
	11_11086	3	53.3	BB	BB	AB	BB	BB	AA	AB	AA	AA	AA	AA	AA	AA	AA	AA	AA	AB	AA
	12_31372	3	54.4	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	AA	BB	BB	AB	AA
	11_20995	3	58.6	BB	BB	AB	BB	BB	AA	AB	AA	AA	AA	AA	AA	AA	BB	AA	AA	AA	BB
	11_21120	3	64.2	AA	AA	AA	AA	AA	BB	AB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
	11_11391	3	65.5	BB	BB	BB	BB	BB	AA	AB	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
DON.17 Chr 4H (21-36 cM)	11_20422	4	24.6	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	AA	BB	AB	AA	BB	BB	BB
	11_20302	4	26.2	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	AA	BB	AB	AA	BB	BB	BB
	11_20777	4	26.7	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	BB	AA	AB	BB	AB	AA	AA
	11_21374	4	28.4	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AB	AA	AA
	11_21122	4	33.4	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	AA	BB	AB	BB	BB	BB	BB
	12_10860	4	36.4	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	BB	AA	AB	AA	AA	AA	AA
DON.18 Chr 4H (40-61 cM)	12_30328	4	41	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	AA	BB	AB	BB	BB	BB	AA
	11_10756	4	48.5	AB	AA	AB	BB	BB	AA	BB	BB	BB	AA	BB	AB	BB	AB	BB	AA	BB	AA
	11_21073	4	48.5	AB	BB	AB	AA	AA	BB	AA	AA	AA	BB	AA	AB	AA	AB	AA	BB	AA	BB
	11_20289	4	50.4	BB	BB	AB	AA	AA	BB	AA	AA	AA	BB	AA	AB	AA	AA	AA	AA	AA	AA
	11_11114	4	54.3	BB	BB	AB	AA	AA	BB	AA	AA	AA	BB	AA	AB	AA	AA	AA	BB	AA	AA
	11_20361	4	59.4	BB	BB	BB	AA	AA	BB	AA	AA	AA	AA	AA	AB	AA	AB	BB	BB	AA	BB
	11_21191	4	61	BB	BB	BB	AA	BB	BB	AA	AA	AA	AA	AA	AB	AA	AB	AA	BB	AA	BB
DON.20 Chr 4H (86-98 cM)	11_20838	4	96.6	AA	BB	AA	AB	AA	BB	BB	BB	BB	BB	BB	AA	BB	AA	AA	AA	AA	BB
DON.29 Chr 6H (42-67 cM)	11_10129	6	42.4	AA	AA	BB	BB	AA	AA	BB	AA	AA	AA	AA	BB	BB	AA	AA	BB	AA	AB
	11_21281	6	43.8	BB	AA	AA	AA	AA	BB	AA	AA	AA	AA	AB	AA	AA	BB	AA	AA	AA	AB
	11_10817	6	45.4	BB	AA	AA	AA	AA	BB	AA	AA	AA	AA	AB	BB	AA	BB	AB	AA	BB	BB
	12_30569	6	51.4	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	BB	BB	AA	BB	AA	AB
	11_21158	6	54	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	AA	AA	BB	AA	BB	AB
	11_20600	6	55	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	BB	BB	AA	BB	AA	AB
	12_10758	6	60.2	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	AA	BB	BB	BB	AA
	11_21069	6	64	AA	AA	AA	AA	AA	AA	AA	AA	AB	AA	AA	AA	AA	BB	AA	AA	AA	AA
	11_20904	6	64.4	BB	BB	AA	BB	BB	AA	AA	BB	AB	AA	BB	BB	AA	BB	BB	BB	BB	AA
	11_20714	6	67	AA	AA	AA	AA	AA	AA	AA	AA	AB	AA	AA	AA	AA	BB	AA	AA	AA	AA
DON.30 Chr 6H (70-77 cM)	11_11349	6	71.1	BB	BB	BB	BB	BB	BB	BB	BB	AB	BB	BB	BB	BB	AA	BB	BB	BB	BB
DON.31 Chr 6H (124 cM)	11_20868	7	125	AA	AB	AA	AA	BB	AA	BB	AB	AA	AA	AA	BB	AA	BB	BB	BB	BB	AA
DON.33 Chr 7H (17-22 cM)	11_21437	7	17.2	AA	AA	BB	BB	AA	AB	BB	BB	BB	AB	BB	AA	AA	AA	AA	BB	AA	AB
DON.35 Chr 7H (60-71 cM)	11_11014	7	60.7	AA	BB	BB	AA	AB	BB	AA	AA	AA	BB	AA	AA	AA	AA	AA	AA	AA	AA

a AA, BB = contrasting homozygous marker aleles, AB = heterozygous marker

☐ = CAP line progeny resulted in one segregating QTL and one homozygous QTL

1

	Position		CAP Parent	ND25681		
marker	cM	Chr		B	F	M
11_20960	74.37	2	AB	BB	BB	AA
12_31394	74.37	2	AB	BB	BB	AA
11_20734	75.18	2	AB	BB	BB	AA
		haplotype:		**1**	**1**	**2**

2

	Position		CAP Parent	FEG148-40									
marker	cM	Chr		C	D	E	H	K	L	M	N	O	
12_30328	40.96	4	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	
11_10756	48.5	4	AB	BB	BB	AA	AA	BB	BB	BB	BB	BB	
11_21073	48.5	4	AB	AA	AA	BB	BB	AA	AA	AA	AA	AA	
11_20289	50.4	4	AB	AA	AA	BB	BB	AA	AA	AA	AA	AA	
11_11114	54.25	4	AB	AA	AA	BB	BB	AA	AA	AA	AA	AA	
11_20361	59.37	4	AB	AA	AA	BB	BB	AA	AA	AA	AA	AA	
11_21191	61.04	4	AB	AA	AA	BB	BB	AA	AA	AA	AA	AA	
		haplotype:		**1**	**1**	**2**	**2**	**1**	**1**	**1**	**1**	**1**	

CAP Parer	ND23899						
	C	D	H	J	L	N	
BB	BB	BB	BB	BB	BB	BB	
AB	AA	BB	BB	BB	BB	AA	
AB	BB	AA	AA	AA	AA	BB	
BB	BB	BB	BB	BB	BB	BB	
BB	BB	BB	BB	BB	BB	BB	
BB	BB	BB	BB	BB	BB	BB	
BB	BB	BB	BB	BB	BB	BB	
	3	**4**	**4**	**4**	**4**	**3**	

3

	Position		CAP Parent	FEG132-63		CAP Parent	ND25732					
marker	cM	Chr		E	O		C	D	E	F	M	N
11_10129	42.36	6	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
11_21281	43.83	6	AA	AA	AA	AB	AA	AA	BB	AA	BB	AA
11_10817	45.44	6	AB	AA	BB	AB	AA	AA	BB	AA	BB	AA
12_30569	51.41	6	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
11_21158	53.95	6	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
11_20600	55	6	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
12_10758	60.23	6	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
11_21069	63.95	6	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
11_20904	64.36	6	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
11_20714	67.04	6	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
		haplotype:		**1**	**2**		**1**	**1**	**3**	**1**	**3**	**1**

4

	Position		CAP Parent	ND25657							
marker	cM	Chr		E	F	I	J	K	L	M	O
11_20868	124.85	6	AB	BB	BB	AA	AA	AA	AA	BB	AA
		haplotype:		**1**	**1**	**2**	**2**	**2**	**2**	**1**	**2**

5

	Position		CAP Parent	ND25684			CAP Parent	ND25728								
marker	cM	Chr		B	I	N		A	B	C	D	E	H	I	J	K
11_21437	17.2	7	AB	BB	AA	AA	AB	AA	AA	BB	AA	BB	BB	AA	AA	BB
		haplotype:		**1**	**2**	**2**		**2**	**2**	**1**	**2**	**1**	**1**	**2**	**2**	**1**

Appendix C. Segregation of non-significant single NIL families for deoxynivalenol (DON) accumulation QTL derived from CAP parent lines for a QTL under investigation. NIL family names are listed above letters A-O representing individual NILs 1) NIL familND25681 testing DON.07 2) NIL families 148-40 and ND23899 testing DON.19 3) NIL families FEG132-63 and ND25732 testing DON.29 4) NIL family ND25657 testing DON.31 5) NIL families ND25684 and ND25728 testing DON.33

Appendix D. *Fusarium graminearum* isolates collected in Minnesota fields used in disease nursery inoculation

Accession Number SGP ID No:	Host Crop	Year	Location[a]	Town / additional information
10110001	Wheat	2010	Marshall, MN	Stephen
10110002	Wheat	2010	Marshall, MN	Stephen
10110003	Barley	2010	Marshall, MN	Stephen
10110004	Wheat	2010	Marshall, MN	S of Argyle, Hwy 75
10110005	Wheat	2010	Marshall, MN	S of Argyle, Hwy 75
10110006	Wheat	2010	Polk, MN	S of Euclid, Hwy 75
10110007	Wheat	2010	Polk, MN	Hwy 9 & County Rd 41
10110008	Wheat	2010	Polk, MN	S of Beltrami, Hwy 9
10110009	Wheat	2010	Polk, MN	S of Beltrami, Hwy 9
10110010	Wheat	2010	Polk, MN	Between Beltrami & Borup, Hwy 9
10110011	Wheat	2010	Norman, MN	S of Borup, Hwy 9
10109001	Wheat	2009	Norman, MN	Hwy 9, S of Borup
10109007	Barley	2009	Clay, MN	Hwy 9 & 80th Ave
10109008	Wheat	2009	Polk, MN	Hwy 2 & Hwy 102
10109009	Wheat	2009	Ottertail, MN	County Rd 82
10109010	Wheat	2009	Norman, MN	County Rd 123 & 250th Ave
10109011	Wheat	2009	Norman, MN	Hwy 9 & Wild Rice River
10109012	Wheat	2009	Clay, MN	Barnsville
10109013	Wheat	2009	Norman, MN	Hwy 9
10108004	Wheat	2008	Polk, MN	N of Euclid, Hwy 75
10108005	Barley	2008	Marshall, MN	N of Alvarado
10108006	Wheat	2008	Marshall, MN	N of Alvarado
10108007	Wheat	2008	Kittson, MN	E of Donaldson, Hwy 11
10108008	Wheat	2008	Kittson, MN	S of Karlstad
10108009	Oat	2008	Polk, MN	Crookston, Hwy 2
10108011	Wheat	2008	Norman, MN	S of Beltrami, Hwy 9
10108012	Wheat	2008	Norman, MN	S of Beltrami, Hwy 9
10108013	Wheat	2008	Norman, MN	Borup
10108016	Wheat	2008	Polk, MN	Erskine, Hwy 59
10108018	Wheat	2008	Red Lake, MN	Extension Trial in Oklee
10108019	Wheat	2008	Polk, MN	240th St S & 310th Ave SW, by Fisher
10108022	Wheat	2008	Polk, MN	Euclid, 240th & 310th Ave
10107001	Wheat	2007	Norman, MN	W of Ada, Hwy 200
10107002	Wheat	2007	Clark, MN	Neillsville
10107003	Wheat	2007	Polk, MN	N of Angus
10107004	Wheat	2007	Marshall, MN	N of Warren
10107005	Wheat	2007	Marshall, MN	Stephen Airport
10107006	Wheat	2007	Marshall, MN	Eagle Point
10107007	Wheat	2007	Polk, MN	Sandsville
10107008	Wheat	2007	Norman, MN	Ada
10107009	Wheat	2007	Norman, MN	Borup, Hwy 9
10106001	Wheat	2006	Norman, MN	Borup
10106002	Corn	2006	Dakota, MN	Rosemount
10105057	Barley	2005	Polk, MN	Fertile
10105064	Barley	2005	Mahnomen, MN	Mahnomen, Hwy 200
10105068	Barley	2005	Dakota, MN	Rosemount
10105008	Wheat	2005	Norman, MN	Borup, Agripro Site
10105014	Wheat	2005	Polk, MN	Angus
10105015	Wheat	2005	Polk, MN	Crookston by Northland Inn
10105037	Wheat	2005	Norman, MN	Borup, Agripro Site

a County, State collected

Appendix E. Deoxynivalenol (DON) concentration of entire field relative to near isogenic line (NIL) family checks (CAP lines) across three locations

	St. Paul (ppm[a])			Crookston (ppm)			Osnabrock (ppm)		
CAP line	mean	SD	range	mean	SD	range	mean	SD	range
Entire field[c]	11.5	4.7	2.8-29.7	32.7	10.1	10.8-70.3	10.6	5.4	0.8-33.9
FEG126-12	15.5	3.1	11.2-19.2	39.2	6.5	31.2-48.6	11.2	4.2	6.6-17.7
FEG132-63	9.9	3.5	5.8-15.3	34.8	4.6	27.4-39.1	6.1	2.5	2.4-9.2
FEG148-40	9.1	2.7	6.7-13.5	24.1	3.4	21.6-30.0	10.7	4.7	6.0-17.0
FEG149-18	12.5	3.4	7.8-17.1	26.9	7.6	19.2-41.2	9.2	3.6	5.0-17.0
FEG168-09	15.0	2.5	14.3-17.1	24.7	7.0	20.1-36.7	8.8	4.8	5.0-17.0
M04-45	12.1	6.0	5.7-20.7	21.5	8.5	15.6-36.0	7.8	4.3	2.0-13.0
ND23899	18.1	5.1	10.9-22.6	54.3	10.0	44.6-70.3	18.2	9.6	9.2-32.1
ND25657	12.5	3.9	7.3-16.8	47.0	5.3	40.8-53.3	14.5	5.6	8.7-23.8
ND25661	8.3	2.6	6.3-12.0	26.9	7.8	20.8-40.2	9.7	2.3	7.5-13.4
ND25665	10.2	3.8	6.7-16.0	38.8	5.2	34.5-47.1	7.8	2.5	5.0-10.5
ND25681	12.4	5.1	8.9-20.8	32.3	1.2	31.1-33.7	6.5	1.3	4.7-8.1
ND25684	11.6	2.2	8.0-13.8	28.4	7.1	21.2-37.8	14.0	5.3	9.8-21.4
ND25691	9.6	2.5	7.3-13.6	51.4	8.7	41.9-61.0	11.0	6.4	6.2-21.9
ND25694	6.3	1.3	5.0-8.3	34.5	4.5	27.9-37.2	4.6	2.5	2.7-8.9
ND25697	11.1	3.4	7.8-16.6	30.8	3.8	24.8-34.6	7.1	3.6	3.4-12.9
ND25728	16.9	4.5	13.2-24.5	29.9	6.5	23.0-39.9	8.2	2.8	5.7-12.4
ND25732	13.4	8.3	4.5-30.4	24.7	5.3	16.7-30.4	8.8	3.1	4.9-17.0

a DON concentration in per million million

b SD = standard deviation

c Entire field values based on data from all lines in field (i.e. CAP parents, parents of CAP lines, and NILs)

☐ = mean of one or both NIL family haplotypes fall outside this range

Appendix F. Fusarium head blight (FHB) severity and heading date (HD) of the entire field relative to near isogenic line (NIL) family checks (CAP lines) across two locations

	St. Paul, MN						Crookston, MN					
	FHB severity (%)			HD (DAP[a])			FHB severity (%)			HD (DAP)		
CAP parent	mean	SD	range	mean	SD[b]	range	mean	SD	range	mean	SD	range
Entire field[c]	4.6	3.1	0.1-18.3	60.7	2.6	56.0-68.0	30.8	14.1	2.2-82.5	50.6	1.4	48.0-56.0
FEG126-12	7.0	3.1	4.1-11.3	61.2	0.4	61.0-62.0	31.7	7.6	25.5-43.0	51.4	0.9	50.0-52.0
FEG132-63	4.3	4.1	1.1-10.5	61.8	1.3	61.0-64.0	30.0	7.9	22.5-42.0	50.8	0.5	50.0-51.0
FEG148-40	5.3	2.5	2.7-8.1	61.2	0.5	61.0-62.0	30.1	11.7	18.8-45.5	49.4	0.6	49.0-50.0
FEG149-18	3.9	2.2	2.2-9.6	60.6	0.5	60.0-61.0	29.4	11.5	15.5-49.0	50.7	1.4	49.0-51.0
FEG168-09	4.6	2.9	2.2-9.6	65.6	0.5	65.0-66.0	31.1	15.3	15.5-49.0	51.8	0.9	51.0-53.0
M04-45	6.4	6.4	1.7-17.0	62.8	1.6	61.0-65.0	50.8	20.6	29.5-82.5	50.2	1.1	49.0-52.0
ND23899	4.3	3.0	1.2-8.2	63.8	0.8	63.0-65.0	42.0	18.9	20.0-63.5	50.8	0.8	50.0-52.0
ND25657	4.7	1.6	2.7-7.2	58.4	0.9	57.0-59.0	29.5	7.1	17.5-35.5	50.6	1.1	49.0-52.0
ND25661	4.5	2.6	2.2-7.6	58.2	1.6	56.0-60.0	25.5	13.5	12.0-48.0	49.8	0.5	49.0-50.0
ND25665	4.4	1.5	2.5-6.3	60.0	0.7	59.0-61.0	47.6	21.9	20.9-66.0	49.8	0.5	49.0-50.0
ND25681	2.6	1.8	0.3-4.7	61.2	1.1	60.0-62.0	16.6	9.2	10.2-32.8	51.4	0.9	50.0-52.0
ND25684	6.7	3.2	3.6-10.6	59.2	1.5	57.0-61.0	40.7	8.4	34.0-53.5	48.6	0.6	48.0-49.0
ND25691	3.1	3.2	0.9-8.6	59.6	1.3	58.0-61.0	35.0	8.4	24.5-44.5	49.8	0.5	49.0-50.0
ND25694	3.2	1.9	1.8-6.4	59.4	0.9	58.0-60.0	30.8	8.1	23.0-42.0	50.0	0.0	50.0-50.0
ND25697	4.9	2.3	2.0-7.7	61.8	1.1	61.0-63.0	13.1	3.7	8.7-17.0	52.2	1.1	51.0-54.0
ND25728	4.7	1.9	2.7-6.8	63.2	2.8	60.0-66.0	23.2	9.3	15.5-35.0	52.0	0.7	51.0-53.0
ND25732	4.2	1.9	2.0-6.9	58.4	0.5	58.0-59.0	32.4	15.4	15.5-49.0	48.8	0.5	48.0-49.0

a heading date in days after planting (DAP)

b SD = standard deviation

c entire field values based on data from all lines in field (i.e. CAP parents, parents of CAP lines, and NILs)

☐ = mean of one or both NIL family haplotypes fall outside this range

Appendix G. Deoxynivalenol (DON) concentrations for near isogenic line (NIL) family haplotypes across three environments

			St Paul DON (ppm[a])				Crookston DON (ppm)				Osnabrock DON (ppm)			
NIL family	QTL	haplotype	n	mean	SD[b]	range	n	mean	SD	range	n	mean	SD	range
ND25681	DON.07	1	10	13.5	3.7	8.4-20.4	10	33.7	6.7	25.7-46.4	10	9.1	3.6	5.9-16.1
	DON.07	2	4	11.9	5.3	6.6-18.8	5	30.1	6.6	21.1-39.1	5	10.5	3.3	6.0-15.0
FEG149-18	DON.10	1	20	8.6	3.0	4.7-13.8	20	26.7	6.1	17.7-40.6	20	9.9	4.7	3.4-19.7
	DON.10	2	15	7.9	2.0	5.6-11.2	15	25.3	5.2	16.7-34.4	15	7.5	4.2	3.7-19.5
ND25697	DON.10	3	5	15.1	1.8	12.5-16.5	5	31.1	2.4	28.9-35.1	5	10.2	6.8	2.9-18.5
	DON.10	4	5	11.7	1.9	9.7-14.5	4	26.6	4.5	21.1-30.3	5	7.2	2.8	3.8-11.6
M04-45	DON.13	1	5	10.0	3.7	17.2-27.9	5	21.9	4.6	17.2-27.9	5	6.8	4.2	3.1-13.7
	DON.13	2	10	7.2	3.4	10.8-26.1	10	16.7	4.3	10.8-26.1	10	7.8	3.9	3.7-16.8
ND25691	DON.13	2	15	14.1	3.2	28.0-58.4	15	46.8	6.8	28.0-58.4	15	12.3	4.5	5.6-21.7
	DON.13	3	30	9.4	2.1	26.0-41.7	29	34.3	4.5	26.0-41.7	30	11.7	3.9	4.6-17.0
ND25661	DON.13	3	4	9.1	2.1	27.7-47.1	5	39.5	7.2	27.7-47.1	5	8.4	2.3	5.9-11.8
	DON.13	4	5	8.4	2.1	17.7-43.4	5	29.7	9.2	17.7-43.4	5	7.1	3.7	1.7-10.8
FEG168-09	DON.17	1	15	13.7	3.2	8.8-23.2	15	25.6	3.8	18.5-31.2	14	9.6	4.3	4.8-20.6
	DON.17	2	20	15.4	2.9	10.9-22.1	19	28.4	4.6	20.8-35.2	20	8.6	4.1	3.1-18.7
FEG148-40	DON.18	1	35	7.0	2.1	3.8-13.0	34	23.5	4.6	16.8-36.2	34	11.7	4.9	5.3-26.1
	DON.18	2	10	8.1	3.2	4.0-13.2	10	24.1	6.5	17.9-40.9	10	13.4	3.9	8.3-18.6
ND23899	DON.18	3	10	18.5	4.9	11.7-28.0	9	49.3	6.5	38.4-57.8	10	13.8	5.5	7.6-25.9
	DON.18	4	20	17.1	5.7	9.6-29.7	19	47.7	5.9	38.3-57.4	20	12.1	4.7	4.7-20.2
FEG132-63	DON.29	1	5	7.8	3.1	3.9-11.0	5	38.2	4.3	33.1-44.1	5	6.4	4.2	2.0-11.0
	DON.29	2	5	7.7	2.7	5.1-11.5	5	35.7	5.7	30.4-44.2	5	8.2	4.6	2.3-14.3
ND25732	DON.29	1	20	7.9	3.5	3.3-14.7	20	26.8	5.6	16.6-39.5	19	10.1	3.1	3.3-15.2
	DON.29	3	10	8.7	3.3	4.5-14.0	9	24.2	6.9	13.5-32.0	10	9.7	3.8	6.0-18.3
ND25657	DON.31	3	15	15.8	4.2	9.4-24.3	15	48.5	9.8	28.6-63.5	15	11.5	3.1	5.1-15.7
	DON.31	4	25	14.7	4.0	7.8-25.3	25	50.2	7.1	34.7-62.3	25	12.8	3.8	6.0-20.5
ND25684	DON.33	1	5	10.3	2.7	7.0-13.4	5	30.2	8.4	21.1-38.0	4	12.2	3.6	7.3-16.2
	DON.33	2	10	9.9	1.6	7.3-12.6	10	28.7	7.0	18.6-36.8	10	12.2	4.5	5.9-18.1
ND25728	DON.33	1	25	14.0	4.7	6.3-25.5	25	27.8	3.5	20.8-34.7	25	8.9	2.4	4.9-14.0
	DON.33	2	20	13.5	3.9	8.8-24.1	20	25.5	4.3	16.5-34.7	20	7.3	2.6	3.4-14.6
FEG126-12	DON.17, DON.18	1	10	11.5	3.1	7.1-16.8	10	33.4	6.7	21.7-41.2	10	9.9	5.3	2.4-17.8
	DON.17, DON.18	2	15	14.4	3.6	7.9-21.8	15	34.9	5.5	25.3-47.1	15	14.9	6.4	5.9-27.5
ND25665	DON.10, DON.20	1	5	10.0	3.6	6.8-15.9	5	39.7	4.7	34.3-45.2	5	10.2	4.2	7.3-17.6
	DON.10, DON.20	2	5	11.0	4.5	7.1-18.6	5	40.4	4.6	34.9-46.2	5	9.1	4.8	4.0-16.0
	DON.10, DON.20	3	5	10.3	3.2	6.3-13.1	5	36.0	4.4	29.7-40.8	5	8.2	2.4	4.9-11.1
ND25694	DON.10, DON.31	1	5	8.5	5.3	5.2-17.6	5	32.7	7.0	21.7-39.2	5	7.9	3.5	3.4-12.8
	DON.10, DON.31	2	10	7.4	2.8	3.6-12.8	10	33.2	5.0	24.9-39.5	10	8.2	4.5	3.2-18.5
	DON.10, DON.31	3	5	7.7	3.5	4.0-12.8	5	36.3	4.1	30.6-40.7	5	6.6	2.9	3.3-10.9
	DON.10, DON.31	4	10	6.8	2.26	4.0-10.9	10	36.0	6.9	25.2-45.3	10	7.5	5.7	2.5-21.9

a DON concentration in parts per million

b SD = standard deviation

Appendix H. NIL family Fusarium head blight (FHB) severity and heading date (HD) performance across two environments

		St. Paul FHB severity (%)				St Paul HD (DAP)[b]				Crookston FHB severity (%)				Crookston HD (DAP)			
NIL family	haplotype	n	mean	SD[a]	range	n	mean	SD	range	n	mean	SD	range	n	mean	SD	range
ND25681	1	10	4.9	3.15	1.2-10.8	10	62.8	1.62	60-65	10	22.4	7.99	10.1-34.5	10	51.7	1.49	50-55
	2	5	4.3	2.81	1.8-8.6	5	61.6	1.95	60-65	5	27.1	3.69	22.0-30.5	5	52.6	1.95	50-55
FEG149-18	1	20	3.8	1.55	1.2-7.1	20	60.3	0.79	59-61	20	26.2	11.67	11.0-56.0	20	49.8	0.77	49-51
	2	15	3.8	2.11	0.6-8.3	15	60.1	0.88	58-61	15	25.8	8.57	13.3-41.0	15	49.7	0.70	49-51
ND25697	1	5	4.1	2.33	2.5-8.0	5	63.0	1.41	61-65	5	16.2	6.85	7.2-25.5	5	51.2	0.45	51-52
	2	5	3.1	1.99	1.0-6.0	5	61.4	1.14	60-63	4	23.6	11.65	15.3-40.5	4	51.0	0.82	50-52
M04-45	1	5	5.4	5.20	1.7-14.0	5	62.0	1.73	61-65	5	38.5	12.47	17.3-27.9	5	50.2	1.30	49-52
	2	10	6.2	5.82	0.5-18.3	10	60.5	1.08	58-62	10	41.9	19.38	10.8-26.1	10	49.7	0.95	49-52
ND25691	2	15	2.6	1.92	0.8-10.1	15	60.9	1.13	59-62	15	35.4	10.45	13.5-53.5	15	49.8	0.41	49-50
	3	29	3.3	2.53	0.5-5.5	30	62.5	1.50	60-65	29	35.2	13.64	11.0-58.5	29	50.8	1.10	49-54
ND25661	3	5	5.7	3.48	3.1-11.4	5	59.4	0.89	58-60	5	28.7	12.20	18.0-42.0	5	51.6	0.89	50-52
	4	5	6.6	4.84	2.9-15.0	5	59.4	0.89	58-60	5	30.2	9.88	18.0-41.5	5	50.2	0.45	50-51
FEG168-09	1	15	3.0	2.45	0.8-10.0	15	64.0	2.85	59-67	15	31.4	12.06	12.0-52.5	15	51.9	0.96	51-54
	2	20	3.9	2.68	1.2-9.1	20	65.5	2.16	59-67	19	32.7	11.40	16.0-50.5	19	52.7	1.29	51-55
FEG148-40	1	35	4.4	2.48	0.8-8.8	35	58.6	1.24	57-61	35	25.2	9.28	10.0-46.5	35	49.8	0.71	48-51
	2	10	3.8	2.68	0.7-9.6	10	57.9	0.88	57-59	10	30.3	8.85	15.5-40.9	10	49.4	0.84	48-51
ND23899	3	10	5.2	5.07	0.8-17.4	10	62.9	0.57	62-64	10	40.6	12.36	25.5-54.5	10	50.8	0.92	50-52
	4	20	5.1	4.13	1.0-16.2	20	62.6	1.67	59-65	19	35.6	12.83	10.0-54.5	19	51.2	0.83	50-52
FEG132-63	1	5	5.2	2.89	2.2-8.9	5	60.8	0.84	60-62	5	28.1	15.10	12.4-50.5	5	51.2	0.84	50-52
	2	5	4.0	3.44	1.0-8.4	5	59.8	0.84	59-61	5	25.4	9.40	15.0-39.0	5	51.2	0.84	50-52
ND25732	1	20	4.2	1.70	1.5-7.3	20	59.7	0.75	58-61	20	34.6	11.53	16.0-61.0	20	49.2	0.70	48-51
	3	10	4.9	1.98	1.7-8.1	10	59.3	0.95	58-61	9	35.3	14.02	13.5-53.0	9	49.0	0.50	48-50
ND25657	3	15	6.2	2.79	2.9-12.2	15	60.6	1.35	58-63	15	35.8	11.22	18.5-57.5	15	51.4	1.18	50-55
	4	25	4.9	1.69	1.7-7.9	25	60.0	1.40	58-64	25	33.4	11.13	14.5-62.5	25	51.5	1.12	49-55
ND25684	1	5	7.2	2.89	5.1-11.3	5	59.4	1.34	58-61	5	43.5	9.87	37.0-55.5	5	49.0	0.71	48-50
	2	10	6.7	2.83	3.1-10.9	10	60.0	0.94	58-61	10	42.7	14.70	26.5-78.5	10	49.1	0.57	48-50
ND25728	1	25	3.5	2.86	0.4-10.9	24	65.6	0.72	64-67	24	22.7	7.35	6.4-33.5	25	52.4	1.08	50-54
	2	20	3.6	2.27	1.2-8.5	19	64.0	2.56	59-67	20	23.5	11.60	5.8-34.5	20	52.6	1.19	51-55
FEG126-12	1	10	4.5	1.31	1.8-6.4	10	58.2	0.42	58-59	10	28.0	12.08	16.0-57.5	10	50.6	1.26	49-52
	2	15	7.9	3.60	7.9-21.8	15	58.6	0.63	58-60	15	36.8	13.73	20.0-71.0	15	50.3	1.16	49-52
ND25665	1	5	7.6	5.27	2.5-14.8	5	58.0	0.71	57-59	5	40.9	24.01	19.9-80.0	5	50.2	1.10	49-52
	2	5	4.6	3.87	1.4-11.3	5	61.0	0.00	61-61	5	37.1	18.16	22.5-57.0	5	50.4	0.55	50-51
	3	5	4.7	2.74	1.8-8.7	5	59.6	0.89	59-61	5	28.5	6.77	21.0-37.5	5	50.4	0.55	50-51
ND25694	1	5	3.8	2.49	2.0-7.6	5	59.6	1.34	58-61	5	37.5	6.67	30.5-45.5	5	50.2	1.10	49-52
	2	10	4.1	3.31	0.7-9.6	10	57.8	0.92	57-59	10	33.1	10.46	18.0-45.5	10	50.0	0.00	50-50
	3	5	4.0	1.87	1.2-5.9	5	57.8	1.30	57-60	5	42.7	13.35	31.5-58.5	5	49.8	0.45	49-50
	4	10	3.7	2.54	1.4-8.3	10	57.8	0.92	57-59	10	34.0	11.82	18.0-50.5	10	50.3	0.67	50-52

a SD = standard deviation

b DAP = days after planting

Appendix I. Significant p-value differences between near isogenic line (NIL) family haplotypes isolating the effect of a single QTL across three locations for Fusarium head blight (FHB), deoxynivalenol (DON) accumulation, and heading date

	# lines per haplotype				DON QTL evaluated[a]									Significant p- values[b]								
														St. Paul, MN			Crookston, MN			Osnabrock, ND	Combined Locations		
NIL family	1	2	3	4	DON.07	DON.10	DON.13	DON.17	DON.18	DON.20	DON.29	DON.31	DON.33	DON	FHB	HD	DON	FHB	HD	DON	DON	FHB	HD
ND25681	2	1			X									-	-	-	-	-	-	-	-	-	-
FEG149-18	4	3				X								-	-	-	-	-	-	0.0398	0.037	-	-
ND25697			1	1		X								-	0.035	-	-	-	-	-	0.006	-	-
M04-45	1	2					X							-	-	-	0.047	-	-	-	-	-	0.0373
ND25691		2	4				X							>0.0001	-	>0.0001	>0.0001	-	>0.0001	-	>0.0001	-	>0.0001
ND25661			1	1			X							-	-	-	0.006	-	0.025	-	0.014	-	-
FEG168-09	3	4						X						-	-	0.021	0.041	-	0.01	-	-	-	0.0116
FEG148-40	7	2							X					-	-	0.038	-	-	-	-	-	-	0.0089
ND23899			2	4					X					-	-	-	-	-	-	-	-	-	-
FEG132-63	1	1									X			-	-	-	-	-	-	-	-	-	-
ND25732	4		2								X			-	-	-	-	-	-	-	-	-	-
ND25657	1	2										X		-	-	-	-	-	-	-	-	-	-
ND25684	5	4											X	-	-	-	-	-	-	-	-	-	-
ND25728	1	1											X	-	-	8E-04	0.008	-	-	0.0463	0.002	-	0.0043

a DON QTL, as defined by Massman et al. (2011)

b Significant markers associated with DON below the $p < 0.05$ threshold

Appendix J. Near isogenic line (NIL) haplotype effects on deoxynivalenol (DON) concentration, Fusarium head blight (FHB) severity, and heading date (HD) across three locations

		DON (ppm)[a]								FHB (% severity)						HD (DAP)[c]					
NIL family	haplotype	SP	effect[b]	CR	effect	OS	effect	Comb	effect	SP	effect	CR	effect	Comb	effect	SP	effect	CR	effect	Comb	effect
ND25681	1	13.5	-12%	33.7	-11%	9.1	15%	18.8	-7%	4.9	-13%	22.4	20%	13.6	14%	62.8	-1.9%	51.7	1.7%	57.3	-0.3%
ND25681	2	12.0		30.1		10.5		17.5		4.3		27.1		15.7		61.6		52.6		57.1	
FEG149-18	1	8.6	-8%	26.7	-5%	9.9	-28%*	15.0	-10%*	3.7	1.6%	26.2	-1%	15.0	-1%	60.3	-0.3%	49.8	-0.1%	55.0	-0.2%
FEG149-18	2	7.9		25.3		7.5		13.6		3.8		25.8		14.8		60.1		49.7		54.9	
ND25697	3	15.1	-25%*	31.1	-15%	10.2	-35%	18.8	-15%*	27.0	-29%*	16.2	38%	10.2	27%	63.0	-2.6%	51.2	-0.4%	57.1	-1.9%
ND25697	4	11.7		26.6		7.2		16.2		25.4		24.0		13.3		61.4		51.0		56.2	
M04-45	1	10.0	-34%	21.9	-28%*	6.8	13%	12.9	-20%	5.4	14%	38.5	8%	21.9	9%	62.0	-2.5%	50.2	-1.0%	56.1	-1.8%*
M04-45	2	7.2		16.8		7.8		10.6		6.2		41.9		24.0		60.5		49.7		55.1	
ND25691	2	9.3	38%***	34.3	31%***	11.7	4%	20.1	14%***	2.6	23%	35.4	-1%	19.0	1%	60.9	2.6%***	49.8	2.0%***	55.3	2.4%**
ND25691	3	14.1		39.5		12.3		23.0		3.3		35.2		19.3		62.5		50.8		55.6	
ND25661	3	9.1	-8%	47.5	-28%**	8.4	-17%	19.0	-23%**	5.7	14%	8.6	-5%	17.2	7%	59.4	0.0%	51.6	-2.8%*	55.5	-1.3%
ND25661	4	8.4		29.7		7.1		15.1		6.6		7.2		18.4		59.4		50.2		54.8	
FEG168-09	1	13.7	12%	25.6	10%*	9.6	-11%	16.3	7%	3.0	26%	31.4	4%	17.2	6%	64.0	2.2%*	51.9	1.4%**	58.0	1.9%*
FEG168-09	2	15.4		28.4		8.6		17.5		3.9		32.7		18.3		65.5		52.7		59.1	
FEG148-40	1	7.0	15%	23.1	4%	11.7	14%	13.9	9%	4.4	-14%	25.2	20%	14.8	14%	58.6	-1.2%*	49.8	-0.9%	54.2	-1.1%**
FEG148-40	2	8.1		24.1		13.4		15.2		3.8		30.3		17.0		57.9		49.4		53.7	
ND23899	3	18.5	-8%	49.3	-3%	13.8	-13%	27.2	-6%	5.2	-1.9%	40.6	-13%	22.9	-12%	62.9	-0.5%	50.8	0.7%	56.9	0.0%
ND23899	4	17.1		47.4		12.1		25.6		5.1		35.6		20.4		62.6		51.2		56.9	
FEG132-63	1	7.8	-0.2%	38.2	-0.2%	6.4	3%	17.5	-2%	5.2	-26%	28.1	-10%	16.6	-12%	60.8	-1.7%	51.2	0.0%	56.0	-0.9%
FEG132-63	2	7.7		35.7		8.2		17.2		4.0		25.4		14.7		59.8		51.2		55.5	
ND25732	1	7.9	9%	26.8	-10%	10.1	-4%	14.9	-6%	4.2	15%	34.6	2%	19.4	3%	59.6	-0.6%	49.2	-0.4%	54.4	-0.5%
ND25732	3	8.7		24.2		9.7		14.0		4.9		35.3		20.1		59.3		49.0		54.2	
ND25657	1	15.8	-7%	48.5	3%	11.5	10%	25.3	2%	6.2	-23%	35.8	-7%	21.0	-9%	60.6	-0.9%	51.4	0.2%	56.0	-0.4%
ND25657	2	14.7		50.2		12.8		25.9		4.9		33.4		19.2		60.0		51.5		55.8	
ND25684	1	10.3	-4%	30.2	-5%	12.2	0.3%	17.6	-4%	7.2	-7.7%	43.5	-2%	25.4	-3%	59.4	1.0%	49.0	0.2%	54.2	0.6%
ND25684	2	9.9		28.7		12.2		16.9		6.7		42.7		24.7		60.0		49.1		54.6	
ND25728	1	14.0	-4%	27.8	-8%**	8.9	-19%*	16.9	-9%**	3.5	3.1%	22.7	3%	13.1	3%	65.6	-2.4%***	52.4	0.4%	59.0	-1.6%**
ND25728	2	13.5		25.5		7.3		15.4		3.6		25.5		13.5		64.0		52.6		58.1	

a DON concentration in parts per million

b Haplotype effect calculated as the difference between means of haplotype classes as a percent of family mean

c DAP = days after planting

*,**,*** = significant difference between haplotypes detected at p-value < 0.05, 0.01, and 0.001, respectively

☐ = direction of effect different than that of other environments