Fine mapping coincident QTL on a chromosome 6H introgression region from unadapted germplasm into elite malting barley

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Lu Yin

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Abstract

Breeders strive to improve a cultivar for multiple traits. This can be challenging when these traits are unfavorably associated. This study fine maps several traits associated with the centromeric region of chromosome 6H by mendelizing the region using near-isogenic lines (NILs). A NIL carrying an introgression from the landrace Chevron for this region was crossed to a malting cultivar Lacey to create 1941 F2 individuals from which recombinant NILs (rNILs) for the introgressed region were developed. We developed a dense genetic map using 249 F4-derived rNILs and phenotyped 101 of them in field and greenhouse trials for fourteen traits. We observed transgressive segregation for *Fusarium* head blight (FHB) severity, grain protein concentration (GPC), days to senescence, normalized difference vegetation index (NDVI), seedling net blotch, and heading date. We detected QTL for eleven traits. For ten of the traits, we detected multiple QTL with opposite allelic effects for each of them. Six traits that showed transgressive segregation mapped to multiple QTL with opposite allelic effects. QTL for deoxynivalenol (DON) accumulation mapped to separate regions to that for GPC and FHB severity, suggesting DON accumulation was likely associated through linkage rather than pleiotropy to GPC and FHB severity. While there was no recombinant line possessing all favorable alleles in this region, we identified two rNILs with numerically lower FHB severity and DON accumulation and GPC similar to Lacey. Seven traits (NDVI, days to senescence, percent stem breakage, GPC, heading date, adult net blotch severity and seedling net blotch severity) mapped to a single region, close to the physical location of the *NAM-1/Gpc-1* gene. This gene is known to encode a NAC

transcription factor that might be involved in nitrogen remobilization from leaves to grains during senescence. Findings in this study about coincident QTL controlling days to senescence, NDVI, percent stem breakage, and GPC might aid in the understanding of nitrogen remobilization process. Our fine mapping study revealed a complex genetic architecture for eleven traits associated with this chromosome 6H region and highlights the need for recombination to uncouple unfavorable linkages in breeding programs.

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Supplementary Figure 1. Marker coverage of each of three genotype-by-sequencing runs conducted on the Gen10 population and marker coverage of the merged genotype-by-sequencing runs of 249 Gen10 rNILs

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Chapter 1 Introduction

Many traits important to crop species are quantitative in nature, and breeders generally want to improve a cultivar for multiple traits. It is frequently observed that the target traits are unfavorably associated to some degree, which creates a bottleneck for many breeding programs.

Barley (*Hordeum vulgare* L.), a self-fertile, diploid (2n=2x=14), widely adapted crop species with large chromosomes (6-8 μm), is extensively studied as a favorite genetic experimental plant (Paulitz & Steffenson, 2011). Barley is mainly used for malt, animal feed and food. From 30% to 40% of the U.S. barley is malted for brewing (Michigan State University Extension, July, 2014). In the Midwest, spring six-row barley is the predominant type of malting barley.

Among the breeding targets for six-row malting barley in the Midwest, *Fusarium* head blight resistance (FHB), bright kernels, and low grain protein concentration (GPC) have been emphasized. A low GPC is desired for less interference with malt extraction, the variety Karl (CIho 15487; Wesenberg et al., 1976) has been used as the source of low GPC in many six-row malting barley breeding programs. Breeding for low GPC using this source has been largely ineffective due to associations of GPC with unfavorable characteristics (See et al., 2002). Breeding for bright kernels, that is kernel discoloration (KD) resistance, in six-row malting barley has mainly relied on Chevron (PI 38061), a landrace introduced from Switzerland. Although it has been used as a source for FHB and KD resistance, Chevron has a relatively high GPC and poor agronomic performance (de la Pena et al., 1999). Efforts have been made to introgress Chevron-derived KD

resistance, namely a major quantitative trait locus (QTL) on chromosome 6HS (Canci et al., 2004; Canci et al., 2003; de la Pena et al., 1999; Canci et al., 2001) into improved malting quality backgrounds. MNBrite is an example of such an effort (Rasmusson et al., 1999), which does have improved KD resistance, although its high GPC renders MNBrite unsuitable for its use in the malting and brewing industries.

Observed phenotypic trait correlations can be due to genetic linkage or pleiotropy. While linkage is breakable upon recombination events, pleiotropy cannot be dissipated through repeated cycles of meiosis (Bernardo, 2010b). Linked traits have important implications in breeding, whereas pleiotropic traits suggest alternative breeding approaches, which focus on other loci that control the traits, are needed.

Parallel to the idea of genetic correlation are coincident QTLs. Given that a quantitative trait maps to several QTLs and each QTL typically maps to intervals as large as 20 cM (Paterson et al., 1988), it is not unusual to observe that the QTLs for different quantitative traits map to a coincident region in the genome.

This study investigated the effectiveness of using the near-isogenic lines (NILs) (Paterson et al., 1990) to elucidate the association of multiple traits coincidentally mapped to the introgression region from Chevron on chromosome 6H into an elite malting background. A series of NILs are generated in a common genetic background except at a target QTL region where the altered phenotype of each NIL is associated with the loss of a specific chromosomal region inside the previously known QTL to narrow down the causal QTL region (Paterson et al., 1990). Firstly, a NIL is generated by backcrossing a donor parent to a recurrent parent while selecting for the donor allele at a

particular QTL. Then a population of recombinant near-isogenic lines (rNILs) is created with differential, overlapping introgressions at the target QTL region by crossing the backcross-derived NIL to the recurrent parent. Lastly, these NILs are self-pollinated for one or more generations for seed increase. The expectations are that a large enough population should generate enough recombination events where QTLs with desirable alleles are separated from QTLs with undesirable alleles, and that advanced DNA sequencing platforms should give dense enough marker coverage to visualize those recombination events.

This fine mapping approach has been used in many plant species to distinguish between linkage and pleiotropy. In barley, Gao et al. (2004) used this method and fine mapped a malting quality trait QTL reducing the interval size from a 30-cM interval to a 2.1-cM interval. Nduulu et al. (2007) used this approach and fine mapped coincident QTL in a region on chromosome 2H for FHB and heading date, suggesting their likely relationship is linkage. They narrowed down the previously 34-cM coincident QTL to an 8-cM QTL for heading date and a 6-cM QTL for FHB.

The rest of this chapter high-lights the significance of studying a multitude of traits of barley that have been reported to associate with the introgression region from Chevron on chromosome 6H.

Fusarium head blight and kernel discoloration

Fusarium head blight (FHB) or scab, caused primarily by the fungus *Fusarium graminearum* (*Gibberella zeae*), is an important cereal disease in temperate regions. Barley spikes can be infected exhibiting dark lesions after the emergence of spikes from

the flag leaves. Infections are favored under moist and warm conditions (Paulitz & Steffenson, 2011). FHB adversely affects barley yield and malting quality. The fungus also produces mycotoxins including deoxynivalenol (DON) which is associated with "gushing" or over-foaming of beer (Paulitz & Steffenson, 2011) and *Fusarium* infections contribute to off-flavors. There are stringent requirements on the DON level in grain destined for malting and brewing. A famous example of the devastating economic and social effects of FHB was the 1993 outbreak in northern Great Plains (Windels, 2000).

Strategies to manage FHB have variable effectiveness. Because *Fusairum* species survive as saprophytes on crop residues, FHB can be managed by cultural practices such as plowing, crop rotation, and avoid excess nitrogen applications to limit lodging. These practices should also reduce the risk for mycotoxin contamination (Nakajima et al., 2008; Steffenson, 2003). FHB can also be partially managed by fungicides, but the adverse environmental effects and development of resistance to fungicides are of concern. Genetic resistance has been an active area of research that complements the above management strategies.

Many genomic regions have been identified for FHB resistance in barley but only a few of them were detected in multiple trials (Massman et al., 2011). One of the QTLs detected by Canci et al. (2004) and Ma et al. (2000) using Chevron-derived bi-parental mapping populations corresponds to the 18 – 54 cM region of chromosome 6H based on the Morex POPSEQ genetic map (Mascher et al., 2013; see Chapter 2 - Materials and Methods for how to the positions were determined).

There are several challenges in mapping the genetic resistance loci to FHB. First, FHB severity is a quantitative trait that is difficult to measure accurately and is often associated with high QTL × environment interaction (Canci et al., 2004; de la Pena et al., 1999; Mesfin et al., 2003). To minimize QTL × environment interaction, multiple-environment screening is recommended. To accurately measure the reaction to FHB, a large enough population, special nurseries and trained labor are required, which requires tremendous resources especially when multiple environments are screened. Additionally, DON accumulation and FHB severity might be controlled by separate loci (Lamb et al., 2009; Smith et al., 2004), so screening for both these traits is needed. Furthermore, the identification of QTL associated with FHB can be confounded by agronomic traits such as heading date and plant height that have been reported to be associated with FHB severity (Massman et al., 2011; Steffenson, 2003).

The NIL fine mapping approach is particularly suitable for studying traits like FHB. When the genetic background is fixed, variability at the introgression region allows researchers to study even small-effect QTLs. This means either fewer individuals are needed (suitable for multiple-environment screening) or a higher resolution can be achieved.

At the 6H locus identified for FHB, coincident QTL of kernel discoloration and heading date were detected (P C Canci et al., 2004; Paulo C Canci et al., 2003; de la Pena et al., 1999). Kernel discoloration (KD) is caused primarily by *Bipolaris sorokiniana,* although other fungi including *F. graminearum* and *Alternaria* spp. and some bacteria may contribute to this complex disease (Anderson & Banttari, 1976). KD results in black

discoloration of kernels. Like FHB, KD is also quantitatively inherited and its infection is favored under humid conditions. While KD can be referred to another disease measured by different methods (C. D. Li et al., 2003), symptoms of the disease discussed here were measured by visual assessment of discolored grain after harvest.

Net blotch

While there is spot-form net blotch with the symptom of elliptical lesions, the net blotch discussed here is net-form net blotch which has the symptom of classical reticulate pattern of necrosis within lesions. Net-form net blotch (hereafter referred to as NB) is caused by hemibiotrophic fungus *Pyrenophora teres* f. *teres* (Paulitz & Steffenson, 2011). *P. teres* mainly infects leaves, although infections still adversely affect kernels diminishing malt extract yield and kernel plumpness (Paulitz & Steffenson, 2011).

Effective and sustainable management methods for *P. teres* are yet to be explored. *P. teres* grows initially on living cells and later on dead tissues and can spread through conidia. Common management practices for NB include tillage, burning crop residue and crop rotation (Paulitz & Steffenson, 2011). NB can also spread through seeds and is favored under cool and moist conditions, therefore NB is also controlled by the use of fungicide-treated seeds. The effectiveness of these management methods varies across environments (Turkington et al., 2006). Foliar fungicides have been successful and routinely used, but fungal resistance has been reported which might limit the use of fungicide treatment as a long-term control measure (Campbell & Crous, 2002).

Host resistance to NB has been deployed. The seedling resistance (typically measured in greenhouse) and the adult plant resistance (measured in the field) seem to

have separate but overlapping genetic control. A region on chromosome 6H near bin 6 was mapped to contain both a major seedling resistance QTL (e.g. Qamar et al., 2008; Cakir et al., 2003; Ma et al., 2004; Richter et al., 1998) and a major adult plant resistance QTL (Cakir et al., 2003; Grewal etal., 2008; Steffenson et al., 1996). The seedling net blotch QTL detected by Ma et al. (2004) using a Chevron-derived population corresponds to position 62 cM on chromosome 6H of the Morex POPSEQ map (Mascher et al., 2013). The 6H seedling resistance QTL alone was reported to provide sufficient field resistance in western Canada and has been routinely selected for in breeding programs (Grewal et al., 2008; Richter et al., 1998). At the University of Minnesota, a genome-wide association mapping study on elite lines from the Barley Coordinated Agricultural Project (Waugh et al., 2009) also identified a seedling net blotch QTL on chromosome 6H (Adhikari, personal communication, 2016).

Grain protein concentration

While soluble protein is important for yeast growth and metabolism in brewing (Rasmusson, 1985), a relatively low (⩽13%) grain protein concentration (GPC) is desirable for malting barley (American Malting Barley Association, June, 2014). There is a negative relationship between GPC and malt extract, which was a priority trait for the six-row malting barley breeding program at the University of Minnesota (Rasmusson, 1985). Additionally, a high GPC leads to brown beer color (Maillard reaction) and increased chill haze formation (Rasmusson, 1985). Studies have identified QTL for GPC in the 38 – 54 cM region of chromosome 6H based on the Morex POPSEQ map (Canci et al., 2003; Mascher et al., 2013).

Yield of soluble extracts is an important trait because the purpose of malting is to convert insoluble grain into soluble malt (Rasmusson, 1985). In this process, endosperm breakdown occurs with cell wall and protein degradation, and starch granules are exposed to the enzymes (Swanston et al., 1995). The enzyme-hydrolyzed starch is then extracted by hot water to yield fermentable sugars. The presence of hordein (the major grain protein in barley) matrix either physically impedes the access of enzymes to the starch, or forms disulfide bonds leading to wort filtration problems (Janes & Skerritt, 1993; Slack et al., 1979). As a result, the steeping time of malting increases and erratic germination occurs because of a high GPC, ultimately reducing malt extract yield (Rasmusson, 1985).

Traditionally GPC is determined as a percentage of grain weight by the Kjeldahl procedure (Rasmusson, 1985). More recently, GPC has been determined using a variety of non-destructive methods such as near-infrared reflectance spectroscopy (NIRS). NIRS has proven to be efficient in measuring the amino acid or nitrogen concentration in multiple plant species including soybean seeds, tomato leaves and orange leaves (Menesatti et al., 2010; Pazdernik et al., 1995; Ulissi et al., 2011).

Studies in barley have detected major QTL on chromosome 6H for GPC. For example, in a bi-parental QTL study, a chromosome 6H microsatellite (SSR) marker *hvm74* explained 40% of the heritable variance for GPC (See et al., 2002). This marker is equivalent to 54 cM on the Morex POPSEQ map (Mascher et al., 2013). Whether the relationship between QTL for GPC and KD on 6H is the result of linkage or pleiotropy is unknown (Canci et al., 2003).

Senescence date, nitrogen remobilization, heading date, and yield

Varieties with high-GPC alleles at the 6H GPC QTL have accelerated whole-plant senescence (Distelfeld et al., 2014). With the Karl-derived low-GPC allele at the 6H region, plants have delayed senescence (Heidlebaugh et al., 2008; Jukanti et al., 2008; Jukanti & Fischer, 2008; Mickelson et al., 2003). Understanding senescence is critical in the understanding of plant productivity, vegetative traits, seed quality traits, biotic and abiotic defense mechanisms (Distelfeld et al., 2014). Accompanying whole-plant leaf senescence is nutrient remobilization to developing grains with organs closest to the grains senesce last such as flag leaves (Distelfeld et al., 2014). Leaf protein degradation plays an important part in senescence (Prins et al., 2008). It is not known how protein degradation products are converted to transportable forms of nitrogen and the genes involved in amino acid metabolism await characterization and discovery (Distelfeld et al., 2014). It is also not known how amino acids are transported from senescing mesophyll cells to the apoplast, so that they can be loaded into phloem and finally delivered to the grains (Distelfeld et al., 2014).

Inferences can be drawn from this wheat *Gpc-B1/ TtNAM-B1* gene to improve our understanding of the orthologous barley 6H GPC QTL (Distelfeld et al., 2008). *Gpc-B1*, associated with high GPC on wheat chromosome 6B short arm, is suggested to have pleiotropic effects on accelerating flag-leaf senescence and nitrogen remobilization (Uauy et al., 2006), even though the effect of *Gpc-B1* on GPC and senescence can be variable for different isolines and environments (Carter, Santra, & Kidwell, 2012). In addition, a functional copy of *Gpc-B1* increases efflux of nitrogen and micronutrients,

especially Fe and Zn, from senescing vegetative tissues to developing grains (Uauy et al., 2006b; Waters et al., 2009). *Gpc-B1* was reported to be a NAC (petunia **N**AM and Arabidopsis **A**TAF1, ATAF2, and **C**UC2) transcription factor (Uauy et al., 2006a; Uauy et al., 2006b). Overexpression of a NAC transcription factor in rice enhances drought and salt tolerance in rice (Hu et al., 2006). Distelfeld et al. (2008) mapped the barley ortholog to a 0.7-cM interval within the 6H GPC QTL, designated *HvNAM-1*, and suggested that it is responsible for the 6H GPC QTL.

The existing literature on the effect of barley 6H locus on nitrogen remobilization is mixed. The presence of high GPC alleles at this locus leads to increased nitrogen remobilization using a Karl/Lewis NIL population (Heidlebaugh et al., 2008; Jukanti et al., 2008; Jukanti & Fischer, 2008). More specifically, Jukanti et al. (2008) and Jukanti & Fischer (2008) reported earlier leaf protein degradation and earlier hordein accumulation in seeds in high-GPC lines, suggesting an increase in phloem nitrogen translocation. They also found that in NILs with high-GPC alleles, many senescence- or N remobilization-related genes were considerably up-regulated. This agrees with previously published work that more than 70% of the grain protein comes from senescing vegetative tissues in crop species (Peoples & Dalling, 1988). Mickelson et al. (2003), also using the Karl/Lewis NIL population, did not detect QTL for nitrogen remobilization at the 6H coincident QTL region, although in half of the environments they detected QTL for leaf nitrogen acquisition and accumulation at this region. Furthermore, Yang et al. (2004) did not detect any protease QTL co-localized at the 6H region, but detected two leaf endopeptidase QTL in half of the environments with variable allelic effects of Karl. This

suggests the effect of the 6H coincident QTL on nitrogen remobilization is not fully understood.

Non-destructive methods are available for measurement of leaf nitrogen. Some examples are GreenSeeker, SPAD-502 chlorophyll meter, and other more expensive methods like visible-NIR (can be more accurate than SPAD), Spectroradiometer, Dual fiber-optic Spectrometer S2000. For example, GreenSeeker© (2012, Trimble Navigation Limited) emits bursts of infrared and red light and measures the ratio of infrared to red light reflected back. The expectations are that chlorophyll in green plants absorbs most of the red light and reflects most of the infrared light, and the greener the plant, the higher the ratio of infrared to red light reflected back.

The existing literature on barley 6H region on heading date is also mixed, and further investigation is needed. The Karl allele at the 6H locus was reported to accelerate flowering in greenhouse settings (Jukanti & Fischer, 2008; Lacerenza et al., 2010), but not in field settings. It could be that water was more limiting in the fields (Jukanti & Fischer, 2008). See et al. (2002) using the Karl/Lewis population did not detect QTL for heading date at the 6H coincident QTL region, and there are inconsistent reported effects of the Chevron allele on heading date (Canci et al., 2004). Heading date, using Chevron-derived population, mapped to 17 – 54 cM on the Morex POPSEQ map (Canci et al., 2004; Mascher et al., 2013).

Another trait that has a debatable connection to the 6H coincident region is yield (reviewed by Distelfeld et al., 2014). Using the Karl/Lewis mapping populations, one study has detected grain yield QTL at the 6H coincident QTL region (Mickelson et al.,

2003), while another study has not (See et al., 2002). More specifically, Jukanti & Fischer (2008) suggested that earlier leaf protein degradation might reduce carbon assimilation, explaining the negative correlation of GPC and yield reported in many studies (e.g. Simmonds, 1995). Additionally, it is observed in wheat (Uauy et al., 2006b) and barley, low GPC lines tend to have larger grains, which is a major determinant of yield. Another study, however, did not find significant differences in yield among NILs with different GPC allele classes at this 6H region (Falcon, personal communication, 2016). Similar studies in wheat did not find differences in grain yield between NILs with different *Gpc-B1* alleles (Brevis et al., 2010; Carter et al., 2012), although Carter et al. (2012) detected significant differences in grain weight.

Despite the inconsistent findings, Distelfeld et al. (2014) gave two hypotheses with regard to the negative correlation between GPC and yield which might help tie all the other traits together at the 6H locus. The first hypothesis was that delayed senescing plants with larger grains (prolonged grain fill) led to a diluted GPC and mineral contents. But Uauy et al. (2006) suggested that instead of a result of a dilution effect, decreased GPC, Fe and Zn concentrations were results of reduced translocation from leaves. The second hypothesis was that the synthesis of storage proteins costs more carbon per unit mass than that of starch synthesis, a critical determinant of yield. The second hypothesis suggests there was a penalty of nitrogen uptake, assimilation and transport. So that when a plant directs more of its energy to protein synthesis, less will be directed towards starch synthesis, and vice versa.

Sequencing technologies

Genotype-by-sequencing (GBS) is a promising time- and cost-efficient next-generation sequencing tool that provides single nucleotide polymorphisms (SNPs) with multiplexing and genome complexity reduction. Next-generation sequencing has recently emerged, and is used to discover large numbers of SNPs. SNPs are useful for visualization of recombination events in the case of fine mapping because they are the most abundant and stable forms of genetic variation in most genomes, and they are cheaper than many conventional markers. Using GBS, a sample containing restriction-digested DNA from an experimental line with a ligated adaptor that has a unique barcode, undergoes polymerase chain reaction and is sequenced simultaneously with other samples (up to 96 samples per channel and up to seven channels per flow cell; Elshire et al., 2011). With appropriate restriction enzymes, repetitive regions can be avoided and low copy regions can be targeted, which is helpful for large-genome species like barley (Elshire et al., 2011). Poland et al., (2012) modified the original single-enzyme approach and used two restriction enzymes for a more uniform and suitable complexity reduction. GBS is especially suitable for barley which has a published yet incomplete physical reference genome that reference is only needed around the restriction sites (Elshire et al., 2011).

The TASSEL (trait analysis by association, evolution and linkage) pipeline can be implemented to call SNPs from raw GBS sequencing data for species with a reference genome or incomplete genome assemblies of contigs (Glaubitz et al., 2014). Sequence tags in raw fastq files are collapsed into a master list of unique tags that have been

observed a certain number of times. Each tag is then aligned to the reference genome and alignment tools such as MAQ (Li et al., 2008), Burrows–Wheeler Transform (BWT) (Burrows and Wheeler, 1994), and the Burrows Wheeler Alignment tool (Li & Durbin, 2009), which is efficient in utilizing alignment of reads to collapsed repeats only once (Li & Durbin, 2009). These tools can have drawbacks as GBS gives large amounts of missing data when sequenced at a low coverage (Glaubitz et al., 2014).

The coincident QTL on barley chromosome 6H represents a typical question in breeding for quantitative traits. In a more general context, it is usually observed that there is yield penalty of disease resistance (e.g. Ortelli et al., 1996). As Brown (2002) put it, it is only worthwhile for a breeder to analyze and try to break such a linkage when the yield penalty in the absence of the target pathogen is not commercially significant.

Chapter 2 Fine mapping coincident QTL on a chromosome 6H introgression region from unadapted germplasm into elite malting barley

INTRODUCTION

Breeders strive to develop cultivars that are improved for multiple traits. Unadapted germplasm has frequently been exploited as donors of favorable traits. However, undesirable characteristics can often be introgressed along with the targeted trait, which is often referred to as linkage drag. It is not uncommon to observe yield depression upon introgression of disease resistance genes, for example leaf rust resistance gene *Lr9* in wheat (Ortelli et al., 1996). This observed unfavorable correlation can be due to genetic linkage or pleiotropy. The basis of genetic correlation has important implications in breeding: trait associations due to linkage can be bred out separately while associations due to pleiotropy cannot be dissipated through repeated cycles of meiosis (Bernardo, 2010b). Resolving which mechanism accounts for the observed trait association can be accomplished through high-resolution QTL mapping.

High-resolution mapping or fine mapping, that is narrowing the causal region for a particular trait or traits, is often achieved through creating a bi-parental population of near-isogenic lines (NILs) and associating the altered phenotype with the loss of a specific chromosomal region within a known QTL region (Paterson et al., 1990). This fine mapping method offers two attractive features. First, genetic effects on phenoytpes can be attributed to a specific region with little to no effect of genetic background. Second, higher resolution mapping is permitted through generating large numbers of recombination events within a specific region, as compared to the whole chromosome.

This fine mapping approach has been used successfully to narrow the QTL for fruit mass and plant yield to 3 cM on chromosome 1 in tomato (Paterson et al., 1990) and a major QTL for *Fusarium* head blight resistance (type II resistance for spreading in the head) to 1.2 cM in wheat (Liu et al., 2006). In the case of coincident traits, this fine mapping approach can be used to recover recombination events between linked genes and distinguish between linkage and pleiotropy.

The resolution of fine mapping replies primarily on the ability to distinguish overlapping chromosomal segments (Paterson et al., 1990), that is, through increasing the number of informative recombination events or the density of markers covering the region of interest. Given the resource requirements to create large populations and the limitations in marker technologies (e.g. a typical QTL region extends for 20 cM or more (Paterson et al., 1988)), finding recombinant lines at a particular coincident QTL with favorable trait combinations has been challenging. Recent fine mapping studies typically use 650 to 1150 progeny NILs (Haggard et al., 2013; Johnson et al., 2012; Nduulu et al., 2007).

Improved SNP technologies, combined with selective phenotyping, offer an attractive approach to conduct fine mapping. For example, Trick et al. (2012) used next generation sequencing to aid in fine mapping and identified causal polymorphisms within a ~30 cM region containing the grain protein concentration *Gpc-B1* gene in wheat. With decreasing costs of high-throughput SNP technologies, it is advantageous to genotype all individuals in the mapping population while phenotyping only selected individuals with

the largest number of recombination events (Jannink, 2005). It can conserve resources and still yield useful results.

Breeding efforts have been made to develop disease resistant, high quality, and high yielding malting barley (*Hordeum vulgare* L.) varieties, but progress has been limited due to unfavorable associations among these traits. Barley, a self-fertile, diploid species, has been used as a model for breeding and genetics studies. In the Midwest of the United States, spring six-row barley has traditionally been used for malting. From 30% to 40% of the U.S. barley production is used for malting (Michigan State University Extension, July, 2014). Chevron (PI 38061), a landrace introduced from Switzerland, has been used as a source of kernel discoloration (KD; caused primarily by *Bipolaris sorokiniana*) resistance in six-row malting barley and is moderately resistant to *Fusarium* head blight (FHB). The presence of FHB and the mycotoxin produced by *Fusarium graminearum*, primarily deoxynivalenol (DON), result in rejection of the grain to be used in malting (Anderson & Banttari, 1976; Paulitz & Steffenson, 2011). However, Chevron has poor agronomic traits and its grain protein concentration (GPC) is much higher than desirable for malting and brewing (de la Pena et al., 1999). A high GPC can physically impede the access of enzymes to the starch, lowering malt extract yields, and forms disulfide bonds leading to wort filtration problems (Slack et al., 1979; Janes & Skerritt, 1993). Thus, Chevron though a source of resistance to KD and FHB has many undesirable agronomic and malting quality traits. The cultivar MNBrite (PI 603050) was developed using a backcrossing strategy selecting for bright colored kernels indicative of the KD resistance derived from Chevron (Rasmusson et al., 1999). Even though it

exhibited good KD resistance, MNBrite also exhibited high GPC, which is why it was not widely accepted by the malting and brewing industry.

Genetic studies with Chevron and Chevron-derived populations showed that large effect QTL for KD, FHB, and GPC mapped similarly to the centromeric region of chromosome 6H (Canci et al., 2003; de la Pena et al., 1999), which includes the *HvNAM-1*/*Gpc-1* gene. *Gpc-B1* in wheat, which influences GPC, is reported to have pleiotropic effects on the senescence date of flag leaves, nitrogen remobilization, and grain size (Uauy et al., 2006). *Gpc-B1* was suggested to be orthologous to *Gpc-1* in barley (Distelfeld et al., 2008). In addition to QTL for KD score, FHB severity, and GPC, QTL for DON accumulation, net-form net blotch (NB) caused by *Pyrenophora teres* f. *teres* (at both seedling and adult plant stages), plant height, heading date, yield, leaf nitrogen at maturity and soluble organic nitrogen at mid-grain fill have been mapped to the same region of barley chromosome 6H (Cakir et al., 2003; Canci et al., 2004; Grewal et al., 2008; Ma et al., 2000; Mickelson et al., 2003; Steffenson et al., 1996). Jukanti et al. (2008), investigating transcriptomes of barley flag leaves and kernels, identified several genes with known and unknown functions with senescence process in NILs differing for the GPC QTL on chromosome 6H. Further evaluations of NILs for the 6H coincident QTL region showed segregation for bacterial leaf streak (BLS) severity and percent stem breakage, suggesting that these traits might also be associated with this region. Table 1 is a summary of seven traits identified using Chevron that mapped to a region extending from 20 cM to 70 cM on chromosome 6H based on the Morex POPSEQ map (see Materials and Methods). In addition, mutant studies have identified loci associated with

lodging and several morphological traits near the centromere of chromosome 6H (Druka et al., 2011; Skov Kristensen et al., 2016).

This study utilized a population of NILs with differential introgressions in the centromeric region on chromosome 6H of Chevron into the malting cultivar Lacey to fine map the fourteen traits potentially associated with this region. The objectives of this study were to: 1) confirm which of the fourteen traits are associated with the centromeric region of chromosome 6H; 2) fine map each of these traits within the 6H region; 3) illuminate relationships among these traits with respect to linkage or pleiotropy; and 4) discover recombinant lines that separate linked genes (e.g. if associated through linkage, it would be possible to improve FHB and KD resistance of Lacey while maintain its GPC and good agronomic traits).

MATERIALS AND METHODS

Parents and the mapping population

A backcrossing and marker-assisted selection scheme was implemented to generate the recombinant near-isogenic lines (rNILs) used in this study (Figure 1). Previously, Chevron was crossed to an elite breeding line M69 to map QTL for FHB, DON, KD, heading date and height (de la Pena et al., 1999). A moderately FHB- and KD-resistant progeny line (MN92-299) was crossed to an elite FHB- and KD-susceptible line (M81), and several of the previously identified QTL were validated (Canci et al., 2004). A progeny line from this validation population, FB11-113, carrying a Chevron introgression spanning the 6H QTL region was crossed and then backcrossed to the cultivar Lacey five times. Throughout these generations of backcrossing, marker-assisted

selection (MAS) was imposed to select for the Chevron allele which spans the 6H QTL interval using five simple sequence repeat (SSR) markers (GBM1021, Bmag0807, Bmag0173, GBM1076 and EBmac0602). This resulted in the generation of the Gen2 near-isogenic lines.

Gen2-129, one Gen2 NIL, was crossed again with Lacey to create a fine mapping population consisting of 1941 F_2's. These F_2's were genotyped and 722 lines that had recombination events among SSR markers, EBmac0602, Bmag0807 and GBM1215, were selected and advanced to the F_3 generation via single-seed descent. These F_3's were genotyped and 249 lines having recombination events among markers, EBmac0602, GBM1063 and GBM1027, were selected and advanced to the F_4 generation via single-seed descent. Seeds harvested from these 249 F_4 rNILs were planted in the greenhouse in 2014 for genotyping and the selfed seeds were sent to New Zealand in 2015 for seed increase. Selective phenotyping was conducted on 101 rNILs returned from New Zealand. This population, derived from 249 F_4 rNILs which trace back to 1941 F_2 NILs (including the non-recombinants), is referred to as Gen10.

To select the 101 rNILs from 249 rNILs that were planted in New Zealand, we conducted a preliminary mapping analysis which gave approximately thirty polymorphic markers spanning the 6H coincident region using the same genotype data as described in the next section except with SNPs called by UNEAK-TASSEL (Lu et al., 2013). Based on the genotypes at these SNP markers, the 249 rNILs were sorted into recombinant classes and redundant lines were removed within each class. This resulted in 101 rNILs

that captured as many recombination events around region of interest (a few lines with missing genotypes were included as potential recombinants).

Genotyping and SNP calling

Each single plant of the 249 $F_{4:5}$ rNILs (Figure 1) was genotyped by genotype-by-sequencing (GBS) at the USDA-ARS Small Grains Genotyping Center in Fargo, ND. Library preparation was done using two restriction enzymes (Poland et al., 2012). *PstI* (CTGCAG) was used as the rare cutting enzyme for complexity reduction in a large genome species like barley. The enzyme *MspI* (CCGG) was selected to recognize more common cutting sites. The sample barcode was attached to the *PstI* overhang and was used for subsequent SNP calling.

To call SNPs from raw fastq data, TASSEL-GBS (the trait analysis by association, evolution and linkage – genotype-by-sequencing) pipeline with default parameters (Glaubitz et al., 2014) was used. The Burrows Wheeler Alignment tool (Li & Durbin, 2009) was implemented to align reads to the Morex whole genome sequencing assembly (Mayer et al., 2012).

GBS did provide dense SNP marker coverage (3874 markers on chromosome 6H), but a lot of the markers were poor quality markers with missing genotypes (only 35 markers had 50% or less missing data among the 249 Gen10 lines and no missing data for the parents; Supplementary Table 1). To obtain a more complete set of SNP genotypes for the 249 Gen10 rNILs on chromosome 6H, two additional GBS runs were conducted on 38 $F_{4:5}$ rNILs and another 13 rNILs (7 $F_{4:5}$ rNILs and 6 $F_{4:8}$ rNILs). A customized R script was used to merge SNP data from these three GBS runs to fill in missing data on

chromosome 6H, so that redundant identical SNP genotypes and non-matching genotypes were considered missing genotypes, where unique genotypes and two out of three times matching genotypes were retained. See Supplementary Table 1 for how many marker each GBS run gave on chromosome 6H and how many high quality markers (i.e. 50% or less missing genotypes among Gen10 lines and no missing genotypes between parents) each GBS run provided. The merged dataset provided 6647 markers on chromosome 6H for 249 Gen10 lines, of which 1118 were high quality markers (Supplementary Figure 1), of which 348 were polymorphic. These 348 markers were expected to cover the region where 249 Gen10 rNILs differed and where recombination occurred (i.e. the introgression region from Chevron).

The SNP genotype of each Gen10 rNIL was then compared to that of Lacey and Chevron to determine whether it was a Chevron (Gen2-129) genotype, a Lacey genotype or a heterozygote. Monomorphic markers, markers with missing Lacey or Chevron genotype, as well as markers with more than 50% missing data of Gen10 rNILs were removed.

Phenotypic evaluations

The selected 101 Gen10 rNILs, as well as Gen2-129, Lacey, Chevron, Gen2-036 (another Gen2 NIL), KLBC4-130i-KK (a NIL with Karl allele at chromosome 6H centromeric region and background in Lacey) and corresponding resistant/susceptible checks were evaluated for 14 traits in four field trials in 2015 and in two field trials in 2016 using randomized complete block designs. $F_{4:7}$ rNILs (Figure 1) were evaluated in Yld (yield), FHB, BLS, and NB trials in 2015, while $F_{4:8}$ rNILs (Figure 1) were evaluated

in Yld and FHB trials in 2016. Yld, FHB, and BLS trials were conducted at Crookston and St. Paul, MN. The NB trials were conducted at Crookston, MN for field screening (adult plant resistance) and in fall and winter greenhouse trials in the Plant Growth Facility on the St. Paul campus of University of Minnesota for greenhouse screenings (seedling resistance). Percent stem breakage was recorded on the single-row plots of the 2015 New Zealand seed increase. A nomenclature with Trial_LocationYear was used to identify a specific environment a trait was measured in. For example, FHB_StP16 refers to the FHB trial at St. Paul in 2016. In FHB and NB trials, each line was planted in a 1.52 m X 0.31 m single-row plot with a Wintersteiger planter (model trm 2200; Salt Lake City, UT), while in Yld trials, each line was planted in a two-row plot and adjacent two-row plots were separated by a single-row plot of winter wheat (each two-row plot was 1.52 m X 0.61 m). BLS trials were planted in 0.31 m X 0.31 m dash plots. Prior to evaluations of GPC, DON and KD, threshed grain samples were cleaned with a Pfeuffer Rational Kornservice Sample cleaner (model SLN3/4).

Evaluations in Yld trials

Heading date, height, lodging, days to senescence, normalized difference vegetation index (NDVI), percent stem breakage, GPC, and yield were measured on the plot basis in the Yld trials in two replications. Heading date was recorded as the number of days after planting when 50% or more of the heads emerged half way from the boot. Height was measured after heading as the height from ground to the tip of the spike (not including awns) on representative plants in the middle of the plot. Lodging was visually scored as the percentage of the plants that bent over severely. Days to senescence was

estimated as the number of days after planting when 90% or more of the peduncles lost green color. A one-time measurement of normalized difference vegetation index (NDVI) was taken using a handheld GreenSeeker© (2012, Trimble Navigation Limited) when there was visible differences of senescence among the plots. Greener plants absorb most of the red light and reflect most of the infrared light and give a higher NDVI reading. Percent stem breakage was recorded just prior to harvest by visual assessment of the percentage of plants that had broken stems. Yield, in g two-row-plot^{-1}, was determined as the weight of harvested, threshed and cleaned seeds and was converted to kg ha^{-1}. Each two-row plot was harvested in Yld trials with Wintersteiger nurserymaster elite small plot combine (Salt Lake City, UT). Grain protein concentration (GPC) was assessed on a 110 ml-sample of cleaned grain using near-infrared reflectance spectroscopy (NIRS) (Perten diode array instrument DA7250 – 1201471; Perten Instrument Inc., Springfield, IL). The calibrated NIRS machine (Barley 20121120.cal) rotationally assessed the wavelength of each sample at every 5 nm from 900 to 1650 nm. GPC was adjusted, where dry matter GPC = raw GPC*100/(100-moisture) (personal communication, Killam, 2016).

Evaluations in FHB trials

In addition to heading date and height, DON accumulation, disease scores for FHB and KD were measured in FHB trials in three replications in 2015 and two replications in 2016. Quest was included in FHB trials as a resistant check. FHB trials were inoculated by two methods: 1) colonized corn inoculum spread on soil surface (grain spawn); 2) macroconidial suspension sprayed onto emerged heads as described by Dill-Macky et al. (2002). In St. Paul, when plants were headed in majority of the plots,

they were sprayed twice in a 3- (2016) or 4-day interval (2015) with a *F. graminearum* macroconidial suspension using a CO_2-powered backpack sprayer. The inoculum was a suspension of 1000 macroconidia/ml which was made from 39 MN isolates in 2015 (Supplementary Table 1a) and 30 MN isolates in 2016 (Supplementary Table 1b) (Dill-Macky Small Grains Pathology lab, University of Minnesota). Mist irrigation was initiated before the first inoculation date and maintained until FHB severity was recorded in these nurseries to facilitate disease development. Further details can be found in Canci et al. (2003). In Crookston, *Fusarium*-colonized corn inoculum was spread on soil surface at a rate of 112 kg ha^{-1} once (2016) or twice in a 9-day interval (2015) before heading using a fertilizer spreader. FHB severity was scored visually four weeks (CR) or two weeks (StP) later. The percent of infected kernels in ten randomly selected spikes per plot was estimated and an average percent severity for each plot was obtained (Dahleen et al., 2003).

KD and DON were measured on grain samples harvested from FHB nurseries after threshing and cleaning. Each single-row plot was harvested with hand sickles, and threshed using a Vogel bundle thresher. A sample of 25 g of cleaned seeds from each plot was measured for DON accumulation, in parts per million (ng μg^{-1}), by gas chromatography/mass spectrometry (Fuentes et al., 2005; Mirocha et al., 1998). KD severity was scored visually by placing each sample on a white paper plate under fluorescent lights using a 1-5 scale (Miles et al., 1987), referencing to a sample of Gen2-129, Lacey, Chevron, Gen2-036, KLBC4-130i-KK, and Quest in that environment. For example, Chevron with the brightest grains was used as the reference of score “1”, while

Lacey and KLBC4-130i-KK with the darkest grains was used as the reference of score "4" or "5" depending on the environment.

Evaluations in NB trials

For net blotch greenhouse screening for seedling resistance, a *Pyrenophora teres* f. *teres* isolate (30115003) collected in Minnesota in 2015 was used (Dill-Macky Small Grains Pathology Laboratory, University of Minnesota). The isolate was grown on V8 juice agar for 10-14 days under 12-h fluorescent lights at room temperature. After culturing, 10-20 ml sterile distilled water was pipetted onto each plate and the conidia were washed and scraped from the agar surface with the aid of an L-shaped glass rod. After filtering the resulting spore suspension through a cheese cloth, the concentration of the conidial suspension was determined and adjusted to 25,000 conidia ml^{-1}. One drop of polysorbate (Tween 20) was added to every 100 ml of inoculum.

Net blotch greenhouse screenings were established in three replications. Five to seven $F_{4:8}$ seeds were planted in 3.81 cm (diameter) X 21.0 cm (height) conetainers filled with Metro-Mix 200 soilless potting mix and grown in a greenhouse at 15-20°C and 18 h photoperiod (fertilized at eight days with Peters 20-20-20 at a rate of 1 tbsp per 200 conetainers). Stander was included as a susceptible check and BT462 was included as a resistant check. When the third leaf emerged, barley seedlings were sprayed till run-off with the inoculum suspension using a hand pump sprayer and were incubated at 100% relative humidity for 24 h. One week after inoculation, net blotch severity on leaves was assessed visually according to Tekauz (1985) using a 1-10 scale with 1 being most resistant and 10 being most susceptible.

For field screening of adult resistance to net blotch, *P. teres* f. *teres* isolate 30199012 (collected in Minnesota in 1999; Dill-Macky Small Grains Pathology Laboratory, University of Minnesota) inoculated seedlings (for methods, see above) of the susceptible barley cultivar, Stander, were used as inoculum in the field. Net blotch infected Stander was transplanted into the gap between plots every other row in the field. Gen10 rNILs, as well as susceptible checks (Robust and Quest) and resistant check (BT462), were at about Zadoks growth scale 2 (3-4 leaf stage) at the time of transplanting. Net blotch severity on leaves was evaluated according to the 0-9 scale (M. Smith Small Grain & Canola Pathology Laboratory, University of Minnesota, Crookston). Besides net blotch severity, NDVI was also taken in the NB_CR15 trial using GreenSeeker©.

Evaluations in BLS trials

Isolate CIX95, a virulent strain of *Xanthomonas translucens* pv. *translucens* (Ishimaru Laboratory, University of Minnesota), was used to inoculate both BLS field nurseries at St. Paul and Crookston. CIX95 was collected in Minnesota in naturally infected barley fields and stored in glycerol nutrient media at -80° C. Stocks were streaked out into single colonies on Wilbrink's Agar (Sands & Fourrest, 1989). After 5 days of incubation at 27 °C, each colony was streaked onto a new plate in a zigzag pattern using a sterile cotton swab, where each plate received 9 streaks. Plates were incubated at 27 °C for 3 days. Inoculum was prepared on-site. Each plate of inoculum was washed into 3 L of a 0.85% NaCl buffer to obtain a concentration of 10^8 cfu ml^{-1}. After 3 g carborundum was added per liter of inoculum (to help wound leaf tissues), a

hand-pump Solo sprayer was used to apply the inoculum to the plot, where every 4 plots received 2 passes of inoculum. The BLS-susceptible checks Stellar-ND and Tradition, and resistant checks Colon and BT462 were included in the BLS trials.

BLS severity on leaves was assessed visually on a whole plot basis using a 1-9 scale, when the plants in most plots were at the mid-dough stage though Chevron was 2-3 days behind (Steffenson Cereal Disease Laboratory, University of Minnesota).

ANOVA and correlations

Following Gomez & Gomez (1984), each trait was tested for suitability of combined-environment/location analysis of phenotypic data with the aid of R (Bell Laboratories, version 3.2.0): 1) analysis of variance (ANOVA) was conducted on single-environment data for each trait, testing for line (fixed) and replication (random) effects; 2) single-environment data was tested for homogeneity of error variance using Levene's test and coefficients of variation; 3) for traits that had a non-significant Levene's test or less than 20% coefficients of variation, combined-environment ANOVA was conducted testing for line (fixed), environment, line and environment interaction and replication within environment (random) effects. Entry mean heritabilities were calculated on single-environment trait data as $\Sigma^2_{ln}/((ln-1)/r*\sigma^2+\Sigma^2_{ln})$ where Σ^2_{ln} is the variance due to lines, ln is the number of lines, r is the number of replications and σ^2 is the variance due to error. Entry mean heritabilities were calculated on combined-environment trait data that passed the Gomez & Gomez (1984) test as $\Sigma^2_{ln}/((ln-1)/(r*l)*\sigma^2+((ln-1)/l*\sigma^2_{l*ln}+\Sigma^2_{ln})$ where σ^2_{l*ln} is the variance due to environment and line interaction and l is the number of environments. Pair-wise individual environment trait data of 101 Gen10 rNILs was

calculated for Pearson correlations using the function “rcorr” in the package “Hmisc” in R.

Linkage map construction and QTL mapping

To construct a genetic map, genotypic data of the 249 rNILs for the 348 polymorphic SNP markers were input into JoinMap®4 as recombinant inbred lines (x=4 for F4s) using the regression mapping algorithm. This algorithm added loci one by one, and goodness-of-fit was calculated for each locus to determine the best position. Markers not mapped to the same linkage group were excluded.

To provide robustness of our QTL results, we evaluated agreement of the linkage map created in the current study to the Morex POPSEQ genetic map (Mascher et al., 2013) and to the most current physical reference of barley (personal communication, N. Stein; IPK Gatersleben). The genetic positions based on the linkage map of the 249 F_4-derived recombinant NILs and parents at the 6H region were calculated into genetic positions based on the 1941 F_2 NILs, assuming the 249 rNILs were the only recombinant lines that were in the F_2 generation. Using the Morex contig name accompanying each GBS SNP marker, we determined its corresponding position on the Morex POPSEQ genetic map. Using the Morex contig name and the base pair position of each SNP on the corresponding contig, we blasted the ± 50 bp sequence surrounding the SNP on the contig to the most current IPK physical reference to obtain a base pair position of each GBS SNP marker. To visualize the relative position of the introgression region to the centromere, BOPA (barley Illumina’s oligo pool assay) SNP markers flanking the 6H

centromeric region (Muñoz-Amatriaín et al., 2011) were blasted to the most current IPK physical reference.

To allow standard comparisons of QTL detected in this study to prior studies, the primer sequences of markers flanking previously reported QTL were blasted to the Morex POPSEQ map (Mascher et al., 2013; IPK BLAST Server with "WGS_Morex_assembly"). Simple sequence repeat (SSR) and restriction length polymorphism (RFLP) primer sequences were obtained from GrainGenes, while BOPA marker sequences were obtained from the The Barley Triticeae Toolbox (https://triticeaetoolbox.org/barley/). For cases with limited marker information, comparisons were made through referencing closely linked markers on the barley bin map (http://barleygenomics.wsu.edu/all-chr.pdf).

To detect QTL, genotypic data with linkage positions of 345 GBS SNP markers that mapped to the same linkage group and phenotypic data for fourteen traits of 101 Gen10 rNILs, together with Lacey and Gen2-129, were input into QTLCartographer Version 2.5_011 (Wang et al., 2012). Composite interval mapping (standard model) was performed using 1-cM walk speed, 500 permutation times, and a 0.05 significance level to determine a LOD significance threshold. A backward regression method was implemented to select five control marker numbers (i.e. cofactors) outside of a 10.0-cM window size of a putative QTL. Gen10 rNILs were input as a population of recombinant inbred lines derived through selfing. A peak exceeding the LOD threshold was considered significant. Dropping the peak LOD score by 1 determined the start and end

positions of the QTL. We defined peaks within 1.4 cM as a single QTL except when these peaks had opposite allele effects.

RESULTS

Phenotypic performance and variation among parents and rNILs

Six of the fourteen traits measured exhibited no significant difference between parents but did exhibit significant variation among progeny rNILs (i.e. transgressive segregation). We observed no significant difference (t-test with unequal variance at 0.05 level) among the parents for FHB severity, GPC, days to senescence, heading date, seedling net blotch and lodging in any environment tested (Table 2). In contrast, significant variation (line effect in ANOVA at 0.05 level) among Gen10 rNILs was observed for all of the traits in at least one of the environments except for BLS severity (Table 2). Specifically, significant variation was observed among rNILs in all environments tested for FHB severity, GPC, days to senescence, NDVI, percent stem breakage, heading date, net blotch (seedling and adult) and KD score. In other words, traits that exhibited transgressive segregation included FHB severity, GPC, days to senescence, NDVI (except environment in NB_CR15), heading date, and seedling net blotch. For performance of individual Gen10 rNILs and checks, see Supplementary Table 2.

Entry mean heritability calculated for each trait in an environment ranged from 0.05 to 0.86. Lodging, FHB severity, BLS severity, heading date, height, yield, and DON accumulation tended to have lower than 0.5 heritability (for more than half of the environments tested). Heritabilities were higher than 0.5 (for more than half of the

environments tested) for days to senescence, NDVI, NB severity (both seedling and adult stage), percent stem breakage and GPC. This latter group of traits, except GPC, were also combined across environments for analysis and their combined heritabililties were greater than 0.60.

A high-resolution genetic map of the chromosome 6H region

Additional genotyping and merging of the three sets of SNP calls resulted in 1118 markers that had 50% or less missing genotype data of 249 Gen10 rNILs and no missing genotype data of Lacey and Gen2-129 (Figure 2), of which 348 markers were polymorphic. Of these 348 markers, 345 mapped to a single linkage group covering a 37.2-cM region of chromosome 6H (hereafter referred to as the Gen10 genetic map) which corresponds to a 44.1-cM region on the Morex POPSEQ map (Mascher et al., 2013), and a 504-Mbp region on the most current physical reference map from IPK (personal communication, N. Stein; Figure 3). There was a linear correspondence between the Gen10 and Morex POPSEQ linkage maps (Figure 3). The Gen10 genetic map corresponded to the physical reference map in a similar fashion as did the Morex POPSEQ map to the physical reference map (Figure 3). Comparisons of the Gen10 genetic map to the physical reference map revealed an area of low recombination from 16.9 – 26.1 cM or 108 – 383 Mbp (Figure 3), which included the centromere of 6H (Figure 2). This 9.16 cM area, which accounted for 24.6% of the Gen10 genetic map at the 6H region, extends for about 275 Mbp or 52.0% of the physical reference map at the 6H region. Marker density was much lower in this region of low recombination. Genotypes for individual Gen10 rNILs are included in Supplementary Table 4.

QTL mapping

Within the 37.2 cM region of chromosome 6H based on the Gen10 genetic map, we detected 34 QTL for eleven out of fourteen traits explaining 3.19 – 37.8% of the phenotypic variation (Figure 4; Table 3). We did not detect any QTL for BLS severity, height, and KD score, while we detected QTL at every environment for days to senescence, adult net blotch severity, and GPC. For all of the eleven traits that we detected QTL except DON accumulation, we detected multiple QTL with allelic effects in the opposite direction (Figure 3). The number of QTL detected for each of the ten traits ranged from two (GPC and yield) to six (heading date). It is important to keep in mind that all subsequent QTL results are based on the Gen10 genetic map, which is in the reverse direction of the physical or POPSEQ reference maps (see Supplementary Figure 2 for QTL peak markers based on the most current IPK physical reference).

Across the 37.2 cM region, there were coincident QTL and singleton QTL. The coincident QTL at around position 30 cM are of special interest because seven traits mapped to this position in at least one environment. The remaining section is organized by traits that mapped coincidentally to the 30 cM position and traits that did not map to the 30 cM position. The former group is further divided into traits that mapped coincidentally in addition to the 30 cM position and traits that mapped only to the 30 cM position. It is important to keep in mind that the number of environments in which each QTL was detected ranged from one to three. A QTL detected in two or three environments presumably is associated with more confidence than a QTL detected only in a single environment.

Traits that mapped coincidentally at around 30 cM are NDVI (NDVI QTL #3), days to senescence (Senesce QTL #2), percent stem breakage (StemBrk QTL #3), GPC (GPC QTL #2), heading date (HD QTL #6), adult net blotch severity (Adult NB QTL #3) and seedling net blotch severity (Seedling NB QTL #3) (Figure 3). In addition, 'just-below-the-threshold' QTL for KD score and yield resided within this region (data not shown). A FHB severity QTL also mapped very near this region. NDVI QTL #3, Senesce QTL #2, StemBrk QTL #3 and GPC QTL #2 were all detected in two environments. This ~30 cM position corresponds to ~49 to 50 cM on the Morex POPSEQ map and ~45 to 51 Mbp on the IPK physical reference map.

Days to senescence and NDVI, as well as GPC and percent stem breakage mapped to coincident QTL in addition to the ~30 cM region. There were two additional coincident regions for days to senescence and NDVI, 27 - 28 cM and 36 cM. NDVI QTL at 36 cM (NDVI QTL #4) was detected in two environments. There was one additional coincident region for percent stem breakage and for GPC, which overlapped with that of days to senescence and NDVI at 27 – 28 cM. Percent stem breakage and NDVI (NDVI_NB_CR15) further mapped to separate regions at 8.2 – 8.4 cM (StemBrk QTL #1) and 19.7 – 22.6 cM (NDVI QTL #1) respectively. In other words, days to senescence (Senesce QTL #1), NDVI (NDVI QTL #2), GPC (GPC QTL #1), and percent stem breakage (StemBrk QTL #2) mapped coincidentally at ~30 cM position and at 27 – 28 cM, where each of these traits showed opposite allele effects to the ~30 cM position.

Only to the ~30 cM position, did seedling and adult net blotch map coincidentally. Seedling and adult net blotch each mapped additionally to two separate

regions. In fact, seedling and adult net blotch mapped to an additional coincident QTL at 4.5 cM but they had opposite allelic effects. The trait correlation between seedling and adult net blotch were weak (Supplementary Table 3). The other seedling net blotch QTL (Seedling NB QTL #2) mapped coincidentally with NDVI QTL #1, where Gen2-129 had positive allele effect on seedling net blotch and negative allele effect on NDVI.

QTL for FHB, heading date, lodging, yield, and DON were detected mostly only in one environment. Heading date and FHB mapped to or close to 30 cM, while lodging, yield, and DON did not. DON mapped to different regions than FHB, and FHB mapped to different regions than heading date except they slightly overlapped at 25.5 – 26.5 cM. There was no QTL that the Chevron allele consistently delayed heading date and lowered FHB severity.

DISCUSSION

Development of NILs at the centromeric region of chromosome 6H was initiated to understand the relationship between FHB and KD resistance and GPC in an effort to breed for six-row spring malting barley with improved disease resistance derived from Chevron while maintaining a suitable GPC (Canci et al., 2004; Canci et al., 2003; de la Pena et al., 1999). Firstly, we discovered that the QTL of several other traits are associated with this introgression region and even more interestingly that multiple QTL associated with each trait often had opposite allelic effects. This finding might have explained the phenotypic transgressive segregation observed for six of the ten traits that we detected multiple QTL with opposite allelic effects. Secondly, inferences about the genetic basis of associations of several traits can be drawn. Unfavorable trait associations

due to linkage can be bred out through recovering and selecting for the lines with favorable recombination events. Unfavorable trait associations due to pleiotropy would require alternative breeding approaches that strive to improve the deficient trait through introgressing and selecting for alleles at other loci. The pleiotropic discoveries nevertheless present the opportunity of promoting our understanding of biological basis of the associated traits. Thirdly, the complex genetic architecture at this introgression region can be hard to interpret and the fine mapping approach using NILs might have limitations for some complex traits. Lastly, there are important implications in breeding for traits that were associated through linkage where rare recombinant lines can be recovered. Confidence in the QTL detected will increase the confidence in breeding decisions.

QTL fractionation with opposite allelic effects

Some of the traits previously reported as single QTL at this region appear to fractionate into two or more QTL with opposite allelic effects in this study. The QTL fractionation phenomenon is not new (Chen & Tanksley, 2004; Edwards & Mackay, 2009; Haggard et al., 2013; Johnson et al., 2012; Lecomte et al., 2004; Studer & Doebley, 2011). Haggard et al. (2013) not only found fractionation of QTL, but also observed the opposite allelic effects of fractionated QTL as observed in this study. It is worth mentioning that Haggard et al. (2013) used composite interval mapping with a narrow (2-cM) window size, given they used 652 NILs (recombinant and non-recombinant) to construct the linkage map. In this study, the equivalent window size is 1.28 cM (after the 10-cM window size of the linkage map constructed by 249 recombinant lines was

calculated back to that of 1941 Gen10 lines). A narrow window size was chosen, assuming there were closely linked QTL in the introgression region. To confirm our results, we experimented with 20-cM (equivalent to 2.57-cM using genetic map of 1941 Gen10 lines) window size and 100-cM (equivalent to 12.8-cM using genetic map of 1941 Gen10 lines) window size (data not shown). The 2.57-cM window size CIM gave very similar as did the 1.28-cM CIM; the 12.8-cM window size CIM detected the exact same QTL as did interval mapping and a few additional QTL. QTL detected by the 12.8-cM window-size CIM, still, showed multiple QTL with opposite allelic effects for stem breakage, heading date, and seedling net blotch.

Closely linked QTL with opposite allelic effects could explain why we did not observe significant differences between the parents, but did observe significant variation among progeny rNILs for FHB severity, GPC, days to senescence, NDVI, heading date, and seedling net blotch. FHB severity and GPC had equal numbers of QTL with opposite allelic effects which suggest the number of QTL with opposite allelic effects might have an effect on the phenotypic outcome. However, days to senescence, NDVI, heading date and seedling net blotch with unequal number of opposite-allelic-effect QTL also exhibited transgressive segregation. The magnitude of the allele effect and the consistency of the QTL detected across environments are likely to also play a role in the expression of transgressive segregation.

Nowadays as the physical reference becomes more and more complete, we have the advantage of confirming the QTL results against the physical reference map. HD QTL #2, #3, and #4 mapped to the similar position, 390 Mbp, on the most current

physical reference map (Supplementary Figure 2). It remains to be investigated for why this was the case and further genotyping validation might be useful. This, nevertheless, was the only case where multiple QTL with opposite allelic effects on Gen10 genetic mapped to the same position on physical reference map.

Pleiotropy or linkage?

Our fine mapping study revealed a more complex genetic relationship for eleven traits associated with the 37.2-cM introgression of chromosome 6H from Chevron into Lacey. Some of the relationships are likely linkage such as between DON accumulation and GPC and between FHB severity and DON accumulation. Other relationships are likely pleiotropy such as among days to senescence, NDVI, percent stem breakage and GPC, which might have physiological significance concerning the *NAM-1/Gpc-1* gene.

The relationship between DON accumulation and GPC is likely linkage. Prior studies have shown that QTL for DON accumulation and for GPC mapped to similar region on chromosome 6H (Canci et al., 2003; Ma et al., 2000). Using the Gen10 population, we observed separate QTL for DON accumulation and for GPC. This suggests that it should be possible to reduce DON levels without increasing GPC (discussed in the next section), which was not attained in the development of MNBrite.

The association between FHB severity and DON accumulation is also likely due to linkage. It is reasonable to expect that FHB severity and DON accumulation are controlled by the same gene since *F. graminearum* infection is expected to result in toxin production by the pathogen. In this study, we resolved these two traits to be separated by at least 0.97 cM at the introgression region on chromosome 6H. In agreement with this

result, Lamb et al. (2009) observed a moderately FHB-resistant line having QTL for improved FHB resistance but not for reduced DON accumulation. Smith et al. (2004) also observed NILs for a DON QTL on chromosome 3H differed significantly for DON accumulation but were similar for FHB severity.

The mechanism for how resistant alleles lower DON accumulation is not known. Smith et al. (2004) hypothesized three mechanisms. Resistant alleles might be involved in degrading DON, eliminating fungal growth after initial infection, or lowering DON production level. A more recent study measured differential expression between Lacey and one Gen2 resistant NIL and found an earlier induction of defense genes in the resistant NIL that were involved in DON detoxification and cell wall fortification (Huang et al., 2016), supporting the first and second proposed mechanisms.

The relationships among GPC, percent stem breakage, days to senescence and NDVI are likely pleiotropy, given their high degree of genetic coincidence, prior evidence on the *NAM-1* gene and their high phenotypic correlations. Days to senescence and NDVI mapped to three coincident positions, two of which were also associated with percent stem breakage and GPC.

In particular, pleiotropy is a likely explanation for the association of these four traits at position 30 cM for several reasons. Firstly, position 30 cM (GPC QTL #2) corresponds to 50.98 Mbp on the most current IPK physical reference map, close to the *NAM-1/Gpc-1* gene which corresponds to 52.56 Mbp. Secondly, all four traits were detected in two environments at 30 cM (GPC QTL #2, Senesce QTL #2, NDVI QTL #3, StemBrk QTL #3). Thirdly, NDVI QTL #3, StemBrk QTL #3, Adult NB QTL #3,

Seedling NB QTL #3 and a KD QTL (at FHB_CR15) at 30 cM were also detected using single marker analysis and/or interval mapping (data not shown). Lastly, the Gen2-129 allele at 30 cM contributed to high GPC, accelerated senescence, high percent stem breakage, and low net blotch severity at adult and seedling stages, as would be expected of the high-GPC allele at the *NAM-1/Gpc-1* gene.

The homolog of *NAM-1/Gpc-1* in wheat, *Gpc-B1/NAM-B1*, is reported to be a NAC (petunia **N**AM and Arabidopsis **A**TAF1, ATAF2, and **C**UC2) transcription factor (Distelfeld et al., 2008; Uauy et al., 2006). The NAC family genes are plant-specific transcriptional regulators involved in pathogen defense, various developmental and morphological processes (Aida et al., 1997; Olsen et al., 2005). In particular, the functional *Gpc-B1/NAM-B1* allele in wheat accelerates senescence, increases remobilization of nutrients including nitrogen, Fe and Zn from leaves to developing grains (Uauy et al., 2006). It is likely that the coincident QTL at position 30 cM was detecting the NAC gene, where Gen2-129 accelerated senescence, remobilized leaf and stem nitrogen to the grains, and facilitated pathogen defense.

While the exact mechanisms of nitrogen remobilization remain to be understood, our study suggests there seems to be expenditure of nitrogen from source to sink. It is known that leaf degradation plays an important part in senescence (Prins et al., 2008). It is not known how protein degradation products are converted to a transportable form of nitrogen (Distelfeld et al., 2014). It is also not known how amino acids can be transported from senescing mesophyll cells to apoplast, so that they can be loaded into the phloem and finally delivered to the grains (Distelfeld et al., 2014). Jukanti et al. (2008) and

Jukanti & Fischer (2008) reported an association between earlier leaf protein degradation and earlier hordein accumulation in grains. Yang et al. (2004) did not detect any protease QTL co-localized at this 6H region, but detected two leaf endopeptidases QTL at this region in half of their environments with variable allelic effect of Karl (CIho 15487; Wesenberg et al., 1976). Based on the genetic results of our study, there were QTL that only controlled days to senescence and NDVI, but not percent stem breakage or GPC, and QTL that only controlled percent stem breakage but not GPC (StemBrk QTL #1 at 8 cM). Based on the phenotypic results of our study, one rNIL, Gen10-093, compared with Lacey had significantly later senescence and lower NDVI (significant in several environments) but non-significantly different percent stem breakage and GPC. Given the prior literature evidence and the findings in our study, it is reasonable to hypothesize that the genes controlling days to senescence in addition to those also controlling GPC might be involved in nitrogen degradation and transportation processes, which were not directly responsible for the protein level in the grains.

High phenotypic correlations among these traits (days to senescence, NDVI, percent stem breakage, and GPC) provide additional support for their genetic coincidence. Days to senescence and NDVI (except in NB_CR15) correlated significantly and positively with each other, and were very similar in their correlations with other traits (Supplementary Table 3). They also correlated significantly and negatively with GPC and percent stem breakage (except in NZ15), which correlated significantly and positively with each other. Because of the high heritabilities of these

four traits, their phenotypic correlations were determined chiefly by their genetic correlations (Bernardo, 2010b).

Breeding implications and limitations

The level of genetic complexity in a narrow genetic base revealed in this study has important implications for breeding to improve multiple traits with regard to selecting recombinants, understanding a complex trait, and demonstrating the effectiveness of the breeding approach used.

Several rare recombinant lines in the introgression region are good examples of potential breeding materials. We searched rNILs for the Gen2-129 allele at 23 cM associated with low DON accumulation and at 26 – 27 cM for low FHB severity and low GPC, while for the Lacey allele at 25, 29, and 30 cM for maintaining FHB severity and GPC (Supplementary Table 4). We were not able to find any recombinants with the Lacey allele at 25 cM and with the corresponding alleles at the other regions. However, two rNILs, Gen10-012 and Gen10-015, did have the Gen2-129 allele at 23 and 26 – 27 cM and the Lacey allele at 29 and 30 cM. Even though not statistically significant, both Gen10-012 and Gen10-015 decreased FHB severity and DON accumulation more than 20% compared with Lacey (except FHB_CR16 for Gen10-015). Both Gen10-012 and Gen10-015 did not differ much from Lacey for yield and GPC (less than 10% difference from Lacey (except Yld_CR16 for Gen10-015). The peak marker, marker #251, associated with both FHB QTL #3 and GPC QTL #1 at position 26 – 27 cM where Gen2-129 contributed a negative effect, is a good example to be used in marker-assisted

selection. To get rid of the remaining linkage drag, further recombination events are necessary.

Interpretation about overall performance of a trait can be complicated. Heading date was mapped to six QTL positions and only one of these, HD QTL #3 at 20.5 cM, was detected in more than one environment. Some of the single-environment QTL do however agree with previous studies. In addition, HD QTL #3, #4 and #5 were also detected using interval mapping. The majority of the 101 rNILs headed within 3 or 4 days and a phenotyping error of ±1 day would have a large effect on the QTL results. A positive relationship between heading date and days to senescence was reported previously using a set of NILs for Karl at the chromosome 6H region (i.e. containing the *NAM-1* gene) (Lacerenza et al., 2010). This is consistent with the result of the effect of Gen2-129 QTL at the 30 cM position on heading date and days to senescence.

Overall, the QTL introgressive breeding approach has been argued to work best for traits with simpler inheritance patterns such as submergence tolerance in rice and cyst nematode resistance in soybean. For complex traits like grain yield whose QTL has minor or inconsistent effects, useful marker-based approaches should rely on increasing the frequency of favorable QTL alleles and predicting genotypic value of inbred lines and hybrids (Bernardo, 2010a). On the other hand, several fine mapping studies using NILs have successfully mapped linked traits in several plant species and St.Clair (2010) argued that MAS worked for a few to many genes, where QTL mapping with dense marker coverage can be precise.

Confidence in the QTL architecture of these traits can help us make more informed breeding decisions. Even though more weight is recommended to be given to the QTL that were detected in two or more environments (e.g. FHB QTL #3 and GPC QTL #1 at position 26 – 27), it does not mean the QTL detected in single environments are not worth considering. Some of the single-environment QTL were consistently detected using different mapping methods (data not shown).

Some QTL were not only detected using composite interval mapping, but also detected using single-marker analysis and/or interval mapping (data not shown). For example, the seedling net blotch QTL at position 30 cM was detected in the NB_WGH15 environment using interval mapping. Adult net blotch QTL at position 30 cM was consistently detected using single-marker analysis and interval mapping. This seedling resistance QTL on 6HS (30 cM) was reported in a previous study using Chevron as a resistance source (Ma et al., 2004). The other two QTL detected for both seedling net blotch on ~6HL were detected previously in a Rika/Kombar cross (Abu Qamar et al., 2008).

It is important to keep in mind its relative position in the whole chromomosome when interpreting the QTL results. Marker #205 (the peak marker associated with NDVI QTL #1) and marker #231 (HD QTL #5) were in the area of low recombination, from 94 to 387 Mbp as discussed above. It is reasonable that this area had low recombination events because it included the centromeric region of chromosome 6H from 181 - 383 Mbp. It is important to note that both 26 - 27 cM and 30 cM positions, which most traits mapped coincidentally, are not in this region.

CONCLUSIONS

The level of genetic complexity in an introgression region of 6H observed in this study highlights the need for recombination to uncouple unfavorably linked genes. This introgression region, covering approximately one third of the genetic distance of chromosome 6H (Mascher et al., 2013), is equivalent to 86% of physical distance of the chromosome. Numerous breeding programs have focused on the other small portions of the genome that are recombination-rich. This study re-shifts our focus onto areas with low recombination and characterized one such area through fine mapping a series of traits using NILs, which allows for the qualitative examination of quantitative traits. One can demonstrate linkage by generating recombinants between these linked loci. Testing in more environments and more precise phenotyping on traits that have higher priorities are possible solutions to recovering recombinant lines. Increased mapping resolution allows for more robust conclusions about the underlying genetic mechanism for trait associations. However, the inability to detect recombinants of associated traits that mapped to coincident loci does not demonstrate pleiotropy. Gene targeted approaches such as mutagenesis, gene silencing, or gene editing maybe needed to unambiguously conclude that associated traits are the result of pleiotropy.

However, the generation of recombination events in regions like this can be challenging and likely requires extensive genotyping and large populations. There was no ideal recombinant line recovered but relative gains in breeding can be made. Yet resources available to breeding programs are often limited, and how to balance between further studies and resources is always a challenge in managing a large population.

Selective phenotyping (Jannink, 2005), high-throughput phenotyping, reduced costs of marker technologies, and selection methods enriched for heterozygotes as potential recombinants are solutions at the other end of the balance to those mentioned above.

In the near future, it would be beneficial to compare our results to the transcriptomic results of Muehlbauer Barley Molecular Genetics Laboratory at University of Minnesota also using Chevron/Lacey-derived NILs. It would be beneficial to see if the similar expression levels are present in the Chevron/Lacey-derived NILs as do the reported expression levels in Karl/Lewis-derived NILs. Uauy et al. (2006) suggested that the reduction in functional protein levels, rather than a complete loss of gene function, may have led to decreased GPC, Fe and Zn concentrations in durum wheat caused by reduced translocation from senescing leaves. Edwards & Mackay (2009) observed that fractionated QTL for *Drosophila* aggressive behavior suggested that extensive epistasis is a big challenge for the understanding of complex traits. Given the already complex genetic relationships among associated traits found in the 6H region, epigenetic modifications and epistasis might add another level of complication. A second useful future experiment would be QTL mapping of Lacey and KLBC4 lines, which have been developed at the barley breeding program at University of Minnesota. KLBC4 lines are NILs with Karl allele at the 6H region in a Lacey genetic background. Comparing the results of current study to KLBC4/Lacey results would add confidence to inferences about associated traits by comparing different alleles at the chromosome 6H region in the same genetic background.

Nevertheless, other alleles and traits are associated with chromosome 6H centromere. In addition to Chevron, Lacey and Karl alleles for the QTL studied, there are a net blotch resistant allele from CIho 5791 (*Rpt5.f*) and an early maturity allele from Atsel (*eam7.g*), and in addition to the traits studied, there are a semidwarf gene (*sdw5*) and a leaf rust resistance gene (*Rph24.an)* associated with chromosome 6H centromere (Franckowiak, personal communication, 2016). The evaluation of recombinant lines in diverse genetic backgrounds is needed before they can be used in breeding and the creation of such lines is only the first step in the breeding process.

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Tables

Table 1. Seven traits mapped to a ~50-cM centromeric region* on chromosome 6H using Chevron or Chevron-derived source in biparental quantitative trait loci (QTL) mapping studies

Trait[a]	Marker region	R^2 (%)[b]	Higher-value allele[c]	Lower-value allele[c]	References
KD	cMWG652a-MWG916	23.0, 6.2	M69	Chevron	de la Pena et al., 1999
	Bmag0500-MWG916	19.7	M69	Chevron	Canci et al., 2003; Canci et al., 2004
	MWG916-Bmag0807	21.5	M69	Chevron	Canci et al., 2003; Canci et al., 2004
	Bmag0807-GMS006	12	M96	MNBrite*	Canci et al., 2003
	HVM14-HVM74	21.5	M96	MNBrite*	Canci et al., 2003
	Bmag0613-Bmac0218	17	M96	MNBrite*	Canci et al., 2003
	Bmag0807-Bmag0173	42	M81	M92-299*	Canci et al., 2004
	Bmag003c-Bmag0613	13.8	M81	M92-299*	Canci et al., 2004
	cMWG652a-Bmag0807	39.5	M81	M92-299*	Canci et al., 2004
	GMS006-ABG458	53.1, 27.0, 46.4, 56.6	Stander	MNS93*	Canci et al., 2004
	ABG458-HVM65	22.8	Stander	MNS93*	Canci et al., 2004
FHB	Bmag0173-Bmag0870	24.6	M69	Chevron	Canci et al., 2004
	cMWG652a-Bmag0807	12.9	M81	M92-299*	Canci et al., 2004
	GMS006-ABG458	20.1	Stander	MNS93*	Canci et al., 2004
	ABG458-HVM65	16.5	Stander	MNS93*	Canci et al., 2004
	WG719d-CDO785d	10.0, 9.7	Stander	Chevron	Ma et al., 2000
DON	WG719d-CDO785d	8.2, 11.9, 7.4	Stander	Chevron	Ma et al., 2000
Seedling NB	ksua3b-WG719d	64.4	Stander	Chevron	Ma et al., 2004
GPC	MWG916-Bmag0807	56.6, 59.2, 59.3	Chevron	M69	Canci et al., 2003
	Bmag0807-GMS006	22.2, 34.8, 28.7	MNBrite*	M96	Canci et al., 2003
	HVM14-HVM74	29.3	MNBrite*	M96	Canci et al., 2003
Heading date	Bmag0807-Bmag0173	21.2	M69	Chevron	Canci et al., 2004
	cMWG652a-Bmag0807	12.3	M92-299*	M81	Canci et al., 2004
Height	CDO686-CDO785d	10.1, 13	Stander	Chevron	Ma et al., 2000

*This region was determined by blasting the primer sequences of for example simple sequence repeat (SSR) markers to Morex POPSEQ map (Mascher et al., 2013).

[a]KD = kernel discoloration, FHB = *Fusarium* head blight, DON = deoxynivalenol, seedling NB = net-form net blotch at the seedling stage, GPC = grain protein concentration;

[b]R^2 = phenotypic variation, in percentage, explained by the QTL in corresponding studies;

[c]Parent that contributed to higher or lower trait value; * = Chevron-derived parents. MNBrite has the pedigree of CIho 9539/`Manker'//`Cree'/3/`Morex'/4/M33/5/2* `Robust'/6/2*M69, where Clho 9539 has the resistant parent Chevron; M92-299 and MNS93 were derived from the Chevron/M69 population, partially FHB and KD resistant.

Table 2. Parent and Gen10 population means, population ranges, variation among Gen10 lines for deoxynivalenol (DON) accumulation, *Fusarium* head blight (FHB) severity, grain protein concentration (GPC), days to senescence, normalized difference vegetation index (NDVI), stem breakage, days to heading, net-form net blotch severity at adult stage (Adult NB), net-form net blotch severity at seedling stage (Seedling NB), lodging, yield, kernel discoloration (KD) score, bacterial leaf streak (BLS) severity, and height

Traits	Trials[a]	Gen2-129[b]	Lacey[b]	Population	Max	Min	P-value[c]	$h^{2(d)}$	Comb $h^{2(f)}$
DON (ng/µg)	FHB_CR15	24.3	24.6	18.3	29.3	10.0	ns	NA	0.64
	FHB_StP15	**10.3**	**16.7**	12.0	22.1	4.0	<0.01	0.38	
FHB (%)	FHB_CR15	16.9	19.4	15.2	22.7	8.6	<0.01	0.33	NA
	FHB_StP15	7.3	17.5	7.2	21.7	1.0	<0.01	0.38	
	FHB_CR16	34.3	42.0	38.5	55.3	24.0	<0.01	0.44	
	FHB_StP16	18.8	19.5	21.4	51.8	8.1	<0.001	0.61	
GPC (%)	Yld_CR15	14.9	12.7	13.9	18.0	11.7	<0.001	0.74	NA
	Yld_StP15	15.6	12.8	14.8	18.0	13.1	<0.001	0.83	
Days to senescence	Yld_CR15	82	82	81	88	79	<0.001	0.73	0.68
	Yld_StP15	78	80	82	92	78	<0.001	0.71	
NDVI (0.00-0.99)	Yld_CR15	0.51	0.58	0.50	0.75	0.37	<0.001	0.62	0.70
	Yld_StP15	0.29	0.31	0.39	0.54	0.24	<0.01	0.44	
	NB_CR15	**0.22**	**0.27**	0.24	0.41	0.14	<0.001	0.71	
	Yld_CR16	0.26	0.32	0.27	0.54	0.18	<0.001	0.78	
	Yld_StP16	0.27	0.33	0.29	0.52	0.18	<0.001	0.75	
Stem breakage (%)	Yld_CR15	60[b]	0[b]	21	60	0	<0.001	0.59	0.80
	NZ15	60[b]	6[b]	26	90	0	NA[e]	NA	
	Yld_CR16	43	33	47	90	5	<0.001	0.68	
	Yld_StP16	**65**	**15**	36	95	5	<0.001	0.86	
Days to heading	Yld_CR15	53	53	53	56	53	<0.05	0.34	NA
	FHB_CR15	58	58	58	62	56	<0.01	0.39	
	FHB_StP15	53	52	53	56	52	<0.001	0.47	
	Yld_CR16	52	51	52	53	51	<0.05	0.31	
	Yld_StP16	55	55	55	59	54	<0.001	0.68	
	FHB_StP16	50	48	48	54	47	<0.001	0.56	
Adult NB (0-9)	NB_CR15	**1.7**	**3.7**	2.8	5.3	1.0	<0.001	0.64	NA
Seedling NB (1-10)	WGH15	4.0	5.3	4.7	6.7	2.7	<0.001	0.59	0.60
	FGH15	5.3	6.3	5.6	7.0	4.0	<0.001	0.62	
Lodging (%)	Yld_CR15	60	10	39	80	10	ns	NA	NA
	Yld_StP15	45	40	49	75	10	<0.05	0.33	
	Yld_CR16	20	10	22	50	5	ns	NA	
	Yld_StP16	35	23	30	65	8	<0.05	0.34	
Yield	Yld_CR15	7639.7	8144.2	7800.7	8899.1	6127.4	<0.01	0.45	0.52

(kg/ha)	Yld_StP15	**4259.8**	**5535.3**	4963.1	6130.0	3503.6	<0.05	0.33	
	Yld_CR16	6531.0	6786.6	7011.5	10018.5	4402.4	ns	NA	
	Yld_StP16	4040.5	4800.7	4246.8	5260.9	3248.0	<0.001	0.71	
KD (1-5)	FHB_CR15	3.8	4.7	4.2	5.0	3.0	<0.001	0.51	NA
	FHB_StP15	**2.7**	**5.0**	4.0	5.0	2.3	<0.001	0.68	
BLS (1-9)	BLS_CR15	7.0	6.3	6.6	7.0	5.8	ns	NA	NA
	BLS_StP15	**7.0**	**5.5**	5.6	6.8	4.3	ns	NA	
Height (cm)	Yld_CR15	**99**	**97**	99	106	92	ns	NA	NA
	FHB_StP15	**100**	**96**	97	103	90	ns	NA	
	Yld_CR16	89	81	88	96	77	<0.01	0.43	
	Yld_StP16	93	87	91	98	85	<0.001	0.50	

[a]Yld = yield trial, FHB = *Fusarium* head blight trial, BLS = bacterial leaf streak trial, NB = net blotch trial; CR = Crookston, MN, StP = St. Paul, MN; 15 = 2015, 16 = 2016.

[b]**Bolded values** indicate significant difference between parents (at 0.05 level) with two-sample unequal variance t-test. Parents in Yld_CR15 and NZ15 for stem breakage were missing and un-replicated and no t-test was performed.

[c]P-value for line effect when fitting line and replication effects for analysis of variance (ANOVA) at alpha level of 0.001, 0.01, 0.05 or not significant.

[d]Single environment entry mean heritability. NA suggested non-significant line effect or error variance being greater than variance due to lines.

[e]In NZ15 for stem breakage, ANOVA was not performed because the trial had a single replication.

[f]Combined environment entry mean heritability fitting line (fixed), environment, replication within environment and line-by-environment (random) effects. NA suggests not passing the Gomez & Gomez (1984) analysis, or error variance greater than line-by-environment variance, or line-by-environment variance greater than line variance.

Table 3. Quantitative trait loci for deoxynivalenol (DON) accumulation, *Fusarium* head blight (FHB) severity, grain protein concentration (GPC), days to senescence, normalized difference vegetation index (NDVI), stem breakage, days to heading, net-form net blotch severity at adult stage (Adult NB), net-form net blotch severity at seedling stage (Seedling NB), lodging, and yield detected at Crookston, MN (CR) and St. Paul, MN (StP) in 2015 and 2016 in the Gen10 population

Trait	QTL ID	Environment[a]	Start pos[b]	Peak pos[b]	End pos[b]	Peak marker ID[c]	Allele effect of Gen2-129[d]	LOD[d]	R^2 (%)[d]
DON	#1	FHB_StP15	22.98	23.22	23.84	217	- 3.27	2.42	15.04
FHB	#1	FHB_StP15	18.59	18.83	18.96	191	- 7.4	3.14	12.85
	#2	FHB_StP16	24.81	25.01	25.41	221	+ 7.2	2.47	36.42
	#3	FHB_StP16	26.58	26.57	26.82	240	- 3.6	2.57	10.85
		FHB_StP15	27.18	27.25	27.43	251	- 5.7	2.26	7.38
	#4	FHB_StP16	28.52	28.75	29.44	276	+ 3.5	2.36	9.22
GPC	#1	Yld_StP15	26.21	26.19	26.70	234	- 0.68	2.76	11.21
		Yld_StP15	27.07	27.31	27.49	251	- 1.24	3.98	17.76
		Yld_CR15	27.31	27.50	27.67	254	- 0.79	2.79	11.61
	#2	Yld_StP15	29.93	30.06	30.17	287	+ 0.6	2.49	9.01
		Yld_CR15	30.35	30.47	30.72	290	+ 1.06	4.62	16.77
		Yld_CR15	30.72	30.96	31.08	296	+ 1.08	4.21	15.24
Senesce	#1	Yld_CR15	27.16	27.21	27.52	251	+ 1.2	2.96	10.56
	#2	Yld_CR15	29.79	30.03	30.52	287	- 0.7	2.69	10.7
		Yld_StP15	29.84	30.39	30.52	290	- 1.7	2.59	13.03
	#3	Yld_StP15	33.64	34.85	35.96	314	- 0.8	2.61	9.34
NDVI	#1	NB_CR15	19.69	21.27	22.55	205	- 0.01	2.51	5.19
	#2	Yld_CR16	27.56	27.67	28.04	256	+ 0.04	3.73	13.94
	#3	Yld_CR16	29.93	30.06	30.17	287	- 0.03	3.17	12.63
		Yld_CR15	29.84	30.39	30.52	290	- 0.05	2.49	14.63
		NB_CR15	30.24	30.47	30.72	291	- 0.03	4.94	11.18
		NB_CR15	30.79	30.93	31.28	295	- 0.03	4.98	12.57
		NB_CR15	31.88	31.91	32.01	312	- 0.02	4.14	9.94
	#4	NB_CR15	36.02	35.94	36.02	323	- 0.03	4.87	10.85
		Yld_CR16	36.21	37.04	37.24	342	- 0.02	2.48	9.40
StemBrk	#1	NZ15	8.22	8.29	8.35	84	- 17	3.28	6.87
	#2	Yld_CR15	27.62	27.91	28.11	261	- 12	2.43	9.27
	#3	NZ15	30.11	30.21	30.72	290	+ 12	14.21	33.03
		Yld_StP16	30.30	30.69	30.97	292	+ 17	2.37	9.31
		NZ15	30.79	31.18	31.33	309	+ 12	14.49	33.35
		Yld_StP16	31.92	32.56	33.52	313	+ 15	2.91	22.91
HD	#1	FHB_CR15	4.63	4.87	5.05	43	- 1.7	4.87	15.21
		FHB_CR15	6.22	6.22	6.27	52	- 1.0	2.85	12.61
	#2	FHB_StP15	14.44	14.44	14.62	145	- 1.0	1.99	17.96

		FHB_StP15	15.11	15.42	15.55	164	- 1.0	3.38	17.97
		FHB_StP15	15.91	15.97	16.04	167	- 1.1	3.55	22.76
		FHB_StP15	16.46	16.84	17.74	172	- 0.3	2.4	9.63
	#3	Yld_StP16	20.29	20.35	20.54	198	+ 1.6	8.63	30.95
		Yld_CR16	19.63	20.36	20.54	198	+ 0.4	2.09	8.17
		Yld_StP16	20.67	20.73	20.85	202	+ 1.6	8.69	30.95
	#4	Yld_StP16	22.31	22.37	22.55	215	- 1.0	5.16	18.11
		Yld_StP16	22.73	22.72	22.92	216	- 1.1	4.89	20.83
	#5	FHB_StP16	25.61	25.72	25.85	226	+ 0.4	3.8	12.41
		FHB_StP16	26.09	26.03	26.27	231	+ 0.4	3.83	12.55
	#6	FHB_StP16	29.69	29.94	29.93	286	- 0.3	1.69	3.34
Adult NB	#1	NB_CR15	1.35	1.64	2.19	25	- 0.3	2.55	3.19
		NB_CR15	2.44	2.57	2.62	32	- 0.3	2.6	3.38
	#2	NB_CR15	4.03	3.96	4.39	37	+ 0.6	4.3	7.14
	#3	NB_CR15	28.77	29.20	29.26	277	- 0.8	13.98	37.8
		NB_CR15	29.75	29.75	29.93	283	- 0.9	9.79	18.81
		NB_CR15	29.93	29.94	30.11	286	- 1.1	11.43	20.47
		NB_CR15	30.48	30.47	30.66	291	- 1.1	13.64	21.9
		NB_CR15	30.79	30.97	31.08	297	- 1.1	13.7	21.9
Seedling NB	#1	NB_FGH15	4.52	5.08	5.79	45	- 0.3	2.63	9.31
	#2	NB_FGH15	18.90	20.31	21.71	197	+ 0.3	2.4	7.89
	#3	NB_FGH15	29.44	29.41	29.62	280	- 0.3	2.51	9.09
		NB_FGH15	30.66	30.69	31.15	292	- 0.5	5.48	18.62
Ldg	#1	Yld_CR15	2.01	2.26	2.63	29	+ 8	5.38	20.07
	#2	Yld_StP15	7.27	7.83	7.89	78	- 17	2.78	11.17
	#3	Yld_StP16	17.74	17.79	18.04	177	+ 10	2.41	9.81
Yld	#1	Yld_StP15	0.00	0.00	0.42	1	- 177	3.23	12.48
	#2	Yld_CR16	2.13	2.26	2.44	29	+ 651	4.08	12.63

[a]Environment refers to a specific Trial_LocationYear combination.

[b]Positions = (linkage map positions of 249 Gen rNILs)*249/1941 in cM; a peak exceeding the LOD score threshold was considered significant. Dropping the peak LOD score by 1 determined the start and end position of the QTL.

[c]Peak marker IDs for reference to GBS SNP markers that are in Supplementary Table 4.

[d]Allele effect of Gen2-129, LOD score and R^2 that is percent phenotypic variation explained by the corresponding QTL all refer to peak marker.

Figures

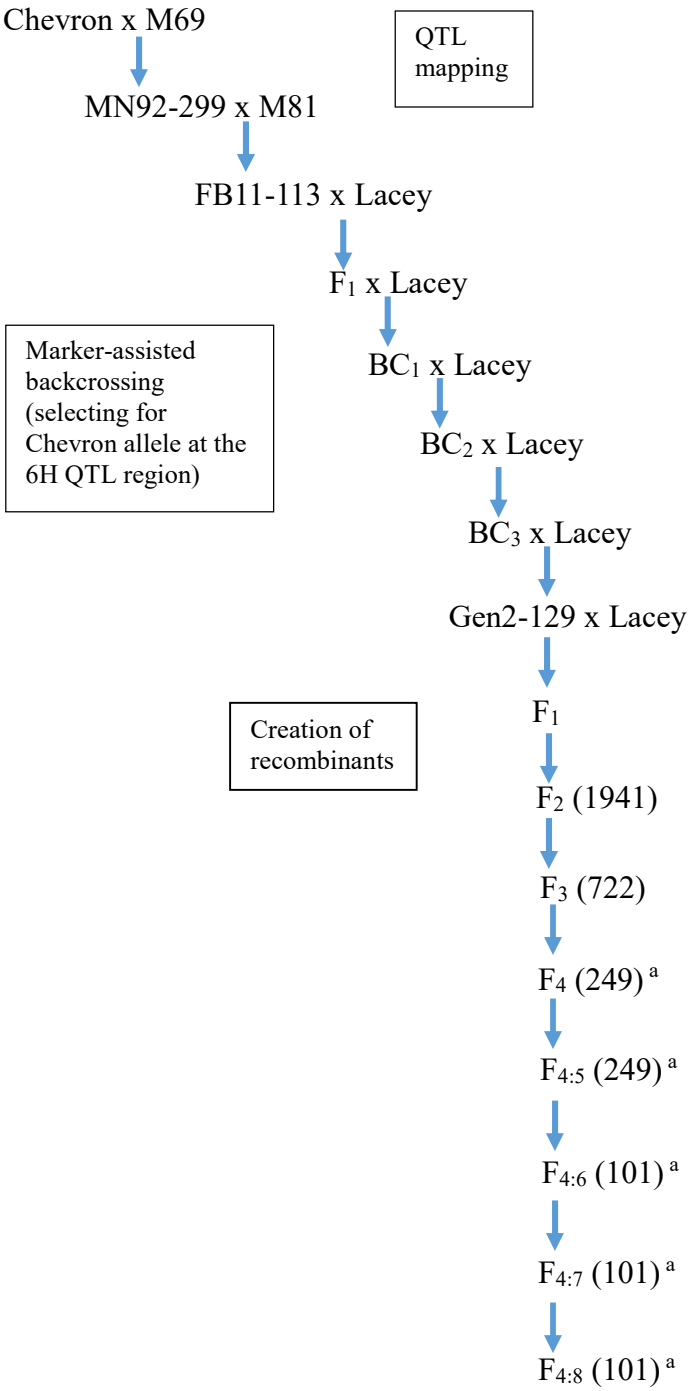

Figure 1. Developmental scheme of Gen10 recombinant near-isogenic lines

Numbers in the parentheses indicate the number of lines in that generation.

[a]$F_{4:5}$ rNILs were genotyped; $F_{4:7}$ rNILs were phenotyped in field trials in 2015; $F_{4:8}$ rNILs were phenotyped in field trials in 2016. Note: 22 of the 249 F_4 rNILs had inadequate seeds. For these 22 lines, F_5 lines were

used as the seed source for subsequent trials (i.e. $F_{5:6}$ rNILs were genotyped; $F_{5:8}$ were phenotyped in field trials in 2015; $F_{5:9}$ were phenotyped in field trials in 2016).

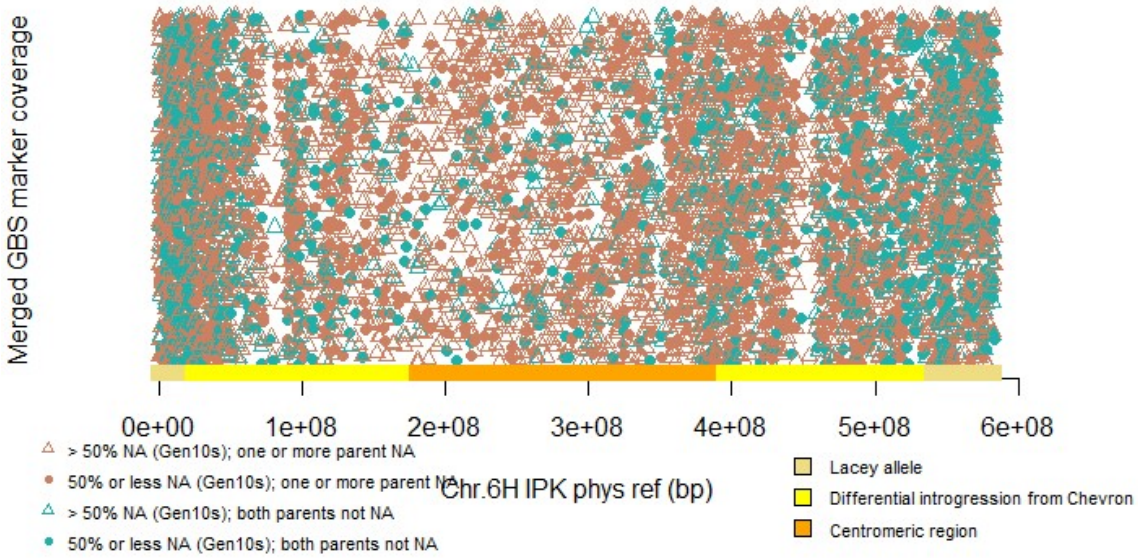

Figure 2. SNP marker coverage of merged genotype-by-sequencing dataset of 249 Gen10 recombinant near-isogenic lines, Lacey and Gen2-129 on chromosome 6H of most current IPK physical reference (bp) 6647 markers of 249 rNILs on chromosome 6H; 1118 markers had 50% or less missing data among Gen10s and no missing data among parents (blue round dots) on chromosome 6H.

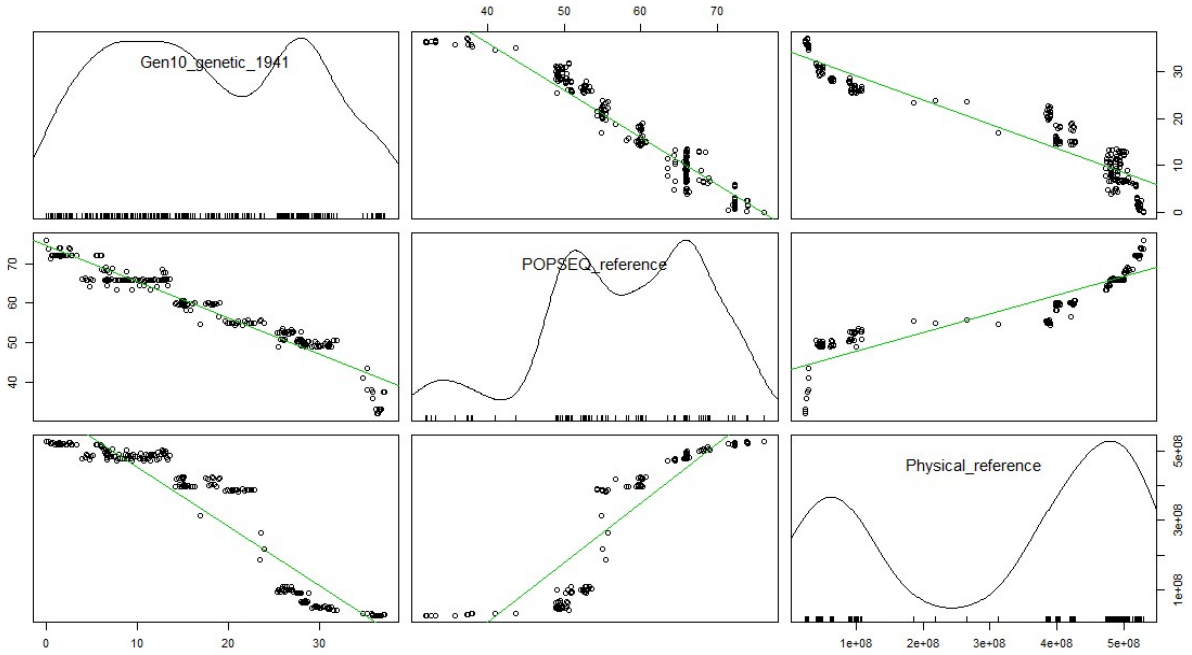

Figure 3. Relationships among SNP markers placed on the Gen10 genetic map (cM; based on 1941 F_2 lines) and Morex POPSEQ genetic map (cM) and the IPK physical reference map (bp)
There was a linear correspondence between the Gen10 and Morex POPSEQ linkage maps (top-middle or middle-left). Gen10 genetic map corresponded to the physical reference map in a similar fashion as did the Morex POPSEQ map to the physical reference map (top-right or bottom-left versus middle-right or bottom-middle). Comparisons of the Gen10 genetic map to the physical reference map revealed an area of low recombination from 16.9 – 26.1 cM or 108 – 383 Mbp, which included the centromere of 6H.

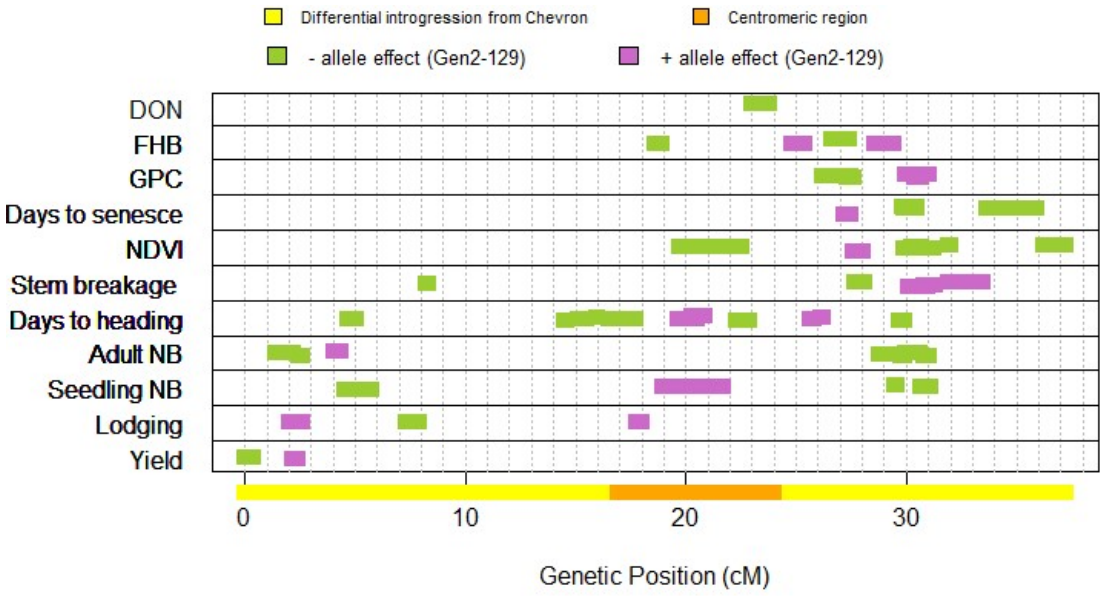

Figure 4. Quantitative trait loci mapped to the 37.2-cM region on chromosome 6H for deoxynivalenol (DON) accumulation, *Fusarium* head blight (FHB) severity, grain protein concentration (GPC), days to senescence, normalized difference vegetation index (NDVI), stem breakage, days to heading, net-form net blotch at adult stage (Adult NB), net-form net blotch severity at seedling stage (Seedling NB), lodging, and yield

Length of the bar corresponded to length of the genetic region of the corresponding significant QTL.

Genetic position was based the linkage map created by 249 Gen10 recombinant near-isogenic lines (rNILs) carrying differential introgression from Chevron, and calculated into the 37.2-cM region on chromosome 6H based on 1941 Gen10 rNILs. NOTE: the orientation of this genetic map was opposite to that of Morex POPSEQ map or most current IPK physical reference.

Jitter function in R was used to differentiate QTL peaks from different environments or the same environments.