GENETIC ARCHITECTURE OF MULTIPLE DISEASE RESISTANCE IN ONE MAIZE CHROMOSOME SEGMENT SUBSTITUTION LINE POPULATION

BY

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THESIS

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ABSTRACT

Maize is one of the most important crops and is grown all over the world. Like other plants, maize is attacked by numerous pathogens. Diseases account for 2 to 15% of maize yield losses annually, and foliar diseases are the most destructive in terms of yield. With an increasing world population, utilizing host plant resistance is an environmentally and economically friendly solution to assure food security. Maize is susceptible to numerous diseases. Thus, breeding multiple disease resistant (MDR) varieties is critical. While the genetic basis of resistance to multiple fungal pathogens has been studied in maize, less is known about the relationship between fungal and bacterial resistance.

Bacterial leaf streak (BLS) is a foliar disease of maize caused by *Xanthomonas vasicola* pv. *vasculorum*. Since the first report of BLS in the United States in 2014, this disease has spread all over the Midwestern corn belt. Little is known about the disease cycle, and consequently, management is difficult. Host resistance will likely play a major role in controlling the disease, as there is no practical chemical control. Thus, we conducted quantitative trait locus (QTL) mapping for BLS resistance in three maize populations: the Z022 (B73 × Oh43 recombinant inbred line) NAM population, the Z023 (B73 × Oh7B recombinant inbred line) NAM population, and the DRIL78 (NC344 × Oh7B chromosome segment substitution line) population. A total of five QTL were detected across two of the mapping populations. One of the detected QTL for BLS resistance in the DRIL78 population, located in chromosomal bin 4.07, overlaps with a region that has also been identified for southern corn leaf blight (SCLB) resistance in this same population. These data will be useful for developing maize varieties resistant to BLS and to mitigate the impact of bacterial leaf streak on maize production.

Goss's bacterial wilt and blight (GW) is an important foliar disease caused by *Clavibacter nebraskensis*. We evaluated an introgression line population, DRIL78, for GW in three different environments and conducted quantitative trait locus (QTL) mapping for each environment separately, as well as for the combined environments. We identified a total of ten QTL across multiple environments. We obtained the phenotypic data from the DRIL78 population for three additional foliar diseases: northern corn leaf blight (NCLB), southern corn leaf blight (SCLB) and gray leaf spot (GLS) and conducted mapping analysis using the same methods. Multivariate analysis was then conducted to identify regions conferring resistance to multiple diseases. We identified 20 chromosomal bins with putative multiple disease effects. We identified five chromosomal regions (bin 1.05, 3.04, 4.06, 8.03, and 9.02) with the strongest statistical support for a role in MDR. By examining the phenotypic effects of each haplotype, we identified several regions associated with increased resistance to multiple diseases and three regions associated with opposite effects for bacterial and fungal diseases. Several promising candidate regions for multiple disease resistance in maize were identified in this study.

The results presented in this thesis are useful for both breeding and to understand the basic biology of host plant resistance. I identified both single disease and MDR QTL, which will serve as a foundation for subsequent fine mapping analysis and can be useful for breeding resistant varieties.

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CHAPTER 1: LITERATURE REVIEW

Maize

Wheat, rice, maize, potato and soybean are five major crops worldwide, contributing to 18.3, 18.9, 5.4, 2.2 and 3.3% of the global human calorie intake respectively in 2013, according to the Food and Agriculture Organization of the United Nations (2018). Maize (*Zea mays* L) is one of the most important crops in the world. The United States, China and Brazil are the top three maize-producing countries, producing 31%, 24% and 8% of the total yield, respectively (Ranum, Peña‐Rosas et al. 2014). Maize can be used for food and industrial products including, for example, starch, fuel alcohol, and sweeteners.

Important diseases of maize

Maize productivity is at risk from pest pressure, including weeds, pathogens and animal pests. Maize is consistently attacked by a variety of pathogens, and the yield loss caused by diseases varies across years and regions. Maize is affected by about 100 pathogens, with a fraction of diseases present in a given environment (Ali and Yan 2012, Munkvold and White 2016). Hence, maize plants need to defend themselves against a wide range of pathogens. The yield loss caused by diseases in the United States ranges from 2 to 15% annually (Munkvold and White 2016). In the United States and Ontario, Canada it is estimated that 13.5% of total production was lost due to diseases in 2015, 10.8% in 2016, and 6.7% in 2017 (Network 2018). There are four categories of maize diseases: root rots and seedling blights, foliar diseases, stalk rots and ear rots. Of the four categories, foliar and aboveground diseases are the most destructive during most years, accounting for about 52% of the total yield loss, according to a survey from 2012 to 2015 of 22 corn-producing US states and Ontario, Canada (Mueller, Wise et al. 2016). In 2017, the disease losses caused by foliar and aboveground diseases were estimated to account for

approximately 49% of the total yield loss due to the diseases (Network 2018). Of the foliar and aboveground diseases, Goss's wilt (GW), gray leaf spot (GLS) and northern corn leaf blight (NCLB) are three of the most destructive diseases (Mueller, Wise et al. 2016, Network 2018). GW is a bacterial disease and GLS and NCLB are fungal diseases. Protecting maize from the infection by pathogens is thus important for food security. The losses due to diseases can be managed in high-density agricultural systems largely by growing resistant germplasm and pesticide applications (in some cases) (Balint-Kurti and Johal 2009). Host plant resistance is thus a crucial tool to protect crops.

Bacterial leaf streak of maize and other selected diseases

A total of five maize foliar diseases were selected for their importance in this study: Southern corn leaf blight (SCLB), NCLB, GLS, GW and bacterial leaf streak (BLS). GLS, GW and NCLB are the three most destructive diseases. GLS is a disease of global importance caused by the necrotrophic fungus *Cercospora zeae-maydis*. It can cause as much as a 50% yield loss (Ward, Stromberg et al. 1999). In the United States and Ontario, Canada from 2012 to 2015, GLS accounts for 5.4% of the total maize yield loss caused by diseases (Mueller, Wise et al. 2016); in 2017, GLS accounted for 17.7% of the total disease loss (Network 2018). GW, also referred to as Goss's bacterial wilt and blight, is caused *by Clavibacter michiganensis* subsp. *nebraskensis* (Vidaver, Gross et al. 1981). Typical symptoms of GW include large, water-soaked, discontinuous lesions. GW accounts for 4.8% of the yield losses caused by diseases of maize in the United States and Ontario, Canada from 2012 to 2015 (Mueller, Wise et al. 2016) and 10.2% in 2017 (Network 2018). Northern corn leaf blight (NCLB) is caused by the ascomycete *Setosphaeria turcica*, and cigar-shaped lesions are characteristic of NCLB. In the

United States and Ontario, Canada, 4.1% of the yield loss from diseases was due to NCLB in 2012 to 2015 (Mueller, Wise et al. 2016) and 10.5% in 2017 (Network 2018).

SCLB, although not as devastating as the other three diseases, has historically been important and is a major issue in some regions. In 1970, the losses in the United States due to an epidemic of SCLB were estimated at nearly one billion dollars (Ullstrup 1972). SCLB has the potential to cause up to 40% yield loss if the varieties are susceptible and the conditions are favorable (Byrnes, Pataky et al. 1989).

Apart from the well-established diseases, there are new maize diseases emerging as potential threats to corn production in the United States. BLS of maize is one such disease. BLS is a foliar disease caused by *Xanthomonas vasicola* pv. *vasculorum*. Developing from small, water-soaked lesions, BLS causes narrow stripe-shaped, 2-3mm wide lesions the infected leaves. The lesions are parallel to leaf veins and are constricted to interveinal spaces (Lang et al. 2017). The symptoms usually spread from lower, older leaves to higher leaves. Following high wind events, the disease can develop in mid and upper leaves of the plant (Jackson-Ziems, Korus, and Adesemoye 2016). The color of the lesions can vary from yellow to brown. In some cases, particularly with some genotypes, the lesions can coalesce and extend the entire length the leaf, and the damage looks similar to drought injury (Munkvold and White 2016). The photosynthetic capacity of the plant is compromised in severe infections, and yield losses are likely. Control depends largely on host resistance, as practical chemical controls do not exist for BLS.

BLS was first reported in South Africa in 1949 (Dyer 1949) and was limited to the African continent until recently. Sixty years after the initial description of the disease, researchers at the Universidad Católica de Córdoba, Argentina detected the symptoms of BLS on maize in northern Argentina in 2010 (Plazas, De Rossi et al. 2018). Since 2010, the disease

expanded from Córdoba Province to other corn-planting provinces in Argentina, affecting all commercial hybrids in the region. The positive identification of BLS in Argentina was officially reported in 2018 (Plazas, De Rossi et al. 2018). It was not until 2014 that BLS was first reported in Nebraska in the United States and subsequently was confirmed in Colorado and Kansas (Korus, Lang et al. 2017). The pathogen range now includes Colorado, Illinois, Iowa, Kansas, Minnesota, Nebraska, Oklahoma, South Dakota, and Texas (Lang, DuCharme et al. 2017, Damicone, Cevallos et al. 2018, Jamann, Plewa et al. 2019).

Initially the casual organism of BLS was named *Xanthomonas campestris* pv. *vasculorum* (Xcv) (Young 1978). Subsequently, studies involving sequence analyses (MLSA), phylogenetic analysis, and whole-genome sequencing have resulted in the reclassification of *Xanthomonas* pathogens (Vauterin, Hoste et al. 1995, Hauben, Vauterin et al. 1997, Dookun, Stead et al. 2000, Aritua, Parkinson et al. 2008). Based on a phylogenetic analysis and a comparison of genome sequences and host ranges, Lang et al. (2017) proposed that the pathogen should be named *X. vasicola* pv. *vasculorum* comb. nov (Cobb 1894; Lang, DuCharme et al. 2017). Detection assays for the positive identification of *X. vasicola* pv. *vasculorum* were developed based on whole-genome sequencing (Lang, DuCharme et al. 2017).

Qualitative and quantitative disease resistance in maize

There are two types of disease resistance in plants: qualitative and quantitative. Qualitative resistance is race-specific and inherited by a single or a few genes with large effects, usually characterized by a gene-for-gene interaction. It is also known as vertical, complete, or major gene-based disease resistance. The gene-for-gene plant interaction involves two basic processes: perception of pathogen attack and the subsequent amplified defense response (Ellis, Dodds et al. 2000, Jones, Vance et al. 2016). Perception requires a receptor with a high degree of

specificity for pathogen strains, namely a disease resistance (R) gene. The term R gene is used to refer to a gene that confers qualitative effects (Poland, Balint-Kurti et al. 2009). R genes associated with specific diseases have been identified in maize. For example, the *Ht* genes (*Ht1*, *Ht2*, *Ht3*, *ht4*, *Htnl*, *Html* and *NN*) have been identified in mostly tropical germplasm and confer qualitative resistance to NCLB (Welz and Geiger 2000). However, the effectiveness of *Ht* genes depends on pathogen race, environmental conditions, factors like light intensity and temperature, which can limit the value of using them (Welz and Geiger 2000).

Quantitative resistance, on the other hand, confers partial effects. Quantitative resistance is usually expressed as a reduction in disease with slowing disease progression, as opposed to the absence of disease. Quantitative disease resistance (QDR), also referred to as polygenic or oligogenic resistance, is usually race non-specific. It is typically conditioned by additive or partially dominant genes (Wisser, Balint-Kurti et al. 2006).

Three are several important reasons for the interest in quantitative disease resistance in maize. First, maize has been a good system for disease resistance studies (Balint-Kurti and Johal 2009). Maize is an important crop and any findings in maize can be directly applied to crop improvement efforts and also provide the framework to increase disease resistance in other species (Balint-Kurti and Johal 2009). The majority of disease resistance developed in the elite maize lines by breeders is quantitative in nature (Wisser, Balint-Kurti et al. 2006). Second, quantitative disease resistance is more durable than qualitative resistance in that qualitative disease resistance mediated by a few genes can be easily overcome by mutation in the pathogen. Third, the availability of R genes is limited in necrotrophic pathosystems. The majority of economically important diseases in maize are caused by necrotrophic pathogens, and resistance to necrotrophic pathogens is mostly quantitative instead of qualitative in nature (Poland, Balint-

Kurti et al. 2009). Qualitative disease resistance usually relies on localized cell death at the point of pathogen penetration and is beneficial to necrotrophic pathogens.

Despite the differences between qualitative and quantitative disease resistance, there is a great deal of gray area between the extremes (Poland, Balint-Kurti et al. 2009). Several studies have questioned whether the loci conditioning the two types of resistance are distinct. For example, Kamoun et al. (1999) found that plant resistance to oomycete plant pathogens, usually associated with the hypersensitive response – a programmed cell death response, is related to all forms of resistance to oomycete pathogens. (Kamoun, Huitema et al. 1999) suggested that there can be some overlap in the genetic mechanisms underlying quantitative and qualitative disease resistance. Considering the wide range of microbial pathogenesis strategies and the corresponding host-defense strategies, Poland et al. (2009) proposed four mechanisms underlying disease-associated QTL (dQTL): 1) genes regulating morphological and developmental phenotypes; 2) pattern-recognition receptors acting in basal defense; 3) component of chemical warfare; 4) defense signal transduction; 5) a weak form of R-genes; 6) a unique set of previously unidentified genes.

Hundreds of QTL have been identified in maize for various diseases. A synthesis of fifty published studies by Wisser et al. (2006) reported 437 dQTL in maize. Wisser et al (2006) found that all the ten maize chromosomes harbor disease-associated QTL, and the confidence intervals of those QTL covered 89% of the maize genome. The distribution of disease-associated QTL is non-random: clusters of resistance loci were identified. With improvement in marker technologies available and the increasing ease of fine-mapping, significant progress in the cloning of genes controlling quantitative disease resistance has been made. Several genes that conditioning quantitative resistance had been cloned in maize: *ZmWAK* is the causal gene

underlying a head smut resistance QTL (Zuo, Chao et al. 2015). Head smut is caused by the fungal pathogen *Sphacelotheca reiliana* (Zuo, Chao et al. 2015). *Htn1*, which confers resistance to NCLB, was cloned and encoded a wall-associated receptor-like kinase (Hurni, Scheuermann et al. 2015).

Multiple disease resistance in maize

There is more than one definition of multiple disease resistance (MDR). MDR can refer to a host plant resistant to two or more diseases or to a gene or allele that confers resistance to more than one pathogen (Zwonitzer, Coles et al. 2010, Wiesner-Hanks and Nelson 2016). An inbred line or variety that has relatively high levels of resistance to two or more diseases is referred to as an MDR line or variety. Multiple disease resistance is under positive selection, and genetic variation conferring resistance to multiple diseases is thus hypothesized to exist naturally. MDR can be mediated by different genetic mechanisms. Wiesner-Hanks and Nelson (2016) proposed four genetic scenarios for MDR in plants: 1) several single disease-associated genes or loci stacked in a single plant genotype, in which genes and loci can be far apart; 2) several single disease-associated genes or loci that are physically linked so that they are always inherited together as a unit from one generation to the next (in linkage disequilibrium); 3) a single gene or locus that confers resistance to multiple diseases with comparable effect sizes; and 4) a single gene or loci that is related to multiple pathogens but with different effect sizes. The third scenario is referred to as even pleiotropy and the fourth scenario is referred to as uneven pleiotropy.

Similar to tightly linked QTL causing positive correlation between diseases, QTLs that are linked in repulsion can cause the negative correlation between diseases, causing opposite effects for different diseases (Wiesner-Hanks and Nelson 2016). Take wheat as an example: the

Sr2 gene is a race non-specific wheat stem rust resistance gene (Hare and RA 1979, Spielmeyer, Sharp et al. 2003) and *Fhb1* is the largest resistance QTL for fusarium head blight (Pumphrey, Bernardo et al. 2007). However, *Sr2* and *Fhb1* are not found together in cultivars because they are linked in repulsion on chromosome 3BS. With the assistance of DNA markers, recombination was detected between *Sr2* and *Fhb1* to couple them in a winter wheat population (Flemmig 2012).

Several studies have identified regions associated with resistance to multiple diseases in maize. The co-localization of dQTL can imply a common genetic basis for MDR. In 1995, McMullen and Simcox (1995) reviewed a number of studies and placed the disease and pest resistance QTL in chromosomal bins. Chromosomal bins are used to describe the map position of QTL or genes. The maize genome was divided into 100 bins, with each chromosome divided into segments of approximately 20 centimorgans each (Gardiner, Coe et al. 1993, Davis, McMullen et al. 1999). By summarizing the dQTL, McMullen and Simcox (1995) found that many of the QTL mapped to the same chromosomal bin locations. All ten chromosomes contained dQTL. The distribution did not fit a random model, and dQTL clustered on all chromosomes, with the exception of chromosomes 7 and 9. Clusters of dQTL in bins 3.04 and 6.01 were tightly linked while other clusters were distributed over 20 to 40 cM regions. On chromosome 3, bins 3.04 and bin 3.05 contain dQTL associated with Fusarium stalk rot and European corn borer and single factor genes for resistance to common rust, wheat streak mosaic virus, and maize mosaic virus. Additional potential resistance gene clusters were reported on chromosomes 1 and 9, where dQTL for resistance to *S. turcica* and leaf-feeding of various lepidopteran insect species (Welz and Geiger 2000).

As more dQTL were identified, additional disease resistance associated clusters were identified. Eleven years later, Wisser et al. (2006) synthesized fifty dQTL publications in maize for 11 different diseases. A total of 437 dQTL, 17 resistance genes and 25 R-gene analogs were reported and placed on a single consensus map. On every chromosome there was co-localization of dQTL for at least two different diseases. Bin 3.04 to 3.05, which had been previously identified as a potential MDR region (McMullen and Simcox 1995), was associated with six diseases. In the region from 315 to 375 cM on chromosomal 2 contained nine of the 11 dQTL; a 50 cM chromosomal region from 450 to 500 cM on chromosome 4 had co-localized dQTL for eight diseases. However, the MDR chromosomal regions from these two synthesis studies were based on different mapping studies using different mapping populations. Different populations have different parental alleles, which constrains the usefulness of the identified MDR regions.

Wisser et al. (2011) screened a diverse maize panel with 253 inbred lines representing much of the global variation among maize inbred lines for three fungal foliar diseases: GLS, NCLB and SCLB. The three fungal pathogens are all in the Dothideomycetes taxonomic class and share characteristics of pathogenesis. High positive (> 0.5) genetic correlations were detected between all pairwise disease combinations. Lines with relatively high resistance or susceptibility to all the three diseases were identified. Select MDR and MDS lines were used as the parental founder lines for chromosome segment substitution line (CSSL) populations. Those CSSL populations, where the recurrent parent was a susceptible and the donor resistant, were used to isolate the loci conferring MDR effects (Lopez-Zuniga, Wolters et al. 2019).

Lopez-Zuniga et al. (2019) mapped resistance to three fungal diseases, SCLB, NCLB, and GLS in eight CSSL populations to identify loci that are associated with multiple diseases. The goal of the study was to confirm the findings from the association mapping and build a

resource useful for understanding multiple disease resistance. A total of eight $BC_3F_{4:5}$ CSSL populations comprising 1,611 lines were developed and evaluated for the three fungal diseases. Those populations are in the same genetic background; thus, four populations share the same recurrent parent Oh7B and the other four populations share the recurrent parent H100. In each population, lines were identified that were significantly more resistant than the recurrent parent for all the diseases. QTL associated with multiple diseases were identified: six QTL were associated with all the three diseases; two QTL were associated with resistance to SCLB and NCLB; seven were related to SCLB and GLS and two QTL for NCLB and GLS. However, due to the marker density and the constraints of the mapping population design, it is not clear if the clustering of QTL in the same chromosomal regions is due to single pleiotropic genes or closely linked.

Individual genes that mediate pleiotropic effects for different pathogens have been identified. The maize panel screened by Wisser et al. (Wisser, Kolkman et al. 2011) shows rapidly decaying linkage disequilibrium within 1 or 2 kb, which is less than the average length of a maize gene, suggests that associated SNPs are caused by variants at specific genes. A glutathione S-transferase (GST) gene on chromosome 7 was found to be associated with modest levels of resistance to all three diseases. Another study from Yang et al. (Yang, He et al. 2017) identified a gene, *ZmCCoAOMT2,* on chromosome 9, which encodes a maize caffeoyl-CoA O-methyltransferase, that underlies the MDR QTL *qMdr9.02*. *qMdr9.02* confers effects for three major maize foliar diseases: NCLB, SCLB and GLS, and *ZmCCoAOMT2* was found to confer quantitative resistance to both SCLB and GLS. The gene accounts for 10% and 6% of the variation of SCLB and GLS, respectively.

Most of the MDR studies focus exclusively on fungal diseases. The shared genetic architecture of fungal and bacterial diseases remains largely unknown. Only a few studies have identified clusters of resistance QTL for both bacterial and fungal diseases in the same genetic background. Chung et al. (2010) used near-isogenic line (NIL) populations to dissect resistance to fungal and bacterial diseases. Selected NILs with QTL associated to the resistance for NCLB were evaluated for anthracnose stalk rot, common rust, common smut and Stewart's wilt, in which Stewart's wilt is a bacterial disease. The results suggested that both $qNLB1.02_{B73}$ and $qNLB1.06_{Tx303}$ were also effective against Stewart's wilt. $qNLB1.02_{B73}$ was also effective against common rust. In a following up study (Jamann, Poland et al. 2014), high-resolution mapping populations were constructed for $qNLB1.06_{Tx303}$ and a candidate gene *pan1* was found to be a susceptibility gene for NCLB and Stewart's wilt. In another study from Jamann et al. (2016), $qNLB1.02_{B73}$ was fine-mapped to a 243-kb interval with four positional candidate genes, and two candidate genes (F-box gene and ZmREM6.3) were found to be highly expressed in the resistant line. A role of *ZmREM6.3* in quantitative disease resistance was suggested in this study.

Future directions

Emerging maize disease can develop into unexpected and very serious pandemic. SCLB caused by race T of *Cochliobolus heterostrophus*, with the aid of favorable climate conditions, developed into a great epidemic in 1970-1971, causing enormous damage in the corn belt in the United States. This SCLB epidemic was attributed to a new race of the fungus that is highly virulent to corn with T-type cytoplasm, which was extensively used in hybrid seed production (Tatum 1971). The evolution of crop pests is not rare and genetic homogeneity in a crop means increased vulnerability to pests. With little information about new diseases, like BLS, limited management options are available. Resistant cultivars are a good way to control new diseases,

and lines with quantitative disease resistance QTL have the advantage of being durable and less race specific. Thus, identifying dQTL conferring effects for BLS is crucial to prevent it from developing into a devastating epidemic.

Furthermore, relatively little is known about the relationship between bacterial and fungal resistance. For the most part, it is unknown whether the same alleles contribute resistance to both bacterial and fungal diseases. In order to increase our understanding of the mechanisms underlying resistance for pathogens from different kingdoms and also provide practical information for future disease-resistance breeding programs, we use introgression line populations with the same genetic background to identify chromosomal regions that are associated with fungal and bacterial disease resistance and evaluate their effects. This thesis will provide information on the genetic architecture of bacterial and fungal disease resistance in maize and the effects of the chromosomal segments on those diseases.

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CHAPTER 2: IDENTIFICATION OF QUANTITATIVE TRAIT LOCI ASSOCIATED WITH BACTERIAL LEAF STREAK OF MAIZE

ABSTRACT

Bacterial leaf streak (BLS), a foliar disease of maize (*Zea mays* L.) caused by *Xanthomonas vasicola* pv. *vasculorum*, recently emerged in the Americas as a disease of major importance. Little is known about the disease cycle, and consequently, management is difficult. No chemical control is available. Host resistance will likely play a major role in controlling the disease, but to date, no data regarding the resistance of maize germplasm to *X. vasicola* pv. *vasculorum* have been published. The objective of this study was to examine the genetic architecture of resistance to BLS. We conducted quantitative trait locus (QTL) mapping for BLS resistance in three maize populations: the Z022 (B73 / Oh43 recombinant inbred line) nested association mapping (NAM) population, the Z023 (B73 / Oh7B recombinant inbred line) NAM population, and the DRIL78 (NC344 / Oh7B chromosome segment substitution line) population. A total of five QTL were detected across two of the mapping populations. Of the QTL detected, one conferred a moderate effect, whereas the others conferred small effects. We also examined the relationship between resistance to BLS and resistance to three foliar diseases of maize, which had been mapped previously. The only significant correlation we found for BLS was with northern corn leaf blight [caused by *Exserohilum turcicum* (Pass.) K. J. Leonard & Suggs] in one of the populations, although two of the five BLS regions were involved in resistance to other diseases. These data will be useful for developing maize varieties resistant to BLS and also mitigating the impact of bacterial leaf streak on maize production.

INTRODUCTION

Bacterial leaf streak (BLS) of maize (*Zea mays* L.), caused by *Xanthomonas vasicola* pv. *vasculorum* (Cobb) comb. nov. (*Xvv*), recently emerged in the Americas, and little is known about host resistance to the disease (Damicone et al., 2018; Jamann et al., 2019; Korus et al., 2017; Leite et al., 2019; Plazas et al., 2018). Bacterial leaf streak was first reported in South Africa in 1949 (Dyer, 1949) and was limited to the African continent until recently. In 2010, researchers detected BLS symptoms in northern Argentina, and since that time, the disease has expanded from Córdoba Province to other provinces in Argentina (Plazas et al., 2018). It was suspected that BLS was present in Brazil for some time, and the disease was recently positively confirmed (Leite et al., 2019). In the United States, BLS was first reported in Nebraska in 2014 and subsequently was confirmed in Colorado and Kansas (Korus et al., 2017). After the initial reports of BLS in the United States, surveys were conducted in Oklahoma and Illinois, and the disease was found in both states (Damicone et al., 2018; Jamann et al., 2019). Currently, the pathogen range includes Colorado, Illinois, Iowa, Kansas, Minnesota, Nebraska, Oklahoma, South Dakota, and Texas (Damicone et al., 2018; Jamann et al., 2019; Lang et al., 2017).

The sudden spread of BLS in North and South America is likely the result of a more virulent novel strain of *Xvv* (Perez-Quintero et al., 2019). The population of *Xvv* that has emerged in North and South America appears to be the result of horizontal genomic acquisitions of multiple gene clusters by *Xvv*, which infects maize, from *X. vasicola* pv. *holcicola* (Xvh), which infects sorghum [*Sorghum bicolor* (L.) Moench] (Perez-Quintero et al., 2019). It is unclear if the genomic acquisitions are specifically responsible for the increase in virulence and spread of the pathogen, but all 22 isolates of *Xvv* from North and South America sequenced to date carry these gene clusters from *Xvh*, whereas the isolates of *Xvv* from South Africa lack these

regions from *Xvh* (Perez-Quintero et al., 2019). The isolates of *Xvv* from North and South America also cause larger lesions on corn than isolates of *Xvv* from South Africa (Perez-Quintero et al., 2019).

Initial research on the disease ecology of BLS in North America reported irrigation use, multiple years of corn in a crop rotation, the corn plant being at the V7 to R2 growth stage (Abendroth et al., 2011) and planting after 2 May as the most important predictors for *Xvv* incidence (Hartman 2018). Corn planted into a previous year's corn residue is at greater risk for the disease, as the pathogen is able to survive in infected corn residue from the previous season (Ortiz-Castro et al., 2018). The disease is often most severe and frequently observed in irrigated fields (Damicone et al., 2018; Hartman 2018) where consistent moisture results in infections from inoculum that survives in residue. The V7 to R2 growth stage is also a good predictor of disease, probably as a result of secondary spread associated with rain and wind dispersal resulting in severe BLS symptoms being observed in both irrigated and nonirrigated fields mid-season (Broders 2017; Ortiz-Castro et al., 2018). Moisture also seems to be important for *Xvv* to enter the plant. In preliminary experiments, *Xvv* cells were shown to cluster around the stomata of corn leaves and appeared to both enter and exit through stomata (Ortiz-Castro et al., 2018). In the same study, green fluorescent protein (GFP)-transformed strains of *Xvv* were found in the intercellular space and not in the vascular tissue. These data support previous inoculation experiments (Korus et al., 2017; Lang et al., 2017; Perez-Quintero et al., 2019; Plazas et al., 2018) that only observed foliar symptoms caused by all evaluated maize-infecting *Xvv* from North and South America. Although the sugarcane (*Saccharum* spp.)-infecting strain does cause vascular infections of maize, recent phylogenomic assessment of the species *X. vasicola* has

determined that the maize-infecting strains of *Xvv* represent a distinct genetic lineage from those infecting sugarcane (Perez-Quintero et al., 2019; Studholme et al., 2019).

Once *Xvv* has successfully entered the leaf, initial symptoms develop as water-soaked lesions and expand into narrow stripe-shaped, 2- to 3-mm-wide lesions on infected leaves that can vary from yellow to brown. Lesions are parallel to leaf veins and are constricted to interveinal spaces. Under high-humidity conditions, yellow droplets of bacterial exudate form on infected areas of the leaf (Coutinho and Wallis, 1991). Korus et al. (2017) reported that lesions can cover 40 to 50% of the leaf area. Because leaf area becomes necrotic, photosynthetic capacity of the plant is compromised in severe infections, and yield losses are likely. However, the true impact of the disease on yield has not yet been successfully quantified.

Successful management of BLS has remained elusive as there are no chemical controls, and initial inoculum continues to increase in no-till corn–corn, corn–sorghum–corn, or corn–soybean [*Glycine max* (L.) Merr.]–corn rotations, which currently cover large numbers of acres in Colorado, Illinois, Iowa, and Nebraska. As with other similar bacterial pathosystems [e.g., Goss's bacterial wilt and leaf blight caused by *Clavibacter michiganensis* (Smith) Davis et al. subsp. *nebraskensis* (Vidaver & Mandel) Davis et al.], it is likely that host resistance will play a major role in controlling the disease. Bacterial leaf streak infects a wide variety of cultivars (Lang et al., 2017; Leite et al., 2019). Symptom type and severity vary among hybrids (Leite et al., 2019; Munkvold and White 2016), indicating there may be genetic control of the plant response to the disease. To date, no studies have been published on the genetic architecture of resistance to BLS, which would facilitate the development of resistant varieties. Understanding the genetic basis of BLS resistance is crucial for controlling the disease.

Several genes have been implicated in multiple disease resistance (MDR) in maize (Jamann et al., 2014; Wisser et al., 2011; Yang et al., 2017). Multiple disease resistance is defined as host resistance to two or more diseases, or a gene or allele that confers resistance to two or more diseases (Wiesner-Hanks and Nelson, 2016). In maize, foliar disease phenotypes are typically significantly correlated within populations, and regions conferring resistance to multiple diseases have been described (Balint-Kurti et al., 2010; Belcher et al., 2012; Chung et al., 2011; Jamann et al., 2016; Lopez-Zuniga et al., 2019; Zwonitzer et al., 2010). Hu et al. (2018) identified a locus for resistance to the Goss's bacterial wilt and the leaf blight pathogen *Clavibacter michiganensis* subsp. *nebraskensis* that was associated with the resistant locus *rp1* to *Puccinia sorghi*, which causes common rust of corn. Consequently, we hypothesized that regions important for resistance to BLS might also be important for other diseases.

Maize is a model for quantitative genetics, and multiple genetic mapping populations are readily available (Wallace et al., 2014). The nested association mapping (NAM) population consists of 25 families of recombinant inbred lines (RILs), where each RIL family was developed by crossing a line chosen to maximize diversity to the inbred line B73 (Buckler et al., 2009; McMullen et al., 2009). The NAM was designed to enable high-power and high-resolution mapping through joint linkage-association analysis (McMullen et al., 2009). The NAM has been used to map resistance to numerous quantitative traits, including resistance to gray leaf spot (GLS, caused by *Cercospora zeae-maydis* Tehon & E. Y. Daniels) (Benson et al., 2015), northern corn leaf blight [NCLB; caused by *Exserohilum turcicum* (Pass.) K. J. Leonard & Suggs] (Poland et al., 2011), and southern corn leaf blight [SCLB; *Bipolaris maydis* (Y. Nisik. & C. Miyake) Shoemaker] (Kump et al., 2011). Three related populations were selected to screen for BLS based on a preliminary evaluation of the NAM parental lines, and the MDR of NC344.

We selected two NAM populations, Z022 (B73 / Oh43) and Z023 (B73 / Oh7B), to screen for their reaction to BLS. Additionally, we screened a complimentary chromosome segment substitution line (CSSL) population, referred to as the disease resistance introgression line 78 (DRIL78) population (NC344 / Oh7B) (Lopez-Zuniga et al., 2019). The populations allowed us to investigate the genetic architecture of resistance to BLS in maize and the relationship between multiple foliar diseases.

Our objectives in this study were (i) to identify quantitative trait loci (QTL) for BLS resistance in the two NAM populations, Z022 and Z023, (ii) to identify QTL underlying BLS resistance in the DRIL78 population, and (iii) to compare resistance to BLS with resistance to other common foliar diseases that occur in the United States, including GLS, SCLB, and NCLB.

MATERIALS AND METHODS

Plant materials

The NAM parents and three mapping populations were evaluated for BLS severity. We did a preliminary experiment to screen the 27 parental lines of the NAM population and selected the Z022 (B73 / Oh43) and Z023 (B73 / Oh7B) populations for further evaluation based on this experiment (Table 1) (Buckler et al., 2009; McMullen et al., 2009; Yu et al., 2008). Both NAM populations are S5 RILs. Seeds of the NAM parents were obtained from the US National Germplasm System, whereas seeds of the RIL populations were obtained from Dr. Paul Scott at Iowa State University. Additionally, DRIL78, a CSSL population with donor introgressions spanning the entire genome, was evaluated. The population was developed by crossing NC344, an MDR line, to Oh7B, a multiple disease susceptible line (Wisser et al., 2011). The nomenclature for the population is as follows: disease resistance introgression line (DRIL) 7

(code for NC344), 8 (code for Oh7B). Lopez-Zuniga et al. (2019) developed the population by crossing NC344 to Oh7B, and the progeny were backcrossed to the recurrent parent (Oh7B) for three generations, followed by four subsequent generations of self-pollination. Seeds of the DRIL78 population were obtained from Dr. Randall Wisser at the University of Delaware.

Experimental design

To evaluate more populations, screenings were conducted in Iowa and Illinois. The NAM populations were evaluated in Iowa, and the DRIL population was evaluated in Illinois. For the Iowa evaluation of the NAM parents and the Z022 and Z023 populations, plants (4 plants per pot) were grown in 946-mL plastic pots filled with a soil mix of peat moss–metro mix–coarse perlite (4:3:4) and placed in a growth chamber at 25 Celsius with a 16-h photoperiod in the Department of Plant Pathology and Microbiology at Iowa State University. All 27 NAM founder lines, 186 lines of the Z022 population, and 182 lines of the Z023 population were evaluated. The plants were fertilized once a week with 200 mL per pot of liquid fertilizer (15–5–15 N–P–K, Miracle-Gro, The Scotts Company), starting 2 wk after planting. Pots (1 pot per line) were arranged in a randomized complete block design, and two independent experimental runs were done for each population tested. A sweet corn variety, NK199, and hybrid variety, DKC 62-08, that both had been observed as susceptible to BLS in the field were used as susceptible controls.

The DRIL78 population was evaluated in the greenhouse at the Plant Care Facility at the University of Illinois at Urbana-Champaign in 2017 to 2018. The room was maintained at 24.5 to 26.5 Celsius during the day and 21 to 23 Celsius at night. Supplemental lighting was provided for 15 h d^{-1}. Seeds were planted in general purpose potting mix (1 soil/1 peat/1 perlite) in a 3.77 L (1-gallon) pot with one plant per pot. Each pot was amended with 8 g Osmocote (15–9–12 N–P–K, Everris). Three seeds were planted per pot and seedlings were thinned to one plant per pot.

Due to limited space, an augmented design was used for the pot arrangement (Federer, 1961). A total of three replications were evaluated, with six blocks arranged in 16 rows and 18 columns per replication. Each replication was run as an independent greenhouse experiment planted on a different date. Included in each replication of the DRIL78 screening were 287 experimental units, including 233 entry lines and nine check lines that were replicated in all six blocks. Check lines were selected based on preliminary screenings and included the two parental lines (NC344 and Oh7B), four BLS-susceptible lines (CML333, M162W, Ky21, and M37W), and three BLS-resistant lines (Mo18W, CML103, and Hp301). Lines were randomized within blocks using the R (version 3.5.1) package "Agricolae" (De Mendiburu, 2014; R Core Team, 2018).

Inoculation and disease evaluation

Somewhat different inoculation and disease evaluation methods were used for the experiments conducted in Iowa and Illinois. For the NAM parents evaluated in Iowa and NAM RIL populations, we modified and optimized an inoculation method reported by Lang et al. (2017). We used *Xvv* strain X14, which was recovered from BLS symptomatic corn leaves collected from a commercial corn field in Iowa. To prepare inoculum, a single colony from a culture of the bacterium on glucose yeast extract (GYE) agar (20 g liter–1 glucose, 10 g liter–1 yeast extract, 20 g liter–1 bacto agar) was grown in 10 mL of GYE broth at 200 rpm at 25 Celsius overnight. Cells of X14 were collected by centrifugation at 5000*g* for 5 min and resuspended in sterile water, and then the inoculum suspension was adjusted to an optical density at 600 nm (OD_{600}) of 0.01. Inoculation occurred when the plants were at the V3 developmental stage (Abendroth et al., 2011). Approximately 0.05 mL of inoculum was infiltrated into of the third leaf of each plant, on both sides of the main vein, 7.5 cm from the leaf tip. Thus, each plant had two lesions. The tip of a 1-mL syringe (with no needle attached) was placed against the

abaxial side, a finger on the adaxial side was used to apply gentle pressure, and the inoculum suspension was gently introduced into the leaf tissue through the stomata (Schaad et al., 1996). Immediately after inoculation, the area of infiltration was delineated with a permanent marker. Inoculated plants were returned to the growth chamber at 25 Celsius with a 16-h photoperiod. Seven days after inoculation, the total length of the third leaf and the total length of any lesion that developed in association with the point of inoculation on the third leaf were measured.

We evaluated the DRIL78 population in Illinois and used a strain of *Xvv*, 16Xvv4.1, that was isolated from diseased leaf material collected in DeKalb County, Illinois, in 2016 (Jamann et al., 2019). A local strain was used because of biosafety considerations. After isolation, the isolate was stored in 30% glycerol at −80 Celsius. The strain was cultured for 2 to 3 d at 28 Celsius in nutrient broth. On the day of inoculation, the cells were suspended in 0.1 M NaCl, and the bacterial suspension was adjusted to an optical density at 600 nm of 0.20 (OD_{600}) using a spectrophotometer. Inoculations were performed at the V4 to V5 stage by cutting the tip of the smallest emerging leaf from the whorl with scissors dipped in the bacterial suspension. After inoculation, the plants were placed in a mist chamber for 18 h. Lesion length was measured from the cut site to the furthest point of the lesion three times post inoculation, at 7, 14, and 21 d after inoculation. Leaf length measurements were not taken. Because of the larger pot size and greenhouse instead of growth chamber conditions used for the NAM populations, plants were scored for more time points for the DRIL population.

Phenotypic data analysis

For the NAM RIL populations, the severity of BLS observed at 7 d after inoculation was calculated as the percentage of total leaf length blighted. The area under disease progress curve (AUDPC) was not calculated for these populations because there was only one data point.

However, we took multiple lesion length measurements for the DRIL78 population, and those were used to calculate AUDPC scores using the following formula:

$$A_k = \sum_{i=1}^{n-1} \frac{(y_i + y_{i+1})(t_{i+1} + t_i)}{2}$$

where y_i refers to individual disease scores, t_i equals time between ratings, and n represents the number of measurements (Wilcoxson et al., 1974). The function "audpc" from R (version 3.5.1) package "Agricolae" was used to calculate the AUDPC scores based on the lesion length measurements using the absolute method (De Mendiburu 2014; R Core Team 2018). The data were not normally distributed, so AUDPC scores were transformed using the one-parameter Box–Cox transformation function in the "MASS" package in R (R Core Team 2018; Ripley et al., 2018). The power parameter lambda value was 0.5858586 and was applied to calculate the transformed AUDPC data. All the subsequent analyses for DRIL78 were performed using the transformed AUDPC data.

For each line, a best linear unbiased predictor (BLUP) was estimated using a model that included the transformed AUDPC data and the significant factors for each population (Supplemental File S1). Significant factors were identified for each population by fitting factors using the lmer() function from the lme4 package in R (version 3.5.1) and determining which factors were significant in the model (Bates et al., 2015; R Core Team 2018). The final model for the Z022 population included genotype and plant (nested within run); for the Z023 population, genotype and lesion (nested within plant and run) were included in the final model; for the DRIL78 population, the final model included genotype, replication, and block nested in replication (Table 2). All factors included in the final models were fitted as random factors.

Pearson's product-moment correlation coefficients for AUDPC between the three different replications of the DRIL78 population were obtained using the R (version 3.5.1) function "cor.test" (R Core Team 2018). SAS software (version 9.4) was used to calculate the heritability for the DRIL78 population. The analysis was done using the PROC MIXED procedure according to Holland et al. (2010). Both the heritability on an individual plot basis and on a line-mean basis were calculated. Due to the limited amount of seed of the NAM RIL populations, those populations were only screened twice, and consequently, a heritability index was not calculated.

Multiple disease resistance data analysis

We examined the correlation between BLS, NCLB, SCLB, and GLS phenotypes for the two NAM RIL populations using phenotypic data reported herein for BLS, and data previously reported for NCLB, SCLB, and GLS (Benson et al., 2015; Kump et al., 2011; Poland et al., 2011). The SCLB index scale used by Kump et al. (2011) was reversed to make it compatible with the other three disease scales, where a higher value indicates a more susceptible line. Similarly, we examined the correlations between BLS, NCLB, SCLB, and GLS phenotypes in the DRIL78 population using data reported in Lopez-Zuniga et al. (2019). Only lines with phenotypes for all the diseases were included in the analysis. For GLS, NCLB, and SCLB lower phenotypic scores indicated more disease and higher phenotypic scores indicated less disease (Lopez-Zuniga et al., 2019). The scale for BLS was different, with lower scores representing less disease on the leaf and higher scores indicating more disease. Consequently, the BLUP values for BLS were inverted by adding a negative sign so that the scales for all the four diseases were consistent. Pearson's product-moment correlation coefficients between diseases were calculated using the R function "cor.test" (version 3.5.1) (R Core Team 2018).

QTL mapping

For the two NAM RIL populations, 7386 single nucleotide polymorphism (SNP) markers (the 0.2 cM resolution linkage map) (Ogut et al., 2015), along with the BLUPs, were used for mapping. Ogut et al. (2015) generated the markers using genotyping-by-sequencing, filtered to a high-quality set of SNP markers, and used the markers to calculate a genetic map. Markers are publicly available at www.panzea.org. A total of 172 individuals in the NAM Z022 population and 163 individuals in NAM Z023 population were included in the analysis. The QTL analysis was conducted using the "qtl" package in R (version 3.5.1) (Broman et al., 2003). The "multiple imputation" interval mapping method was used in this study, using imputations with a step size of 1 and 0.005 error probability. Significance thresholds were determined by performing 1000 permutations for each population and an experiment-wide error rate of 0.10. The interval estimate of the location of the QTL was obtained by Bayes credible interval method using the "bayesint" function. Intervals were expanded to the nearest flanking markers. The percentage variance explained by the QTL and the QTL effect sizes were estimated using the "fitqtl" function.

A total of 194 lines from the DRIL78 population, including the recurrent parent, were used for analysis. The inclusion of the recurrent parent improves mapping power. A total of 241 genotyped SNPs with map locations based on the IBM4 map were used for mapping (Fu et al., 2006; Lopez-Zuniga et al., 2019). The genotypic dataset was generated using the Pioneer Illumina publicplex platform using 765 SNP markers and is available as supplemental data from Lopez-Zuniga et al. (2019). The DRIL78 BLUPs were used as the phenotypic dataset. The QTL analysis was performed using ICIMapping 4.0.6.0 and the "CSL: mapping of additive and digenic epistasis genes with chromosome segment substitution lines" function was used (Meng et

al., 2015). To control multicollinearity, the "by condition number" parameter was set as 1000. The "RSTEP-LRT-ADD" mapping method was used for QTL mapping, which enabled a likelihood ratio test based on stepwise regression analysis for determining the most significant markers (Meng et al., 2015). A total of 1000 permutations were conducted to determine the logarithm of odds (LOD) threshold representing an experiment-wide error rate of 0.10. Markers with LOD scores above the permutated threshold are reported as significant markers. Physical marker positions are based on the B73 RefGen_v3 sequence (Schnable et al., 2009).

RESULTS

Phenotypic distributions

We conducted a pre-experiment to determine which populations to evaluate for BLS. The mean percentage leaf blight on the 27 NAM parent lines ranged from 13.5% for Ki11 to 92.4% for Oh7B (Table 1). The common NAM parent B73, placed among the top five most resistant lines, with 19.8% of the leaf length blighted. The NAM parent lines Oh43 and Oh7B had the least resistance to *Xvv* of all lines tested. Consequently, RIL subpopulations Z022 (B73 X Oh43) and Z023 (B73 X Oh7B) were selected for further analysis. We evaluated the DRIL78 population because we knew that Oh7B was susceptible to BLS and NC344 had resistance to other common foliar diseases (Wisser et al., 2011), and we conjectured that it would also be resistant to BLS. To maximize the number of populations evaluated, the NAM populations were evaluated in Iowa and the DRIL population was evaluated in Illinois with slightly different methods and a different *Xvv* strain.

The populations showed a wide range of disease phenotypes. All three populations showed transgressive segregation (Figure 1), regardless of the inoculation method. In the Z022

population, the mean percentage of leaf blighted by the pathogen was 44.4% and ranged from 5.00 to 100%. In the Z023 population, the mean percentage of leaf blight was 56.9% and ranged from 0.80 to 100%. The BLUPs of the Z022 population exhibited a bell-shaped distribution (Figure 1). The two parental lines for the Z022 NAM population were symmetrically located, with the resistant parent B73 having a BLUP value of 24.7 and the susceptible parent Oh43 having a value of 64.5 (Figure 1). The BLUP values of the Z023 RIL population were skewed toward susceptibility (Figure 1). The DRIL78 population showed a wide range of lesion lengths, from 0.10 to 57.6 cm.

In all the three populations, genotype accounted for the highest proportion of the variance (Table 2). The heritability on an individual plot basis for the DRIL78 population was 0.231, and the heritability on a line–mean basis was 0.417. The correlations between the three replications, where each replication was a separate run, were all highly significant based on Pearson's correlation test, with correlations ranging from 0.23 to 0.30. Heritability was not calculated for the Z022 and Z023 populations, as there were only two replications. Pearson's correlation coefficients show the positive relationship between the two runs of each NAM population, with the value being 0.23 for Z022 and 0.29 for Z023.

Linkage mapping

A total of five significant QTL were identified across the three mapping populations (Table 3), with one QTL identified in the Z023 population, and four QTL identified in the DRIL78 population. No significant QTL were identified in the Z022 population. The largest effect QTL was located on chromosome 5 in the Z023 population, with a LOD score of 7.44, and accounted for 18.9% of the variation in the population. The other QTL, all identified in the DRIL78 population, were of smaller effect, with a maximum of 6.7% of the variance explained.

Both the resistant and susceptible parents were found to carry resistant alleles in the DRIL78 population. In the Z023 population, the allele from the resistant parent B73 conferred resistance for the chromosome 5 QTL.

We tested the effect of the phenotypic measurement parameter, lesion length, or percentage of leaf length blighted on our mapping results. Using the NAM populations for which both phenotypic measures were available, we conducted QTL mapping using BLUPs calculated based on lesion length data and compared it with the results generated using percentage of leaf length blighted. We did not identify any significant QTL using lesion length, indicating that percentage of lesion length blighted was a better phenotypic measure for either these populations, or for the method used to screen these populations (infiltration of *Xvv* into the third leaf of V4 plants). We did not collect percentage of leaf length blighted for the DRIL78 population.

Relationship between resistance to bacterial leaf streak and resistance to other foliar diseases

Pearson's correlation coefficients were calculated among BLS, SCLB, NCLB, and GLS in the NAM and DRIL78 populations (Table 4). In the NAM populations, the three fungal diseases were highly positively correlated, with correlation coefficients ranging from 0.460 to 0.621 ($P < 0.0001$), but BLS was not significantly correlated with any of the fungal diseases ($P > 0.05$). In the DRIL78 population, positive correlations between NCLB and SCLB (coefficient = 0.158, $P = 0.029$) and between NCLB and GLS (coefficient = 0.152, $P = 0.037$) were detected. Bacterial leaf streak was positively correlated with NCLB (coefficient = 0.235, $P = 0.001$), indicating that lines that are resistant to NCLB tend to be resistant to BLS as well. A strong relationship was not detected between resistance to BLS and SCLB or GLS.

To further examine the relationships among BLS, NCLB, SCLB, and GLS, we compared QTL locations for the four diseases. The same marker (PZA01187-1) on chromosome 4 that was significant for BLS in DRIL78 was also significant for SCLB in the same population (Lopez-Zuniga et al., 2019). For both diseases, the NC344 allele increases resistance and the percentages of variance explained by this QTL were similar (5.11% for BLS and 5.81% for SCLB). The Z023 chromosome 5 QTL region included significant genome-wide association mapping associations for SCLB (Kump et al., 2011) and NCLB (Poland et al., 2011) in the NAM.

DISCUSSION

This is the first study to evaluate maize germplasm for resistance to *Xvv*. To increase the number of populations evaluated, we conducted disease screenings in two states. Due to local constraints, we used different strains and slightly different methods at each location. It is important to note that we observed transgressive segregation in all populations, regardless of inoculation method. Although different strains were used to screen different populations, no significant differences in genetic diversity or virulence among North American isolates of *Xvv* have been identified (Perez-Quintero et al., 2019), so we do not expect differences between data from the two states to be due to the pathogen isolates used. There are no standardized methods for BLS evaluation, as this is a recently emerged disease. Lang et al. (2017) compared leaf infiltration with stab injection into stems and found that disease symptoms were less variable with leaf infiltrations. Previous studies have not examined cut assays. The bacterium enters through natural openings and wounds and then moves in the intercellular space and does not enter the vasculature (Ortiz-Castro et al., 2018). Although one inoculation method introduces bacteria to the vasculature (cut) and the other does not (infiltration), both methods are forcibly introducing the bacteria into the leaf tissue, and similar phenotypic rankings are expected. We

found similar correlations between replications for both inoculation methods, indicating similar reproducibility between the two inoculation methods. It is possible that different resistance mechanisms are at play between cut and infiltration inoculations, and this may help explain the lack of overlap between NAM and DRIL QTL.

Using three mapping populations, we identified five significant QTL across five chromosomes in two populations. The Z022 QTL on chromosome 5 explained 18.9% of the phenotypic variation in the population and was the largest effect QTL we identified. This QTL could be useful for marker-assisted selection. The DRIL78 QTL located on chromosomes 2 and 4 had negative additive values, indicating that the resistant donor (NC344) alleles increased resistance, whereas for the other two QTL, the allele from the resistant parent increased susceptibility. It is common for a resistant parent to contribute susceptibility alleles or for favorable alleles to be conferred by lines with unfavorable phenotypes (Balint-Kurti et al., 2007; Tanksley et al., 1996). We examined whether any of the identified regions harbored genes that might be expected to confer resistance to this disease but did not identify any strong candidate genes. Although transgressive segregation was detected in all three populations, no significant QTL were detected in the Z022 population. Two possible reasons could explain this: (i) the phenotypic difference between the parental lines in the Z022 population was smaller than that of the Z023 population, and (ii) BLS resistance is a polygenic trait, and we did not have sufficient power to detect small effects in this population. Overall, resistance to BLS appears to be controlled by multiple loci, and we detected several QTL with minor to moderate effect sizes. This is similar to the genetic architecture of resistance to many other diseases in maize (Wisser et al., 2006).

It is interesting to note that we did not identify any QTL in common across all three populations. Several explanations are possible: (i) only the parents of one of the populations segregate for resistant and susceptible alleles, so QTL are not identified in the other populations; (ii) QTL conferred small effects and due to inherent differences among populations, and low power LOD scores may have remained below the threshold for detection in other populations; (iii) low marker coverage may have impeded QTL identification in the DRIL78 population; and (iv) populations were evaluated in different environments using different techniques. Using the same environmental conditions, inoculation method, and phenotyping method may result in overlap in QTL findings. We did not want to contribute to the spread of this pathogen, so we conducted all of our screenings in controlled conditions. It would be interesting to compare mapping results in field conditions with those in controlled conditions.

Phenotyping method can affect mapping results, so we examined how the two different phenotypic measurement parameters, lesion length and percentage of leaf length with lesions, influenced mapping results. For the NAM populations, percentage of leaf length covered by lesion was a more robust phenotype. We detected QTL using lesion length percentage, but not using lesion length. Further testing is required to determine the interaction between inoculation method and phenotypic measurement parameter. It is possible we may have obtained different QTL mapping results if we had used percentage lesion length as the phenotype for the DRIL78 population. Nevertheless, we did identify four QTL for this population, more than for the other two populations combined. This suggests that both phenotypic measurement parameters are valid.

We examined the relationship between BLS and other common foliar diseases in the United States to determine whether lines resistant to other diseases with known genetic

architectures and confirmed resistance genes were also resistant to BLS. High positive correlations for the three foliar fungal diseases (NCLB, SCLB, and GLS) have been shown previously, indicating that lines resistant to one of these diseases tend to be resistant to the other diseases as well (Wisser et al., 2011). No significant correlations were found with our BLS data from the NAM populations we screened. For the DRIL78 population, resistance to BLS was significantly positively correlated with resistance with NCLB (coefficient = 0.235, $P = 0.001$), but not SCLB or GLS. It is common for different populations to have different correlations for similar diseases, and this may result from the different genetic structure of the populations. In maize, the relationship between fungal and bacterial diseases has been explored. Previously, Cooper et al. (2018) observed a significant correlation among Goss's bacterial wilt, leaf blight, and NCLB, but not among Goss's bacterial wilt, leaf blight, and GLS or SCLB. The lack of correlation between disease phenotypes may be due to different diseases being controlled by different genetic mechanisms.

We examined whether any regions that confer resistance to BLS also conferred resistance to NCLB, SCLB, or GLS. Two MDR regions were identified, one in the Z023 population, and the other in the DRIL78 population. In the Z023 population, the confidence interval for the BLS QTL encompassed significant genome-wide association mapping associations for resistance to SCLB and NCLB in the NAM populations (Kump et al., 2011; Poland et al., 2011). The 4.07 region identified for BLS resistance in the DRIL78 population overlaps with a region that has also been identified for SCLB resistance in this same population (Lopez-Zuniga et al., 2019). For both diseases, the NC344 allele confers resistance. Interestingly, however, the phenotypic data for SCLB and BLS were not correlated in the DRIL78 population. The lack of correlation may be due to the small effect size of this QTL and lack of overlap for other QTL in this population.

The colocalization of QTL may be a false positive, which is supported by the absence of a correlation at the population level. These BLS QTL did not colocalize with any known MDR genes. These findings suggest that the relationship between measurements of resistance to multiple foliar diseases is complex. The perception of bacteria and fungi by the innate immune system differs, and thus different resistance mechanisms to these different pathogens are possible. A closer study of the relationship between resistance to bacterial and resistance to fungal foliar pathogens is warranted.

This study is the first report on the genetic architecture of resistance to BLS. The family-mean basis heritability for DRIL78 (NC344 / Oh7B) population was 0.417, indicating that progress can be made in breeding for this disease. Furthermore, we report five BLS resistance QTL located on chromosomes 1 through 5 across two mapping populations. We did not detect any large-effect QTL but one moderate-effect QTL and several small-effect QTL, indicating that BLS resistance is polygenic. The BLS QTL in bin 4.07 in the DRIL78 population was previously reported as effective against SCLB (Lopez-Zuniga et al., 2019), whereas the Z023 BLS resistance QTL in bin 5.05 has been reported as responsible for resistance to NCLB and SCLB (Kump et al., 2011; Poland et al., 2011). Overall, this research improves our understanding of BLS resistance in maize, which can be useful for future maize disease resistance studies and breeding for BLS resistance.

TABLES AND FIGURE

Table 1. Mean percentage leaf blight and standard deviation of the 27 maize nested association mapping (NAM) parent lines inoculated with *X. vasicola* pv. *vasculorum.*

NAM parent	Mean percentage leaf blight†	SD
	%	
Ki11	13.5	4.2
Ki3	17.0	7.3
CML 103	17.2	6.9
M37W	18.0	10.3
B73	19.8	5.9
CML 228	20.8	9.8
Mo18W	24.1	9.8
B97	27.7	14.9
Tzi 8	28.8	17.3
M162W	31.3	13.4
NC350	32.3	13.4
Mo17	32.3	43.4
DKC 62-08	32.5	23.0
Ky21	34.9	12.9
CML 52	35.3	13.8
Il14H	36.7	14.4
Ms71	37.3	17.4
Tx303	37.7	18.2
CML 322	37.8	13.4
CML 277	42.1	12.8
CML 69	42.8	12.7
CML 333	43.3	12.6
P39	48.3	13.0
CML 247	52.5	19.8
NC358	52.6	22.0

Table 1. (cont.)

HP301	55.1	15.3
KN199	59.2	24.6
Oh43	79.3	11.8
Oh7B	92.4	7.1

† Calculated as the percentage of total leaf length blighted.

Table 2. Variance component estimates and their standard errors for factors included in the analysis. Factors, variance estimates, and standard errors included in the mixed model for the intermated NC344 Oh7b introgression line (DRIL78) population, NAM Z022 (B73 Oh43) population, and Z023 (B73 Oh7B) population are shown. All factors included in this table were significantly different from zero ($P < 0.05$).

Population	Factor	Variance	SE
DRIL78	Genotype	31.5	5.61
	Replication	4.21	2.05
	Replication: block	9.50	3.08
	Error	82.2	9.07
NAM Z022	Genotype	219	14.8
	Run: plant	6.61	2.57
	Error	280	16.7
NAM Z023	Genotype	244	15.6
	Run: plant: lesion†	2.16	1.47
	Error	298	17.2

† Each plant had two lesions. The lesions were on the same leaf and were located on either side of the midrib.

Table 3. Significant quantitative trait loci (QTL) detected in the NC344 Oh7b introgression line (DRIL78) population and the NAM Z023 (B73 Oh7B) population.

Population	Chr.†	Physical position‡	Bin§	Peak marker	LOD¶	*A*#	R^2††
		bp					
DRIL78	2	20,542,104	2.03	PHM4425-25	3.12	−1.38	6.67
DRIL78	4	178,611,318	4.07	PZA01187-1	2.76	−1.86	5.11
DRIL78	3	206,100,399	3.08	PHM9672-9	2.70	1.76	4.96
DRIL78	1	144,982,957	1.05	PHM4695-5	2.66	1.61	4.90
NAM Z023	5	183,863,194	5.05	M3912	7.44	6.80	18.9

† Chr., chromosome.

‡ Physical position of peak marker (RefGen_v3).

§ Bin, chromosome bin location for significant QTL (Davis et al. 1999).

¶ LOD, logarithm of odds value at the position of the peak likelihood of the QTL. A permutation test (1000 permutation replicates) was used to determine the LOD threshold. All QTL reported were significant with a Type I error value of 0.10.

A, additive effect estimates of the detected QTL. Effects are in terms of the disease rating scale used. For the DRIL78 population, a negative value indicates that the NC344 allele increases resistance; for the NAM Z023 population, a positive value indicates the B73 allele increases resistance.

† R^2, percentage of variance explained by the marker associated QTL.

Table 4. Correlation for multiple disease resistance in the nested association mapping (NAM) recombinant inbred line populations and the NC344 Oh7B introgression line (DRIL78) population. Pearson's correlations were calculated by comparing best linear unbiased predictors for bacterial leaf streak, southern corn leaf blight, northern corn leaf blight, and gray leaf spot. Data were curated from this study and previous studies for the correlation analysis (Benson et al. 2015; Kump et al. 2011; Lopez-Zuniga et al. 2019; Poland et al. 2011)

Population(s)	Disease	Southern corn leaf blight	Northern corn leaf blight	Gray leaf spot
DRIL78	Bacterial leaf streak	−0.099	0.235**	0.075
	Southern corn leaf blight		0.158*	0.044
	Northern corn leaf blight			0.152*
NAM Z022 & Z023	Bacterial leaf streak	−0.003	−0.022	0.079
	Southern corn leaf blight		0.621****	0.460****
	Northern corn leaf blight			0.448****

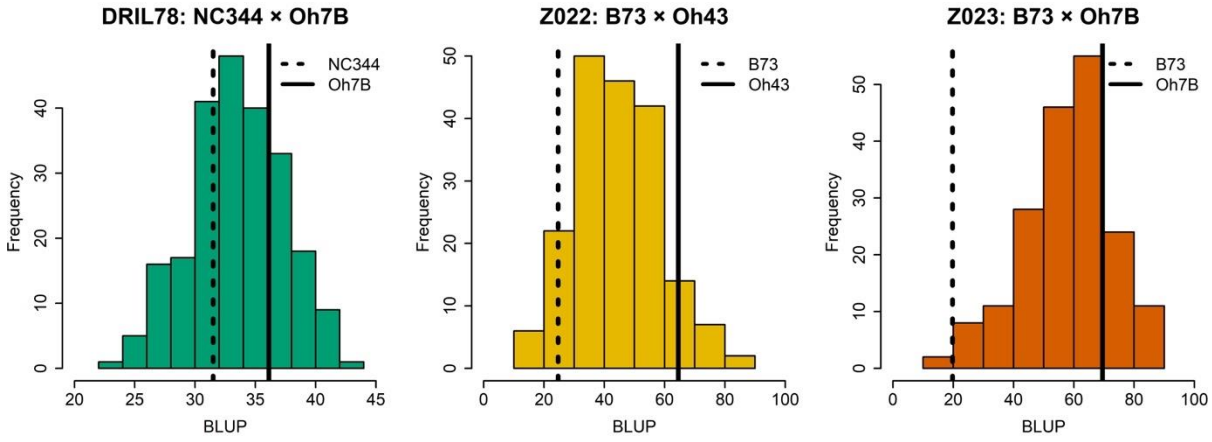
* Significant at the 0.05 probability level ($0.01 < P < 0.05$).

** Significant at the 0.01 probability level ($0.001 < P < 0.01$).

*** Significant at the 0.001 probability level ($0.0001 < P < 0.001$).

**** Significant at the 0.0001 probability level ($P < 0.0001$).

Figure 1. Phenotypic distributions for bacterial leaf streak (BLS). Lesion and leaf lengths were assessed and best linear unbiased predictors (BLUPs) were calculated based on those measurements for the three populations (DRIL78, Z022, and Z023). The phenotypic data shown are expressed as BLUPs, including the intercept.



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CHAPTER 3: IDENTIFICATION OF LOCI THAT CONFER RESISTANCE TO BACTERIAL AND FUNGAL DISEASES OF MAIZE

ABSTRACT

Crops are hosts to numerous plant pathogenic microorganisms. Maize has several major disease issues; thus, breeding multiple disease resistant (MDR) varieties is critical. While the genetic basis of resistance to multiple fungal pathogens has been studied in maize, less is known about the relationship between fungal and bacterial resistance. In this study, we evaluated a disease resistance introgression line (DRIL) population for the foliar disease Goss's bacterial wilt and blight (GW) and conducted quantitative trait locus (QTL) mapping. We identified a total of ten QTL across multiple environments. We then combined our GW data with data on four additional foliar diseases (northern corn leaf blight, southern corn leaf blight, gray leaf spot, and bacterial leaf streak) and conducted multivariate analysis to identify regions conferring resistance to multiple diseases. We identified 20 chromosomal bins with putative multiple disease effects. We examined the five chromosomal regions (bin 1.05, 3.04, 4.06, 8.03, and 9.02) with the strongest statistical support. By examining how each haplotype effected each disease, we identified several regions associated with increased resistance to multiple diseases and three regions associated with opposite effects for bacterial and fungal diseases. In summary, we identified several promising candidate regions for multiple disease resistance in maize and specific DRILs to expedite interrogation.

INTRODUCTION

Plants need to defend themselves from many pathogenic microbes present in their environment. Furthermore, the widespread cultivation of varieties with limited genetic diversity increases the risk of pathogen attack (Strange and Scott 2005). Crops are seldom attacked by just

a single pathogen, and thus, breeding is usually conducted for resistance to multiple pathogens (Khush 1989, Ceballos, Deutsch et al. 1991). Multiple disease resistance (MDR) is defined as host plant resistance to more than one disease and is controlled by single to many genes (Nene 1988, Wiesner-Hanks and Nelson 2016). Despite the widespread need across many crops for multiple disease resistant varieties, little is known about the genetic determinants of MDR. A few cloned disease resistance quantitative trait loci (QTL) have been shown to provide protection against multiple diseases including *Lr34* and *Lr67* in wheat (Krattinger, Lagudah et al. 2009, Moore, Herrera-Foessel et al. 2015) and *GH3-2* in rice (Fu, Liu et al. 2011). Genes conferring resistance to multiple diseases include those that encode signaling pathways, pathogen recognition, hormone-associated defense initiation, antimicrobial peptides, sugar signaling and partitioning pathways, and cell death-related pathways (Wiesner-Hanks and Nelson 2016). A more thorough understanding of MDR in crops will facilitate the development of varieties resistant to multiple diseases.

Maize is a staple cereal affected by over 32 major diseases that can cause substantial yield losses (Mueller, Wise et al. 2016, Munkvold and White 2016). Foliar diseases can cause significant production constraints, particularly in conducive environments. A survey from 2012 to 2015 showed that foliar diseases of maize lead to the largest estimated yield losses in the northern U.S. corn belt in non-drought years (Mueller, Wise et al. 2016). Pesticides are available to manage fungal foliar diseases but are costly and have environmental impacts (Bartlett, Clough et al. 2002, Paul, Madden et al. 2011). No labeled effective chemical control is available for the major bacterial foliar diseases. An effective and environmentally benign method of disease management is host plant resistance (Nelson, Wiesner-Hanks et al. 2018). The heritability for foliar diseases are moderate to high, indicating breeding to develop resistant varieties is possible

(Ceballos, Deutsch et al. 1991, Dingerdissen, Geiger et al. 1996, Zwonitzer, Coles et al. 2010, Lopez-Zuniga, Wolters et al. 2019).

Many MDR mapping studies in maize have focused on fungal diseases, and less is known about the relationship between resistance to fungal and bacterial diseases. In a synthesis study Wisser, Balint-Kurti et al. (2006) examined the relationship between fungal, bacterial, and viral resistance and identified loci that conferred resistance to fungal and bacterial diseases. Subsequent studies identified regions, and even genes, that confer resistance to the three most significant fungal foliar diseases – southern corn leaf blight (SCLB), northern corn leaf blight (NCLB), and gray leaf spot (GLS) (Zwonitzer, Coles et al. 2010, Belcher, Zwonitzer et al. 2012, Yang, He et al. 2017, Lopez-Zuniga, Wolters et al. 2019). Relatively few regions have been identified that confer resistance to both a fungal and a bacterial pathogen in maize (Chung, Longfellow et al. 2010, Chung, Poland et al. 2011, Jamann, Poland et al. 2014, Jamann, Luo et al. 2016, Hu, Ren et al. 2018, Qiu, Kaiser et al. 2019). In this study, we focused on two bacterial diseases bacterial leaf streak (BLS) and Goss's bacterial wilt and blight (GW), as well as three fungal diseases: SCLB, NCLB, and GLS.

Goss's wilt and bacterial blight is one of the most destructive foliar diseases of maize (Mueller, Wise et al. 2016) and is caused by *Clavibacter nebraskensis* (Li, Tambong et al. 2018). The blight phase of the disease is characterized by water-soaked tan to gray linear lesions with irregular margins parallel to, but not bounded by, leaf veins. The bacteria colonize the xylem, and vascular wilt symptoms can develop in susceptible lines (Schuster 1975, Jackson, Harveson et al. 2007, Mbofung, Sernett et al. 2016). The bacteria usually enter the leaves through wounds, but can also enter through natural openings in the absence of wounding in high-humidity conditions (Mallowa, Mbofung et al. 2016). First identified in 1969 (Schuster, Compton et al.

1972), GW is now found throughout the midwestern United States and in Canada (Schuster 1975, Malvick, Syverson et al. 2010, Howard, Harding et al. 2015, Singh, Hollier et al. 2015, Mueller, Wise et al. 2016).

Bacterial leaf streak caused by *Xanthomonas vasicola* pv. *vasculorum* (*Xvv*), is an emerging disease in the Americas (Korus, Lang et al. 2017, Damicone, Cevallos et al. 2018, Jamann, Plewa et al. 2019, Leite, Custodio et al. 2019). The bacteria enter and exit through wounds and stomata to colonize intercellular spaces, but do not enter the vasculature (Ortiz-Castro, Jacobs et al. 2018). NCLB, GLS, and SCLB are among the most important fungal foliar diseases. NCLB is of global importance and is caused by the hemibiotrophic pathogen, *Exserohilum turcicum*. In inoculated trials using susceptible germplasm, NCLB caused a 30 to 62% grain yield reduction (Raymundo and Hooker 1981, Perkins and Pedersen 1987). Humid conditions and moderate temperatures favor NCLB development. Gray leaf spot is also of global importance and is caused by the necrotrophic fungi *Cercospora zeae-maydis* and *Cercospora zeina*. It can cause as much as a 50% yield loss (Ward, Stromberg et al. 1999) and develops quickly in high humidity conditions. Southern corn leaf blight, caused by *Bipolaris maydis*, is usually found in hot and humid regions and can cause up to a 40% yield loss if the varieties are susceptible and the conditions favorable (Byrnes, Pataky et al. 1989). All the diseases are favored by high humidity environments. There are overlapping pathogenesis and tissue-level pathogen localization between diseases. For example, the pathogens causing NCLB and GW both colonize the xylem (Chung, Longfellow et al. 2010, Mbofung, Sernett et al. 2016) and for both BLS and GLS the pathogen enters the stomata (Beckman and Payne 1982, Ortiz-Castro, Jacobs et al. 2018).

We conducted linkage mapping for GW in a disease resistance introgression line (DRIL) population. We selected a DRIL population because it was developed to study multiple disease resistance (Lopez-Zuniga, Wolters et al. 2019). Data for BLS (Qiu, Kaiser et al. 2019), SCLB, NCLB, and GLS (Lopez-Zuniga, Wolters et al. 2019) were combined with the GW data to examine MDR. We evaluated the DRIL78 population, which is an ideal population for this study, as the donor line NC344 is resistant and the recurrent parent Oh7B susceptible for all the diseases studied (Cooper, Rice et al. 2019, Lopez-Zuniga, Wolters et al. 2019, Qiu, Kaiser et al. 2019). Thus, we hypothesized that we could identify regions for resistance to fungal and bacterial pathogens in this population.

Multivariate analysis was used to identify potential MDR loci. Multivariate analysis based on Mahalanobis distance (Md) has been used for genome scans in both human and plant studies (Tian, Gregersen et al. 2008, Lotterhos, Card et al. 2017, Luu, Bazin et al. 2017). In this study, we used Md to combine the mapping results from the five diseases. Md is not trait-specific; instead, it is a test for outlier markers across all traits and takes multiple mapping result datasets into consideration. The outlier markers, reported as putative MDR markers, are those that do not follow the pattern of the majority of the data point cloud (Rousseeuw and Van Zomeren 1990).

The overall objective of this study was to compare the genomic basis of resistance to fungal and bacterial diseases in maize. Mapping was conducted for GW using phenotypic data collected in three environments and combined with previously published studies for BLS, NCLB, SCLB, and GLS (Lopez-Zuniga, Wolters et al. 2019, Qiu, Kaiser et al. 2019). Here, we: 1) identify novel QTL associated with GW through linkage mapping; 2) explore the relationship

between the five diseases in this population; and 3) estimate the effect of potential MDR haplotypes on the five diseases.

MATERIALS AND METHODS

Plant materials

Chromosome segment substitution population DRIL78 is an ideal population for multiple disease evaluation, as the parents are multiple disease resistant (NC344) and multiple disease susceptible (Oh7B) (Wisser, Kolkman et al. 2011, Cooper, Rice et al. 2019, Lopez-Zuniga, Wolters et al. 2019, Qiu, Kaiser et al. 2019). This population was developed by a cross between NC344 and Oh7B, three generations of backcrosses, and four subsequent generations of self-pollinating via single-seed descent to obtain $BC_3F_{4:5}$ lines (Lopez-Zuniga, Wolters et al. 2019).

Phenotypic evaluation

The DRIL78 population was planted in three environments: Urbana 2016, Monmouth 2017, and Urbana 2017. The Urbana trials were conducted at the University of Illinois Crop Science Research and Education Center South Farms located in Urbana, IL. The Monmouth trial was conducted at the University of Illinois Monmouth Research Station located in Monmouth, IL. In Urbana 2016, 260 lines were evaluated for GW in one replication. In 2017, 229 and 233 lines were evaluated in Monmouth and Urbana, respectively, each with two replications. Differences in the number of lines evaluated was due to seed availability. For Monmouth and Urbana 2017, we generated an incomplete block design using the agricolae package in R (Version 3.5.1) (R Core Team 2018, de Mendiburu and de Mendiburu 2019). For Monmouth 2017 and Urbana 2017, Oh7B was included in each block, along with the resistant check line NC344 or NC258. NC344 was not included in every block due to seed availability. For Urbana 2016, we used an augmented incomplete block design with one replication. In this location, the

parental lines NC344 and Oh7B were included in each block. Seed was machine planted with 20 kernels per plot. Plots were 3.2 meters with 0.76 m alleys between each plot and row spacing of 0.762 meters. Fields were managed using standard agronomic practices for central Illinois.

Disease evaluation

Clavibacter michiganensis subsp. *nebraskensis* isolate 16Cmn001 was used for the GW inoculations. We inoculated the plants twice, once at the V4 stage and a second time at the V7 stage (Abendroth, Elmore et al. 2011), as described by Cooper, Balint-Kurti et al. (2018). We assessed the extent of necrosis using a visual percentage rating on a per plot basis with 5% intervals starting about two weeks after the first inoculation date. A rating of 0% represented no disease in the plot, while 100% indicated that all the foliage was necrotic (Poland and Nelson 2011). In Urbana 2016, two visual ratings were taken 17 days apart; in Urbana 2017, two ratings were taken 18 days apart; in Monmouth 2017, three ratings were taken with 8 and 9 days between ratings. We calculated the area under the disease progress curve (AUDPC) scores for each plot in R (Version 3.5.1) (R Core Team 2018) using the *audpc* function in the agricolae package (de Mendiburu and de Mendiburu 2019) (File S1).

Statistical analysis

Least Square Means (LSMeans) were estimated for GW for each environment (2016 Urbana, 2017 Urbana, and 2017 Monmouth) and for the combined multienvironment dataset using AUDPC values and the *lmer* function in the R package lme4 (Doran, Bliese et al. 2007). Linear mixed models were constructed for each environment and the combined dataset and are listed below:

$$\text{Urbana 2016: } Y_{ijk} = \mu + G_i + B_j + \epsilon_{ijk};$$

$$\text{Urbana 2017, Monmouth 2017: } Y_{ijkl} = \mu + G_i + R_j + B(R)_{(j)k} + \epsilon_{ijkl};$$

Combined dataset: $Y_{ijklm} = \mu + G_i + E_j + GE_{ij} + R(E)_{(j)k} + B\big(R(E)\big)_{(jk)l} + \epsilon_{ijklm}$;

where μ is the overall mean, *G* is the fixed genotype (introgression line) effect, *B* is the random blocking effect, *R* is the random replication effect, *E* is the random environment effect, and *GE* is the random genotype-by-environment interaction effect. Blocks are nested within replications within environments. Only significant factors were included in the models. We examined the skewness of the data using the *skewness* function from the e1071 package (Dimitriadou, Hornik et al. 2009). Heritability on both a plot and family-means basis were calculated for GW with SAS (version 9.4) using PROC MIXED, as described by Holland, Nyquist et al. (2003).

We calculated LSMeans for the BLS data based on the raw measurements from Qiu, Kaiser et al. (2019). The model included genotype as a fixed factor, and replication and block nested within replication as random factors. We obtained LSMeans for SCLB, NCLB, and GLS from Lopez-Zuniga, Wolters et al. (2019).

Multiple comparison tests were conducted using the LSMeans calculated for each disease individually to identify the lines that were significantly different from the recurrent parent Oh7B using the function *glht*, with a Dunnett's *p*-value adjustment, in the package multcomp in R (Hothorn, Bretz et al. 2016).

Disease Correlations

We conducted Pearson's product-moment correlation tests between LSMeans for the diseases (ten total comparisons) in R using the *cor.test* function. The parent lines were not included. SCLB, NCLB, and GLS were rated using a 1-9 rating scale, where 1 indicated 100% leaf area affected by the pathogen and 9 indicated no disease; BLS phenotypes were lesion length measurements where small values indicate shorter lesions; GW ratings were rated using a percentage scale based on the severity of the disease with 0% suggesting no disease. To have a

uniform scale for correlation analysis, we multiplied the BLS and GW LSMeans values by -1. With this modification, low values indicated more severe infections for all datasets.

Linkage mapping

A total of 190 lines, including the recurrent parent Oh7B, were shared across all five datasets. We used the LSMeans for 190 lines and 237 single nucleotide markers from Lopez-Zuniga, Wolters et al. (2019) to conduct linkage mapping for each of the five diseases (File S2). The software ICIMapping 4.0.6.0 with the options "CSL" and "RSTEP-LRT-ADD" mapping were used to conduct QTL analysis (Meng, Li et al. 2015). We conducted 1000 permutations with a 0.10 Type I error rate to determine the logarithm of odds (LOD) threshold. We recalculated the LOD threshold for each disease. The physical positions of markers with LOD values exceeding the threshold are reported based on B73 RefGen_v3 coordinates (Schnable, Ware et al. 2009).

Multivariate analysis

We conducted multivariate analysis to identify QTL associated with more than one disease using the methods described in Lopez-Zuniga, Wolters et al. (2019). The five diseases each served as a variable and the “robust Mahalanobis distance” method was used to combine the five variates to detect outlier markers. In this study, Mahalanobis distance (Md) was calculated based on the five negative log10 *p*-values of the LOD scores derived from the five single-disease mapping results. Outlier markers were detected based on *p*-values for Md. The detailed steps of multivariate analysis are described below: (i) conduct linkage mapping analysis with ICIMaping for each trait in the population independently; (ii) obtain trait-specific, permutation-based LOD thresholds and trait-specific marker LOD values from the mapping results; (iii) calculate *p*-values for each marker for each disease based on the following function:

to account for the variation in LOD significance thresholds between different mapping studies (Nyholt 2000); (iv) convert *p*-values into negative log10 *p*-values; (v) calculate Mahalanobis distance based on negative log *p*-values (Md-p) for each of the diseases in R with *OutlierMahdist* function in rrcovHD package (Todorov 2018), as described by Lotterhos, Card et al. (2017); (vi) calculate *p*-values for Md-p for each marker (Rousseeuw and Van Zomeren 1990). To control for multiple comparisons, the false discovery rate (FDR) was calculated by adjusing the *p*-values using the "BH" method (Benjamini and Hochberg (1995) with the *p.adjust* function in R. Markers were declared to be significant using a 1% FDR.

Haplotype effect calculation

The maize genome has previously been divided into 100 bins which we used here to delineate disease resistance-associated segments of the genome (Davis, McMullen et al. 1999). The chromosomal bin for each marker that passed the 1% FDR Md-p test and the single-disease linkage mapping analysis was recorded. We considered bins with at least three significant Md-p markers as candidate MDR regions. The selected MDR regions were delimited by the position of the two flanking significant markers. To calculate the haplotype effect for each region, we identified lines with introgressions in the MDR regions and then calculated, using the raw AUDPC data, the difference between the mean AUDPC for those lines and the mean AUDPC for the recurrent parent Oh7B (Belcher, Zwonitzer et al. 2012). Because different scales were used for each disease and we wanted to compare between diseases, we standardized the haplotype effect by Oh7B.

Finally, we conducted a *t*-test using the percentage change to determine whether there was a significant difference between Oh7B phenotype and introgression line effect. The null hypothesis was that there is no difference between Oh7B and the haplotype effect (percent change=0).

RESULTS

Characterization of germplasm

As expected, the recurrent parent Oh7B was the susceptible parent for all the diseases we examined. Of the five diseases, the parents were the most phenotypically similar for BLS. Using Dunnett's multiple comparison test, we detected significant differences between the donor and recurrent parent for all diseases except BLS. Similar to what has been reported previously for fungal disease phenotypes (Lopez-Zuniga, Wolters et al. 2019), there was substantial transgressive segregation for the bacterial diseases (Figure 2). Like the fungal diseases, the DRIL78 population included lines with transgressive segregation for GW only in the direction of susceptibility, indicating NC344 may donate alleles for both resistance and susceptibility. In contrast, transgressive segregation for BLS occurred in both directions, suggesting that resistance to BLS in NC344 and Oh7B is conditioned by complementary sets of alleles. Using our data, we calculated the heritability for GW: heritability on a plot basis was 0.53 (s.e.= 0.03) and on a family-mean basis was 0.78 (s.e. = 0.02), indicating that progress can be made from inbred line evaluations in breeding for this disease.

Using a Dunnett's multiple test comparison, we examined whether there were DRILs that were significantly more resistant or susceptible than the recurrent parent. For GW, 16 of the 258 lines, or 6.2% of the lines tested, were significantly different than Oh7B (Table 5). Despite the presence of transgressive segregants for susceptibility to BLS, none of the DRILs were

significantly more susceptible than Oh7B; however, three lines were significantly more resistant than Oh7B.

Correlation between diseases

We tested pairwise correlations among the five diseases. A total of five out of ten pairwise correlation tests were significant ($P < 0.05$); the two bacterial diseases were not significantly correlated. Of the correlations that were significant, coefficients ranged from 0.15 to 0.31 (Table 6). For the three fungal diseases, as previously reported, resistance to NCLB was significantly and positively correlated with resistance to SCLB and GLS, while the correlation between resistance to GLS and SCLB was positive but not significant (Lopez-Zuniga, Wolters et al. 2019). Here, we found significant and positive correlations among pairs of bacterial and fungal diseases (GW and NCLB; GW and GLS; BLS and NCLB). Given the genomic structure of the DRIL population, these correlations suggest that loci conditioning MDR to bacterial and fungal diseases exist in this population.

Identification of multiple disease resistant lines

The correlations between diseases suggested that MDR loci exist in this population, so we tested whether the same DRILs that were significantly more resistant or susceptible than the recurrent parent for multiple diseases. Only 5.3% of the lines (10 of 189 lines) were significantly different than Oh7B for more than one disease, and there were seven unique two disease combinations. Only one line was significantly different than Oh7B for the combination of the two bacterial diseases. There were four bacterial/fungal disease combinations, all of which included GW, with seven lines that were significantly more resistant to the combination of a bacterial and fungal pathogen. The remaining two lines were significantly different than Oh7B for a combination of two fungal diseases (SCLB and GLS; NCLB and GLS). For NCLB and

SCLB there were lines that were resistant to the respective fungal disease, but susceptible to GW. No lines were significantly different than Oh7B for more than two diseases.

GW linkage mapping

The genotype and environment interaction accounted for some variance; thus, single environment mapping analysis was also conducted for GW. We conducted linkage mapping for GW for three individual environments, as well as the combined dataset. A total of ten QTL on chromosomes 1 through 6, and 9 were detected (Table 7). Six of the QTL were stable, as they were consistently detected across multiple environments or in the combined dataset. The QTL detected in chromosomal bin 2.07 (*qGW2.07*; peak marker PHM14412-4) was detected in all three individual environments and the combined dataset. The QTL in chromosomal bin 3.06, 4.06 and 9.02 were detected in more than one environment, and the additive effect estimates and percentage of variance explained by these QTL were similar across datasets.

We examined the additive effect estimates and percentage of variance explained by the significant markers. The GW QTL were of small effect, with the largest-effect QTL, namely *qGW2.07*, accounting for 8.96% of the phenotypic variation in the combined dataset. The other QTL explained from 3.73% to 8.84% of the phenotypic variance. The QTL detected on chromosomes 2, 3 and 9 had negative additive effect estimates, indicating that the NC344 allele confers resistance. The QTL with positive additive effect estimates on chromosome 1, 3, 4 and 6 indicate that the Oh7B allele confers resistance. On chromosome 3, two QTL were identified within the same bin. NC344 conferred the resistant allele for both QTL on bin 3.04.

Multivariate multiple disease mapping

Across all diseases, we detected 18 significant markers in the single-trait mapping, with two markers for BLS, five for GW, four for SCLB, three for NCLB and six for GLS. The

markers detected in the single-trait mapping were designated “single-trait markers.” Among the 18 single-trait markers, two were shared by multiple diseases (GW and SCLB; GW, and GLS) (Figure 3). Across the single-trait analyses, chromosomes 1 through 4 were all associated with more than one disease (Figure 3).

Multivariate analysis was conducted to detect MDR regions using the robust Mahalanobis distance method (Rousseeuw 1985, Rousseeuw and Van Zomeren 1990). At a 1% false discovery rate, 54 out of 237 markers were detected as related to one or more diseases. The 54 significant markers included all 18 single-trait markers. Several regions emerged as likely MDR candidates. We identified the largest number of significant markers on chromosomes 1 (10 significant markers), 3 (8 significant markers), and 8 (9 significant markers). On chromosome 4, 6 and 10, several markers exceeded the multi-trait threshold, indicating that even markers with relatively low LOD scores for individual diseases can have a high multi-trait Md value (Lopez-Zuniga, Wolters et al. 2019). We observed four co-localized QTL in bin 8.03 and three in bin 9.02. The two regions with markers that were identified for more than one disease in the single trait analysis, specifically bin 3.04 (GW and SCLB) and bin 4.06 (GW and GLS), were also detected in the Md test. In all, five regions with the strongest statistical support, and that have been examined in previous studies, were selected to examine their role in resistance to multiple diseases.

Haplotype effect analysis

We hypothesized that some haplotypes may have opposite effects on bacterial and fungal diseases, e.g., a region may confer resistance to a fungal disease but susceptibility to a bacterial disease. We selected MDR regions located in bins 1.05, 3.04, 4.06, 8.03 and 9.02 to test this hypothesis. We estimated the effect of the haplotype at each of the selected regions, referred to

as the haplotype effect, on disease severity for each of the diseases (Figure 4). The MDR region at bin 8.03 was associated with resistance to GW, NCLB and GLS; bin 9.02 was associated with resistance to GW, SCLB and GLS. While the introgressions conditioned resistance relative to Oh7B for these two bins, the effect sizes varied. These may be examples of uneven pleiotropy, whereby an MDR locus has varying effect sizes for different diseases (Wiesner-Hanks and Nelson 2016), or tight linkage. Some regions conferred contrasting effects for the diseases examined: the haplotypes at bins 1.05, 3.04 and 4.06 had an opposite effect for GW as compared to the other diseases. The NC344 haplotype at bin 1.05 was associated with resistance to SCLB and GLS, but susceptibility to GW. The introgressions in bin 3.04 conferred resistance to all the three fungal diseases, but susceptibility to GW. Lines with introgressions at bin 4.06 were more resistant to BLS and SCLB, but more susceptible to GW as compared to Oh7B.

DISCUSSION

The heritability of GW resistance in this population was relatively high and on par with previous GW studies (Ngong-Nassah 1992, Singh, Andersen et al. 2016, Cooper, Balint-Kurti et al. 2018). High heritability has been reported for the three fungal diseases for this population, namely 0.76 for SCLB, 0.75 for NCLB, and 0.59 for GLS (Lopez-Zuniga, Wolters et al. 2019), indicating that progress can be made from inbred line evaluations in breeding for these diseases. Bacterial leaf streak had the lowest heritability of the diseases examined in this population: 0.42 (Qiu, Kaiser et al. 2019). The GW QTL we identified were relatively stable across multiple environments in the single trait analysis. The QTL in bins 1.05, 2.07 and 9.02 were consistently detected and colocalized with previously identified QTL (Singh, Andersen et al. 2016, Cooper, Balint-Kurti et al. 2018).

A central objective of this study was to investigate the relationship between resistance for multiple diseases. Previous studies demonstrated that resistance for the three fungal diseases, namely SCLB, NCLB, and GLS, are correlated with each other. For instance, high positive (>0.5) genetic correlations were detected in a diversity panel between resistance to all the pairwise fungal disease combinations in 253 inbred maize lines (Wisser, Kolkman et al. 2011). The DRIL78 correlations for the fungal diseases are not as strong compared to other populations, as no correlation was detected between resistance to SCLB and GLS (Lopez-Zuniga, Wolters et al. 2019). Resistance between these two diseases are typically significantly and highly correlated (Zwonitzer, Coles et al. 2010). We previously reported a significant positive correlation between resistance to a bacterial (GW) and a fungal disease (NCLB) in a different population (Cooper, Balint-Kurti et al. 2018). The significant relationship indicates the possibility of MDR in this population.

Despite the differences between fungal and bacterial pathogens, some of the pathogens can infect the same tissue types, specifically the vasculature. SCLB and GLS are non-vascular diseases (Beckman and Payne 1982, Minker, Biedrzycki et al. 2018), while GW and NCLB are vascular diseases (Mbofung, Sernett et al. 2016, Minker, Biedrzycki et al. 2018). Only one vascular/vascular (NCLB and GW) disease correlation combination was identified. Most combinations were of a vascular and non-vascular disease (NCLB with BLS, NCLB with SCLB, NCLB with GLS, and GLS with GW), indicating that either resistance may be linked but not pleiotropic or that there is another resistance mechanism at play that does not interfere with the pathogen's growth within specific plant tissues.

We found evidence of regions conferring resistance to more than one disease from the single disease analysis. The same marker was effective for two disease combinations,

specifically for the combination of GW and SCLB in bin 3.04 and for the combination of GW and GLS in bin 4.06. This is consistent with the Dunnett's multiple comparison test, where lines effective against these two disease combinations were identified. The Pearson's product correlation coefficients were significant for the combination of GW and GLS. Interestingly, in both instances, the QTL protect against a combination of a vascular bacterial disease and a non-vascular fungal disease.

To examine MDR in the DRIL78 population, multi-disease post-mapping analysis based on Md was conducted. All 18 of the markers detected in the single-trait mapping analysis were significant in the Md analysis. One possible explanation for this is that significant Md values can arise only due to one trait so that if a marker was highly significant for one disease, it would be identified as an MDR marker as well. The fundamental idea of the Md approach is to identify outliers in multivariate space, and outliers can occur in any one of the dimensions (the five disease-trait dimensions in our case). For the 36 novel markers from the multivariate analysis, LOD values were not high enough to exceed the LOD threshold in the single-trait mapping analysis. However, when combining the five diseases together, creating a new variable Md-p, and testing for Md-p outliers, led to the identification of the additional markers. Lopez-Zuniga, Wolters et al. (2019) also noted this phenomenon when testing for MDR markers using an Md approach.

We found that disease-associated QTL were distributed across all 10 chromosomes, but the QTL were not evenly distributed. This is in consistent with previous synthesis studies on the genomic distribution of disease QTL in maize (Wisser, Balint-Kurti et al. 2006). Based on the distribution of the single-trait and multi-trait QTL, we focused on five MDR regions to investigate further. Of these five regions, bins 1.05, 3.04, 8.03 and 9.02 have been reported

previously to be related to multiple diseases (McMullen and Simcox 1995, Wisser, Balint-Kurti et al. 2006, Ali, Pan et al. 2013, Cooper, Balint-Kurti et al. 2018, Lopez-Zuniga, Wolters et al. 2019) in other populations. Lopez-Zuniga, Wolters et al. (2019) identified bin 1.05 for resistance to SCLB, NCLB and GLS, and bin 3.04 for SCLB and GLS. Another study in maize utilizing near-isogenic lines found that bin 3.03-3.04 and bin 9.02-9.03 were associated with SCLB, NCLB and GLS resistance (Belcher, Zwonitzer et al. 2012). In addition to the three selected fungal diseases, bin 3.04 was also found to harbor QTL conferring resistance to European corn borer, Fusarium stalk rot, common rust and maize mosaic diseases (McMullen and Simcox 1995).

We hypothesized that allele effect sizes differed at each locus for each disease and that some QTL had contrasting effects for different diseases. We found that some regions were associated with resistance to one disease and susceptibility to another, which is consistent with previous findings in other studies (Belcher, Zwonitzer et al. 2012). The introduction of resistance for one disease might unintentionally introduce susceptibility for a second disease. Fine mapping is required to determine whether the same gene is conferring resistance to one disease and susceptibility to another.

The mechanisms underlying MDR in this population remain elusive. Of the combinations of diseases identified using the Dunnett's and the multivariate tests, there was no clear pattern of pathogen kingdom or pathogenesis process in the MDR disease combinations. Thus, if there is a pleiotropic gene underlying these regions, the mechanism is not obviously associated with pathogen kingdom or the growth of the pathogen in the vasculature. It is important to note, however, that our study does not have the resolution to resolve these QTL to single genes, and it is likely that several of these cases are due to linkage, not pleiotropy.

In summary, a total of five QTL associated with resistance to GW in the combined-environment mapping study were identified, one of which was consistent across all individual environments and the combined-environment mapping analysis. By combining GW mapping results with published data for NCLB, SCLB, GLS (Lopez-Zuniga, Wolters et al. 2019) and BLS (Qiu, Kaiser et al. 2019), we identified genomic regions associated with multiple disease resistance. Two markers were identified in the independent single-trait mapping analysis as conferring effects for two diseases. A total of 36 MDR-related markers were identified in the multivariate analysis. Disease QTL were distributed across all ten chromosomes, and we focused on five regions with QTL clustering. We found strong support for multiple disease resistance QTL at 1.05, 3.04, 4.06, 8.03 and 9.02 across multiple analyses. We found evidence of uneven pleiotropy and of QTL conferring contrasting effects for different diseases. This work deepens our understanding of multiple disease resistance in maize and the relationship between fungal and bacterial disease resistance.

TABLES AND FIGURES

Table 5. Dunnett's multiple comparison test for five traits in DRIL78 population. A Dunnett's multiple comparison test was conducted to identify lines that were significantly different than the recurrent parent Oh7B. For two disease combinations, all lines were more resistant to both diseases in the combination, except where noted otherwise.

Disease	Population Size	Total # of lines significantly different than Oh7B (# more resistant/# more susceptible)
BLS	229	3 (3/0)
GW	258	16 (3/13)
SCLB	216	23 (23/0)
NCLB	216	6 (6/0)
GLS	216	10 (10/0)
BLS/NCLB	189	2
BLS/GW	189	1
SCLB/GLS	189	1
SCLB/GW	189	2*
NCLB/GLS	189	1
NCLB/GW	189	2**
GLS/GW	189	1

* Both lines were more resistant to SCLB, but more susceptible to GW.

** Both lines were more resistant to NCLB. Of those, one line was more resistant to GW, while the other was more susceptible to GW.

Table 6. Pairwise correlation coefficients for LSMeans in DRIL78 population. Phenotypic correlations were examined between the five diseases examined in this study.

Disease	BLS	SCLB	NCLB	GLS
GW	0.12	- 0.11	0.31 ***	0.24 ***
BLS		- 0.11	0.23 **	0.06
SCLB			0.16 *	0.05
NCLB				0.15 *

* 0.05

** 0.01

*** 0.001

Table 7. Significant QTL detected in DRIL78 population for GW across all environments.

Peak marker	Chr. §	cM	Position ¶	Bin§§	Environment	LOD†	Add‡	PVE(%)#
PHM12633-15	1	116.2	103,835,578	1.05	Combined	3.69	53.72	4.84
PHM14412-4	2	127.4	203,610,640	2.07	Combined	6.58	-63.65	8.96
					Urbana 2016	3.36	-71.34	6.16
					Urbana 2017	3.57	-55.10	5.39
					Monmouth 2017	4.40	-61.68	6.62
PZA00348-11	3	68.94	32,780,891	3.04	Combined	3.38	49.76	4.42
PHM5502-31	3	78.21	68,060,067	3.04	Monmouth 2017	3.32	65.67	5.00
PHM1959-26	3	105.64	170,153,721	3.06	Urbana 2016	4.26	-82.89	7.90
					Monmouth 2017	5.77	-79.25	8.84
PHM15864-8	4	87.18	151,565,558	4.06	Combined	2.83	57.70	3.73
					Urbana 2017	3.28	74.74	5.02
PZA03092-7	5	64.27	12,049,611	5.02	Urbana 2016	3.24	-91.96	5.99
PHM5529-4	6	126.27	167,219,234	6.08	Urbana 2017	4.83	56.39	4.83
PHM5185-13	9	47.48	18,905,238	9.02	Combined	4.76	-72.02	6.38
					Monmouth 2017	3.22	-73.97	4.81
PZA00588-2	9	61.08	62,366,576	9.03	Urbana 2017	5.52	-74.93	8.54

§ Chromosome.

¶ The physical position (RefGen_v3) of significant markers.

§§ Chromosomal bin location of significant QTL (Davis, McMullen et al. 1999).

† LOD value at the position of the peak likelihood of the QTL. A permutation test was conducted to determine the LOD threshold for the significant markers.

‡ Additive effect estimates of the detected QTL. Effects are in terms of the disease rating scale used. A negative value indicates that the donor allele increases the disease resistance of the line in the population.

Percentage of the phenotypic variance explained by the detected QTL.

Figure 2. Phenotypic distribution for DRIL78 (NC344×Oh7B) population of five traits. The two vertical lines indicate the least square means (LSMeans) of the two parental lines. The recurrent susceptible parent Oh7B is represented by the solid line and the donor resistant parent NC344 is represented by the dashed line. The LSMeans for BLS and GW were based on the lesion length measurement and percent leaf diseased where lower numbers indicate less disease; the LSMeans of SCLB, NCLB and GLS were based on a 1 to 9 scale where lower numbers indicate more disease.

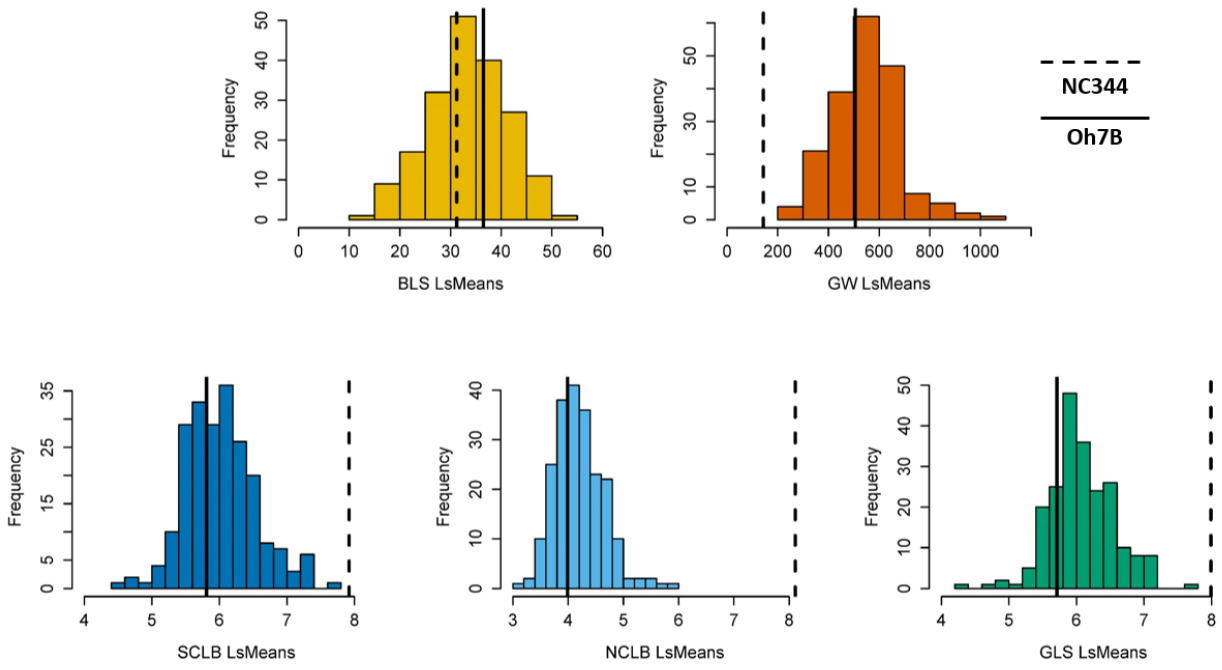


Figure 3. Manhattan plot for multivariate analysis. The mapping results for the two bacterial diseases are represented with warm colors and the three fungal diseases in cold colors. The GW&SCLB and GW&GLS symbols indicate that the same SNP is significantly associated with both diseases. The MO symbol corresponds to the markers that were not significant in the single-trait mapping analysis but were significant in the multi-trait composite analysis. The dotted line indicates the 1% FDR for the Md statistic. The dashed line represents the Md value for the minimum LOD threshold for the five mapping analyses.

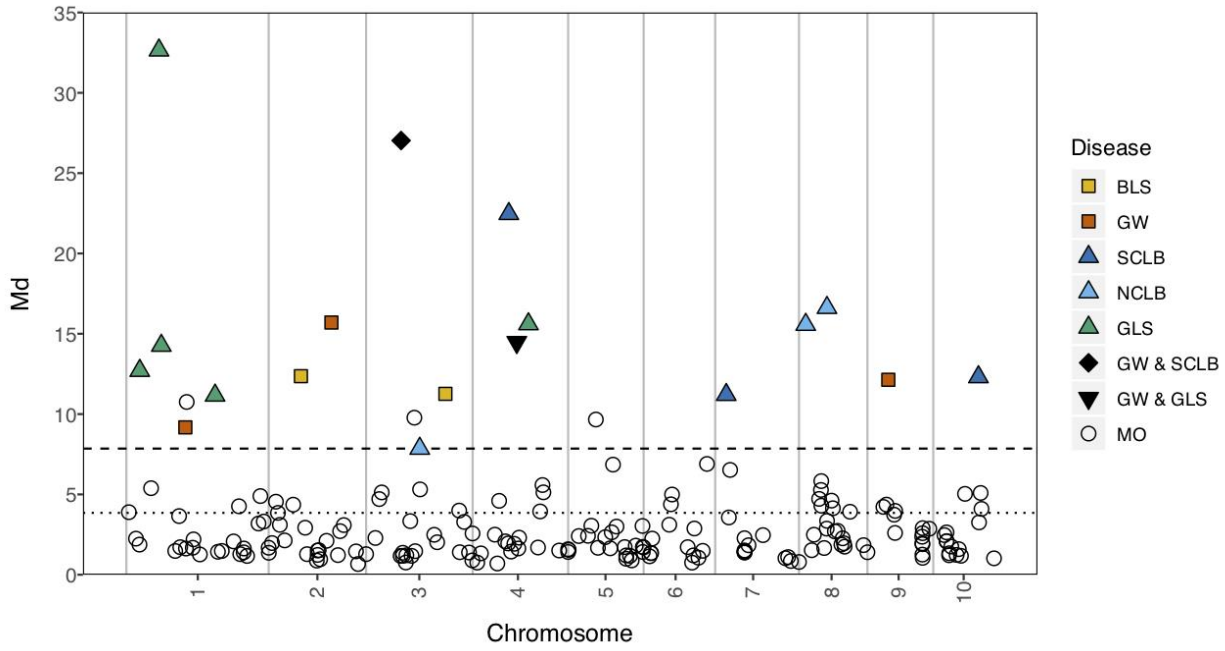
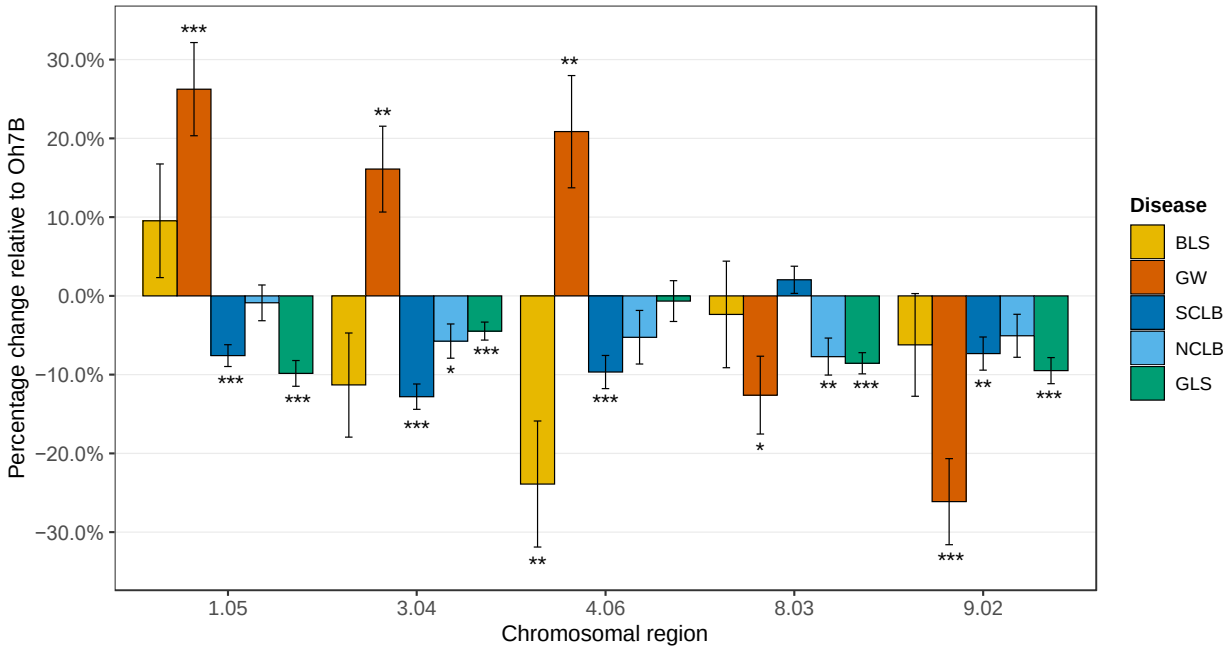


Figure 4. Estimation of haplotype effect. The x-axis indicates the selected genomic regions, and the y-axis indicated the percentage change of disease severity of lines with an introgression at that region. The negative percentage value indicates that lines with an introgression in this region were more resistant than Oh7B and a positive value indicates that the lines were more susceptible. A *t*-test was conducted to examine the significance of bin effect. * indicates the 0.05 significance level; ** indicates the 0.01 significance level and *** indicates that p-value was smaller than 0.001.



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