

ABSTRACT

SANTA CRUZ HIDALGO, JOSE HERNAN. Fine-Mapping and Agronomic Evaluation of a Quantitative Trait Locus Conferring Resistance to Southern Corn Leaf Blight Caused by *Cochliobolus heterostrophus*. (Under the direction of Peter J. Balint-Kurti and Matthew Krakowsky).

Southern leaf blight (SLB) caused by the fungal pathogen *Cochliobolus heterostrophus* is a disease in southeastern US and in many hot and humid areas in the world. Most genetic disease resistance deployed in maize hybrids is quantitative in nature; however, quantitative disease resistance remains poorly understood. The work in this thesis concerns the characterization of a disease resistance quantitative trait locus (dQTL) for SLB resistance that we have called the *6a* locus. The resistance allele at *6a* was first identified in the highly SLB-resistant maize line NC292 and was subsequently introgressed into the commonly used maize line B73 to produce the B73-6A near isogenic line (NIL). Experiments carried out in both growth chamber and field suggested that SLB resistance conferred by *6a* was due to a single recessive resistance locus. Specific objectives of this research were the fine-mapping the *6a* locus and cloning of the underlying gene and evaluating the yield and fitness effects of the 6A introgression (i.e. the introgression in B73-6A that carries the *6a* locus) under both high and low disease pressure.

By mapping in an F_{2:3} segregating population derived from a cross between B73 and B73-6A, we delimited the region carrying the SLB resistance gene at the *6a* locus to a ~300 Kb region. This region contained 24 predicted genes, five of which belonged to the amino acid transporter gene family. One of these transporters, lysine-histidine transporter-1 gene (LHT1) was thought to be a good candidate to underlie the observed SLB resistance.

We undertook two approaches to prove the role of LHT1 in SLB resistance. The first approach was to knock out the (dominant) disease susceptibility gene using the *Activator/Dissociation (Ac/Ds)* transposon tagging system. More than 8000 F₁s from a cross between plants carrying a *Ds* closely linked to *6a* and B73-6A were screened for SLB resistance in the field. Ten plants were identified as resistant and subsequent progeny testing showed that one of these plants appeared to be a genuine knockout of the susceptibility gene at the *6a* locus. We have also shown that this plant carries the *Ds* transposon and that the LHT1 gene appears to be disrupted. Work to identify the *Ds* insertion site is ongoing. The second approach was to transiently suppress the expression of *LHT1* using virus-induced gene silencing. Oh7B plants were infected with *Brome Mosaic Virus* (BMV) vectors carrying different fragments of *LHT1* (BMV-LHT1). Subsequently, the plants were inoculated with SLB. Five of 48 BMV-LHT1- inoculated plants showed areas with SLB resistant reaction similar to B73-6A while none of the control plants did.

We evaluated the effect of introgression 6A and another introgression conferring SLB resistance (introgression 3B on chromosome 3) on yield and other agronomic traits under both SLB disease pressure and disease-free conditions. Isohybrid triplets were developed by crossing B73, B73-3B (a B73 NIL carrying the 3B introgression) and B73-6A to several inbred lines (testers) and tested for SLB resistance. Only triplets in which the B73-6A or B73-3B hybrids significantly differed in resistance from the B73 check hybrids were selected for subsequent yield trial evaluation. These hybrids were evaluated according to a strip-split plot design in which identical blocks grown side by side were either inoculated with SLB or sprayed with a fungicide to protect them from disease. In the presence of SLB, averaged across testers, the 3B introgression was associated with an approximately 3% increase in

yield while introgression 6A did not confer any significant yield effect. In the absence of SLB, both introgressions were associated with some reduction in yield but this reduction was only statistically significant for introgression 6A.

Fine-Mapping and Agronomic Evaluation of a Quantitative Trait Locus Conferring
Resistance to Southern Corn Leaf Blight Caused by
Cochliobolus heterostrophus

by
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A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Plant Pathology

Raleigh, North Carolina

2012

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DEDICATION

To my beloved family and friends...

Para mi querida familia y amigos...

BIOGRAPHY

Jose Santa Cruz Hidalgo was born in Lima, Peru in 1979, to Raymundo Santa Cruz and Rosa Hidalgo. He spent eleven years in the “San Jose” Marists Catholic School in El Callao, Peru where he developed his love for scientific research and agriculture at an early stage. Also at an early stage, he decided to travel abroad to pursue graduate studies. With that in mind, he took English as a second language during high school. Following his passion for research, he enrolled at the National Agricultural University “La Molina” in Lima, Peru to pursue a career in Agronomy. During those years, he developed a profound interest in genetics, molecular biology and biotechnology, reason why he expanded his career with several classes of microbial, animal and plant genetics. After graduation, he decided to follow a career in research and got a scholarship to do his bachelors’s thesis research in the International Potato Center (CIP). While working at CIP, he worked at both the crop protection area under the supervision of Dr. Luis Salazar and the crop improvement and germplasm area under the supervision of Eng. Alberto Salas. He developed an increasing interest in host-pathogen interactions and disease resistance while working with both viruses and potatoes at CIP. His B.S. thesis titled - Genetic characterization of *Solanum* wild potato species for resistance to potato virus Y. His experience at CIP was defining into his decision to apply to the Plant Pathology graduate program at Penn State University in central Pennsylvania, US. In the summer of 2005, Jose began a Master’s degree in plant pathology under the guidance of Drs. Barbara Christ and Kathleen Haynes to study quantitative resistance on potatoes. At Penn State, he also got the chance to work as an administrative

assistant at the College of Agricultural Sciences under the direction of Mrs. Deanna Behring. This proved to be one of the most gratifying experiences at Penn State. He completed his M.S. thesis titled - Effects of one cycle of recurrent selection for early blight resistance in a diploid hybrid *Solanum phureja*-*S. stenotomum* population in Summer 2008. Right after finishing his M.S. degree, he changed gears and decided to move to the south. He enrolled at NC State University in Raleigh, North Carolina to pursue a Ph.D. program in plant pathology and plant breeding. At NC State, he switched potatoes for maize and studied its genetics of quantitative disease resistance against Southern Corn Leaf Blight under the supervision of Drs. Balint-Kurti and Krakowsky. During the period 2008-2011, he was awarded a fellowship from the Monsanto Fellows in Plant Breeding program to achieve his Ph.D. During those years in grad school in the US, Jose has met many fellow students, professors and coworkers that have had a profound effect on his professional and personal life. For that, he will always be grateful.

ACKNOWLEDGMENTS

A page is not enough to express my appreciation and gratitude to all the people who have made this Ph.D. research possible. I would like to especially thank the members of my advisory committee: Drs. Peter Balint-Kurti, Matthew Krakowsky, Gary Payne and James Holland, whose guidance and friendship have been truly inspiring. There are numerous other colleagues who have contributed with various aspects of the research. Thanks to Dr. Consuelo Arellano, David Rhyne, William Hill, Wayne Dillard, Dale Dowden, Larissa Benavente and Jared Smith for their important expertise. Abbey Sutton, Adisu Negeri, Ed Duren, Ryan Parks, Jennifer Patton-Ozkurt, and Randy Wisser for their help throughout the process. I would like to express my appreciation to Dr. Major Goodman for being an inspiration through the process of the Ph.D. program. Thanks to Cathy Herring and the staff of Central Crops Research Station, the NCSU Sandhills Research Station, the Peanut Belt Research Station, the Cunningham Research Station for their expertise in the field experiments. Also thanks to Janet Shurtleff, Carole Saravitz and the staff of the NCSU Phytotron. Thanks you to the Monsanto Fellows in Plant Breeding Fellowship for funding my graduate studies, to USDA-ARS, the NCSU Plant Pathology Dept., Center for Plant Breeding and Applied Plant Genomics, Corn Growers Association of North Carolina for supporting corn research. Finally, thanks to all the fellow grad students who have been important throughout this process: Araby Belcher, Ashley Zearfoss, Katie Neufeld, Kristen Kump, Jill Recker, Charlie Zila, Jake Delheimer, Mahendra Dia, Adam Call and all the folks from the Plant Pathology, Crop Science and Horticulture Depts.

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1. CHAPTER I

Literature Review

1.1. Introduction

Maize is one of the most important crops in the world, along with rice and wheat. The increasing demand for a more environmental friendly alternative to gasoline has increased the importance of corn as a biofuel. The US government has announced that the US production of biofuels is projected to be increased up to 35 billion gallons by 2017, boosting expected corn production and demand between 22.6 and 36% (IBISWorld <http://clients.ibisworld.com>). Yield is affected by many problems in the field. Drought, insects and diseases are among the most serious problems. Losses caused by some diseases can be harmful for maize production. Prevention of disease by means of using genetic resistance has been proved to be the most effective environmentally friendly form of control. One of the most useful methods of disease control is the use of host plant genetic resistance.

Many genes conferring resistance to several diseases have been identified and cloned in many crops; however, qualitative resistance has often proved to be short-lived, being easily overcome by pathogens. Furthermore, most of the disease resistance found and used in maize is quantitative in nature. The mechanisms involved in this resistance are not well understood; therefore, it becomes a priority to understand the molecular mechanisms behind quantitative disease resistance (QDR). In that sense, maize becomes a valuable system to

study QDR due to the plethora of genetic tools available in the system, and the whole-genome sequencing efforts.

1.2. Southern Leaf Blight

Southern leaf blight (SLB) can be found worldwide (Ullstrup, 1970), but it is mainly a problem in tropical and sub-tropical regions such as India, Latin America and Africa; as well as many other hot and humid areas in the world including the southeastern US. It is caused by the ascomycete *Cochliobolus heterostrophus* (Drechs.) Drechs., which is the scientific name for the teleomorph or sexual stage. The anamorph or asexual stage is recognized as *Bipolaris maydis* (Nisikado) Shoemaker. *Helminthosporium maydis* is an older name which can still be found in earlier literature.

The disease cycle starts when the overwintering mycelia on the soil and crop debris start the infection. Conidia can be transported to the leaves by wind or water splash. Conidia germinate and hyphae penetrate through stomata or directly through the epidermis. Then hypha invades the chlorenchyma and proliferates; but does not invade the vascular tissue (Jennings and Ullstrup, 1957; White, 1999). The fungus produces degrading enzymes that attack and kill the cells. SLB lesions can range in color from tan to brown with brown borders to dark reddish brown borders. SLB lesions can be elliptical to irregular and range in size from 0.6 to 6 mm in width by 0.6 to 22 mm in length (White, 1999). Then the fungus starts producing conidia that potentially start another cycle. SLB is a polycyclic disease and the asexual spores can produce many cycles during a season if the right conditions are present. During the sexual stage, pseudothecia are produced but this is rarely seen in nature

(Turgeon and Lu, 2000). Warm temperatures (25-32°C) and high humidity are the optimal conditions for the disease. Under these conditions, the fungus can complete its life cycle in 2-3 days. Moisture is also essential for initial infection (White, 1999).

There are two main physiological races of the pathogen (O and T) based on the symptoms they induce on maize, toxin production, optimum growth temperature, among others traits (Hooker *et al.*, 1970b). This classification is heavily based on cytoplasm specificity of the corn plants they infect. Corn possessing the Texas cytoplasmic male sterility (called Texas sterile cytoplasm or T-cytoplasm) is highly susceptible to race T, while corn carrying normal cytoplasm (N-cytoplasm) is susceptible to race O. *C. heterostrophus* race T produces T-toxin, a group of linear polyketides required for high virulence on T-cytoplasm corn. T-toxin interacts with a specific receptor protein (URF13) located in the inner membrane of mitochondria from maize carrying T-cytoplasm. The toxin disorganizes the mitochondrial membrane, making it more porous and less selective. It eventually disrupts the membrane and kills the cell. N-cytoplasm does not contain the gene that produces URF13; therefore, N-cytoplasm maize is not sensitive to the toxin.

Yield losses of economic importance caused by SLB are rare in the US because most corn grown has some level of resistance (Hooker *et al.*, 1970b). It was given little attention in the US until the 1970 SLB epidemic caused by race T of the pathogen (Hooker *et al.*, 1970a; Hooker *et al.*, 1970b; Hooker, 1972). The presence of favorable environmental conditions for disease development and rapid spread, and the intensive maize production containing T-cytoplasm were the right combination that triggered the 1970 SLB epidemics.

1.3. Disease Resistance

One of the most useful methods of disease control is the use of host plant genetic resistance. Genetic resistance can be classified in two broad main classes: qualitative resistance and quantitative disease resistance (here termed QDR). Qualitative resistance is characterized by a complete or near complete reduction of the disease. On the other hand, QDR is characterized by a partial inhibition of the pathogen. This classification is sometimes too simple as more information about the complex nature of disease resistance is further studied that challenges it.

1.3.1. Qualitative Disease Resistance

Qualitative disease resistance is also known as monogenic or vertical resistance. It is typically governed by a single gene with a major effect, and it is normally race-specific. It is usually not developmentally or environmentally influenced, and it is often associated with resistance against biotrophic pathogens. Many genes conferring resistance to several diseases have been identified and cloned in many crops; however, qualitative resistance has often proved to be short-lived, being easily overcome by pathogens (Van der Plank, 1982; Agrios, 2005).

1.3.1.1. Genetic Molecular Basis of Qualitative Resistance

The 'gene for gene' interaction described by Flor (1971), at its simple definition, is when an *R* gene product in the plant interacts with the corresponding pathogen avirulence (*Avr*) gene product, triggering resistance. When the plant does not carry the *R* gene, it is

susceptible to the pathogen. If the pathogen does not produce the avirulence gene product, it escapes the *R* gene-mediated recognition and therefore, the plant is susceptible to the pathogen attack (Flor, 1971) (Fig. 1.1). This interaction triggers a chain of downstream events that activates a series of defense mechanisms that culminates in resistance (Flor, 1971; Dangl and Jones, 2001; Jones and Dangl, 2006). These events usually involve a hypersensitive response (HR), oxidative burst and other loss of cell integrity related mechanisms. HR is defined as a quick controlled form of programmed cell death that is usually localized in the site of infection (Heath, 2000; Bent and Mackey, 2007).

Pathogen effectors are small molecules that are delivered into the host cell. Some of them are avirulence gene products and can be recognized by the *R* gene product in a 'gene for gene' manner. Some *R* genes can recognize these effectors directly, but some *R* genes can recognize an indirect interaction between the effector and the *R* gene product. The 'guard' hypothesis states that the *R* gene protein detects the interaction of the effector target with the target itself or any modification of the host target activity or structure (Van der Biezen and Jones, 1998; Jones and Dangl, 2006). For example, RIN4 is an *Arabidopsis thaliana* protein that interacts with different *Pseudomonas syringae* effectors: AvrRpm1, AvrB, and AvrRpt2. These effectors are found in different pathogenic *P. syringae* strains and their sequences are unrelated (Grant *et al.*, 2006). *R* genes *Rpm1* and *Rps2* from *A. thaliana*, each encodes a plasma membrane-associated protein. RPM1 and RPS2 have both a nucleotide binding (NB) and leucine-rich repeats (LRR) domains but do not directly recognize the effectors. RPM1 and RPS2, instead, recognize the modifications of RIN4 by the effectors. AvrRpm1 and AvrB hyperphosphorylate RIN4. RPM1 is activated by this hyperphosphorylation and triggers HR

(Mackey *et al.*, 2002). AvrRpt2 affects RIN4 in a different way. AvrRpt2 is a cysteine protease and cleaves RIN4 at two different sites. RPS2 recognizes this cleavage (Axtell *et al.*, 2003; Day *et al.*, 2005; Kim *et al.*, 2005).

But not all qualitative resistance work in this way. Resistance gene *Hm1* (*Helminthosporium maydis1*) against *Cochliobolus carbonum* produces a toxin reductase that inactivates the HC toxin produced by the pathogen (Johal and Briggs, 1992). Transcription activator-like (TAL) effectors are a type of effectors that are also delivered into the host cell by pathogenic bacteria. These activators mimic the host transcription factors, directly bind to its DNA to manipulate and reprogram the host cell (Schornack *et al.*, 2006; Yang *et al.*, 2006; Kay *et al.*, 2007). AvrBs3 is a TAL effector delivered by *Xanthomonas campestris* pv *vesicatoria* that activates the *upa* box in promoter of the cell size regulator Upa20 among other genes (Kay *et al.*, 2007). This results in an advantage for the pathogen and contributes to its virulence. Bs3 is a flavin monooxygenase that confers resistance against *X. c.* pv *vesicatoria* in pepper plants. *Bs3* possesses an *upa* box in its promoter which is also recognized by the AvrBs3. So AvrBs3 activates *Bs3* expression, leading to HR (Römer *et al.*, 2007). Even though there is much progress in understanding the molecular mechanisms of qualitative resistance, there is still many aspects that need to be clarified to assess a durable resistance.

1.3.2. Quantitative Disease Resistance (QDR)

QDR usually involves several genes and is race-nonspecific. This resistance is normally influenced by the environment and by the plant development. Depending on the pathosystem, QDR is also known as minor effect, polygenic, rate-reducing, incomplete, multigenic, partial, race-nonspecific, general, adult-plant, field, durable or horizontal resistance (Agrios, 2005). Unlike qualitative resistance, QDR is often regarded as durable resistance and it is often associated with resistance against necrotrophs, though there are QDR against biotrophs as well. Even though there are pathosystems where qualitative resistance have been durable and satisfactory, most of the durable resistance used for breeding purposes is quantitative in nature.

QDR is controlled by many small effect loci or disease resistance quantitative trait loci (dQTL), each having an additive effect. It is suggested that each dQTL works at different levels of the plant-pathogen interaction, thus reducing the attack, adherence, entrance, growth and multiplication of a pathogen (Agrios, 2005). The phenotype may include different mechanisms of resistance such as slow-spreading, reduced sporulation, and an overall partial resistance against the disease (Van der Plank, 1975). Because of this variability, QDR can be difficult to detect for breeding purposes. While mapping and cloning genes controlling qualitative resistance have been easy to achieve, it is a more difficult task to map and clone dQTLs. Some of the reasons that makes this task difficult stems from the number of loci and their small effects, as well as the interaction with the environment and interaction with the plant development (Lynch and Walsh, 1998; Mackay, 2001). Therefore, to identify a

particular dQTL, different mapping methods involving sophisticated statistical analyses and experimental designs across different environments have been developed.

1.3.2.1. Molecular Mechanisms of QDR

Although QDR has been used by plant breeders to protect major crops from diseases, little work has been achieved in the study of the identity and mechanisms of the genes responsible for QDR (Balint-Kurti and Johal, 2008). While several major effect genes for resistance have been mapped and cloned, only a few genes which underlie dQTLs have been identified. The genes underlying five dQTLs have been cloned. *Pi21*, a recessive rice gene against rice blast, produces a protein with a heavy metal-transport/detoxification protein domain in the N-terminal region (Fukuoka *et al.*, 2009). Resistance is conferred by *pi21* recessive allele. This defective allele has a couple of single nucleotide deletions in a proline-rich protein domain. It is hypothesized that this proline-rich domain is involved in protein-protein interactions and that resistant *pi27* allele somehow competes and inhibits this interaction. A germin-like gene family (*OsGLP*) conferring broad spectrum resistance in rice against *Magnaporthe oryzae* and *Rhizoctonia solani* is thought to enhance basal defense responses. The family is predicted to produce enzymes with superoxide dismutase activity. These enzymes are known to be involved in the generation of H₂O₂ (Manosalva *et al.*, 2009). In wheat, the high temperature dependent gene *Yr36* against wheat stripe rust encodes a protein containing a predicted kinase domain and a predicted steroidogenic acute regulatory protein-related lipid transfer or START domain. These domains suggest a role in lipid trafficking, metabolism and sensing (Fu *et al.*, 2009). It is suggested that *Yr36* is involved in

regulation of plant defense based on the similarity with *A. thaliana* defense protein against powdery mildew pathogen. Wheat gene *Lr34* against diseases caused by leaf rust fungus *Puccinia triticina*, stripe rust pathogen *P. striiformis*, and the powdery mildew fungus *Blumeria graminis* produces a protein similar in structure to the multi-drug resistance subfamily of ATP-binding cassette (ABC) transporters. These proteins are known to confer resistance to different drugs. Its similarity to *A. thaliana* gene *PEN3* suggests that *Lr34* may be involved in the export of metabolites that affect fungal growth as well (Krattinger *et al.*, 2009). In maize, *Rcg1* against anthracnose stalk rot and leaf blight is predicted to be a R gene (Frey, 2006).

1.3.3. Plant Immune System

The classification of resistance into qualitative and QDR is based on the reaction of the different pathogen strains on the plant host. At a molecular level, there are two different criteria to classify the plant immune system (Fig. 1.2). The first criterion uses transmembrane pattern recognition receptors (PRRs) that respond to pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) (Chisholm *et al.*, 2006). PAMPs are small molecular motifs that are highly conserved among pathogens. PAMPs are important to the pathogen to survive and thrive in the host environment: e.g. structural components of cell walls such as chitin, extracellular polysaccharides, and flagellum among others (Jones and Dangl, 2006). PAMPs are recognized by conserved defense receptors (PRRs) resulting in PTI (PAMP-Triggered Immunity) (Jones and Dangl, 2006). Flagellin is a common example of a PAMP and probably the most well studied system in PTI (Zipfel *et al.*, 2004). Some bacteria

possess a flagellum, which help them swim their way through their environment looking for nutrients. The flagellum is composed of a protein named flagellin. One component of this flagellin, *flg22*, is recognized by the FLS2 receptor in *Arabidopsis*. FLS2 is a receptor-like kinase with a leucine-rich repeat domain (LRR). This recognition leads to the activation of basal host defense responses such as callose deposition, activation of pathogenesis related (PR) genes, and activation of the salicylic acid (SA) pathway.

Pathogens have evolved different mechanism to circumvent PTI. Pathogens deliver small molecules called effectors that among other things, suppress the basal defense responses. Bacterial effectors are the best characterized effectors. These effectors are delivered through the Type 3 secretion system (TTSS). Bacteria inject many effectors with different functions, many of them unknown. Some of them suppress PTI at different levels of the defense response (Fig. 1.2). For example, as mentioned above, the PAMP flagellin *flg22* is recognized by the FLS2 receptor in *Arabidopsis*, triggering PTI. But bacteria deliver many effectors through the TTSS, one of them being AvrPto. AvrPto inhibits the kinase of FLS2 receptor, resulting in susceptibility to the pathogen (Xiang *et al.*, 2008).

The second criterion involves the recognition of pathogen effectors by the host R genes products. Effector Triggered Immunity (ETI) encompasses the defense responses activated by the R protein recognition of the effector or its function in the cell, and it represents qualitative disease resistance (See section 1.3.1.1.).

1.4. Genetic Basis of SLB Resistance

Resistance to *C. heterostrophus* race T is due to the lack of the toxin receptor site. As previously mentioned, race T produces T-toxin which interacts with a specific receptor protein (URF13). URF13 is located in the inner membrane of mitochondria only in maize carrying T-cytoplasm. The toxin then disorganizes the mitochondrial membrane and kills the cell. N-cytoplasm does not contain the gene that encodes URF13; therefore, maize carrying N-cytoplasm is resistant to the race T.

SLB qualitative disease resistance has been found in maize. A single recessive gene for resistance against *C. heterostrophus*, *rhm1* (resistance to *Helminthosporium maydis*), has been identified in a Nigerian breeding stock (Craig and Daniel-Kalio, 1968; Craig and Fajemisin, 1969). Resistance locus *rhm1* was subsequently mapped to the distal end of the short arm of chromosome 6 in maize (Neuffer and Calvert, 1975; Zaitlin *et al.*, 1993). This resistance is expressed in juvenile and adult plants, but it is only partially effective after anthesis (Thompson and Bergquist, 1984). Based on transposon tagging experiments, Chang and Peterson (1995) suggested that the resistance conferred by *rhm1* locus is actually two closely linked genes. Crossing a homozygous susceptible *Rhm1* line containing a transposable element to a homozygous resistant *rhm1* line resulted in a F₁ progeny with a higher number of resistant mutants than the expected number based on the mutation rates of the transposable element used. To account for this higher mutation rate, they proposed that *rhm1* locus was in fact two linked loci (*rhm1* and *rhm2*), in which, resistance is shown only when both genes are homozygous recessive *rhm1 rhm2/rhm1 rhm2*. This higher number of

mutants would have arisen by recombination events on the susceptible *Rhm1 rhm2/rhm1 Rhm2* parent. See Fig. 1.3 for details.

Most of SLB resistance used in maize is quantitative in nature. Several studies have reported quantitative SLB resistance. Pate and Harvey (1954) first described partial resistance found in US germplasm. Subsequent studies on inheritance demonstrated that this quantitative resistance is additive (Lim and Hooker, 1976; Thompson and Bergquist, 1984; Burnette and White, 1985). Holley and Goodman (1989) identified additive and recessive types of gene action working with different selected inbred lines from tropical hybrid maize derivatives. The new sources of resistance were identified by topcrossing to SLB resistant NC250 line and susceptible B73. It is suggested that the resistance identified is controlled by relatively few genes that worked together epistatically with the resistance genes in NC250.

The development of molecular markers facilitated the mapping of QTLs. Several authors using populations derived from different crosses have identified many SLB dQTLs. Using three different populations (ADENT x B73rhm, B73rhm x NC250A and NC250A x B73) and 80 restriction fragment length polymorphism (RFLPs) markers, Bubeck (1991) identified 7 dQTLs conferring SLB resistance. Using composite interval mapping, Carson *et al.* (2004) identified 11 dQTLs in a recombinant inbred line (RIL) population F_{2:7} derived from a B73 x Mo17 cross. The same population was examined for juvenile plant resistance to SLB by Balint-Kurti and Carson (2006). 6 dQTLs on chromosomes 1, 2, 3, 6, 7, and 8 were identified. The intermated B73 x Mo17 (IBM) population is an advanced intercross RIL population from the B73 x Mo17 cross composed of 302 F_{7:8} RILs. This population was allowed to intermate four times prior to the derivation of inbred lines, thus increasing the

number of recombination events compared to other RIL populations. The IBM population therefore provides greater mapping resolution compared to any other RIL population (Sharopova *et al.*, 2002; Balint-Kurti *et al.*, 2007). Using the IBM population, Balint-Kurti *et al.* (2007) identified 4 common QTLs in all the four environments tested: two on chromosome 3 and one each on chromosomes 1 and 8. Several dQTLs have been identified in other populations. Balint-Kurti *et al.* (2006) identified 7 dQTLs in a RIL population from the cross NC300 x B104 and two of the strongest dQTLs were found on chromosomes 3 and 9. This study was the first report of a major effect dQTL for resistance to SLB on chromosome 9. In another experiment, Balint-Kurti *et al.* (2008) identified different dQTLs while working with two different RIL populations. Using a RIL derived from the H99 x B73 cross, 2 dQTLs were identified on chromosome 3 and one on each of the chromosomes 6 and 8; while only one dQTL on chromosome 2 was detected using the RIL population from the cross B73 x B52. These QTL mapping studies suggested that there are two ‘hotspots’ for SLB resistance loci on chromosome 3 and 6 (Balint-Kurti *et al.*, 2008). Zwonitzer *et al.* (2009) mapped 9 dQTLs for SLB resistance using two $F_{2:3}$ populations derived from two different crosses: B73rhm1 x NC250A and NC250A x B73. See section 1.5.1 for details.

The nested association mapping (NAM) is a group of 5,000 RILs derived from crosses between the inbred line B73 to a set of 25 other inbreds (founder inbreds) (McMullen *et al.*, 2009). Recently, a joint-linkage analysis on this NAM population identified 32 dQTLs with small additive effects on SLB resistance (Kump *et al.*, 2011). Using genome-wide association tests, Kump *et al.* also identified 51 SNPs (single nucleotide polymorphisms) markers within and outside the intervals of the previously identified 32 dQTLs that were

associated with variation for SLB resistance. From the 32 dQTL, 5 contained no SNPs, 21 contained a single SNP and 6 contained two SNPs. These SNPs were located within or near sequences, some of them homolog to genes involved in disease resistance (Kump *et al.*, 2011). For a comprehensive summary of the SLB dQTLs identified in these publications, refer to supplementary figure 4 in Kump *et al.* (2011).

1.5. Relevant Background Information on the Project

1.5.1. Plant Materials

The populations to be used in this project were derived from an original cross between SLB-susceptible maize line B73 and a highly SLB-resistant line NC250P, generated at the NCSU maize breeding program. NCSU-released inbred lines NC250 and NC250A are sister lines derived from NC250P as a common progenitor. These SLB resistant lines were all developed from the cross (Nigeria Composite A-Rb x B37) x B37 (Thompson and Bergquist, 1984). B73 is an inbred line with good agronomic qualities derived by recurrent selection of a randomly mated Iowa Stiff Stalk Synthetic line (BSSS) at Iowa State University (Russell, 1972). Inbred line B37 was developed from a earlier cycle of selection of the same Iowa Stiff Stalk Synthetic line (Russell *et al.*, 1971). NC292 and NC330 are sister near-isogenic lines (NILs) that were obtained by backcrossing the NC250P several times to B73 (three and four times for NC292 and NC330, respectively) and selecting for SLB resistance at each generation . Therefore, NC292 and NC330 are agronomically similar to B73, but highly resistant to SLB. A genome-wide analysis identified twelve small NC250P-derived chromosomal regions (introgressions) that were different in B73 compared to NC292 and

NC330. Eight of the twelve introgressions were shared between NC292 and NC330 (Zwonitzer, 2008). One of these introgressions, located in chromosome 6, was named introgression 6A. B73-6A is a B73-background NIL carrying the NC250P introgression 6A. This NIL was developed from a NC292 x B73 cross. F₁s were then backcrossed to B73, followed by two generations of selfing in order to generate BC₁F₃ progeny. During the process, BC₁F₁ plants were genotyped with SSR markers to select families with few introgressions and these plants were self-pollinated to produce the BC₁F₂. A second genotyping was performed on these individuals to select for homozygosity for this NC250P introgression (Zwonitzer *et al.*, 2009).

Two F_{2:3} populations derived from two different crosses: B73rhml x NC250A and NC250A x B73, were used to generate a linkage map and to map disease resistance QTLs associated with SLB resistance (Zwonitzer *et al.*, 2009). The original crosses were first described by Bubeck (1991). B73rhml used in the B73rhml x NC250A is a B73-background NIL provided by R. R. Bergquist (Pfister Hybrid Corn Company, El Paso, IL). Resistance in this B73rhml lines can be traced back to the same highly resistant Nigerian composite line from where NC250P was derived (Craig and Daniel-Kalio, 1968; Craig and Fajemisin, 1969; Smith and Hooker, 1973; Thompson and Bergquist, 1984; Holley and Goodman, 1989). Therefore, the parents used for mapping are essentially the same parents that were used to develop NC292 and NC330. Nine dQTLs for SLB resistance were mapped using both populations combined, but only 4 colocalized with introgressions found to come from the resistant parent NC250P in NC292 and NC330. Introgressions 3B, 6A and 9B, found in chromosomes 3, 6 and 9, respectively, had a significant effect on resistant to SLB compared

to the susceptible line B73. From these, the QTL present in introgression 6A was the only having a significant effect on SLB resistance in both adult and juvenile plants (Belcher *et al.*, 2011).

1.5.2. Cost of Resistance

There are several studies on how much induction of resistance costs to the host; however, most of them deal with induced qualitative resistance like the ones conferred by R genes. The effect of QDR in the plant fitness is little studied. Korves and Bergelson (2004) demonstrated that there was a cost of resistance in *A. thaliana* when there was not intraspecific competition. Resistant *A. thaliana* plants actually produced fewer seed than susceptible plants under disease pressure. Orgil *et al.* (2007) tested *RPW8* locus which contains two genes that together confer broad spectrum resistance in *A. thaliana*. Using a transgenic approach, they determined that *RPW8* locus have a negative effect on fitness when plants are not infected. This cost of resistance can be attributable to the expression of *RPW8* genes. Tian *et al.* (2003) showed that there is around 9% of yield reduction (as total seed production) due to the presence of R gene *RPM1* in uninoculated *A. thaliana* plants. The study used a pair of transgenic lines that were identical, except for the presence of *RPM1* gene. The gene was inserted in a non coding region thus avoiding any other effect except the effect from *RPM1*.

Systemic acquired resistance (SAR) is a basal induced mechanism commonly present in all angiosperms. Some studies have demonstrated a cost effect of artificially induced SAR. Dietrich *et al.* (2005) used the chemical compound benzothiadiazole to artificially activate

SAR pathway. Results showed a significant reduction in *A. thaliana* plants growth after being treated with benzothiadiazole. Heidel *et al.* (2004) tested different mutations affecting different points of SAR activation pathway associated with disease resistance. They concluded that these genes are associated with a cost on fitness in the field, but failed to show any effects on growth chamber experiments. While most of the publications address single gene effects on fitness, few studies have worked with QDR. Michell-Olds and Bradley (1996) studied the cost on fitness on *Brassica rapa* lines selected for resistance to 2 different pathogens. These lines were recurrent selected for QDR against *Leptosphaeria maculans* and *Peronospora parasitica*. *L. maculans* QDR had no effect on growth rate, but QDR against *Peronospora parasitica* had a negative effect on growth rate in the absence of disease. Frey *et al.* (2011) did not find any significant cost of fitness in maize for having the dQTL *Rcg1*, against *Colletotrichum graminicola*. Working with near-isogenic hybrids, they concluded that *Rcg1* did not confer a cost in yield under disease-free conditions in the field when compared to hybrids without *Rcg1*.

1.5.3. AC/DS Transposon Elements

Since they were first discovered by McClintock (McClintock, 1948, 1949), transposable elements (TE) have been widely characterized using classical genetics and molecular techniques (Brutnell and Conrad, 2003). The *Activator (Ac)* and the *Dissociation (Ds)* elements are transposable element belonging to the class II “cut and paste” (non-replicative) DNA transposons. *Ac* is a 4565-bp autonomous element capable of catalyzing its own transposition (McClintock, 1949). *Ac* encodes a 3.5-kb open reading frame that encodes

an 807-amino-acid transposase essential for *Ac* and *Ds* transposition (Fedoroff *et al.*, 1983). While the *Ac* is an autonomous TE, *Ds* is a non-autonomous TE and depends on the supply of transposase from other autonomous TEs in the genome (Kunze *et al.*, 1997). In contrast to the sequence of *Ac* elements, *Ds* sequences are more variable. But both *Ac* and *Ds* share common sequences: the terminal inverted repeats (TIR) and a 240 bp of subterminal sequences. These sequences are critical for recognition and binding of the transposase and transposition of both *Ac* and *Ds* (Coupland *et al.*, 1988). *Ac* elements can transpose themselves at a low frequency (2-4% of the progeny will inherit a newly transposed *Ac*) (Brutnell and Dellaporta, 1994). 60% of the transpositions occur to genetically linked sites (within 10cM of the donor) (Van Schaik and Brink, 1959; Greenblatt, 1984; Dooner and Belachew, 1989).

Tom Brutnell's lab at Cornell University have developed a collection of lines each containing a *Ds* at a unique position every 1 cM, and a defective *Ac* element (named immobilized *Ac* or *im:Ac*) incapable of transposition but able to encode the transposase (Vollbrecht *et al.*, 2010). This *im:Ac* element arose due to an aberrant transposition event in which their flanking regions have been truncated, being unable to be recognized by the transposase. This *Ac/Ds* system is a great tool for regional mutagenesis (Brutnell and Conrad, 2003; Kolkman *et al.*, 2005; Ahern *et al.*, 2009; Vollbrecht *et al.*, 2010).

1.5.4. Post-Transcriptional Gene Silencing and Virus-Induced Gene Silencing

Post-transcriptional gene silencing (PTGS) was first discovered by several groups working in different model organisms. With the development of transgene technology, many organisms were engineered with exogenous genes to enhance certain traits. In plants, petunia plants were transformed with a gene to increase the purple color of the flowers. Plants failed to express the transgene and even silenced the endogenous copy of the gene. This silencing of the endogenous copy was referred as 'cosuppression' in plants (Lindbo and Dougherty, 1992; Jorgensen *et al.*, 1996; Que and Jorgensen, 1998) and it was one of the first reports of gene silencing. PTGS is a complex mechanism involving many enzymes and processes, but the basic mechanism of PTGS can be summarized as follows. Silencing is initiated when the double stranded RNA (dsRNA) is recognized by the plant dicer-like ribonucleases. These enzymes process the dsRNA into single stranded RNA (ssRNA) of about 21 to 24 nucleotides (Llave, 2010). RNA-dependent RNA polymerases then recognize the ssRNA and use it as a template to generate more dsRNA. These dsRNA are used as a template by the RNA-induced silencing complex (RISC). In a sequence-specific manner, RISC degrades endogenous messenger RNA or viral dsRNA. As a result, the virus and/or the endogenous mRNA transcripts become silenced (Robertson, 2004; Voinnet, 2005; Llave, 2010).

Virus-induced gene silencing (VIGS) refers to the use of a viral RNA as a carrier of a fragment of interest that be silenced. By infecting with this viral RNA carrying the fragment of interest, the silencing machinery can be used to target not only the virus but also the corresponding endogenous host transcripts (and closely related sequences). VIGS has been successfully used in many crops to silence genes of interest, especially in many dicots. In

monocots, the first report of the use of VIGS to silence endogenous genes was on barley (Holzberg *et al.*, 2002). Holzberg *et al.* successfully used *Barley stripe mosaic virus* (BSMV) to transiently silence phytoene desaturase (*pds*) gene, among other genes. PDS is required for synthesizing carotenoids, and produces a clear phenotype on leaves. This phenotype, characterized by photobleached sectors on leaves, makes it a good reporter of silencing activity. More importantly, VIGS have been used to silence resistance gene *Lr21* in wheat (Scofield *et al.*, 2005). Scofield *et al.* successfully silenced the resistance gene turning a resistant wheat plant into susceptible when inoculated with wheat leaf rust. *Rice tungro bacilliform virus* (RTBV) has also been implemented for VIGS in rice plants (Purkayastha *et al.*, 2010). Ding *et al.* (2006) successfully implemented a VIGS system for maize, barley and rice plants using *Brome mosaic virus* (BMV). BMV is a positive-strand tripartite RNA virus and the type member of the genus *Bromovirus*. More recently, this system was successfully used to characterize the role of candidate genes involved in the interaction between the biotrophic fungus *Ustilago maydis* and maize inbred line Va35 plants using BMV (van der Linde *et al.*, 2010).

The vascular puncture inoculation (VPI) was first developed by Louie (1995) as an efficient way to mechanically inoculate and transmit maize viruses using a jeweler engraving tool. VPI delivers purified viral RNA and DNA as effectively as virions (virus nucleic acid plus proteinic envelop) when inoculated at high concentrations. This eliminates the need for virion preparations before inoculation (Redinbaugh *et al.*, 2001).

1.6. Research Objectives

The overall goal of this research proposal is to use the available tools to study these loci accounting for quantitative resistance against SLB in maize. Specific objectives include fine-mapping introgression 6A, and evaluating yield and fitness for possible future use of this resistance.

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		Pathogen	
		Avr+	Avr-
Host	R_	Resistance (Incompatible)	Disease (compatible)
	rr	Disease (compatible)	Disease (compatible)

Figure 1.1 Schematic representation of the “gene for gene” interaction described by H.H. Flor. When the *R* gene is present in the host plant and its gene product interacts directly or indirectly with a corresponding pathogen effector (*Avr* gene), an incompatible reaction (resistance) is seen. If the pathogen ‘loses’ the effector (*Avr*-), it escapes the *R* gene-mediated recognition; therefore, the plant is susceptible to the pathogen attack (compatible reaction). When the plant host does not carry the *R* gene (*rr*), it is susceptible to the pathogen with or without *Avr* gene (compatible).

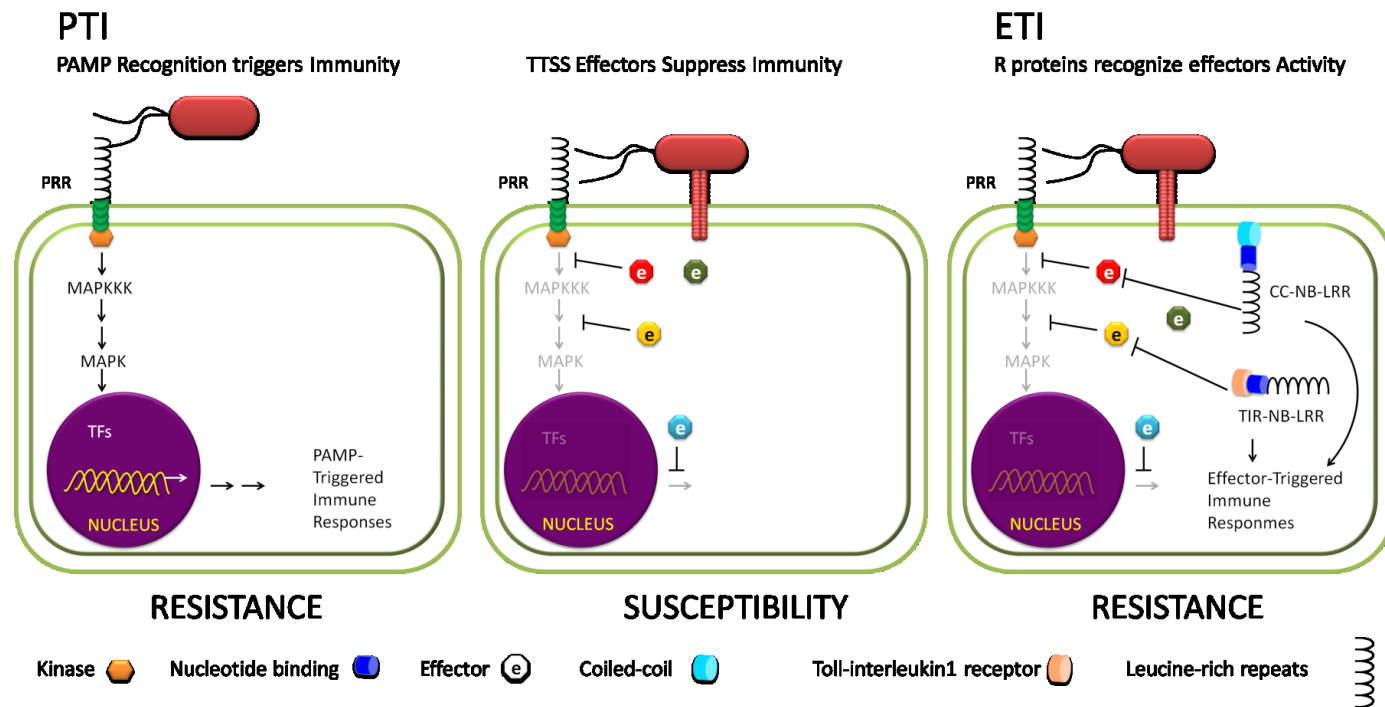


Figure 1.2 The plant immune system. Left to right, PAMP-triggered immunity (PTI): bacterial pathogen-associated molecular pattern (PAMP), flagellin, is recognized by the pattern recognition receptor (PRR) leading to mitogen-associated protein kinases (MAPK and MAPKKK) cascades and transcription factors (TFs) activation of basal responses. Pathogenic bacteria inject effectors through the type 3 secretion system (TTSS) that target different host proteins to suppress PTI. Effector-triggered immunity (ETI): Plant R proteins (e.g.: CC-NB-LRR and TIR-NB-LRR) recognize the effector or the effector activity and trigger ETI responses such as HR. Based on Chisholm *et al.*, 2006.

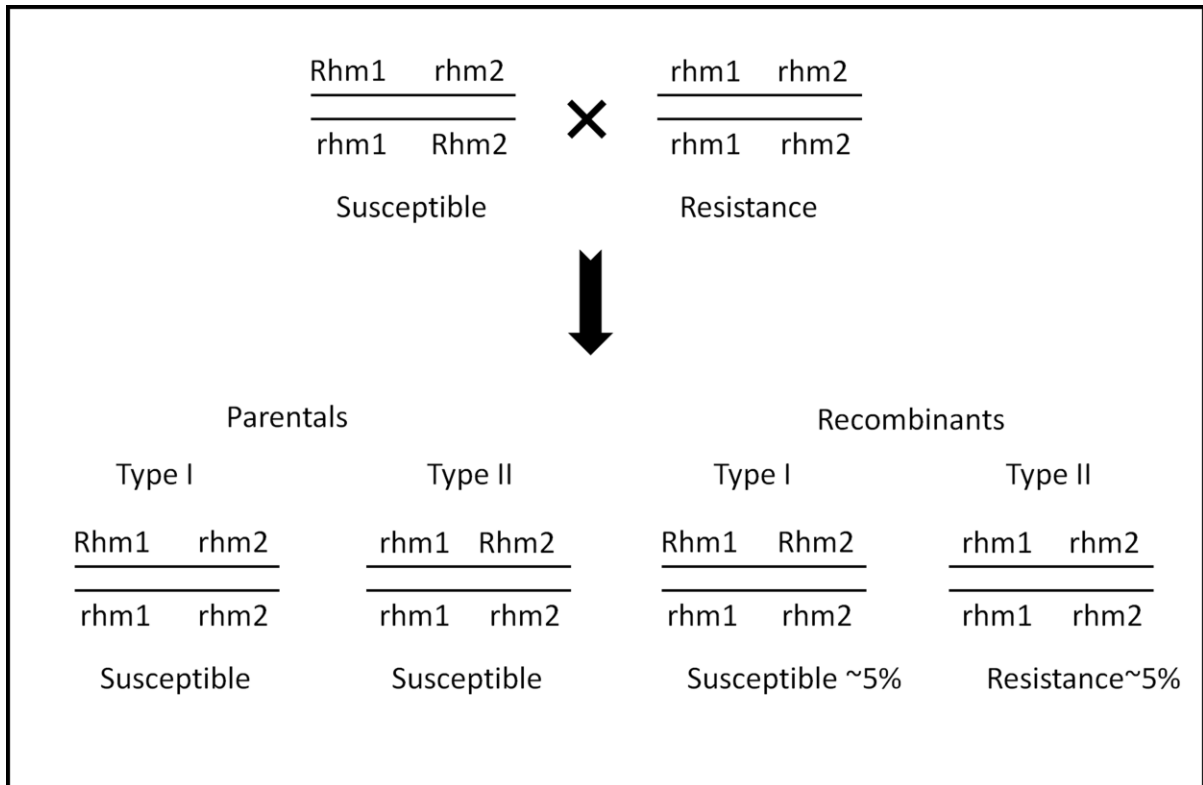


Figure 1.3 Model for SLB resistance controlled by two linked recessive genes. Recombination between the two genes *rh_{m1}* and *rh_{m2}* produced approximately 5% resistant seedlings in the progeny (recombinants type II). Based on the two-gene model from Chang and Peterson, 1995.

2. CHAPTER II

Fine-Mapping a Quantitative Trait Locus Conferring

Resistance to Southern Leaf Blight Caused by *Cochliobolus heterostrophus*

2.1. Abstract

Southern leaf blight (SLB) caused by *Cochliobolus heterostrophus* is a disease in southeastern US, as well as many hot and humid areas in the world. To investigate quantitative disease resistance (QDR) to SLB, we have used a pair of B73-background near isogenic lines (NILs). One is the highly susceptible line B73 and the other is B73 containing an introgression (B73-6A) in the short arm of chromosome 6 derived from a highly resistant line NC292. Introgression 6A co-localizes with a disease resistance quantitative trait loci (dQTL) (*6a* locus) for resistance against SLB. Experiments carried out in both growth chamber and field suggested that SLB resistance from B73-6A might be due to a single recessive locus. The objective of this study was to fine-map introgression 6A and to determine the possible candidate genes underlying this *6a* locus for future cloning. Using both publicly available and newly designed markers, fifteen families from the cross of B73 and B73-6A were found to contain a recombination event within the introgression 6A. These recombinants were used to narrow down the candidate region to a ~300 Kb region. The region contained 12 filtered genes (genes that were selected after transposon and orthology screens were applied) and 12 other gene loci, composed of evidence-based genes and non-overlapping predicted genes (predicted using Fgenesh gene model software

(www.softberry.com). Five of the genes belong to the amino acid transporter gene family. Furthermore, one of these transporters GRMZM2G127328 has been described as a lysine histidine transporter-1 gene (LHT1) and may be involved in the SLB resistance conferred by gene *rhm1*. *LHT1* may be a good candidate for cloning experiments.

2.2. Introduction

Maize (*Zea mays* L. ssp. *mays*) is affected by many problems in commercial fields. Yield losses caused by some diseases can be detrimental for maize production. One of the most useful methods of disease control is the use of host plant genetic resistance. Genetic resistance can be classified into qualitative or quantitative resistance. The former, also known as monogenic or vertical resistance, is generally governed by a single gene (R genes), and is normally race-specific. It acts in a 'gene for gene' interaction manner. The 'gene for gene' interaction described by Flor (1971), at its simplest, is when an R gene product in the plant interacts with the corresponding pathogen effector product, triggering resistance. Many R genes conferring qualitative resistance to several diseases have been cloned. However, this type of resistance has often proved to be short-lived, being easily overcome by pathogens. On the other hand, QDR is characterized by a partial inhibition of the pathogen and is usually race-nonspecific. It usually involves several genes and it is often influenced by the environment. Most of the disease resistance found and deployed in maize is quantitative in nature, but the genetic and functional basis of plant quantitative disease resistance is poorly understood (Poland *et al.*, 2009).

Southern leaf blight (SLB) caused by *Cochliobolus heterostrophus* (Drechs.) Drechs. (anamorph = *Bipolaris maydis* (Nisikado) Shoemaker), is a common disease in southeastern US, as well as many hot and humid areas in the world. It was the causal agent of the SLB epidemic caused by race T of the pathogen in 1970; however, yield losses are rare in the US because most corn grown has some level of resistance (Hooker *et al.*, 1970b). Several studies have reported quantitative resistance against this pathogen in US germplasm (Pate and

Harvey, 1954; Hooker *et al.*, 1970a; Lim, 1975; Lim and Hooker, 1976; Thompson and Bergquist, 1984; Burnette and White, 1985; Holley and Goodman, 1989; Balint-Kurti *et al.*, 2006). However, SLB is still a problem in other parts of the world; especially in tropical and sub-tropical regions such as India, Latin America and Africa, with the potential to cause significant losses (White, 1999; Shah *et al.*, 2007).

To investigate QDR to SLB, we have used a pair of B73-background near isogenic lines (NILs). One is the highly susceptible line B73 and the other is B73 containing an introgression (B73-6A) in the short arm of chromosome 6 derived from a highly resistant line NC292 (Zwonitzer *et al.*, 2009). Introgression 6A co-localizes with a disease quantitative trait loci (dQTL) (*6a* locus) for resistance against SLB (Zwonitzer *et al.*, 2009). Introgression 6A is about 11Mb and has a significant effect on both field and growth chamber juvenile plant resistance to SLB. The resistance locus *rhm1*, as well as *6a*, has been mapped in the same region of the short arm of chromosome 6 (Zaitlin *et al.*, 1993). *rhm1* acts as a single recessive gene for resistance against SLB and has an effect on juvenile and adult plants (Thompson and Bergquist, 1984). Resistances in both B73-6A and a B73-background NIL carrying *rhm1*, B73rhm1, can be traced back to the same highly resistant Nigerian breeding stock (Craig and Daniel-Kalio, 1968; Craig and Fajemisin, 1969; Smith and Hooker, 1973; Thompson and Bergquist, 1984; Holley and Goodman, 1989).

Proposed mechanisms for quantitative disease resistance include passive and active defenses. All of these mechanisms have been linked to dQTLs in other plant-pathogen systems, but the precise modes of action in maize still needs to be elucidated. Recently, the maize gene *Rcg1* was cloned from a dQTL against anthracnose stalk rot and leaf blight

caused by *Colletotrichum graminicola*. *Rcg1* is predicted to encode a protein that share similarities to an R gene product because it produces NB-LRR (nucleotide-binding-leucine-rich repeat) motifs (Frey, 2006). These proteins are thought to be directly or indirectly involved in pathogen protein recognition within host cell. The objective of this study was to fine-map introgression 6A and to determine the possible candidate genes underlying this *6a* locus for future cloning.

2.3. Materials and Methods

2.3.1. Plant Materials

The populations to be used in this project were derived from a cross between SLB-susceptible maize line B73 and a highly SLB-resistant line NC292. B73 is an inbred line with good agronomic qualities derived by recurrent selection of an Iowa Stiff Stalk Synthetic line (Russell, 1972). NC292 is a NIL to B73 developed through the North Carolina State University breeding program. NC292 is agronomically similar to B73, but highly resistant to SLB (Fig. 2.1). Further details on the development of this material can be found elsewhere (Zwonitzer, 2008; Belcher, 2009). A genome-wide analysis of NC292 and B73 identified 12 introgressions that differentiated NC292 from B73 (Zwonitzer, 2008). B73-6A is a B73-background NIL developed from marker-assisted selection of B73 x (NC292 x B73) backcross BC₂F₂ progeny. B73-6A is homozygous for the NC292-derived introgression 6A located on the distal end of the short arm of chromosome 6 (Fig. 2.2A). F₂ plants were generated from a cross of B73-6A and B73. F_{2,3} families were generated by self-pollinating the resulting F₂ individuals and were screened for recombinants. Resistances in B73-6A can

be traced back to a highly resistant Nigerian composite line (Craig and Daniel-Kalio, 1968; Craig and Fajemisin, 1969; Smith and Hooker, 1973; Thompson and Bergquist, 1984; Holley and Goodman, 1989).

2.3.2. Growth Chamber Experiments

In order to study the inheritance of the resistance in B73-6A, a set of 48 F₂ plants were screened in growth chamber experiments at the North Carolina State University phytotron. This F₂ population is derived from the cross of B73-6A to B73. Plants were planted and grown in 600ml Styrofoam cups in a 1:2 peat-lite and gravel mixture. Plants were maintained in a 3 x 3 x 2.13 meter growth chamber, with a day length of 14 h per 24 h cycle (6am-8pm) and a constant temperature of 22°C. Plants were watered overhead twice daily with a nutrient solution according to the Phytotron Procedural Manual (Saravitz *et al.*, 2009).

2.3.2.1. Fungal Growth and Inoculation in Growth Chambers

Cultures of *C. heterostrophus* race O were grown on PDA plates from stocks frozen in glycerol. They were kept at a constant temperature of 22°C with a 12hr darkness/12hr fluorescent daylight cycle. Cultures were used for inoculation at 10-13 days after plating. Spores were dislodged by washing three times with chilled inoculation solution (0.05% agar; 0.05% Tween-20) and a paintbrush. The suspension was then filtered with two layers of cheesecloth. A concentration of 10⁴ spores/ml was used to spray-inoculate the third and fourth leaf of the juvenile corn plants using a Paasche H airbrush and a Paasche D200R air

compressor set at 23 psi (Belcher, 2009). Clear plastic autoclave bags were used to enclose the plants just immediately after plants had dried from inoculation. They remained enclosed for approximately 16 h in order to create the free moisture required for proper spore germination. B73 and NC292 were used as susceptible and resistant controls, respectively. Plants were scored as resistant if their reaction was similar to the resistant control line NC292 and susceptible if their reaction was similar to the susceptible control line B73 (Fig. 2.3).

2.3.3. Field Trials

Approximately 331 and 187 F_{2:3} families were evaluated on a plot basis for SLB resistance during summer 2009, and 2010, respectively. Each plot represented one family derived from self-pollinating each F₂ plant. Experiment was laid out in two replications in a randomized complete block design, in the North Carolina State University Central Crops Research Station in Clayton, North Carolina. Plots were planted as single rows with a length of 2 m, and 0.97 m between rows and an alley of 0.6 m after each plot. Plots contained one family (8 plants). 169 F₂ plants were also evaluated during summer 2009 on a single plant basis.

2.3.3.1. Fungal Growth, Inoculation and Rating of Field Trials

Plots were artificially inoculated following procedures described elsewhere (Carson, 1998; Carson *et al.*, 2004). Plants were inoculated at the four-to six-leaf stage by placing approximately 20 sorghum grains infested with *C. heterostrophus* race O into the leaf whorl, followed by overhead irrigation to provide free moisture to favor fungal growth. All SLB

ratings were made using a 1-9 scale on each family (on a plot basis) using increments of 0.5 with 9 being more symptomless and 1 being completely dead. Ear leaf and the leaf above were scored approximately every 7 days for a total of 4 and 3 assessments in 2009 and 2010, respectively. Disease assessments were done in July 8, 15, 22, and 29 in 2009; and in July 12, 19, and 27 in 2010. These ratings were used to calculate the standard area under the disease progress curve (sAUDPC) on a plot basis. sAUDPC was obtained by dividing AUDPC values by the total number of days of evaluation (Balint-Kurti *et al.*, 2007). AUDPC values were calculated as previously explained (Shaner and Finney, 1977). The average value of two consecutive ratings was multiplied by the number of days between the ratings. These values were then summed over all intervals. Also, to facilitate the phenotyping of the F₂ plants, segregation data per family was taken. If all the family was resistant, all susceptible, or was segregating approximately 3:1; the F₂ plant it comes from was classified as homozygous resistant, homozygous susceptible, or heterozygous respectively for the introgression 6A (Fig. 2.4).

2.3.4. DNA Extraction

Total nucleic acid was extracted from the F₂ plants using a modified CTAB extraction procedure (Saghai-Marooif *et al.*, 1984; Zwonitzer *et al.*, 2009). All the DNA extraction procedures were performed in a 96-well format. Approximately 100 mg of leaf tissue was ground using liquid nitrogen and Retsch® Mixer Mill MM301 Retsch GmbH & Co. (Haan, Germany). 600 µl of CTAB buffer was added to each sample. Samples were then incubated at 65° C for 30 min and mixed by inversion for approximately 20 min. 400 µl of

chloroform:isoamyl alcohol 24:1 (v/v) was used to eliminate proteins by centrifuging at 3473 x g for 10 min. 400 µl of iced-cold isopropanol was used to precipitate nucleic acids for 1 hour at -20° C. Washing was performed twice with 75% alcohol and the total nucleic acids were re-suspended in 100 µl of 1X TE. For single nucleotide polymorphism (SNP) marker purposes, total nucleic acid was diluted with nuclease free water to a concentration of approximately 10 ng/µl.

2.3.5. Genome Scan

A genome scan of the B73-background NILs, B73, and B73-6A was performed using Illumina's high-throughput MaizeSNP50 v1 BeadChip which interrogates 56,110 markers derived from the B73 reference genome. The 'Infinium HD Ultra' assay (Steemers *et al.*, 2006) was executed on 200 ng of each maize genomic DNA sample at a concentration of 50ng/ul and hybridized to BeadChips. Sample intensities were detected on Illumina's iScan array scanning instrument and genotypes called with Illumina's GenomeStudio v2009.2 data analysis software. A total of 984 'Intensity Only' SNPs, 1319 SNPs with a cluster separation <0.25, and 99 SNPs with an AB R Mean <0.25 were removed from the analysis.

2.3.6. Molecular Markers

Two types of markers, simple sequence repeats (SSR) and SNP, were used to genotype 520 F_{2:3} families in order to narrow down the region between markers umc2068 and umc2311 (Fig. 2.2) (Zwonitzer, 2008). SSR Markers bnlg2243, umc2515, umc1018 were selected from a previous study and were known to be polymorphic between B73 and

B73-6A (Zwonitzer, 2008). Marker bnlg2243 was used to genotype F₂ plants for studies of inheritance in growth chamber experiments.

The SNP polymorphisms used were identified in a number of ways: SNP marker PHM15961.13 was developed from data stored at www.panzea.org/index.html. PUT-163a-5739956-2305 is a SNP marker from the SNP chip genome scan from Illumina's high-throughput MaizeSNP50 v1 BeadChip mentioned above. The remaining SNP markers were developed from B73-Mo17 SNPs available in www.maizegdb.org. Since Mo17 was not one of the parents, SNP polymorphisms were checked in a pair of parent controls: B73, B73-6A. A total of 65 SNPs evenly spread over the introgression 6A were selected. From the 65 SNPs, only 21 were polymorphic between B73 and B73-6A and showed good quality for genotyping based on a clear resolution of the genotypes in the fluorescence scanning with a LightCycler® 480. Primers were designed for KASPar® genotyping using the primer-picker tool from www.kbioscience.co.uk/. Two allele-specific oligonucleotides of about 40 nt in length and 1 common reverse oligonucleotide of about 20 nt in length were designed based on the SNP polymorphism and its flanking 50 nt at each side. Details information on markers used in this study can be found in table 2.1.

2.3.7. Genotyping

PCR reactions for SSR markers followed procedures described elsewhere (Schuelke, 2000; Kirigwi *et al.*, 2008) with the difference that for markers bnlg2243 and umc2515, no labeled primer was used since the polymorphism could be resolved in agarose gels. The PCR amplification profile consisted of 3 min at 95°C, followed by 30 cycles of 30 s at 95°C, 45 s

at the optimum annealing temperature [Ta] for each primer pair, 45 s at 72°C, followed by 10 cycles of 30 s at 95°C, 45 s at 53°C, 45 s at 72°C, and a final extension step of 10 min at 72°C. Only for marker umc1018, 1ul of PCR product to 11.99 µl of Hi-Di formamide (Applied Biosystems, Foster City, CA) and 0.01 ul LIZ size standard (Applied Biosystems, Foster City, CA) and the PCR products were resolved using an ABI3130 genetic analyzer. Markers bnlg2243 and umc2515 amplification products were separated on a 3% agarose gel with 1X TBE (90 mM Tris, 90mM Boric Acid, 20mM EDTA), and stained with 1 µl ethidium bromide (0.5 µg/ml). A 100 bp DNA ladder (Invitrogen) was used as a molecular marker. Products were visualized using a UV transilluminator.

KASPar® genotyping was used for all SNP markers. Assay mix for each SNP was prepared by dissolving the two allele-specific and the reverse primers in nuclease-free water to a final concentration of 12 µM for each allele-specific primer and 30 µM for the reverse oligonucleotide. KASPar® amplifications were performed in a 96- and 384-well format. With a 96-well format, reactions were carried out in a total volume of 8 µl containing 10 ng DNA, 0.11 µl of the previously prepared assay mix and 4 µl of 2X reaction mix according to the manufacturer's guidelines (KBioscience, 2011). When using a 384-well format, reactions were prepared using only half of each component. PCR conditions were 94°C for 15 min, followed by 20 cycles of 94°C for 10 s, 57°C for 5 s, 72°C for 10 s, and 26 cycles of 94°C for 10 s, 57°C for 20 s, 72°C for 40 s. All PCR amplifications were carried out on a PTC-225 Peltier Thermal Cycler (ML Research Inc. Waltertown, MA). Fluorescence scanning was done in a LightCycler® 480 Multiwell Plate 96, and data analysis with the Endpoint Genotyping Software v.1.5.0 (Roche Applied Science. Indianapolis, IN).

2.4. Results

2.4.1. Genome Scan of the B73-background NILs

We used the illumina MaizeSNP50 v1 BeadChip to interrogate 56,110 markers, comparing the original B73 and B73-6A. The analysis identified only two regions on chromosome 6 that differed between B73 and B73-6A (Table 2.2 and Fig. 2.5). The larger region corresponded to introgression 6A. This region, ranging from 3.7 to 9.5 Mb on chromosome 6, was within the originally-defined region between markers umc2068 and umc2311 (Approximately from 2.3 to 13 Mb in Fig. 2.2B) (Zwonitzer *et al.*, 2009). These introgression regions were homozygous.

2.4.2. Growth Chamber Experiments

Forty-eight F₂ plants from a cross between B73 and B73-6A were infected with SLB in growth chamber experiments. The F₂ plants segregated into 2 distinct phenotypes: 36 susceptible and 12 resistant, a ratio of 3 susceptible: 1 resistant, implying that SLB resistance present in B73-6A may be controlled by a recessive allele (*6a*). Plants also segregated for the bnlg2243 SSR marker genotype in a 1:2:1 ratio ($\chi^2 = 1.125$ pr > 0.5698). All resistant plants were homozygous for the introgression 6A. Susceptible plants were either homozygous dominant (9 plants) for the B73-specific allele or heterozygous (27 plants).

2.4.3. Field Trials

Approximately 520 F_{2,3} families were evaluated for SLB resistance in two reps in a randomized complete block design during summer 2009 and 2010, on the North Carolina

State University Central Crops Research Station in Clayton, North Carolina. Distribution of sAUDPC values from the 520 $F_{2:3}$ families was bimodal with two groupings of susceptible and resistant plants in approximately a 3 to 1 ratio (Fig. 2.6). Segregation data within the $F_{2:3}$ families were recorded and used to help determine the genotype of the original F_2 individual from which the family was derived. If all the family was resistant, all susceptible, or was segregating approximately 3:1; the F_2 plant it comes from was classified as homozygous resistant, homozygous susceptible, or heterozygous, respectively, for the introgression 6A (Fig. 2.4). Using the family segregation data, 245 families were determined to have originated from F_2 plants heterozygous for the introgression 6A, 137 were determined to have originated from resistant plants (homozygous for the introgression 6A) and 132 were determined to have originated from F_2 plants homozygous for the B73 allele (p-value for $X^2 = 0.5439$). This is in good agreement with the growth chamber experiments suggesting that SLB resistance derived from NC292 may be due to a single recessive locus. X^2 tests were statistically significant for the Mendelian genotypic segregation 1:2:1 in both years (p-values 0.3303 and 0.8712 for 2009 and 2010, respectively).

The distribution of the last scores taken at the end of season followed similar patterns with more extreme values compared to sAUDPC values (Fig. 2.7). F_2 plants did not show a clear bimodal distribution.

2.4.4. Fine-Mapping 6a

A total of 65 new SNP markers were designed and tested. 21 of these markers were polymorphic between B73 and B73-6A and the alleles could be easily differentiated with

good resolution on the KASPar® genotyping system. These 21 markers, plus 5 that had been previously developed, were used to genotype the F₂ parents of each of the 520 F_{2:3} families that had been phenotyped (Table 2.1). As mentioned above, segregation for resistance within the F_{2:3} families was recorded and used to help determine the genotype of the original F₂ individual with regard to the *6a* locus. Using the publicly available sequence of B73, markers were mapped onto the genome. Fifteen families were found to contain a recombination event within the region of interest which was between the markers *umc2068* and *umc2311*, and they were used to narrow down the candidate region to a ~300 Kb region (Fig. 2.4 and 2.8). The region contained 12 filtered genes (genes that were selected after transposon and orthology screens were applied) and other 12 gene loci, composed of evidence-based genes and non-overlapping Fgenesh models genes (based on the gene model predictor software from www.softberry.com) (www.maizesequence.org)

The complete list of these genes and their features is show in table 2.3. Five of the genes belong to the amino acid transporter gene family. These genes are described as transmembrane proteins that transport amino acids through the cell membrane. Furthermore, GRMZM2G127328 has been described as a *lysine histidine transporter-1* gene (LHT1).

2.5. Discussion

QDR has always been used in maize; however, the molecular mechanisms behind it are not well understood (Poland *et al.*, 2009). While several major-effect genes for resistance have been cloned, only a few genes which underlie dQTLs have been identified. *Pi21*, a recessive rice gene against rice blast, produces a protein with a heavy metal-

transport/detoxification protein domain in the N-terminal region (Fukuoka *et al.*, 2009). A germin-like gene family (*OsGLP*) conferring broad spectrum resistance in rice is thought to be involved in the generation of H₂O₂ (Manosalva *et al.*, 2009). In wheat, the high temperature dependent gene *Yr36* against wheat stripe rust encodes a predicted kinase domain and a predicted steroidogenic acute regulatory protein-related lipid transfer or START domain. These domains suggest a role in lipid trafficking (Fu *et al.*, 2009). Wheat gene *Lr34* against diseases caused by leaf rust fungus *Puccinia triticina*, stripe rust pathogen *P. striiformis*, and the powdery mildew fungus *Blumeria graminis* produces a protein similar in structure to the multi-drug resistance subfamily of ATP-binding cassette transporters. This protein is produced in susceptible plants. The resistant alleles showed three polymorphisms (Krattinger *et al.*, 2009). In maize, *Rcg1* against anthracnose stalk rot and leaf blight is predicted to encode a protein that share similarities to an R-gene product (Frey, 2006).

SLB resistance is mostly quantitative in nature; however, a major resistance locus, *rhm1*, has been mapped in chromosome 6, in the region of the 6a locus, but not been cloned (Craig and Daniel-Kalio, 1968; Craig and Fajemisin, 1969). Here we report the fine-mapping of 6a locus for SLB resistance on the short arm of chromosome 6 of maize.

In order to fine-map any quantitative trait locus, it is helpful to be able to score it on a single plant basis (mendelize the locus). Both growth chamber and field experiments showed the Mendelian segregation of 6A. Growth chamber screening of young F₂ plants was accomplished readily; however, scoring the phenotype in the field was not as easy probably due to environmental variation. Using F_{2:3} families helped to increase the accuracy of phenotyping compared to using F₂ populations due to the fact that multiple plants per row

and multiple reps could be evaluated. For susceptible families, by noting the segregation within a row (i.e. if there were a few resistant plants interspersed among the susceptible plants) we were also able to determine whether the original susceptible parent was homozygous or heterozygous for the susceptibility allele. This would not have been possible to determine by looking at the F₂ phenotypes and it helped considerably in providing information for fine mapping.

Using the KASPar® genotyping was an efficient way to score SNPs. Even though the polymorphisms that were originally identified represented differences between Mo17 and B73 lines, 21 of the 65 SNPs were also found to be polymorphic between NC292 and B73. Fifteen recombinants were found within the introgression 6A and used to narrow down the location of the resistance gene to a region of ~300-kb which contains 12 filtered genes (that were selected after transposon and orthology screens were applied) and other 12 predicted genes (composed of evidence-based genes and non-overlapping Fgenesh models genes) (Table 2.3). Five of the filtered genes belong to the amino acid transporter gene superfamily and contain transmembrane domains. The amino acid transporter superfamily contains proteins with a function of moving amino acids through the cell membrane. Other gene domains ranged from bHLH (basic Helix-Loop-Helix) domains, typical of transcription factors, to acid phosphatase/vanadium dependent haloperoxidase domains of unknown functions. A clustering of resistant gene analogues (RGAs) could also be found in the distal end of the small arm of chromosome 6 (Quint *et al.*, 2003). *Rxo1* gene (reaction to *Xanthomonas oryzae*), a R gene conferring resistance to the bacterial stripe disease *Burkholderia andropogonis* and resistance reaction to the rice bacterial streak pathogen

Xanthomonas oryzae pv. *oryzicola* was found very close to our region of interest (Zhao *et al.*, 2004; Zhao *et al.*, 2005). *Rxo1* and its 4 pseudogenes were found within the introgression; however, we were able to rule them out of the region of interest in this study.

LHT1 is an amino acid transporter that, in *Arabidopsis thaliana*, possesses high affinity for certain amino acids. According to Chen and Bush (1997), *LHT1* occurs as a single copy gene in *Arabidopsis* and possesses 9 to 10 putative transmembrane domains. Also, *LHT1* is expressed in all tissues in *Arabidopsis*, especially in young leaves, flowers and siliques; as compared to other amino acid transporters that are mostly confined to the vascular tissues (Chen and Bush, 1997). It is known that plants not only obtain the nitrogen needed for their metabolism and structure from inorganic sources, but plants can also uptake free amino acids and recycle them into new compounds. LHT1 is involved in the uptake of amino acids through the roots and their transportation to the leaf mesophyll (Hirner *et al.*, 2006). Recent studies in *A. thaliana* have shown that LHT1 is also involved in a complex mechanism of basal defense in which it acts as a modulator of the plant defense response conferring broad spectrum defense to diseases (Liu *et al.*, 2010). LHT1 has a preference for D-amino acids and is associated with H₂O₂/NO accumulation, callose deposition, and programmed cell death at the site of penetration. When a plant cell is infected, it creates a redox imbalance, which in turn, switches off cytosolic glutamine (Gln) biosynthesis in the cell. This signal triggers salicylic acid- (SA) dependent defense responses. It is suggested that LHT1 will actively compensate this by uptaking apoplastic Gln into the cell, thus blocking SA-dependent defense response. Even though there is some indication that LHT1 has a role in defense response, SA-dependent response is usually associated with active response

against biotrophs and hemibiotrophs and *lht1* mutants failed to control necrotrophic pathogen on *A. thaliana*. Herein, *C. heterostrophus* is a necrotroph to our knowledge, and the hypothesis of the association between Gln uptake into the cell and modulation of SA-dependent response does not necessarily explain SLB resistance. The pathogen may be acting as a biotroph in early stages of infection and may be suppressing plant immunity by modulating the expression of *LHT1*. It is believed that resistance conferred by *LHT1* in *A. thaliana* is not because of pathogen failure, but for enhanced host defense responses (Liu *et al.*, 2010). But other mechanisms may be affecting the fungal growth in our pathosystem. *LHT1* may still be involved in susceptibility in maize, though the exact mechanism still needs to be elucidated.

The resistance locus *rhm1* has been mapped in the same region of the short arm of chromosome 6 (Zaitlin *et al.*, 1993). *rhm1*, like the *6a* locus, acts as a single recessive gene for resistance against SLB. Resistances in both B73rhm1 and B73-6A can be traced back to the same highly resistant Nigerian breeding stock (Craig and Daniel-Kalio, 1968; Craig and Fajemisin, 1969; Smith and Hooker, 1973; Thompson and Bergquist, 1984; Holley and Goodman, 1989). Furthermore, neither the *6a* locus or *rhm1* inhibit fungal germination, penetration, or growth; but both work similarly in reducing fungal growth after 48 hours (Gao *et al.*, 2005; Belcher, 2009). Recent studies have shown that *LHT1* maize analog gene may be the gene underlying *rhm1* resistance (Zhao *et al.*, 2012). Fine-mapping of *rhm1* using the H95rhm line has revealed the *LHT1* maize analog gene as the only candidate. All these points would indicate that the *6a* locus and *rhm1* are the same gene. Furthermore, they have failed to genetically complement each other in an experiment using a mapping population

derived from a cross between a B73rhm1 line and NC250A (a line containing introgression 6A) (Belcher, 2009). Two markers within the previously identified dQTL in introgression 6A by Zwonitzer *et al.* (2009) were used to detect differences in the genotypic classes in this population. Fisher's protected least square difference test was used to compare the disease severity least-squares means from the genotypic classes (homozygous for the B73rhm1 allele (*rhm1*), homozygous for the NC250A allele (*6a*) and heterozygous. There were no significant differences among the genotypic classes. No association between resistance and either allele could be detected in the region where the dQTL is located, suggesting that *6a* and *rhm1* may represent the same recessive gene.

There are several amino acid transporter predicted gene sequences close to LHT1 that share similarity with it and that would fit the two-gene model. In a tagging experiment using two lines, a susceptible homozygous *Rhm1* line containing a transposable element and a resistant *rhm1* line, Chang and Peterson (1995) reported that the F₁ progeny yielded a higher number of resistant mutants than the expected based on the mutation rates of the transposon element. To account for this higher mutation rate, it was proposed that *rhm1* locus was in fact two loci: *rhm1* and *rhm2*, and that the resistance is shown only when both genes are homozygous recessive *rhm1 rhm2/rhm1 rhm2*. It was hypothesized that this higher number of mutants may have arisen due to recombination events between the two genes in the susceptible *Rhm1 rhm2/rhm1 Rhm2* parent. Unfortunately, the fact that *6a* locus does not complement *rhm1* contradicts this hypothesis and we may be dealing with two different alleles of the same *LHT1* gene. Further analysis is required to know if they are in fact the same gene or two separate alleles of the same recessive resistance gene.

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Table 2.1 Markers used in fine-mapping. SNP markers were developed for KASPar® genotyping and each marker comprised two allele-specific forward primers and a common reverse primer. Newly developed SNP markers are coded with the letter M

Marker	Primer	Sequence (5' - 3')	Polymorphism ¹	T _m (°C)
umc1018	F	GAACGGATATTGGAACCTGTGC	CT(7), 120	61.4
	R	GAACGGATATTGGAACCTGTGC		61.4
bnlg2243	F	ATCTATCACGACGAACGGGA	AG(16), 121	59.0
	R	ATCTCCCTAGCTCGCTCTCC		58.6
umc2515	F	TCGATCTGCACAGATGAGTCAGTA	CGC(n),131	66.0
	R	GCTAGGAGGGCGCTAAATCGAG		66.0
PHM15961.13	F	GAAGGTGACCAAGTTCATGCTTTATTTATGTAACAGATGTTTGGCAACAC	[C/G]	80.1
	F	GAAGGTCTGGAGTCAACGGATTTATTTATGTAACAGATGTTTGGCAACAG		81.1
	R	ACGAAGTGGACAGTGAGCAAATTAACAT		69.5
PUT-163a-5739956-2305	F	GAAGGTGACCAAGTTCATGCTGGTCTGCGACCAAAGGCCATT	[A/G]	83.5
	F	GAAGGTCTGGAGTCAACGGATTGGTCTGCGACCAAAGGCCATC		85.2
	R	CAGTAAAGAAAGATATTTCTGCCTCCCTA		62.1
M1	F	GAAGGTGACCAAGTTCATGCTGCAACTTTCTCACAGCTCACATGTA	[T/C]	83.5
	F	GAAGGTCTGGAGTCAACGGATTCAACTTTCTCACAGCTCACATGTG		84.5
	R	AGTTGTGGGCTAAGAAACAGTTGTTGTA		67.1
M2	F	GAAGGTGACCAAGTTCATGCTAAGATCTATAGGTTGCATTTTTATGACTTG	[G/C]	78.5
	F	GAAGGTCTGGAGTCAACGGATTAAGATCTATAGGTTGCATTTTTATGACTTC		79.1
	R	TCCAGTGACAGTATCAGGCTGCAA		69.9
M3	F	GAAGGTGACCAAGTTCATGCTTTCTCTCGGATGTCATCCTGT	[A/G]	79.4
	F	GAAGGTCTGGAGTCAACGGATTCTTTCTCTCGGATGTCATCCTGC		82.5
	R	GATTCTTCTGCAGTTTACATGTCTTCCTT		63.0

Table 2.1 Continued

Marker	Primer	Sequence (5' - 3')	Polymorphism ¹	T_m (°C)
M4	F	GAAGGTGACCAAGTTCATGCTGGTGCGCTGGGCGACGTC	[G/C]	89.5
	F	GAAGGTCGGAGTCAACGGATTGGTGCGCTGGGCGACGTG		91.0
	R	CCTGGATCTCGAGGACCACGTT		70.6
M5	F	GAAGGTGACCAAGTTCATGCTAAGTACTACGGCGAGAAATTGCTCA	[A/G]	81.8
	F	GAAGGTCGGAGTCAACGGATTGTACTACGGCGAGAAATTGCTCG		84.9
	R	GAAAGGCTTCTGCCCCACCGAA		73.9
M6	F	GAAGGTGACCAAGTTCATGCTAATCATTGTGCGAGGCCGCCACA	[T/C]	86.6
	F	GAAGGTCGGAGTCAACGGATTATTGTGCGAGGCCGCCACG		89.3
	R	TGTCCTCCTCGCACAGCTCCAA		73.5
M7	F	GAAGGTGACCAAGTTCATGCTGAAGACCCCGCCATTGCCATA	[T/A]	86.7
	F	GAAGGTCGGAGTCAACGGATTGAAGACCCCGCCATTGCCATT		88.1
	R	GAGAGAGAACGTGAGATGCCGCT		70.1
M8	F	GAAGGTGACCAAGTTCATGCTGGCTAGGCAATTAGCTACCCG	[C/A]	82.9
	F	GAAGGTCGGAGTCAACGGATTGGGCTAGGCAATTAGCTACCCT		83.4
	R	AGACACACAACAAGCAAAGTCTAGCTAAGTT		65.9
M9	F	GAAGGTGACCAAGTTCATGCTCGGCATATGAATGTTGAACAGGGA	[T/G]	85.0
	F	GAAGGTCGGAGTCAACGGATTGGCATATGAATGTTGAACAGGGC		85.6
	R	AGGCTTGTTCCAGAAACCATTATCTAA		68.6
M10	F	GAAGGTGACCAAGTTCATGCTGCAGACAGGATGGATCTGCTATT	[T/C]	83.3
	F	GAAGGTCGGAGTCAACGGATTGCAGACAGGATGGATCTGCTATC		85.0
	R	CATTTTGATGAAGGGGAGAGCCGAT		72.3
M11	F	GAAGGTGACCAAGTTCATGCTTGTAGCTTGGCCATCTGCTG	[C/A]	83.7
	F	GAAGGTCGGAGTCAACGGATTGCTTGTAGCTTGGCCATCTGCTT		85.8
	R	CATGGCCGAGCTGATGCGCAA		77.5

Table 2.1 Continued

Marker	Primer	Sequence (5' - 3')	Polymorphism ¹	T _m (°C)
M12	F	GAAGGTGACCAAGTTCATGCTGACTTTGTGTTGTTTGTTCATGATCTAAT	[A/C]	78.6
	F	GAAGGTCGGAGTCAACGGATTGACTTTGTGTTGTTTGTTCATGATCTAAG		80.0
	R	CCAGATGGGAAGCTTTACCTGCTT		64.5
M13	F	GAAGGTGACCAAGTTCATGCTAAAATGGCTAGTTCACGCACAAGG	[C/T]	82.7
	F	GAAGGTCGGAGTCAACGGATTGAAAATGGCTAGTTCACGCACAAGA		85.3
	R	TTGGTAGGATTAGATGTATCCGAATGCAT		69.0
M14	F	GAAGGTGACCAAGTTCATGCTAACAACACAAGGTTATCCACTTAACTG	[C/T]	76.9
	F	GAAGGTCGGAGTCAACGGATTCAAACAACACAAGGTTATCCACTTAACTA		78.8
	R	GGAACCAGCTCAACACTCAATAGCAT		64.5
M15	F	GAAGGTGACCAAGTTCATGCTCAGGTCCGGCCGTGGTCA	[T/C]	88.3
	F	GAAGGTCGGAGTCAACGGATTGAGGTCCGGCCGTGGTCCG		89.9
	R	AGGCGGATGGCCTGCTACAGAA		72.7
M16	F	GAAGGTGACCAAGTTCATGCTGTTGAGGGAGGGCACCA	[A/G]	82.2
	F	GAAGGTCGGAGTCAACGGATTCTGTTGAGGGAGGGCACCG		84.5
	R	GCGCTCCAACCTGGCGTCCAT		71.4
M17	F	GAAGGTGACCAAGTTCATGCTCAGTAAGCGTAGCAGGCACGA	[A/G]	84.2
	F	GAAGGTCGGAGTCAACGGATTGAGTAAGCGTAGCAGGCACGG		85.8
	R	ACCACGCAGGAACACATGCAAACAT		73.8
M18	F	GAAGGTGACCAAGTTCATGCTGAGGAGGGTGCGGCTGACTAT	[A/G]	85.3
	F	GAAGGTCGGAGTCAACGGATTGGAGGGTGCGGCTGACTAC		86.5
	R	GCCTCTTGAACCTTCTCGAATGGGAT		70.0
M19	F	GAAGGTGACCAAGTTCATGCTGTGCAGGATTTGCCAGGAGGAT	[T/C]	85.9
	F	GAAGGTCGGAGTCAACGGATTGCAGGATTTGCCAGGAGGAC		86.5
	R	AGGCGCAGGGGGCCTCCAT		76.2

Table 2.1 Continued

Marker	Primer	Sequence (5' - 3')	Polymorphism ¹	T_m (°C)
M20	F	GAAGGTGACCAAGTTCATGCTACACCGGCAATTCCTCACCGA	[A/G]	85.9
	F	GAAGGTCGGAGTCAACGGATTCACCGGCAATTCCTCACCGG		88.6
	R	CACGTGATGCGGCCCGGTT		78.2
M21	F	GAAGGTGACCAAGTTCATGCTGTGTCGTTTCAGGTGTATGCCTTTA	[A/C]	83.1
	F	GAAGGTCGGAGTCAACGGATTGTCGTTTCAGGTGTATGCCTTTC		84.2
	R	CAACAGACAGCTTTAGAGGCAAACTTTT		68.5

Table 2.2 Introgressions present in the B73-6A line relative to B73 based on the genome scan. Genome scan was done using illumina MaizeSNP50 v1 BeadChip that interrogated 56,110 markers. Introgression start and end points are given in base pairs (bp) according to the B73 Refgen v1 positions (based on www.maizegdb.org).

Chromosome	Introgression Start (bp)	Bin	Introgression End (bp)	Bin	Introgression size (bp)
6	3,779,023	6.00	9,565,617	6.01	5,786,594
6	28,198,317	6.01	28,446,784	6.01	248,467

Table 2.3 List of genes present in the introgression 6A after fine-mapping. G1-11 = filtered genes/syntelog, G12 = filtered genes/ortholog, G13-18 = sequence similar to known proteins, G19-24 = sequence not similar to known proteins. Gene start and end points are given in base pairs (bp) according to B73 AGPv2 positions in chromosome 6 (based on from maizesequence.org)

Gene	Start (bp)	End (bp)	Gene ID	TN*	Gene Name	Other Names	Gene type	Features
G19	6903467	6903595	GRMZM2G591073	1	NA	NA	Novel pseudogene	Low complexity
G20	6904584	6904704	GRMZM2G591072	1	Zm.50082	NA	Known pseudogene	NA
G21	6913575	6913820	AC211622.4_FG002	1	NA	NA	Novel protein coding	Low complexity
G12	6914289	6914735	GRMZM2G481280	1	NA	NA	Novel protein coding	Basic Helix-Loop-Helix binding domain superfamily typical of eukaryotic transcription factors
G22	6933845	6933961	GRMZM2G510515	1	NA	NA	Novel transposable element	Low complexity sequence with predicted transmembrane helices
G23	6979048	6979722	AC194149.3_FG007	1	NA	NA	Novel protein coding	Low complexity
G13	6980314	6981000	GRMZM2G142152	1	NA	NA	Novel protein coding	Zinc finger domain
G14	7029048	7030417	GRMZM2G127356	1	NA	NA	Novel protein coding	Zinc finger domain
G24	7033794	7035041	GRMZM2G426116	1	NA	NA	Novel pseudogene	Predicted cleavage site
G1	7036171	7039824	GRMZM2G127342	1	NA	NA	Novel protein coding	Amino acid transporter, transmembrane

Table 2.3 Continued

Gene	start	end	Gene ID	TN*	Gene Name	Other Names	Gene type	Features
G2	7045096	7047616	GRMZM2G127328	1	LOC100281437	LHT1	Known protein coding	Amino acid transporter, transmembrane
G3	7051859	7059857	GRMZM2G127294	1	NA	NA	Novel protein coding	Amino acid transporter, transmembrane
G15	7062850	7065027	GRMZM2G125191	1	NA	NA	Novel transposable element	Zinc ring finger; DnaJ peptide-binding domain superfamily
G16	7094066	7095657	GRMZM2G517007	1	NA	NA	Novel protein coding	Nucleotidyltransferase superfamily domain
G4	7106675	7111297	GRMZM2G018262	1	NA	NA	Novel protein coding	Proline rich extensin signature, transmembrane
G5	7124203	7126755	GRMZM2G127338	2	LOC100384362	Zm.154108	Known protein coding	Amino acid transporter, transmembrane
G6	7128592	7131540	GRMZM2G429322	1	Zm.31998	NA	Known protein coding	Amino acid transporter, transmembrane with short-chain dehydrogenase/reductase signature
G7	7132205	7137741	GRMZM2G127299	1	Zm.20529	NA	Known protein coding	Bromodomain; coiled coils domain
G17	7132235	7133171	GRMZM5G868156	1	C4J261_maize	NA	Known protein coding	NA
G8	7146681	7149063	GRMZM2G057258	5	LOC100284364	COPMM2_maize	2 Known protein coding, 3 novel protein coding	Acid phosphatase/vanadium-dependent haloperoxidase domain of unknown function
G18	7146800	7147359	GRMZM5G800924	2	C0PLC6_maize	C0HFV4_maize	Known protein coding	NodO calcium binding signature
G9	7149982	7154478	GRMZM2G057091	5	LOC100217132	C0HHU6_maize	3 Known protein coding; 2 novel protein coding	DnaJ molecular chaperone domain; transmembrane

Table 2.3 Continued

Gene	start	end	Gene ID	TN*	Gene Name	Other Names	Gene type	Features
G10	7154710	7159074	GRMZM2G057026	1	NA	NA	Novel protein coding	Coiled coil domain
G11	7159492	7160258	GRMZM2G056961	1	LOC100275427	NA	Known protein coding	Low complexity

*Number of transcripts



Figure 2.1 Reaction to SLB of the inbred lines B73, NC292, and B73-6A. The photographs above represent ear leaves taken at the North Carolina State University Central Crops Research Station in Clayton, North Carolina from B73 (A), NC292 (B), and B73-6A (C). All pictures were taken in the same field under same conditions.

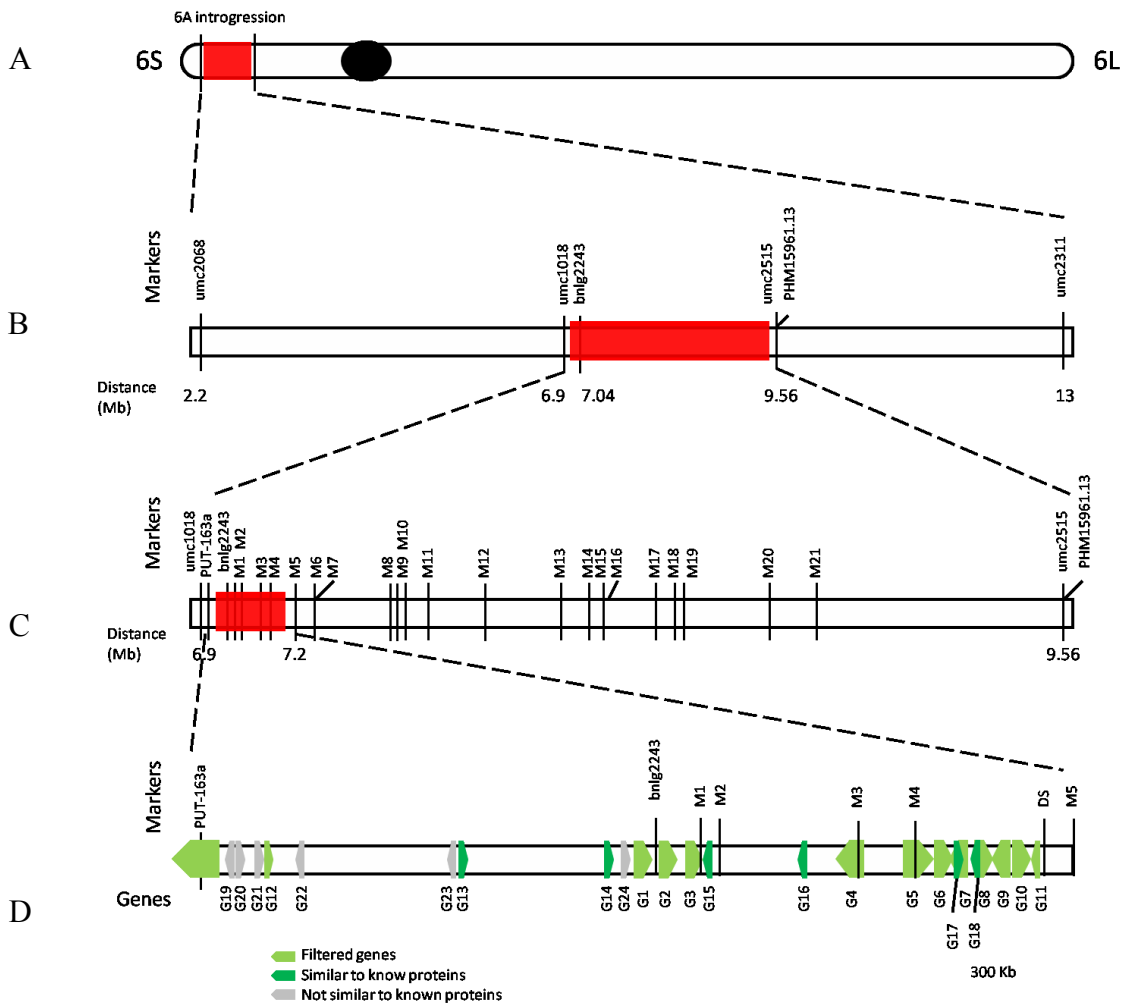


Figure 2.2 Fine-mapping of introgression 6A. (A) Location of introgression 6A on distal end of short arm of chromosome 6. (B) Coarse-mapping of introgression 6A with publicly available markers. (C) Fine-mapping of introgression 6A with SNP markers developed in this study. (D) Annotation of the candidate region containing 24 genes based on B73 AGPv2 positions in chromosome 6 (from maizesequence.org). Red rectangles represent the introgression 6A from highly resistance NC292 line. White rectangles represent B73 chromosome regions.

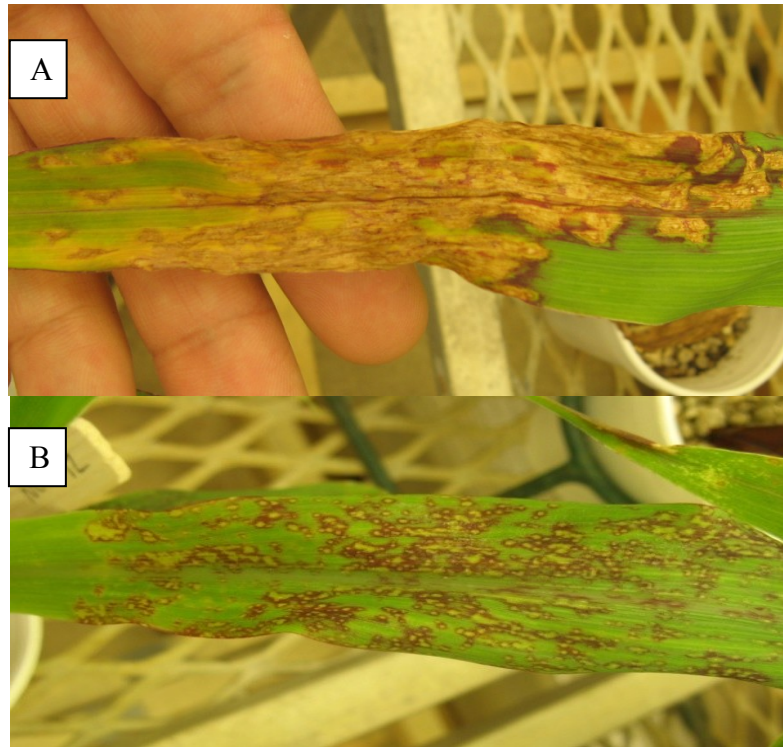


Figure 2.3 Reaction to SLB in growth chamber experiments. Phenotype of susceptible parent B73 (A) and resistant parent NC292 (B) four days after SLB inoculation.

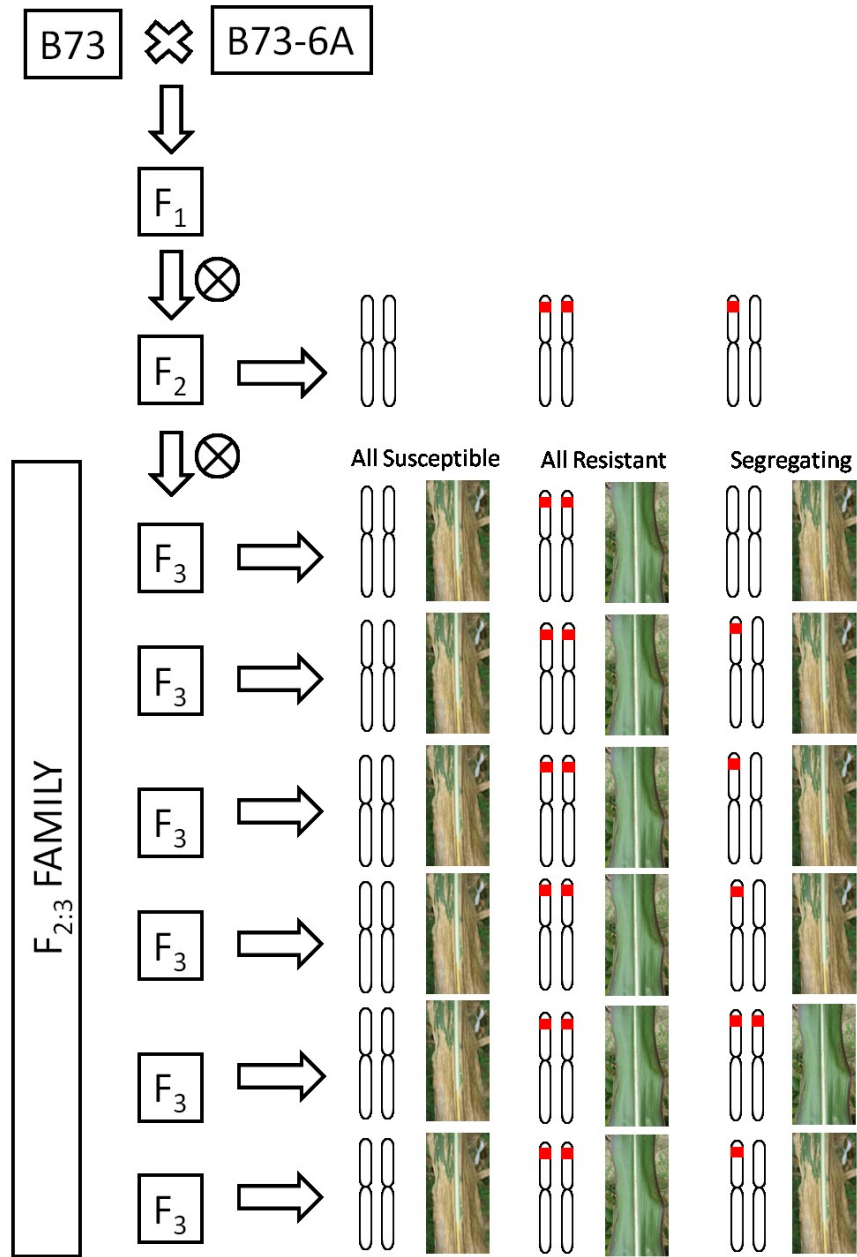


Figure 2.4 F_{2:3} family segregation. Field phenotyping was done on F_{2:3} families to facilitate the phenotyping of the F₂ plants. Segregation data per family was assessed in Clayton 2009 and 2010. If all the family was all resistant, all susceptible, or was segregating approximately 3:1; the F₂ plant it comes from was classified as homozygous resistant, homozygous susceptible, or heterozygous respectively for the introgression 6A (indicated as a red square on the chromosome 6).

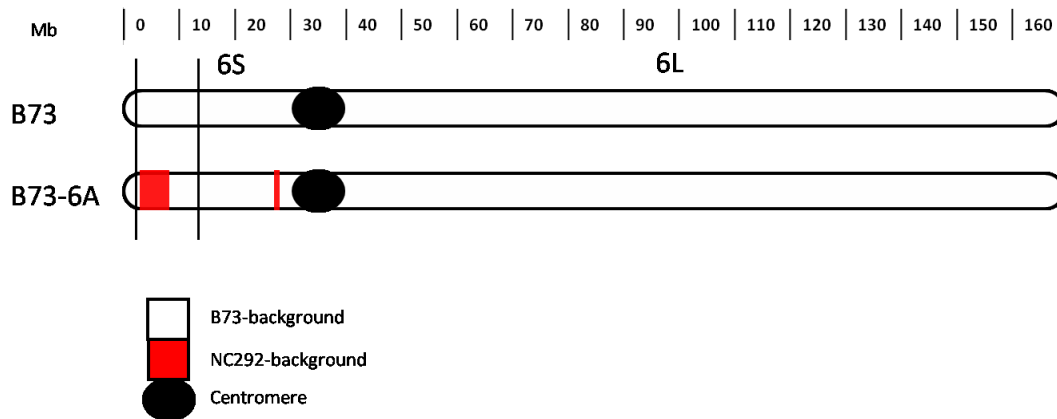


Figure 2.5 Comparison of chromosome 6 from B73-background NILs based on the genome scan. Genome scan was done using illumina MaizeSNP50 v1 BeadChip that interrogated 56,110 markers. B73 and B73-6A show different introgressions coming from the highly resistant line NC292. White regions represent B73 chromosome 6. Red regions represent introgressions from highly resistant NC292 line. Vertical lines across the two chromosomes represent the introgression 6A before the fine-mapping experiment of this study. Distances are shown in Megabases above the chromosomes. 6S and 6L represent the short and long arm of chromosome 6 of maize, respectively.

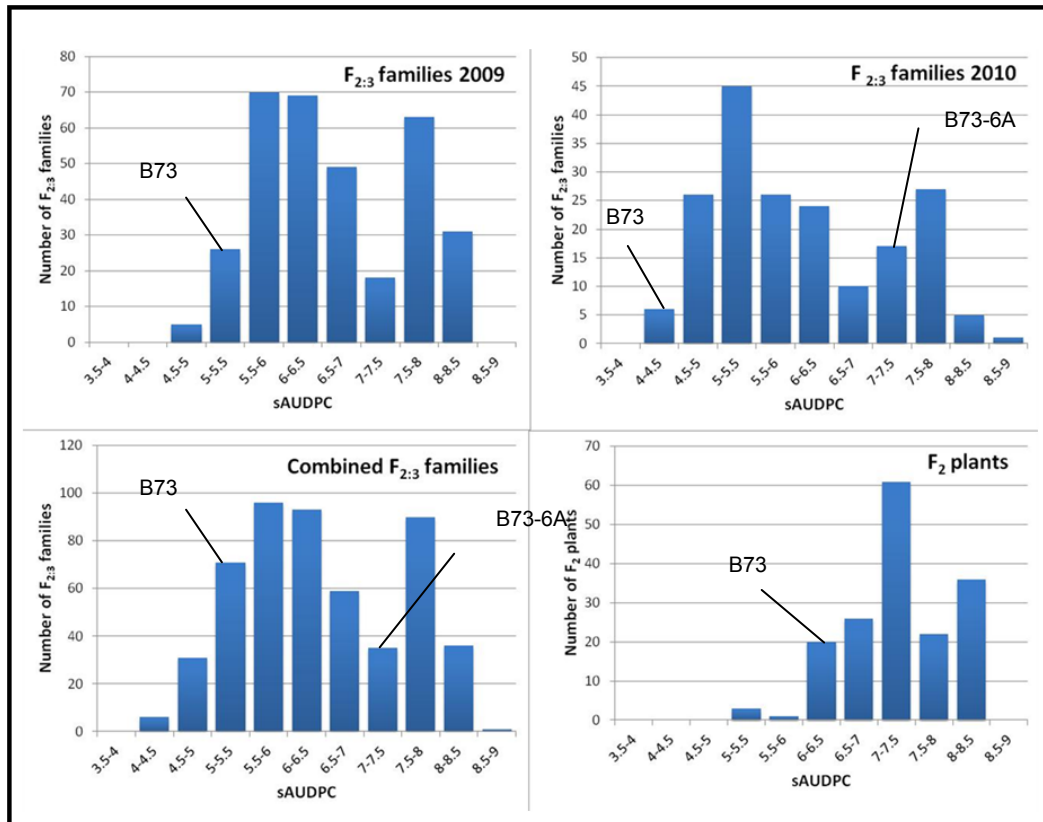


Figure 2.6 Distribution of sAUDPC values of F_{2:3} families in 2009, 2010, combined years and F₂ plants. Populations were obtained from a cross between B73 and B73-6A. Field phenotyping data was assessed using a 1-9 scale rating, with 1 being killed by disease and 9 being completely symptom-free.

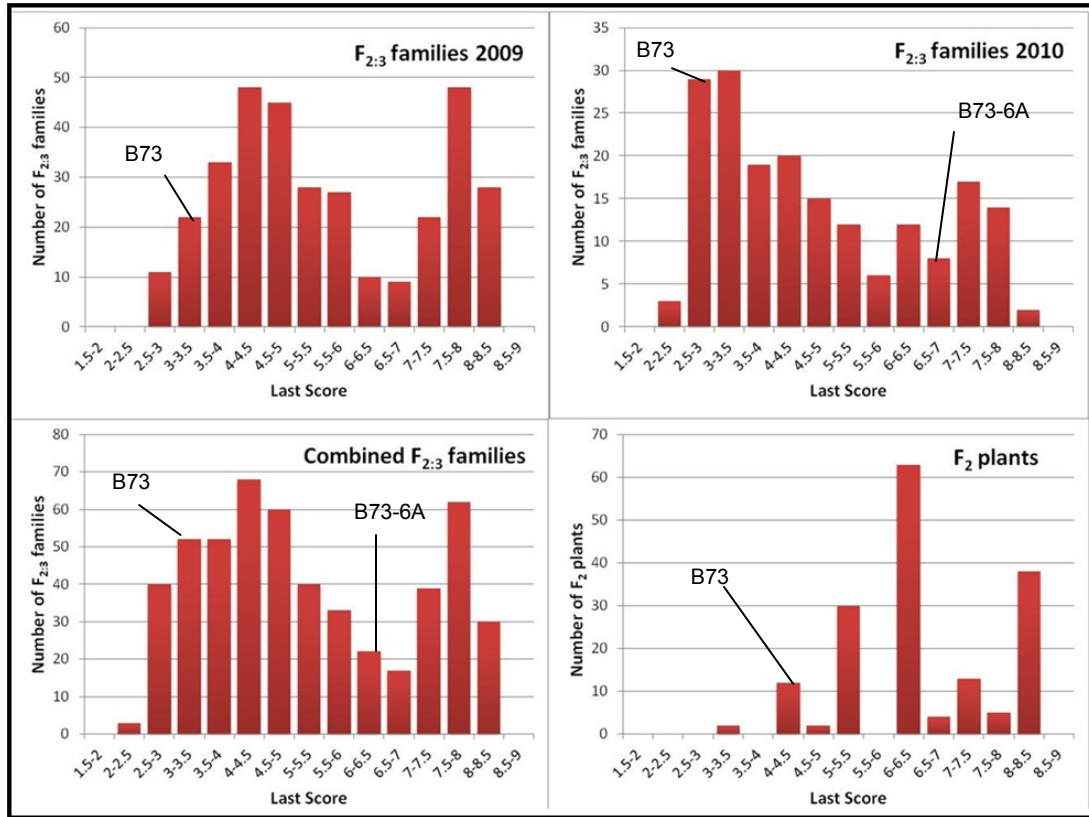


Figure 2.7 Distribution of final scores of the end of the season of F_{2:3} families in 2009, 2010, combined years and F₂ plants. Populations were obtained from a cross between B73 and B73-6A. Field phenotyping data was assessed using a 1-9 scale rating, with 1 being killed by disease and 9 being completely symptom-free.

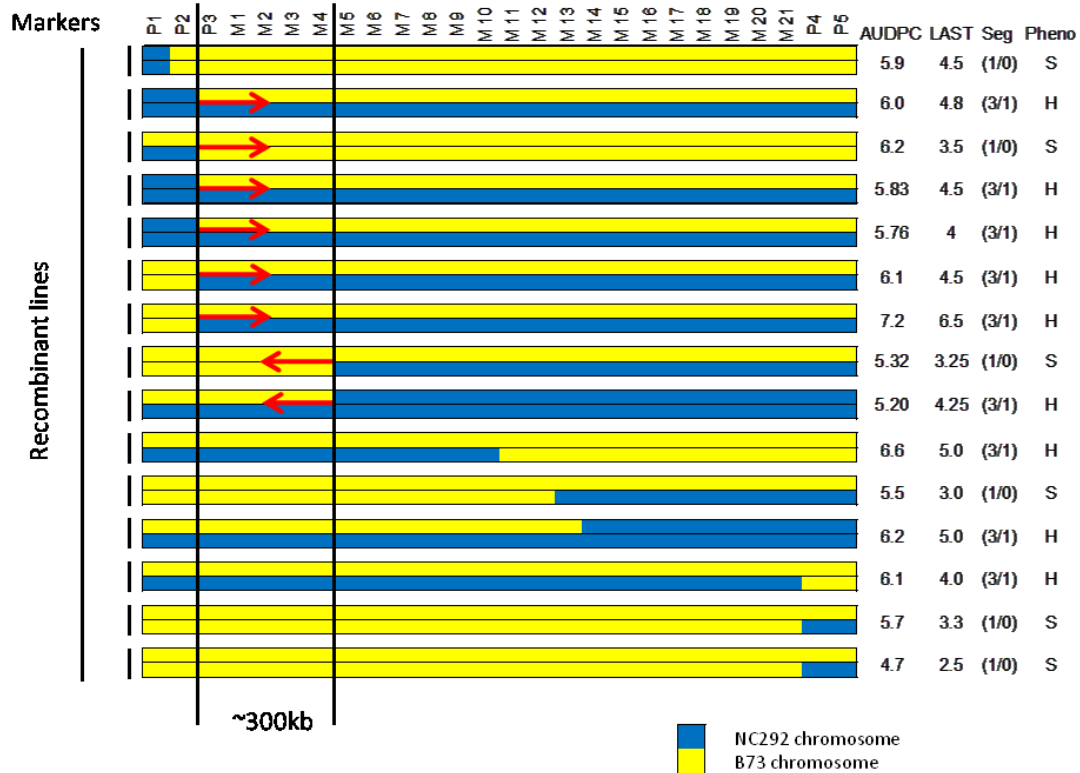


Figure 2.8 Graphical genotype and phenotype of recombinant lines. The graphical genotypes of the 15 recombinant F₂ individuals. Yellow and blue bars indicate from B73 and NC292 chromosome respectively. AUDPC values, last scores, segregation in the respective F_{2:3} family and the phenotype are shown to the right of each chromosome. Red arrows indicate where the region of interest is located. P1-5 are umc1018, PUT-163a-5739956-2305, bnlg2243, umc2515 and PHM15961.13, respectively.

3. CHAPTER III

Using *Ac/Dc* Transposon Tagging Mutagenesis and Virus-Induced Gene Silencing (VIGS) to Verify Identity of a Southern Leaf Blight Resistance Gene of Maize

3.1. Abstract

B73-6A is a B73-background near isogenic inbred line containing an introgression (6A) derived from a highly southern leaf blight (SLB) resistant inbred line NC292. A disease QTL (*6a*) against SLB that co-localizes with introgression 6A is suggested to be the SLB resistance gene *rhm1*. Both loci *rhm1* and *6a* have been mapped to the same region of the short arm of chromosome 6 and both act as single recessive genes. Both sources of resistance can be traced back to the same highly resistant breeding stock. Recent fine-mapping studies have shown that a transmembrane amino-acid transporter analog to the *Arabidopsis thaliana* lysine histidine transporter 1 (LHT1) may be the gene underlying *rhm1* resistance. It is suggested to be a loss-of-function resistance. Our objective is to describe two approaches to study the identity and function of the locus underlying the resistance from NC292-derived introgression 6A. The first approach was to knock out the susceptibility gene using the *Activator/Dissociation (Ac/Ds)* system. More than 8000 F₁s from a cross between plants carrying the *Ds* in the W22 background and B73-6A were screened for SLB resistance in the fields. As expected, most of the plants were susceptible to SLB, except for 10 plants showing resistance compared to susceptible lines. Using primers designed to amplify different portions of *LHT1*, an amplicon size polymorphism of around 350 bp was detected in exon 2

between W22 and B73-6A in a single plant. This plant was crossed to B73-6A, and all of its progeny were resistant. These results suggested that this F₁ may have a *Ds* insertion within *LHT1*. The second approach was to transiently suppress the expression of *LHT1* using virus-induced gene silencing (VIGS). Oh7B plants were infected with *Brome Mosaic Virus* (BMV) vectors carrying different fragments of *LHT1* (BMV-LHT1). Subsequently, the plants were inoculated with SLB. Five out of 48 BMV-LHT1- inoculated plants showed sectors of the leaves with SLB resistant reaction similar to B73-6A. Virus symptoms in Oh7B line were intense, making more challenging to have a clear assessment of this resistance reaction.

3.2. Introduction

Yield is affected by many problems in the field, from which some diseases can drastically reduce maize production. One of the most useful methods of disease control is the use of host plant genetic resistance. Many genes conferring resistance to several diseases have been identified and cloned in many crops; however, single-gene resistance has often proved to be short-lived, being easily overcome by pathogens. On the other hand, quantitative disease resistance (QDR) is characterized by a partial inhibition of the pathogen, is race-nonspecific and often regarded as durable. It usually involves several genes and it is often influenced by the environment. Most of the SLB disease resistance found and used in maize is quantitative in nature, though little is known about the identity and function of the genes that underlie it. B73-6A is a B73-background near isogenic line (NIL) containing an introgression on the short arm of chromosome 6 derived from a highly resistant line NC292. Introgression 6A carries a QTL (*6a* locus) for resistance against SLB. There is some evidence that the gene underlying SLB resistance in introgression 6A is an allele of the previously identified SLB resistance gene *rhm1*. Resistance locus *rhm1* has been mapped in the same region of the short arm of chromosome 6 as the *6a* locus (Zaitlin *et al.*, 1993). *rhm1*, as *6a* locus, acts as a single recessive gene for resistance against SLB. Resistances in both B73*rhm1* and B73-6A can be traced back to the same highly resistant Nigerian breeding stock (Craig and Daniel-Kalio, 1968; Craig and Fajemisin, 1969; Smith and Hooker, 1973; Thompson and Bergquist, 1984; Holley and Goodman, 1989). Also, *6a* locus and *rhm1* do not inhibit fungal germination, penetration, or growth; but both work similarly in reducing fungal growth after 48 hours (Gao *et al.*, 2005; Belcher, 2009). Both loci have failed to

genetically complement each other in an experiment using a mapping population derived from a cross between a B73rhm1 line and NC250A (a line containing introgression 6A) (Belcher, 2009). Two markers within the previously identified dQTL in introgression 6A by Zwonitzer (2009) were used to detect differences in the genotypic classes in this population. Fisher's protected least square difference test was used to compare the disease severity least-squares means from the genotypic classes (homozygous for the B73rhm1 allele (*rhm1*), homozygous for the NC250A allele (*6a*) and heterozygous. There were no significant differences among the genotypic classes. No association between resistance and either allele could be detected in the region where the dQTL is located, suggesting that *6a* and *rhm1* may represent the same recessive gene.

Recent studies have shown that a transmembrane amino-acid transporter analog to the *Arabidopsis thaliana* lysine histidine transporter 1 (*LHT1*) may be the gene underlying *rhm1* resistance (Zhao *et al.*, 2012). Fine-mapping of *rhm1* using H95rhm line has revealed maize *LHT1* gene as the only candidate (Zhao *et al.*, 2012). Resistance against SLB conferred by *LHT1* is shown wherever the mutated version of the gene, *lht1*, is present in homozygous condition.

The *Activator* (*Ac*) and the *Dissociation* (*Ds*) elements are transposable elements (TE) belonging to the class II "cut and paste" transposons. While the *Ac* is an autonomous TE (can produce its own transposase and transpose itself), *Ds* is a non-autonomous TE and depends on the supply of transposase from other autonomous TEs in the genome (Kunze *et al.*, 1997). *Ac* elements can transpose themselves at a low frequency (2-4% of the progeny will inherit a newly transposed *Ac*) (Brutnell and Dellaporta, 1994). 60% of the transpositions occur to

genetically linked sites (within 10cM of the donor) (Van Schaik and Brink, 1959; Greenblatt, 1984; Dooner and Belachew, 1989). Tom Brutnell's lab at Cornell University has developed a collection of lines each containing a single *Ds* at a unique position every 1 cM in the maize genome and a defective *Ac* element (named immobilized *Ac* or *im:Ac*) incapable of transposing itself but able to encode the transposase (Vollbrecht *et al.*, 2010). This element arose due to an aberrant transposition event in which the flanking regions necessary for recognition by the transposase have been truncated. So, *im:Ac* cannot be recognized by its own enzyme. Because *Ds* elements have been distributed throughout the maize genome and due to the fact that 60% of the transpositions occur to genetically linked sites, this *Ac/Ds* system becomes a great tool for regional mutagenesis and fine-scale genetic mapping (Brutnell and Conrad, 2003; Kolkman *et al.*, 2005; Ahern *et al.*, 2009; Vollbrecht *et al.*, 2010).

Virus-induced gene silencing (VIGS) is a widely-used tool that exploits the intrinsic post transcriptional gene silencing (PTGS) defense response against RNA virus infections. Please refer to Chapter 1 for current review of PTGS and VIGS. By inserting a fragment of a gene of interest into the *Brome mosaic virus* (BMV) genome and inoculating corn with this modified virus, the plant triggers PTGS in order to degrade the corresponding endogenous transcripts (and closely related sequences). VIGS using viruses such BMV has been used for functional gene studies in species such as barley (Holzberg *et al.*, 2002), wheat (Scofield *et al.*, 2005) and rice (Ding *et al.*, 2006; Purkayastha *et al.*, 2010). In corn, this system was used successfully to silence the phytoene desaturase (*pds*) gene in cultivar Va35 (van der Linde *et al.*, 2010). This VIGS system has been used to study the interaction between the biotroph

fungus *Ustilago maydis* and Va35 maize plants. In our lab, the system have been successfully adapted to inbred line Oh7b in which *pds* has been efficiently suppressed (Louie, 1995; Redinbaugh *et al.*, 2001)

Here we describe two techniques to investigate the gene responsible for this resistance: using the *Activator/Dissociation (Ac/Ds)* transposon elements to knock out the susceptibility gene *LHT1* and VIGS to transiently suppress the post transcriptional expression of *LHT1*.

3.3. Material and Methods

3.3.1. Plant Material

Four W22-background lines carrying a *Ds* element on the proximity of our region of interest were used for tagging the gene involved in resistance. I.W06.0147, B.S06.0473, I.S06.0835 and B.S05.0354 were selected from the collection of lines from the Boyce Thompson Institute affiliated to Cornell University (www.plantgdb.org/prj/AcDsTagging). These lines were also carrying the defective immobilized-*Ac (im:Ac)* TE (Refer to chapter 1). B73-6A is a B73-background NIL developed from marker-assisted selection of B73 x (NC292 x B73) backcross BC₂F₂ progeny. B73-6A is homozygous for the NC292-derived introgression 6A located on the distal end of the short arm of chromosome 6. Only lines carrying both the *Ds* and *im:Ac* (W22-*Ac/Ds*) were crossed as a male to B73-6A to generate over 8000 F₁ plants (Fig. 3.1).

Oh7B was the inbred line used herein in the VIGS experiment. Oh7B has been demonstrated to be compatible with BMV-VIGS system in corn (L. Benavente, Pers. com.).

In contrast to the photobleaching reaction on Va35, the photobleaching in Oh7B lines spreads over large sectors of the leaves (L. Benavente, Pers. com.).

3.3.2. Activator/Dissociation Transposon Tagging

3.3.2.1. Activator/Dissociation Lines Characterization

We received 10 kernels of each of the 4 W22-*Ac/Ds* lines segregating 1:1 for presence: absence of *Ds*. Kernels were planted in the Clayton in 2009. Twenty-nine out of 40 plants successfully germinated and tissue samples were collected. The presence of the *Ds* elements was checked using PCR and primers based on the sequence of the *Ds* and the flanking regions. Flanking regions and *Ds* primers JSR03 and JSR05 were obtained from www.plantgdb.org/prj/AcDsTagging (Table 3.1). Thirteen out of the 29 plants that germinated contained a *Ds* element. These plants were self-pollinated. Progenies from these plants were planted in Homestead, FL during the winter season 2009/2010. Each of these progenies was segregating for the *Ds*. So, Individuals from each of these progenies were checked for the presence of the *Ds*. Also, the excision of the *Ds* was also checked in these plants. Primers designed based on the flanking regions on both sides of *Ds* insertion were also used to detect the excision of the *Ds*. This allowed us to identify if the *Ds* was homozygous or heterozygous. Only one plant from W22-*Ac/Ds* line B.S06.0473 and 5 from I.S06.0835 were homozygous for the *Ds* (Fig. 3.2). These plants were self-pollinated. B.S06.0473 could not be used because of poor seed production. So, only W22-*Ac/Ds* line I.S06.0835 was used as males to cross to B73-6A line for the tagging experiment in order to double the chance of a transposition event. All PCR reactions used the following cycle; 3 min

at 95°C, followed by 35 cycles of 30 s at 95°C, 45 s at the optimum annealing temperature [Ta] for each primer combination, 45 s at 72°C, and a final extension step of 10 min at 72°C. Amplification products were separated on a 1.2% agarose gel and visualized in a UV transilluminator as previously described in chapter II. The presence of the *im:Ac* was checked by the activity of the transposase on a reporter gene. This activity was expressed as a variegated pattern in the aleurone (Brutnell and Conrad, 2003). Only plants showing this variegated pattern were used for the tagging experiment.

3.3.2.2. Field Screening of Resistance

More than 8000 heterozygous F₁ plants from a cross of the W22-*Ac/Ds* line I.S06.0835 to the resistant line B73-6A were screened for resistance at the NCSU Central Crops Research Station in Clayton during summer 2011 in an isolated crossing block. Plots were artificially inoculated with SLB by placing approximately twenty sorghum grains infected with *Cochliobolus heterostrophus* Race 0 in the whorl of plants at the 4-6 leaf stage (Carson, 1998; Carson *et al.*, 2004). Following inoculation, overhead irrigation was applied to provide moisture for fungal growth. All F₁ plants were treated as females and were detasseled. One row of resistant line B73-6A was planted as male every four rows of females to insure progeny in every resistant plant observed.

3.3.2.3. DNA Extraction and Detection of Polymorphism

Total nucleic acid was extracted from the all plants from this study using a modified CTAB extraction procedure (Saghai-Marooof *et al.*, 1984; Zwonitzer *et al.*, 2009). DNA extraction was performed as previously described in chapter II. If we needed to check the genotype of the seed before planting, DNA extraction was performed from seed using the modified Mogg and Bond protocol (Mogg and Bond, 2003 ; Coles, 2009). All of the DNA extraction procedures were performed in a 96-well format. Two 48-well ELISA type plates were used to store the kernels. Each position in the extraction plate had a corresponding position in the ELISA plates. The 48-well plates were aligned so they accurately matched the positions in the 96-well plates. Seeds were chipped at the distal end on the endosperm with commercially available cat nail clippers. After placing the endosperm pieces into a single well of the 96-well plate, the same seed was placed into the corresponding well in the 48-well ELISA plate. To avoid cross-contamination, cutting for each kernel was done in a different piece of wax paper. Endosperm tissue was ground using Retsch® Mixer Mill MM301 Retsch GmbH & Co. (Haan, Germany) with a #2 Bismuth shotgun BB in each well. 400 µl of extraction buffer containing 100mM Tris, 50 mM EDTA, 500 mM NaCl, 0.7% sodium dodecyl sulfate, 50µg/mL Proteinase K, and 50 µg/mL RNase was added to each sample. Samples were then incubated overnight in an oven at 37° C. The following day, 260 µL of 5 M NaCl was added and mixed thoroughly for about 10 seconds. Then samples were centrifuged 2576 g for 10 min. 500 µl of iced-cold isopropanol was used to precipitate nucleic acids for 1 hour at -20° C. Washing was performed twice with ice cold 70% ethanol and the total nucleic acids were resuspended in 150 µl of 1X TE.

Based on the assumption that the susceptibility gene was *LHT1*, primers designed to amplify a portion of this gene were used to detect size polymorphisms within the sequence (Fig. 3.3). Primers were designed to target approximately every 250 bp along the sequence. B73, W22 were used as a susceptible control and B73-6A as the resistant control. All the primers used in this study can be found in table 3.1.

3.3.3. Virus-Induced Gene Silencing

All experiments were conducted on Oh7B line. All experiments were carried out at the North Carolina State University phytotron and all plants were planted and grown as previously explained in chapter II and following procedures from the Phytotron Procedural Manual (Saravitz *et al.*, 2009). Plants were maintained in a 3 x 3 x 2.13 meter growth chamber, with a day length of 12 h per 24 h cycle (6am-6pm) and a constant temperature of 22°C.

3.3.3.1. Construction of Infectious BMV Vectors

Primers specifically designed for the *LHT1* gene were generated based on exonic regions. Two set of primers ZmLht1F2 - ZmLht1R1 (treatment A/E) and ZmLht1F7 - ZmLht1R4 (treatment D), amplified a specific fragment on the *LHT1*. Other two set of primers, ZmLht1F4-ZmLht1R3 (treatment B) and ZmLht1F5-ZmLht1R3 (treatment C) amplified regions conserved in *LHT1* and its orthologues in the maize genome. The complete sequences and details of the primers and treatments can be found in table 3.1. The schematic procedure has been summarized in figure 3.4. Primers were tested in B73 for amplification of

the correct amplicon. PCR products were separated on a 1.2% agarose gel and the correct sizes of the expected amplicon were checked. cDNA synthesis was performed on previously extracted total RNA from Oh7B line and provided by Larissa Benavente. Total RNA was extracted using Trizol (Life Technologies, Carlsbad, CA) as recommended. RNA integrity was determined on a 1.5% agarose gel and quantified on the Nanodrop. For Rreverse transcriptase-PCR (RT-PCR), 1 µg of total RNA was used as template in a 20 µl reaction containing 2.5 µM oligo dT(20) (Life Technologies, Carlsbad, CA), 250 ng of random primers (Life Technologies, Carlsbad, CA), 0.5 mM of dNTP (Promega, Madison, WI), 10 mM DTT, and 200 U of M-MLV (Life Technologies, Carlsbad, CA), according to manufacturer's recommendations, for 2 h at 37°C. The cDNAs were digested with *NcoI* and *AvrII* enzymes for 2h at 37°C and inserted into a plasmid vector carrying RNA3. The plasmid vector pB3m was provided by Larissa Benavente. Plasmids pB3m carrying each of the 4 *LHT1* fragments were transformed into *E. coli* following standard molecular biology methods according to Sambrook *et al.* (1989).

3.3.3.2. *In-vitro* Transcription

To test the authenticity of the insert, plasmids were sent to be sequenced by Eton bioscience, Inc. The *p3Bm* plasmids carrying the correct fragments sequences, as well as plasmids *pF1-11*, *pF2-2* containing BMV RNA1 and RNA2 DNA sequences, were individually digested with *SpeI* or *PshAI* (New England Biolabs, Ipswich, MA) for 2 h at 37°C and heat-inactivated. The linearized plasmids were treated with 100 µg/ml Proteinase K at 50°C for 30 min, purified by phenol chloroform extraction and resuspended in RNase-free

water. The mMESSAGE mMACHINE T3 kit (Life Technologies, Carlsbad, CA) was used for the production of infectious virus transcripts following manufacturer's recommendations. For production of *in vitro* transcripts, 1 µg of linearized plasmid was used as template as recommended. The transcripts were precipitated using lithium chloride and resuspended in RNase-free water. RNA integrity was confirmed by agarose gel electrophoresis and quantified using the Nanodrop. The inoculums consisted of 4 µl of transcript mix containing 1.5 µg of each transcript (RNA 1, RNA 2, and RNA 3 harboring of the fragments from maize *LHT1* gene) (Ding *et al.*, 2006).

3.3.3.3. Virus Infection Using the Vascular Puncture Inoculation

The vascular puncture inoculation (VPI) procedure was used to infect maize seeds with BMV (Louie, 1995). 12 seeds for each of the 4 LHT1 treatments were pre-soaked at 30°C in water for 4h. Kernels were then incubated in moist paper towels at 22°C for additional 4h (Redinbaugh *et al.*, 2001). The inoculums, which consisted of 4 µl of transcript mix containing 1.5 µg of each virus particle (RNA 1, RNA 2, and RNA 3/LHT1 fragment), was pushed 1-2 mm into the scutellum alongside the embryo toward the underlying vascular bundle using the engraving tool. The engraving tool was constructed by fixing five 'minuten' insect pins (0.2 mm, stainless steel) to the flattened end of 10-gauge copper wire. The assembled pins were mounted onto an engraving tool following manufacturer's specifications (Ideal Industries Inc., Sycamore, IL, USA) (Louie, 1995). The virus is inoculated by pushing the insect pins into the scutellum of germinating kernels. To deliver the virus to the vascular tissue, the engraving tool must be tilted so it penetrates under the embryo (Louie, 1995).

Inoculations were performed on a single side of the scutellum. Seeds were incubated for 2 days on moist paper towels at 30 °C in the dark, planted on soil and transferred to the growth chamber. Controls consisted of mock (water only) inoculated plants, plants inoculated with the BMV-GUS (RNA 3 carrying a fragment of the *GUS* gene) as a control of virus infection, and plants inoculated with BMV-PDS (RNA 3 carrying a fragment of the *pds* gene) as a silencing control.

3.3.3.4. SLB Inoculation

SLB inoculation was performed 14 days after planting as previously described in chapter II. Twelve plants for each of the 4 LHT1-fragment treatments were randomized and spray-inoculated with *C. heterostrophus* race 0. Mock, BMV-GUS and BMV-PDS inoculated plants were also SLB inoculated. Non-BMV-infected B73 and NC292 were used as SLB susceptible and resistant controls, respectively. SLB Scoring was performed 3 days after SLB inoculation as previously described in chapter II. Plants were scored as resistant if their reaction was similar to the resistant control line NC292 and susceptible if their reaction was similar to the susceptible control line B73.

3.4. Results

3.4.1. Activator/Dissociation Transposon Tagging

3.4.1.1. Activator/Dissociation Lines Characterization

We received 10 kernels of each of the 4 W22-*Ac/Ds* lines segregating for *Ds*. The presence of the *Ds* elements in these plants was checked using PCR and primers based on the sequence of the *Ds* and the flanking regions. Only W22-*Ac/Ds* line I.S06.0835 was used as males to cross to B73-6A line for the tagging experiment.

We also confirmed that these lines were susceptible to SLB in growth chamber experiments. We hypothesized that W22 lines carried the same susceptibility allele as B73.

W22-*Ac/Ds* were crossed as males to the SLB resistant B73-6A. We hypothesized that if a transposition occurred within the W22 susceptibility allele, it would render the plant resistant. The procedure is explained in detail in figure 3.1. Only 2-4% of the progeny will inherit a newly transposed *Ds* element. Thus, approximately 8000 F₁s were produced.

3.4.1.2. Field Screening of Resistance

More than 8000 F₁s from a cross B73-6A x W22-*Ac/Ds* I.S06.0835 were planted in Clayton in an isolated crossing block. All F₁ plants were detasseled and were allowed to cross to resistant B73-6A line to secure a progeny in every resistant plant found. All F₁ plants were screened for resistance. Using the *Ac/Ds* system, the *im:Ac* element might transpose the *Ds* into the dominant susceptibility gene. Most of the 8000 F₁s screened were susceptible to SLB when artificially inoculated. Ten plants showed some resistance compared to susceptible line B73 and susceptible F₁s (Fig. 3.5) (Table 3.2).

Amplification of regions of *LHT1* was performed to look for polymorphisms (Fig. 3.3). There was a polymorphism of around 350 bp on third exon (exon 2 based on www.maizesequence.org) between B73 and B73-6A (Fig. 3.6). Parent NC292 also showed the B73-6A allele while W22 showed the B73 allele. All the heterozygous F₁s checked presented two bands corresponding to the B73 allele and the B73-6A allele, except for one plant, PBK4A. No other polymorphism was found in this plant and the plant was highly resistant in the field (Fig. 3.5). Progeny from the cross of B73-6A to each of these resistant plants were inoculated with SLB in a growth chamber experiment. Selfed progeny only of plant PBK4A were uniformly resistant to SLB (14 of 14 plants; Table 3.2).

3.4.2. Virus-Induced Gene Silencing

Our goal was to test the effect on SLB resistance when we transiently silenced *LHT1* in maize using the VIGS system. The BMV VIGS system has been standardized in our lab with the phytoene desaturase (*pds*) gene used as a visual reporter on inbred line Oh7B (L. Benavente, pers. com.). 12 seeds were inoculated with BMV carrying one of each of the 4 treatments. Subsequently, we spray-inoculated the plants with SLB to assess resistance. Over 55% of the BMV-PDS-infected plants (5 out of 9) showed silencing of the *pds* gene as suggested by the photobleaching phenotype (Table 3.3 and fig 3.7). The four remaining plants showed virus symptoms but no photobleaching. No photobleaching phenotype was seen in any other treatment. Eight out of 9 BMV-GUS-infected plants showed typical symptoms of BMV (Table 3.3). Plants were stunted and the mosaic pattern was present on the leaves as soon as the plant emerged (Fig. 3.7). The remaining plant was probably an

escape from the VPI technique. The mock-infected controls showed no BMV symptoms. BMV infection using VPI was successful in all the four treatments with an inoculation success percentage of at least 78%. By silencing *LHT1*, we would expect regions on the leaf to show a reaction similar to the resistant lines B73-6A or NC292 after SLB inoculation. Resistant B73-6A and susceptible controls Oh7B and B73 reacted to SLB as expected (Fig. 3.8A). BMV-GUS-, BMV-PDS- and Mock-infected plants were all susceptible when inoculated with SLB. For treatments BMV-C, D and A/E, five plants showed sectors of the SLB inoculated leaves that had a reaction similar to the resistant line B73-6A (Table 3.3). Some of the plants showing this reaction are shown in figures 3.8C-E.

3.5. Discussion

Quantitative resistance is the most useful method to control plant disease; however, the exact mechanism of quantitative resistance is not well understood. While several major effect genes for resistance have been mapped and cloned, only a few genes which underlie dQTLs have been identified (See Chapter 1).

At Brutnell's lab at Cornell University, a set of W22-background lines have been developed each containing a *Ds* element at a unique position (Brutnell and Conrad, 2003; Ahern *et al.*, 2009; Vollbrecht *et al.*, 2010). These lines may be used as a tool for regional mutagenesis. By crossing these W22-*Ac/Ds* lines to resistant line B73-6A, we were expecting to disrupt the susceptibility gene. But since transposition only occur in 2-4% of the progeny, many thousands of plants needed to be screened in order to find one transposition event. We were able to obtain over 8000 F₁ plants from a cross B73-6A x W22-*Ac/Ds* I.S06.0835. The

fastest way to screen so many plants was in the field. The ten resistant plants we identified were comparatively more resistant than the susceptible control.

There is some evidence that the gene behind this SLB resistance in B73-6A line is the SLB resistance gene *rhm1* (See chapter 2). Recent studies have shown that a transmembrane amino-acid transporter analog to the *AtLHT1* may be the gene underlying *rhm1* resistance (Zhao *et al.*, 2012). We hypothesized that *LHT1* confers susceptibility to SLB and it is the loss-of-function that confers resistance in B73-6A and NC292. However, if resistant selections really carried a mutation in the W22 susceptible allele of the gene underlying the resistance conferred by the 6A introgression then, the progeny of a cross of these F₁ selections to resistant B73-6A line should be all resistant (Table 3.3). Surprisingly, the progenies of these selected candidate plants to B73-6A segregated for SLB resistance (progenies included both resistant and susceptible plants). Only one of these lines, PBK4A, produced all resistant progeny (14 out 14). This suggests that other plants were false positives and were not resistant. One explanation for the false positives could be that environment was not conducive for the development of the disease in the field. This is unlikely, since plants were selected from a field where all plants were inoculated and infection was evenly distributed. Plants surrounding the selected resistant plants were highly infected. Maybe they were escapes of inoculation and the disease just did not develop as fast as in the other neighboring F₁s.

To further check the background of these F₁s, a polymorphic region on exon 2 was amplified. This region showed a polymorphism of over 350bp. The allele from susceptible W22 line is about the same size as the B73 allele. The B73-6A allele is approximately 350

bp larger than B73. Any F₁ from a cross between these two lines (B73-6A x W22-AC/Ds) should produce two bands. All of the candidate F₁ plants produced two bands, except PBK4A. This plant yielded only the B73-6A allele. If a *Ds* insertion was to be found within the W22 version of the *LHT1*, a band of around 3Kb (that includes the *Ds*) would be present as well. However, no such a band was found. One explanation for the absence of a second band that includes the whole *Ds* sequence could be the competition between short and long sequences for the primers. A short sequence will amplify faster than a much longer sequence, leading to competition for the primers, thus potentially reducing amplification of the longer sequence. The fact that plant PBK4A looked like any of the other F₁ hybrids, produced spotted seeds (thus containing the *im:Ac*), and produced a resistant progeny when crossed to B73-6A makes it a good candidate for having a *Ds* insertion.

The VIGS system has been used for functional gene studies in several species (Holzberg *et al.*, 2002; Scofield *et al.*, 2005; Ding *et al.*, 2006; Purkayastha *et al.*, 2010). In the controls for our study, the *pds* gene was effectively silenced in more than half of the seeds we inoculated. This is in agreement with the findings from Benavente (L. Benavente; Pers. comm.). Silenced *pds* is expressed as white sectors covering large sectors of the leaves of Oh7B. Similar symptoms could be seen in our experiment. Virus symptoms in Oh7B line were very intense in our experiment. Plants were too stunted and leaves were too little for a clear assessment of the disease. According to Benavente (L. Benavente pers. com.) experiments, stunting is more severe in the Oh7b line compared to other testers tested. As a consequence, it was a challenge to have a clear assessment of the SLB resistance phenotype, and to set that reaction apart from the mosaic symptoms caused by the virus. The fact that

silencing is transient and restricted to some sectors of the leaf blade works better for a phenotype such as the *pds* gene. It was a more challenging process for the *LHT1*. Using a plant that does not get too much symptoms from the virus may be needed to confirm that *LHT1* has been silenced.

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Table 3.1 List of primers used in this study. Primer Name for detection of transposition is composed of the gene name followed by the exon number or region the primer was located (3UTR = 3'-untranslated region, 5UPS = 5'-upstream sequence). *For VIGS primers only, the treatment if the fragment being silenced, the size of the fragment in bp and some notes on the characteristics of the sequence amplified (unique sequence for LHT1 or conserved sequence among LHT1 and its orthologues).

<i>Primer Name</i>	<i>Direction</i>	<i>SEQUENCE (5' - 3')</i>	<i>T_m (°C)</i>	<i>Treatment*</i>	<i>Size* (bp)</i>	<i>Notes*</i>
PRIMERS TO AMPLIFY THE DS FROM ORIGINAL LINES						
JSR05-F	Forward	CGTCCC GCAAGTTAAATATGA	63.5			
JSR03-R	Reverse	CGATCGGGATAAACTAACAAAATC	64.1			
IW060147-R	Reverse	TCAGAGATGCATGGAAGCTG	64.2			
BS060473-R	Reverse	CGGCGAGGACGAGTAGTAGTAG	63.9			
IS060835-F	Forward	GCTGAAGAAGTTCACGACAC	63.8			
BS050354-F	Forward	TTAAGAGCACCGTACCATTGC	63.7			
PRIMERS TO DETECT THE HOMOZYGOUS STATE						
IW060147-F	Forward	AGCTTCATT CAGGGGTGTT	64.6			
BS060473-F	Forward	CCCTACACACGAGGGAAAAA	63.7			
IS060835-R	Reverse	CGTCGAGTAGCTGCAACAAG	63.7			
BS050354-R	Reverse	CTTAAGTCTTGCACGCACCA	63.9			
PRIMERS TO DETECT POLYMORPHISMS ON LHT1						
GRMZM2G127328.exon2	Reverse	GGTCTGTTAAGGGTGACGA				
GRMZM2G127328.3UTR	Reverse	AGAACTCGCACTGGACGCTGTAAT	68.9			
GRMZM2G127328.exon6	Forward	TCAGCCTCTCATGGTTCACCAACT	70.0			
GRMZM2G127328.exon2	Forward	ATGTCGCTCAGCTACTCGACCATC	69.6			
GRMZM2G127328.exon7	Reverse	TCGAAGAAGGAAACGTGATGGCT	72.3			
GRMZM2G127328.exon7	Forward	TAGCCATCACGTTCCCTTCTTCGAC	72.2			

Table 3.1 Continued

<i>Primer Name</i>	<i>Direction</i>	<i>SEQUENCE (5' - 3')</i>	<i>T_m (°C)</i>	<i>Treatment*</i>	<i>Size* (bp)</i>	<i>Notes*</i>
GRMZM2G127328.exon3	Forward	TGCCCGTGTTCGACATGATAGAGA	71.6			
GRMZM2G127328.exon5	Forward	ATCGACAACCTGGCTTCCCATCA	69.8			
GRMZM2G127328.exon2a	Reverse	TCACCATGTACACGATGTTACCC	69.8			
GRMZM2G127328.exon4	Forward	AGTCCAGCCGTCCTGGAGATGA	75.0			
GRMZM2G127328.exon5	Reverse	ACCATGGCCGTGACGTTGTGGAA	76.2			
GRMZM2G127328.5UPS	Forward	TCTCTCTCTCGTCTTCGCCATCCA	72.3			
GRMZM2G127328.3UTRa	Reverse	ACATATATGAAGCACAACTCTTCCCT	64.7			
GRMZM2G127328.exon6	Reverse	AACCGTTTGGGCTTGTAGATTGCG	72.1			
PRIMERS FOR VIGS*						
ZmLht1F2	Forward	CATACCTAGGGTCTTCGCCATCCACCATCC	77.5	A/E	217	unique in 5'UTR
ZmLht1R1	Reverse	CATACCATGGACCACCACTTGGCATTCGGC	81.2	A/E		
ZmLht1F4	Forward	CATACCTAGGCTCACGCTCCGTCTCATTTG	75.4	B	348	conserved
ZmLht1F5	Forward	CAGACCTAGGGCGTTCACAATGTTTCATAGC	74.1	C	312	conserved
ZmLht1R	Reverse	CATACCATGGCTGGTGAAGCAAGGGGTTTG	78.9	B & C		
ZmLht1F7	Forward	CAGACCTAGGCTTGCTTCACCAGTAACTG	71.7	D	274	unique in 3'UTR
ZmLht1R4	Reverse	GACTCCATGGCAGGAACATTCTCATCAACC	76.2	D		

Table 3.2 Table showing the details of the resistant plants from the *Ac/Ds* experiment.
Plants were screened for SLB resistance in the field.

F1 clone	Field Phenotype	LHT1 allele	Progeny seed color	Progeny (Sus/Res)
3D	HIGHLY RESISTANT	Het	variegated	NA
4A	RESISTANT	Het	variegated	2/6
PBK4A	HIGHLY RESISTANT	B73-6A	variegated	0/14
7B	MODERATELY RESISTANT	Het	-	-
22B	RESISTANT	Het	variegated spotted	6/2
25A	MODERATELY RESISTANT	Het	variegated spotted	4/4
28C	MODERATELY RESISTANT	Het	-	5/3
31B	MODERATELY RESISTANT	Het	variegated	6/2
31C	RESISTANT	Het	NA	NA
PBK31A	HIGHLY RESISTANT	Het	variegated	8/0
Controls				
F1 Sus	SUSCEPTIBLE	Het	variegated	5/2
B73-6A	HIGHLY RESISTANT	B73-6A	yellow	0/2
B73	SUSCEPTIBLE	B73	yellow	2/0

Table 3.3 VIGS experiment of SLB resistance. Ratios of BMV-VIGS treatments using the VPI procedure. The ratios indicate number of seeds displaying virus symptoms, photobleaching phenotype and SLB susceptible and resistant reaction based on NC292 resistant control and B73 susceptible control. Virus ratio is based on the number of plants showing virus symptoms out of total seed germinated. Photobleaching is the number of plant showing that phenotype based on total plants showing virus symptoms. SLB reactions are based on the number of plants showing susceptible or resistant reaction out of all the plant showing virus symptoms. BMV-GUS is the control for virus infection. BMV-PDS is the control for gene silencing of the *pds* gene. Mock = inoculation with water (no virus control). BMV-B through E are the different treatments of LHT1 fragments tested in this study.

<i>Treatment</i>	<i>Germination</i>	<i>Virus</i>	<i>Photo bleaching</i>	<i>SLB</i>	
				<i>Sus</i>	<i>Res</i>
Mock	16/18	0/16	NA	all	0
BMV-GUS	9/12	8/9	0/8	8/8	0/8
BMV-PDS	10/10	9/10	5/9	9/9	0/9
BMV-B	9/12	7/9	0/7	7/7	0/7
BMV-C	10/12	9/10	0/9	7/9	2/9
BMV-D	11/12	11/11	0/11	9/11	2/11
BMV-E	11/12	9/11	0/9	8/9	1/9
Res control	12/12	0/12	NA	0	all
Sus control	8/12	0/8	NA	all	0

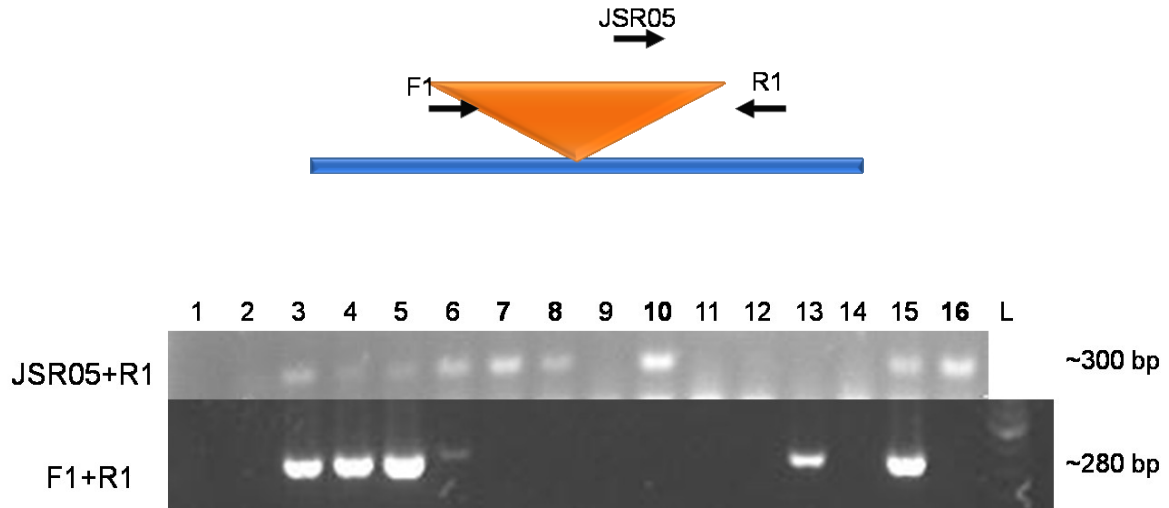


Figure 3.2 Detection of *Ds* element on W22 lines. PCR amplification of *Ds* insertion using *Ds*-specific primer JSR05 and primers designed based on the flanking region (R1) (Upper gel). To detect the homozygous condition of the *Ds*, amplification was performed with primers F1 and R1 designed from the flanking regions (Lower gel). Individuals 7, 8, 10 and 16 showed the amplification of the *Ds*, but no amplification using F1+R1. These four individuals are homozygous for the *Ds*. Individuals showing only the F1+R1 band are homozygous for the W22 allele and have no *Ds*. Individuals showing the two bands are heterozygous. *Ds* transposable element is represented by the orange triangle. Flanking regions are represented by the blue rectangle. L = 1 Kb plus DNA ladder. Primers are represented by black arrows.

GRMZM2G127328

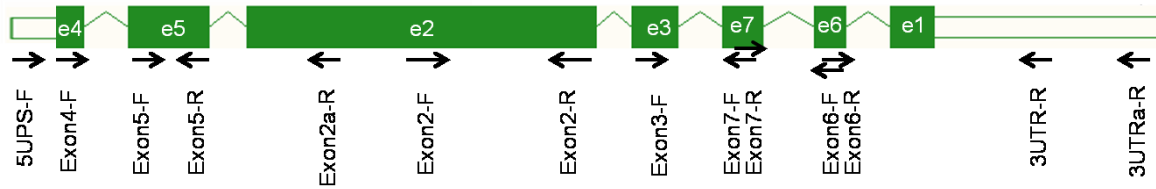


Figure 3.3 Schematic diagram of GRMZM2G127328 (LHT1) genomic structure of B73. Green boxes indicate exons 1 through 7 and introns are represented by green lines. Empty boxes represent the 5'-upstream sequence (5UPS) and the downstream 3'-untranslated region (3UTR). Primers to detect polymorphisms along LHT1 are represented by arrows and are coded by the exon number or region is located, followed by R (Reverse) or F (Forward). Gene image based on www.maizesequence.org

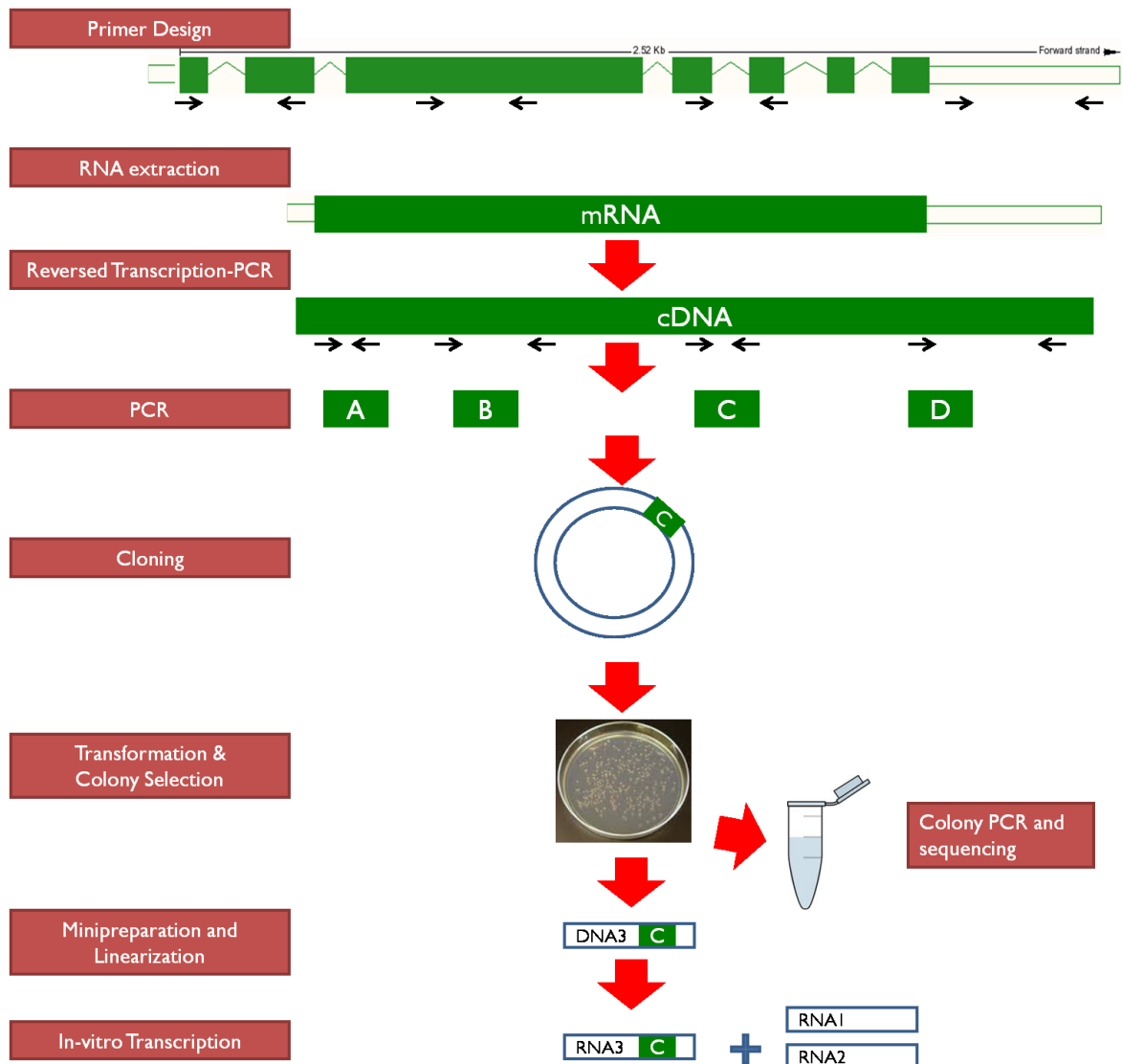


Figure 3.4 Construction of infectious BMV vectors for VIGS. Schematic procedure to generate the BMV vectors carrying the fragments for VIGS. RNA1, RNA2, RNA3 are the RNA particles which form the tripartite BMV. Treatments A/E, B, C and D are represented by green rectangles with their corresponding letter. The scheme represents only the development of the construct with C treatment from the cloning step. The other treatments BMV-B, BMV-D, BMV-A/E, as well as control BMV-PDS and BMV-GUS followed the same protocol.

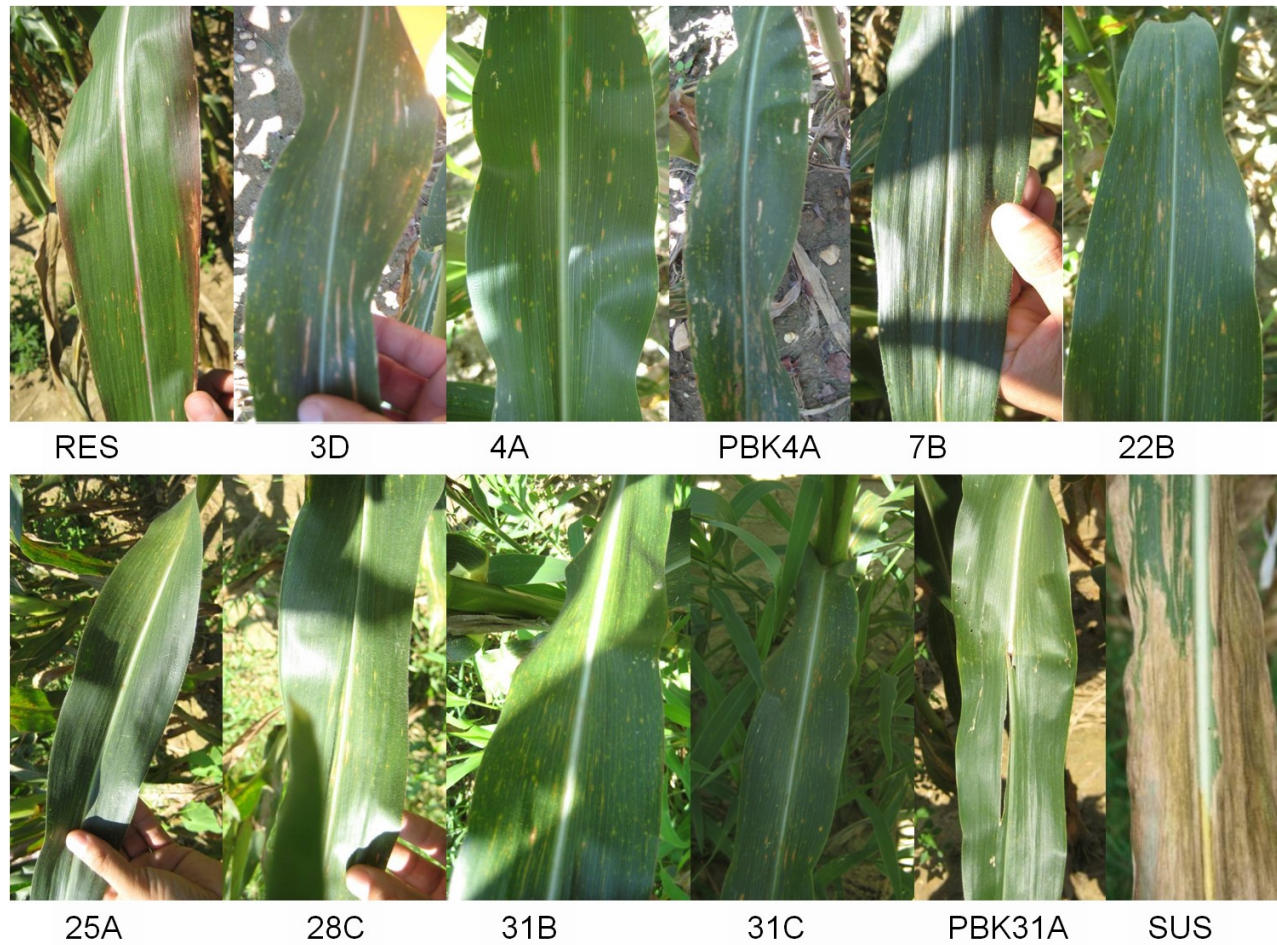


Figure 3.5 Phenotypes of the 10 resistant plants from the *Ac/Ds* transposon tagging experiment. A resistant B73-6A and a susceptible F₁ individual are showed as a comparison to the resistant lines.

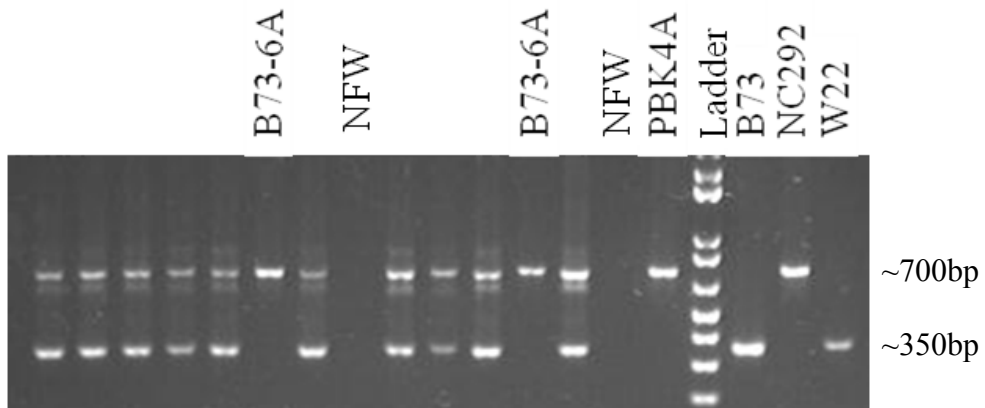


Figure 3.6 PCR amplification of exon2 of the LHT1 gene. Amplification was performed with primers GRMZM2G127328. Exon2-F and Exon2-R. PCR products were run in a 1.2% agarose gel. Polymorphism of around 350 bp was observed between B73 and B73-6A. Only PBK4A is labeled. All the other F₁s are not labeled and showed two bands from both the B73 and B73-6A alleles. 1 Kb plus DNA ladder was run along the samples.

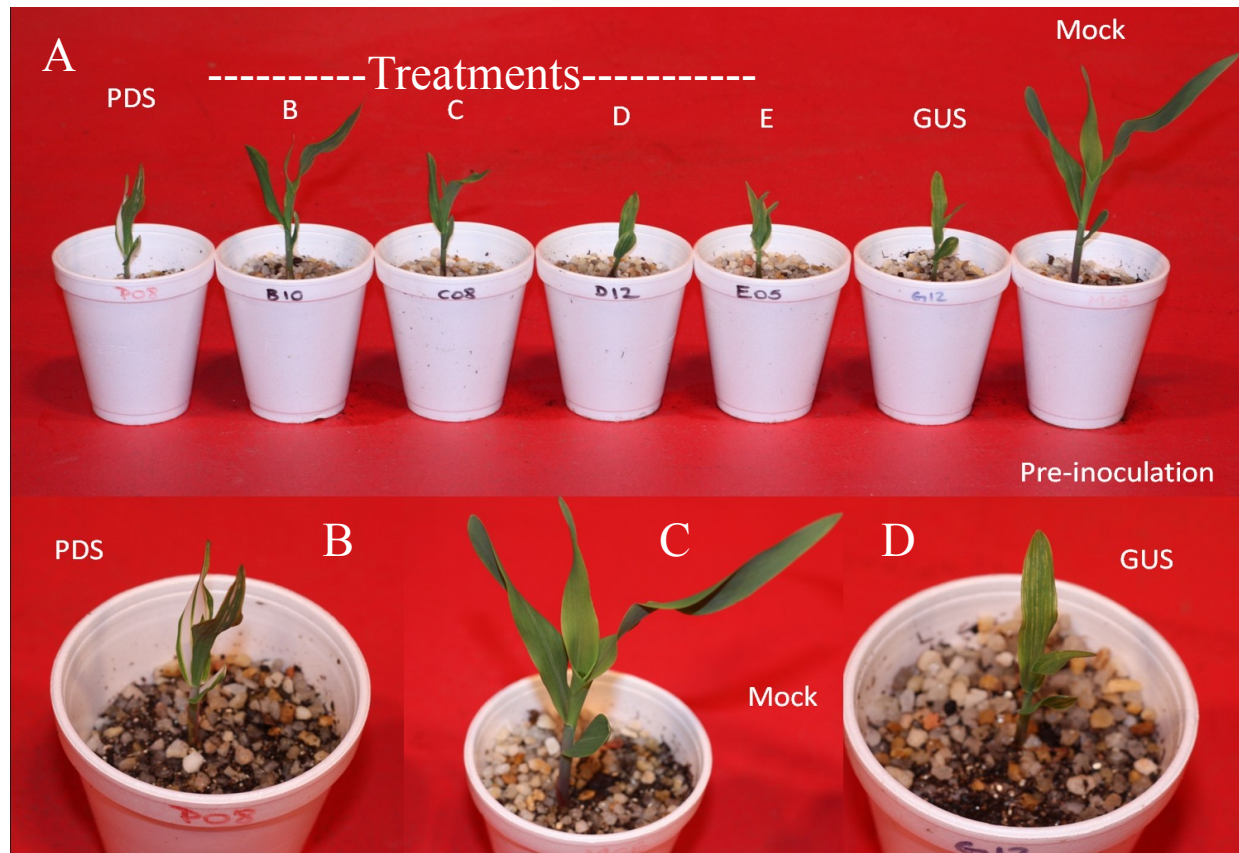


Figure 3.7 Reactions from control lines and treatments previous inoculation with *C. heterostrophus*. A) reactions from the four treatments (B,C, D and E) compared to the BMV-GUS-, BMV-PDS-, and mock-inoculated controls. B) Oh7B plant inoculated with BMV-PDS showing the photobleaching phenotype. C) mock-inoculated Oh7B plant. D) Typical mosaic and dwarfing symptoms of Oh7B inoculated with BMV-GUS.

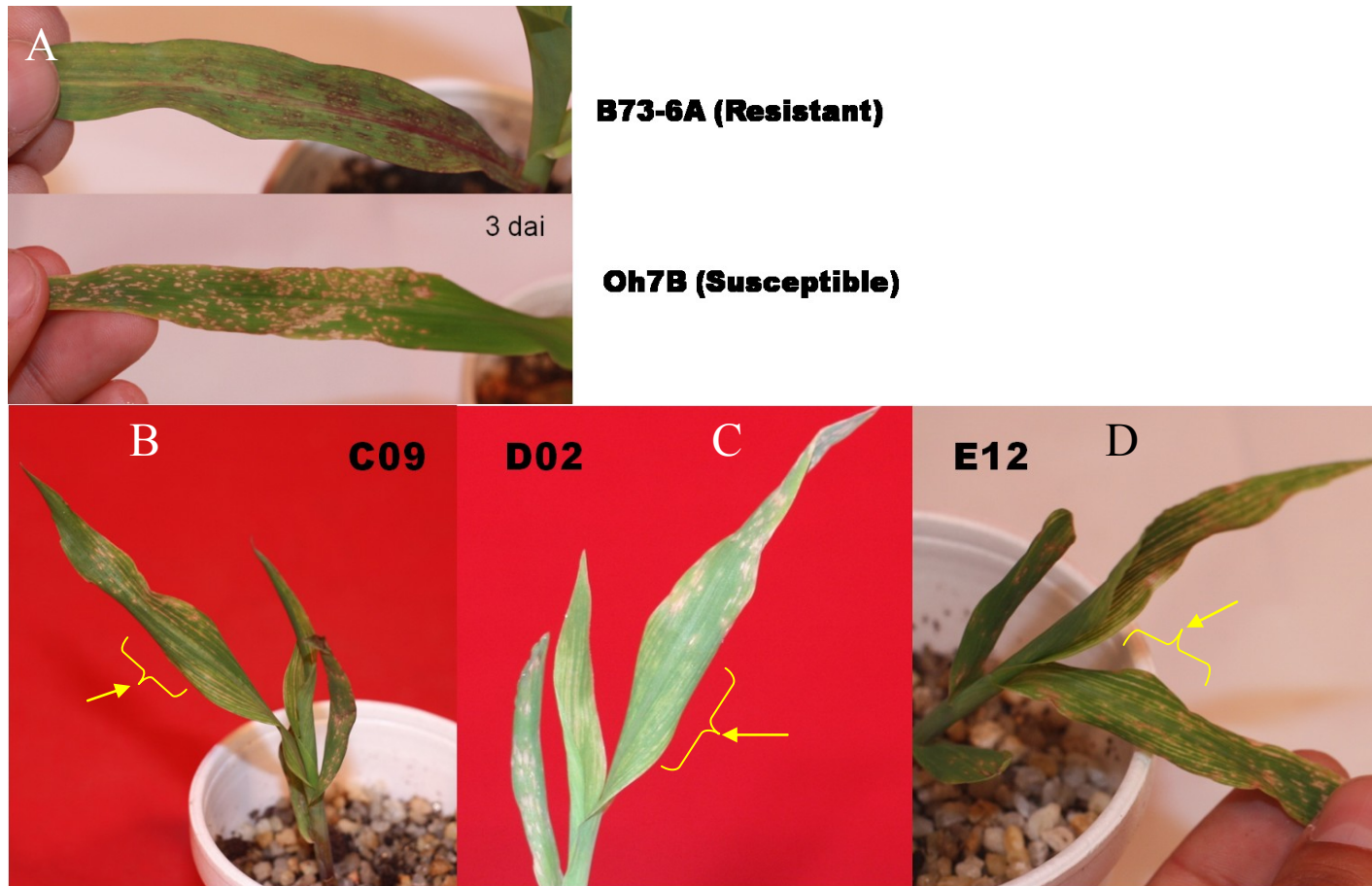


Figure 3.8 Reactions from control lines and treatments to inoculation with *C. heterostrophus*. A) Typical resistant and susceptible reactions of B73-6A and Oh7B, respectively, to SLB inoculation in growth chamber experiments 3 days after inoculation (dai); B-D) Reaction of some plants from treatment C, D and E showing some sectors of the leaf blade with similar reaction to resistant line B73-6A indicated by the yellow arrows.

4. CHAPTER IV

Evaluation of Two Southern Leaf Blight Resistance QTL for their Effect on Yield and Disease Resistance in Isogenic Maize Hybrids

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Pathology, North Carolina State University, Raleigh, NC 27695.

4.1. Abstract

B73-3B and B73-6A are two near-isogenic lines (NILs) in the background of the commonly-used maize inbred B73, each carrying one introgression with a southern leaf blight (SLB) resistance QTL from the highly SLB-resistant line NC292. The objective of this research was to evaluate the impact of each QTL for yield and fitness under both SLB disease pressure and disease-free conditions. We developed isohybrid triplets by crossing B73, B73-3B and B73-6A to several inbred lines (testers). We selected the isohybrid triplets in which the B73-6A or B73-3B hybrids significantly differed in SLB resistance from the B73 check hybrids for multi-environment yield trials conducted under artificially inoculated and disease-free (fungicide-sprayed) conditions. Results from this study identified significant differences between the yields of inoculated and sprayed plots, as well as significant differences between control hybrids and those containing either NC292 introgression. In the presence of the SLB disease pressure, introgression 3B was associated with an approximately 3% yield increase. No significant advantage in yield was associated with introgression 6A in the presence of SLB across all testers. However, this was likely because the resistance conferred by 6A was only fully expressed in 4 of the 10 hybrids tested. Hybrids NC250 x B73-6A and Va35rhm x B73-6A yielded significantly more than their B73 hybrid counterparts in the presence of SLB. Results suggested that both introgressions might confer a cost in fitness in the absence of SLB. But only introgression 6A was associated with a statistically significant yield reduction.

4.2. Introduction

The causal agent of SLB, the ascomycete *Cochliobolus heterostrophus* (Drechs.) Drechs. (anamorph = *Bipolaris maydis* (Nisikado) Shoemaker), penetrates and colonizes the intercellular spaces of the leaf, ultimately causing necrotic lesions. This disease lesion phenotype is easily visually assessed, and is highly heritable (Balint-Kurti *et al.*, 2007; Zwonitzer *et al.*, 2009; Kump *et al.*, 2011).

In general, though not in every case (e.g. Belcher *et al.*, 2011), quantitative disease resistance (QDR) in plants is controlled by many small-effect quantitative trait loci (QTL) (Wisser *et al.*, 2006; Poland *et al.*, 2011). Although the additive effects of these disease resistance QTL (dQTL) do create large phenotypic differences in aggregate, little is known about how much individual dQTL contribute to differences in agronomic traits, especially yield, in inbred lines and hybrids (Frey *et al.*, 2011). Because most dQTL have small and somewhat variable phenotypic effects, field experiments of a conventional scale would likely lack adequate power to detect their potentially correlated effects on agronomic characteristics. To investigate effects of single QTL thus requires studying the QTL of larger magnitude in a pathosystem for which host resistance is highly heritable, and thus less sensitive to environmental perturbation. Two loci, that we have called *6a* and *3b* and are associated with resistance to SLB of maize, fit these qualifications (Zwonitzer *et al.*, 2009; Belcher *et al.*, 2011).

B73-3B and B73-6A are two near isogenic lines (NILs) in the background of the commonly-used maize line B73. Each carries a single introgression derived from the highly SLB resistant line NC292 referred to as “3B” and “6A”. These introgressions carry,

respectively, the SLB resistance alleles at the *3b* and *6a* loci (Belcher *et al.*, 2011) located in bins 3.04 and 6.01 of the maize genome map (Davis *et al.*, 1999).

Several studies have identified strong-effect SLB dQTL in bins 3.04 and 6.01 (Bubeck, 1991; Balint-Kurti *et al.*, 2007; Balint-Kurti *et al.*, 2008; Zwonitzer *et al.*, 2009; Kump *et al.*, 2011). The 3.04 SLB dQTL has been identified at almost precisely the same locus in all these studies. In every case the susceptibility was derived from B73 but the resistance allele derived from a number of different lines, in particular NC292 or its progenitor NC250 (Bubeck, 1991; Zwonitzer *et al.*, 2009) and Mo17 (Balint-Kurti *et al.*, 2007; Kump *et al.*, 2011). Zwonitzer *et al.* (2009) identified these as the two major SLB dQTL in a NC250A x B73 F_{2:3} population and further showed that in each case the resistance allele, which was derived from NC250A a direct progenitor of NC292, was recessive to the susceptibility allele derived from B73.

There are several studies investigating the cost of induction of resistance to the host; however, most of them deal with induced monogenic resistance conferred by major resistance (R) genes. Korves and Bergelson (2004) demonstrated that there was a yield cost associated with the resistance gene *Rps2* even in the presence of disease in *Arabidopsis thaliana* when there was not competition with other *Arabidopsis* plants. Orgil *et al.* (2007) examined the *RPW8* locus, which contains two genes that together confer broad spectrum resistance in *A. thaliana*. Using a transgenic approach, they determined that the *RPW8* locus had a negative effect on fitness when plants were not infected. Tian *et al.* (2003) reported a 9% of yield reduction (as total seed production) due to the presence of the major R gene *RPMI* in uninoculated *A. thaliana* plants. At least two studies have examined the costs of

QDR. Mitchell-Olds and Bradley (1996) studied the cost on fitness of *Brassica rapa* lines selected for resistance to different pathogens. These lines were recurrently selected for QDR against *Leptosphaeria maculans* and *Peronospora parasitica*. *L. maculans*. They observed that QDR against *Peronospora parasitica* had a negative effect on growth rate in the absence of disease. Working with near-isogenic hybrids, Frey *et al.* (2011) concluded that the maize major anthracnose stalk rot resistance QTL *Rcg1* does not confer a yield cost under disease-free conditions in the field when compared to hybrids without *Rcg1*.

Here we report the results of our investigation into the agronomic effects of the 3B and 6A introgressions. Our objective was to estimate the effects of these two genomic introgressions on SLB resistance, yield, and other agronomic traits in inbred and hybrid material under infected and disease free conditions.

4.3. Materials and Methods

4.3.1. Plant Material

NC250 is a highly SLB resistant, yellow dent, inbred line developed at the NCSU maize breeding program from the cross (Nigeria Composite A-Rb x B37) x B37 (Thompson and Bergquist, 1984). B73 is an inbred line with good agronomic qualities derived by recurrent selection (Cycle 5) within the Iowa Stiff Stalk Synthetic population (Russell, 1972). B37 is an inbred line from an earlier selection cycle of the population. NC292 is a NIL that was obtained by backcrossing B73 four times to NC250P (the progenitor of NC250), followed by several cycles of ear-to-row selfing to derive the line. Importantly, selection for SLB resistance was performed at each generation. Therefore, NC292 is agronomically

similar to B73, but highly resistant to SLB. B73-3B and B73-6A are two B73-background NILs, each containing one introgression from NC292. NC292-specific introgressions were isolated by backcrossing NC292 to B73 twice and then using simple sequence repeats (SSR) marker-assisted selection to derive a NIL containing a single introgression (either introgression “3B” or introgression “6A”). Further details on the development of this material can be found elsewhere (Zwonitzer *et al.*, 2009; Belcher *et al.*, 2011).

Inbred line Mo17 was developed by M. S. Zuber at the University of Missouri. It was released in 1964 (Zuber, 1973; Troyer, 1999) and it is most famous for its use in the very popular hybrid B73 × Mo17. B73-Mo17.3B is a NIL containing the Mo17 3B introgression and was derived through backcrossing the Mo17 x B73 F₁ to B73 four times and using marker assisted selection to ensure maintenance of 3B introgression and purging of other Mo17 alleles. For the pilot study, 3B F_{2:3} families were derived by crossing the B73-Mo17.3B NIL to B73, and then selfing to the F₃ generation. 6A F_{2:3} families were generated by self-pollinating the resulting F₂ individuals from the B73-6A x B73 cross. Isogenic hybrids were developed by crossing B73-3B and B73-6A, to several inbred testers (Table 4.1). Due to asynchrony in flowering, some testers were delay-planted to match the B73 line maturity. B73 was also crossed to the testers to create a relatively susceptible comparison hybrid.

4.3.2. Field Trials

4.3.2.1. Pilot Study on Near-Isogenic Lines

A pilot study on near-isogenic lines was performed at the NCSU Central Crops Research Station in Clayton (CL), NC. 955 $F_{2:3}$ families from B73 x B73-Mo17.3B NIL cross, and 520 $F_{2:3}$ families from the B73 x B73-6A cross were planted in two independent experiments. Each experiment was treated as a large block, where different $F_{2:3}$ family entries were planted according to an incomplete block design. Each single-row plot of eight to twelve plants contained a different $F_{2:3}$ family that was either homozygous for the B73 allele, homozygous for either the 6A introgression or the 3B introgression, or segregating. The experiments from this pilot study were artificially inoculated with SLB and scored as described below. Using markers that segregate for either 3B or 6A introgressions, we identified instances where a family homozygous for B73 allele and a family homozygous for the introgression were planted in pairs of plots no more than two rows distant from each other. In each case, that pair of families was harvested. Forty-nine pairs of 3B introgression families were hand-harvested during summer 2009 and 50 pairs of 6A introgression families were hand-harvested during summer 2009 and summer 2010. Yield was estimated by measuring the average weight per primary ear (g), and 50-kernel weight (g).

4.3.2.2. Preliminary SLB Evaluation and Selection of Different Isogenic Triplet-Tester Combinations

Isogenic hybrids were developed by crossing B73, B73-3B and B73-6A to fifteen tester lines or testers (Table 4.1). Resistance conferred by the introgressions is recessive with

respect to the corresponding B73 allele, therefore we evaluated the F₁ progenies (isohybrids) for SLB resistance to identify testers that permitted expression of the differences in disease resistance between B73 and one or both introgression lines. Seed for this disease trial was generated during the summer of 2009 at Clayton, NC. Isohybrid sets from the fifteen testers were evaluated during the winter of 2009-2010 in Homestead, FL. Fifteen seeds of each of the three isogenic hybrids, as well as their parent lines were planted in three replications of a split plot design with tester as a whole plot. Whole plots consisted of B73, the tester inbred, B73-3B, B73-6A and three isohybrids: B73 x tester, B73-3B x tester, and B73-6A x tester, if the seed of all entries was available. Whole plots within a rep and entries within whole-plots were completely randomized. Entry plot size was 3.6m in length with row spacing of 0.91 m and an alley after each plot of approximately 0.91m. Disease pressure was applied via the artificial inoculation method described below.

Triplets of isogenic hybrids showing significant differences in resistance between the B73x tester control hybrid and either the B73-3B x tester or B73-6A x tester hybrids (or both) at the $\alpha=.05$ level were selected for additional disease and agronomic trait evaluation. A control tester that produced no significant difference between isohybrids was also selected. Ten testers were thus represented in the disease & agronomic evaluation (Table.4.1).

4.3.2.3. Disease & Agronomic Evaluation of Different Isogenic Triplet-Tester Combinations

During the summers of 2010 and 2011, selected isohybrid triplets were evaluated in two row plots according to a strip-split plot design (Steel *et al.*, 1997). The whole plot factor

applied to strips was artificial inoculation versus fungicide control (to prohibit spread of the disease). Whole plot strips were planted adjacent to each other but separated by 4 rows of a common commercial hybrid to avoid spread of the disease from the inoculated plot to the fungicide-control plot. Inoculations were performed as described below. Each whole plot was divided into four replications of ten sub-plots of isogenic hybrids. Within a subplot defined by the tester, the three isogenic hybrids (B73 x tester, B73-3B x tester, B73-6A x tester) were randomly assigned to experimental units (sub-subplots). To reiterate, the whole plot factor (inoculation treatment) tests the effect of disease development; the subplot factor (tester) tests the effect of the tester; the sub-subplot factor (introgression) tests the differences between hybrids derived from B73 or the nearly-isogenic introgression lines (3B or 6A).

Experiments were planted at three locations in North Carolina during both 2010 and 2011. In 2010 experiments were planted at the NCSU Sandhills Research Station in Jackson Springs (SH), the Peanut Belt Research Station in Woodville-Lewiston (LE) and the NCSU Central Crops Research Station in Clayton (CL). In 2011, experiments were planted at the LE, CL, and the Cunningham Research Station in Kinston (KI). Thus, a total of 6 North Carolina environments were represented in this study: SH10, LE10, CL10, LE11, CL11 and KI11. Experiments were planted on April 6, 8, and 12, 2010 for SH, CL and LE locations, respectively; and on April 14, 15 and 19, 2011 for LE, CL and KI, respectively. Experimental units consisted of two 4.88-m rows, with spacing of 1.83 m at all locations except Lewiston, NC, where row spacing was 1.93 m. Plots were planted with 44 seeds/plot with a population density of approximately 45,000 plants/ha for all locations, except for LE where density was of approximately 50,000 plants/ha. In 2011, stand counts (ST) were taken at the four leaf

stage, and approximately 10% of the plots at LE, CL, and KI exhibited poor germination. To foster inter-row competition, all plots with germination rates lower than 50% were replanted with the hybrid Pioneer 3394 in these environments. In the analysis, data from these plots were considered missing.

Measurements on several agronomic traits were recorded on each sub-subplot. Days to silking (DTS) and days to anthesis (DTA) were estimated as the number of days from planting to when half of the plants in the plot were silking or shedding pollen, respectively. DTS and DTA were recorded at CL only. After grain fill, plant and ear heights of four plants from the middle of the plot were recorded to the nearest 5 cm. Plant height was measured to the height of the flag leaf, and ear height was measured at the node from which the ear emerged. The four measurements were averaged for the analysis of the traits. Immediately prior to harvesting, the number of lodged plants per plot was counted, and percentage of erect plants was calculated by subtracting this number from the total stand count, and subsequently dividing by the total stand. Grain was mechanically harvested and measured, at approximately four months after planting. Plot weights and moisture readings were recorded. Yield measurements were standardized based on a stand of forty plants at 14.5% grain moisture and then scaled up to t/ha according to the plot size and planting density.

4.3.3. SLB Inoculation and Fungicide Application

All experiments that were artificially inoculated with SLB followed procedures described by Carson *et al.* (Carson, 1998; Carson *et al.*, 2004). Approximately 20 *C. heterostrophus* Race 0- infected sorghum grains were placed in the whorl of plants at the 4-6

leaf stage. Sorghum grains were infected with a mixed isolate population. Following inoculation, overhead irrigation was applied to provide moisture for fungal growth.

For the preliminary disease evaluation of the isohybrids, plots were inoculated on October 29, 2009 in the Homestead, Florida environment.

For the disease and agronomic evaluation of isohybrids, one of the whole factor plots was inoculated, while the other whole plot was sprayed with fungicide in each of the 6 environments. Plots were inoculated with SLB on May 12, 13 and 19, 2010 for SH, CL and LE, respectively. In 2011, CL experiments were inoculated on May 17 and LE and KI experiments were inoculated on May 18. The remaining whole plots were sprayed with fungicide approximately every 3 weeks, starting on the day their corresponding whole plots were artificially inoculated. Headline ® (BASF) was applied at a rate of 9 oz/acre for the first two applications, and Folicur ® (Bayer Crop Science) at a rate of 6 oz/acre was used for the third application in both years.

4.3.4. Disease Rating

All plots were scored at least three different times after flowering, on a scale of 1-9, on which 9 is a highly resistant plant and 1 is a highly susceptible or dead plant. sAUDPC (standard area under the disease progress curve) was obtained by dividing AUDPC values by the total number of days of evaluation (Balint-Kurti *et al.*, 2007). AUDPC values were calculated as previously explained (Shaner and Finney, 1977). The average value of three consecutive ratings was multiplied by the number of days between the ratings. These values were then summed over all intervals.

For the preliminary disease evaluation of the isohybrids, SLB was scored on the following dates November 22, 2009; December 19, 2009; and January 5, 2010.

For the disease and agronomic evaluation of isohybrids, isohybrids were scored again for the presence of SLB symptoms approximately every seven days in 2010 and 2011. In 2010, disease assessments were conducted on July 2, 9, 16 for CL and SH (with a fourth assessment on the 23rd for CL); and on July 8, 16 and 23 for LE. In 2011, SLB was assessed on July 12, 19, 25 for CL and Jul 13, 20, 27 for LE and KI. These ratings were used to calculate sAUDPC on a per-plot basis as described above. In the blocks sprayed with fungicide there was no disease development and so no SLB evaluation was performed.

4.3.5. Statistical Analyses

For the pilot study on isogenic lines, each pair of families (one family homozygous for B73 allele and one family homozygous for the introgression) was considered dependent samples. Therefore, paired t-tests (Proc T-test) from SAS v. 9.1.3 (SAS Institute, 2004) were used to analyze each measurement: average weight per ear (g/ear), and 50-kernel weight in grams (50K wt).

To evaluate the statistical significance of the differences in disease resistance between the isogenic hybrids in the preliminary evaluation in FL in 2009, sAUDPC scores were analyzed using Proc Mixed in SAS v. 9.1.3 (SAS Institute, 2004). The sAUDPC score of an isohybrid plot was modeled as the sum of the fixed effects of its introgression (or lack thereof), its tester alleles, and the interaction between its introgression and tester, as well as random effects due to block and residual. The pdiff option of the LSmeans statement from

SAS was used to estimate the differences between isohybrids within a tester combination, and evaluate this difference for statistical significance. Only these pre-planned comparisons were conducted; thus, no experimentwise error correction was used.

In the disease and agronomic evaluation of the isohybrids, disease scores were only available for the inoculated plots. Thus, sAUDPC scores were analyzed again using Proc Mixed, only this time, a split plot design with tester as the whole plot and introgression treatment as the subplot was used. Statistical analysis of the agronomic traits was performed using the general linear model (GLM) and mixed procedures in SAS version 9.2 (SAS Institute, 2004). The whole plot (inoculation treatment), sub-plot (tester), sub-sub-plot treatments (presence or absence of the 3B or 6A introgression), and the tester-introgression interaction effects were treated as fixed. All other effects i.e., year-location combinations (environments), replications, and their interactions with fixed effect terms were considered random. The GLM procedure was used to test the random effects of the full model. After dropping the non-significant random effect, each agronomic trait was modeled as the sum of the fixed effects of its introgression (or lack thereof), its tester alleles, the inoculation treatment and the interaction among the three factors, as well as the significant random effects (Table 4.6). Percent stand out of 40 plants was included as a fixed covariate. Statistical models were considered for each trait separately.

4.3.6. Genotyping

To verify that families chosen for the pilot study were homozygous for the Mo17 introgression or B73 alleles, tissue from F_{2,3} families was genotyped at DNA markers

surrounding the 3B introgression. DNA was extracted according to a modified CTAB protocol (Doyle and Doyle, 1987). Tissue was sent to Pioneer Hi-Bred International and DuPont Crop Genetics for genotyping. Single nucleotide polymorphism (SNP) markers PZB7039, PZA3524, PZA13887, PZA12576, PZA17767, PZA4145 and PZA5816 were genotyped at Pioneer Hi-Bred International using PCR and a probe-based detection method. SSR marker UMC2000 and indel marker IDP6793 were genotyped at DuPont Crop Genetics via PCR and visualization on agarose gel. Homozygosity for the introgression 6A or B73 allele in the F_{2,3} families selected for the pilot study was verified by genotyping total nucleic acid using a SSR marker bnlg2243 located in introgression 6A. Total nucleic acid was extracted using a modified CTAB extraction procedure (Saghai-Marooif *et al.*, 1984; Zwonitzer *et al.*, 2009). SSR genotyping was performed following procedures described elsewhere (Schuelke, 2000; Kirigwi *et al.*, 2008) and visualized on agarose gel.

4.4. Results

4.4.1. Pilot Study on near-isogenic lines

A pilot study to test the effect of an individual introgression, either 3B or 6A, on yield in inbred lines was conducted under SLB disease pressure. 955 $F_{2:3}$ families from B73 x B73-Mo17.3B NIL cross, and 520 $F_{2:3}$ families from the B73 x B73-6A cross were planted in two independent experiments where each single-row plot of 8-12 plants contained a different $F_{2:3}$ family that was either homozygous for the B73 allele, homozygous for the either the 6A introgression or the 3B introgression, or segregating. The experiments from this pilot study were artificially inoculated to SLB pressure and scored. Forty-nine (3B) or fifty (6A) adjacent pairs of resistant (homozygous for introgression) and susceptible families (homozygous for B73 alleles) were hand-harvested and yield was estimated.

Families carrying the 3B introgression had an average yield advantage of 12% over families lacking the introgression (12% for both measurements: 1.2g for 50-kernel and 8.8g for weight /ear measurements) (Table 4.2). Similarly, families carrying the 6A introgression had an average yield advantage of approximately 8% over families lacking this introgression over two years (8% for both measurements: 0.8g for 50-kernel weight and 5g for weight /ear) (Table 4.2). The difference in SLB disease resistance between families carrying the 3B and 6A introgression and the families without any introgression was 1.4 and 3 points, respectively, on a 1-9 scale.

4.4.2. Preliminary SLB Evaluation and Selection of Different Isogenic Triplet-Tester Combinations

Since the 3B and 6A introgressions act recessively with respect to the B73 allele, we first needed to identify a set of hybrids in which their phenotypic effects were expressed. With that objective, isogenic hybrids were produced by crossing B73, B73-3B and B73-6A, to 15 inbred testers. Isohybrid sets from the fifteen testers were evaluated for SLB resistance in three replications of a randomized incomplete block design during the winter of 2009-2010 in Homestead, FL. Blocks consisted of B73, the tester inbred, B73-3B, B73-6A and the three isohybrids: B73 x tester, B73-3B x tester, and B73-6A x tester. Isohybrids carrying the 3B and 6A introgressions were significantly more SLB resistant than the corresponding B73-background hybrid control in 9 out of 15 testers and 5 out of the 15 testers (NIL-tester combinations) respectively at the $\alpha=0.05$ level (Table.4.1). All five of the testers showing a difference for 6A were amongst the nine showing a difference for 3B. These nine isogenic triplet-tester combinations were selected for additional disease and agronomic trait evaluation. Isohybrids developed from B97 all had similar levels of disease resistance, and were included as a negative control (Table.4.1).

4.4.3. Disease & Agronomic Evaluation of Different Isogenic Triplet-Tester Combinations

The selected isohybrid triplets were evaluated in two-row plots according to a strip-split plot design. The whole plot factor (inoculation treatment) tested the effect of disease development; the sub-plot factor (tester) tested the effect of possessing the inbred tester

haplotype; the sub-sub-plot factor (introgression treatment) tested the effect of having B73 alleles at both loci vs. containing NC292 alleles at either the 3B or 6A introgressions.

4.4.3.1. Disease Evaluation

For analysis of disease resistance only the artificially inoculated plots were taken into account. Thus, the whole plot factor (tester treatment) tested the effect of the inbred tester haplotype and the sub-plot factor (introgression treatment) tested the effect of the 3B or 6A introgressions compared to the nearly isogenic B73 control in hybrid combinations. Results largely agreed with the previous preliminary SLB evaluation (see above) but there were some differences (Table 4.3).

Across testers, the mean sAUDPC value for the isohybrids without any introgression was 6.8, significantly lower (less resistant) than either mean value for the isohybrids carrying 3B and 6A introgressions (7.6 and 7.4, respectively; Table 4.4 and Fig. 4.1). Averaged across introgression treatments, the testers' mean sAUDPC values ranged from 6.2 for the B97 background to 8.1 for the NC350 background (Table 4.4). B97 was included as a control tester based on its suppression of resistance from both introgression lines in the preliminary disease screening of hybrids.

The significant tester by introgression interaction indicated that the presence of either introgression reduced the amount of disease by different degrees across the different testers (Fig. 4.2). Just as in the preliminary experiment the effect of introgression 3B was highly significant at the $\alpha=0.01$ for all nine testers tested not including control B97 (Table 4.5, fig.4.2). The effect of 6A on SLB resistance was significant in only 6 testers sets (Table 4.5,

fig. 4.2). Surprisingly these six crosses did not include all the 5 identified in the preliminary experiment; no significant resistance effects were detected for introgression 6A in crosses with Oh43 and NC350 whereas there had been a significant effect in the preliminary experiment. Contrastingly significant effects associated with the 6A introgression were detected in crosses with Va35, CML333 and H95 where no such effects had been identified previously. The effect of 6A on SLB resistance was highly significant at the $\alpha=.01$ level in crosses with H95, H95rh_m, NC250 and Va35rh_m. The entire list of pairwise contrasts is presented in table S4.1.

4.4.3.2. Agronomic Evaluation

We next determined the effect of the 3B and 6A introgressions on yield and other agronomic traits in hybrids both in the presence and absence of SLB disease. Agronomic traits evaluated included yield, moisture content, plant height, ear height, lodging (percent erect plants at harvest), and flowering time (DTS and DTA). For all agronomic traits measured, the tester effect was significant, but the inoculation effect was not (Table 4.6 and S4.2). The interaction of inoculation and introgression treatments was significant for yield (Table 4.6). For ear height, significant effects were found for the interaction between tester and introgression factors. For moisture content, the introgression term and the interaction between tester and introgression factors were significant at $\alpha=.1$ level. There was a significant inoculation by tester by introgression effect on the percentage of erect plants (Table S4.2).

Across all testers and introgression treatments, the mean yield of the fungicide-sprayed whole plot was 0.28 t/ha more (approximately 5%) than the mean yield of the SLB-infected plot, but the difference was not significant (Table 4.7). Averaged across all treatments, the hybrid means for the different testers ranged from 5.4 t/ha for H95rhm isohybrids to 6.54 t/ha for Mo17 isohybrids. LS means for yield and other agronomic traits can be found in Table S4.4.

In the absence of SLB and averaged across testers, mean yield for the hybrids carrying introgression 6A was approximately 0.172 t/ha (about 3%) less than the control B73-background hybrids without any introgression. This difference was significant at a 5% level. The 3B introgression was associated with a 0.133 t/ha reduction in yield in the absence of SLB but, this difference was not statistically significant (Table 4.7 and fig. 4.3).

In the presence of SLB and averaged across testers, mean yield for the hybrids carrying introgression 3B was approximately 0.19 t/ha (about 3%) more than the control B73-background hybrids without any introgression (Table 4.7 and fig.4.3). In contrast, introgression 6A did not show any significant overall effect on yield compared to the control (Table 4.7 and fig.4.3) under disease pressure. However this lack of yield effect is a little misleading as the 6A introgression, unlike the 3B introgression, did not confer significant levels of resistance in all the crosses. Introgression 6A conferred the highest amount of resistance in crosses with NC250, Va35rhm and H95rhm (Fig. 4.2). In these crosses introgression 6A was associated with yield increases of were 0.526 and 0.573 and 0.336 t/ha (about 10%, 10% and 6%) respectively. For NC250 and Va35rhm, these increases were statistically significant (Fig. 4.4).

4.5. Discussion

SLB resistance is mostly quantitative in nature and most maize grown in the US has some level of resistance. Several studies have reported quantitative resistance against SLB in US germplasm (Pate and Harvey, 1954; Hooker *et al.*, 1970; Lim, 1975; Lim and Hooker, 1976; Thompson and Bergquist, 1984; Burnette and White, 1985; Holley and Goodman, 1989; Balint-Kurti *et al.*, 2006). Although QDR against SLB has been used in maize breeding for a long time, little is known about how much individual QTLs may contribute to differences in agronomic traits, such as yield, in inbred lines and hybrids, and in the presence and absence of disease pressure. Yield losses as high as 38% to 46% have been found in hybrid maize inoculated with *C. heterostrophus* under experimental conditions (Fisher *et al.*, 1976; Byrnes *et al.*, 1989). To investigate individual effects of two QTL in both inbred and hybrid backgrounds, we chose QTLs with relatively large additive effects in a pathosystem for which host resistance is known to be highly heritable. The lines B73-3B and B73-6A each contain one introgression from highly SLB resistant line NC292 in a B73 background. Each of these introgressions carries a SLB resistance QTL with a relatively large effect. To study the effects of introgressions 3B and 6A on agronomic traits, we used isogenic hybrids, differing only by the presence or absence of each introgression.

We discerned a yield effect associated with each introgression during a pilot study comparing F_{2:3} families homozygous for either introgression to F_{2:3} families without either introgression (essentially inbred isolines). In this study, we found that there is a significant yield advantage when isolines carrying either introgression were infected with SLB. This result suggests that the SLB resistance conferred by these introgressions may correlate with

an increase in grain weight in inbred lines under disease pressure. It should be noted that this preliminary study used the Mo17 allele at 3B as the source of resistance while the other experiments reported here used the NC292 allele. For our purposes, we are assuming that the genes underlying the resistance at 3B are identical in Mo17 and B73, based on that fact that they precisely colocalize, that they are both recessive to the B73 allele and that they have similar effect estimates (Zwonitzer *et al.*, 2009 and unpublished data), but this remains to be proven.

Since corn is commercially produced as a hybrid, a more relevant measure of the economic impact of these introgressions would be a yield advantage in hybrids rather than in inbreds. However, the resistances conferred by introgressions 3B and 6A are recessive relative to their respective B73 alleles, so we first needed to identify a set of hybrids in which their phenotypic effects were expressed. Therefore, we developed and challenged 15 sets of isohybrids triplets (carrying introgression 3B, 6A or no introgression) from a diverse set of testers with SLB to determine whether we could detect resistance effects of 3B and 6A across different backgrounds. In our preliminary experiments we observed significant resistance effects in 5 out of 15 backgrounds for 6A and 9 for 3B (Table 4.1). All 5 backgrounds in which resistance associated with 6A was expressed were among the 9 in which 3B resistance was expressed. In the main experiment assessing agronomic effects, resistance effects associated with 3B were again detected in the same 9 backgrounds (Fig. 4.2) but there were some differences in the backgrounds in which resistance associated with 6A was expressed. Significant resistance effects were detected for 6A in crosses with Oh43 and NC350 the preliminary but not in the main experiment; whereas in crosses with Va35, CML333 and

H95, 6A effects were detected in the main but not in the preliminary experiment. The backgrounds in which 6A was associated with the largest effects on SLB resistance, namely H95rhm, NC250 and Va35rhm, were consistent across the two experiments. It is not clear why we observed these differences but contributing factors likely include the well known environmental dependence of QTL and the fact that these two experiments were performed in quite different environments; the preliminary experiment took place in Homestead FL, during the winter of 2009-10 while the main experiment took place during the summers of 2010 and 2011 over several different locations in North Carolina. Disease pressure in Homestead was substantially higher than in North Carolina and the corresponding effects of both introgressions were on average substantially higher for the preliminary compared to the main experiment (compare the LS mean Diff values in Table 4.1 and the estimate values in Table 4.5). In addition, the preliminary experiment had 3 replications for each set of germplasm whereas the main experiment was much more highly replicated (24 reps per entry), thus the power of the statistical analysis used in the preliminary study may have contributed to the disagreement on the results.

The introgressions in B73-3B and B73-6A were initially derived from NC250P (a progenitor of NC250 and NC292, see Zwonitzer *et al.* (2009)), thus the NC250 tester possesses both of the regions tested in this study. Hybrids derived from a cross of NC250 with either B73-3B or B73-6A were therefore homozygous for their respective introgressions and as expected were significantly more resistant than the NC250 x B73 hybrid. It is worth noting that the hybrids derived from crosses of B73-6A to testers carrying the SLB resistance gene *rhm* (H95rhm and Va35rhm) also showed high levels of resistance. Not only Va35rhm

x B73-6A and H95rhm x B73-6A were significantly more resistant than their respective isohybrids without introgression 6A, but the hybrid Va35rhm x B73-6A and H95rhm x B73-6A were more resistant than their respective Va35 x B73-6A and H95 x B73-6A (1.6 and 0.8 sAUDPC units on the 1-9 scale). Both *rhm* and introgression 6A map to the same distal region of the short arm of chromosome 6. Since introgression 6A is recessive to the corresponding allele in B73, this suggests that the tester allele complements the 6A effect, allowing its expression in the hybrid. This observation agrees with the hypothesis that *rhm* and 6A could be the same gene (Belcher, 2009).

Heterosis, a phenomenon common in outcrossing species, causes hybrid plants to be taller, higher-yielding and more vigorous than inbred plants. Physiological differences due to heterosis, such as tougher cell wall structures, greater accumulation of disease-fighting metabolites, or taller plants (and the fungus takes more time to infect upper leaves), could have also accounted for resistance against SLB in hybrids. This is supported by the fact that there are modest but highly significant correlations between the sAUDPC values and yield under both SLB pressure (~37%) and no disease (~25%) (Table S4.5). The correlation under disease-free conditions suggests that differential levels of heterosis in the different hybrids are influencing both yield and disease resistance.

While the disease-free block yielded on average 0.28 t/ha (~5%) more than the SLB-infected block, but this difference was not statistically significant. This was surprising to us; however, this was not the main goal of the experiment and the design was not best to capture these differences. The primary disadvantage in a strip split plot design is the loss in precision in the whole plot factor compared to the sub- and sub-sub-plot factors. The effects of the

whole plot factor are tested using the environment-by-whole plot interaction variance, which has few degrees of freedom, and thus low power (Steel *et al.*, 1997). Also the whole plot experimental units are larger than the sub- and sub-sub-plots units, thus increasing the error variance. Another reason of this result could have been attributed to the weather in both years of the trials. Hot dry summers in some environments could have delayed the spread the disease on hybrids; therefore, reducing the overall difference in yield between SLB infected and disease-free conditions.

Introgression and inoculation effects exhibited significant interactions. In the presence of the SLB disease pressure, mean yield for the hybrids carrying introgression 3B across all testers was approximately 3% more than the control B73-background hybrids implying that the protection given by the resistance present in introgression 3B may be responsible of this reduction in yield loss. Even though all the hybrid combinations with B73-3B were significantly more resistant to SLB compared to the B73 hybrid control, only hybrid Va35rh_m x B73-3B showed significantly more yield than the corresponding hybrid control (Va35rh_m x B73). In contrast, no significant advantage in yield was detected with introgression 6A across all testers in the presence of SLB. Only four out of the 10 testers tested in this study showed increases in resistance associated with introgression 6A that were significant at the 1% level (as compared to 9 for 3B). So, we would not necessarily expect yield benefits from having the introgression 6A in the presence of SLB in the other 6 testers. Out of the 4 testers that did show a highly significant resistance advantage associated with 6A, two of them, crosses with NC250 and Va35rh_m showed a significant yield gain

associated with the presence of 6A under infected conditions. This result suggests that the resistance conferred by introgression 6A can indeed protect yield during SLB infection.

Rather than a yield advantage, a yield penalty associated with the presence of genetic disease resistance has been noted in several studies, especially in the absence of pressure from the corresponding disease. (Tian *et al.*, 2003; Korves and Bergelson, 2004; Orgil *et al.*, 2007). The prevalence of susceptibility alleles in natural populations has suggested a cost on fitness due to these resistance loci (Parker, 1992; Bergelson and Purrington, 1996). While most studies have addressed major gene effects on fitness (Tian *et al.*, 2003; Heidel *et al.*, 2004; Korves and Bergelson, 2004; Dietrich *et al.*, 2005; Orgil *et al.*, 2007), a few studies have worked with QDR (Mitchell-Olds and Bradley, 1996; Frey *et al.*, 2011). In the absence of SLB, mean yield for the hybrids carrying introgression 6A was significantly (3%) less than the control B73-background hybrids without any introgression suggesting a cost of resistance while the 3B introgression was associated with a smaller (~2%) yield reduction which was not statistically significant. Within testers, most testers hybrid combinations showed a reduction in yield associated with the 6A introgression. Some tester hybrid combinations showed a reduction of up to 0.442 t/ha (e.g. NC250).

One explanation for the yield penalty associated with the introgressions is of course linkage drag. We have genotyped both B73-3B and B73-6A with a set of >50,000 SNP markers (data not shown) and consequently have a good measure of the size of the two introgressions. The 6A introgression is almost 11 Mb (out of a total genome size of ~2500 Mb) while the 3B introgression is ~5.6 Mb. Clearly both these introgressions likely carry a large number of genes, some of which may be deleterious to yield.

The yield cost associated with 6A (and possibly also with 3B) could be also ascribed to some sort of physiological cost of having the disease resistance gene. A constitutively active defense response or high basal defense level has been associated with yield penalties in many different studies (Tian *et al.*, 2003; Korves and Bergelson, 2004; Orgil *et al.*, 2007). This is certainly a possibility. To address this, it is instructive to inspect the individual testers with respect to resistance and yield. If expression of resistance itself were the reason for the reduction in yield in the absence of disease then one would expect that hybrids in which 6A was associated with high levels of SLB resistance (corresponding presumably to robust phenotypic expression of the resistance gene) would have correspondingly high yield penalties associated with the presence of 6A in the absence of disease pressure (compare figures 4.2 and 4.4). While there is not a completely consistent pattern, the four testers in which 6A has no significant effect on resistance, B97, Mo17, NC350 and Oh43 (see Fig. 4.2) have relatively low yield penalties associated with 6A (+0.02, -0.2, +0.09 and -0.2 tha^{-1} , respectively) while for the 4 testers in which 6A has a highly significant effect on resistance (H95, H95rhm, NC250 and Va35rhm, the effects associated on yield associated with 6A are somewhat larger (-0.4, -0.2, -0.4 and -0.03 tha^{-1} respectively). The rank correlation between resistance associated with 6A and yield penalty associated with 6A under no disease pressure amongst the 10 hybrid testers is -0.60 which is significant at the 10% level (Table S4.5). The corresponding figure for introgression 3B is -0.59, again significant at the 10% level (Table S4.5). So we have some level of evidence supporting the contention that the modest yield penalties associated with 6A and possibly 3B in disease-free conditions might be associated with expression of the disease resistance mechanisms themselves.

Other agronomic traits, including plant height, ear height, flowering time, percentage of erect plants at harvest and moisture did not seem to be influenced by the presence or absence of any of the introgressions tested. There were no significant differences for these traits under SLB pressure and disease-free conditions.

The disease resistance loci present in the introgressions tested can be used for breeding purposes, though as we have demonstrated this resistance may not be expressed across all testers. Our results suggest that both the 3B and 6A introgressions can protect yield during southern leaf blight epidemics though they may have modest yield penalties in the absence of the disease. If the yield penalties are due in whole or in part to linkage drag rather than expression of the resistance itself, then smaller introgressions at these loci could be used which still confer the SLB resistance phenotype but have smaller associated yield penalties.

Acknowledgements

The authors would like to thank the following people who helped with various aspects of the research: William Hill, Wayne Dillard, Dale Dowden, Abbey Sutton, and David Rhyne. We thank Cathy Herring and the staff of Central Crops Research Station, the NCSU Sandhills Research Station, the Peanut Belt Research Station, the Cunningham Research Station for their expert help. We thank Janet Shurtleff, Carole Saravitz and the staff of the NCSU Phytotron for the use of their facilities. This work was funded by the USDA-ARS and NCSU. J. Santa-Cruz's fellowship is funded by Monsanto, Inc.

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Table 4.1 Testers used in this study to produce isohybrids triplets and preliminary SLB evaluation and selection of different isogenic triplet-tester combinations. Mean difference of sAUDPC values for isohybrids with and without introgressions 3B and 6A. The ten testers included in the agronomic evaluation are highlighted.

Tester	3B				6A			
	LS Mean Diff	Std Error	t-value	p-value	LS Mean Diff	Std Error	t-value	p-value
B97	-0.61	0.32	-1.93	0.0552*	-0.50	0.32	-1.57	0.1183
CML103	0.33	0.39	0.86	0.3905	-0.07	0.32	-0.22	0.8234
CML333	1.26	0.45	2.82	0.0054***	-0.35	0.45	-0.79	0.4329
CML69	0.80	0.45	1.78	0.0765*	-0.61	0.45	-1.37	0.1727
H95	1.74	0.32	5.50	<.0001***	0.41	0.32	1.29	0.1991
H95rhm	1.33	0.32	4.20	<.0001***	1.75	0.32	5.54	<.0001**
Ky21	-0.13	0.45	-0.30	0.7646	-0.03	0.45	-0.07	0.9419
LH85 §	0.22	0.32	0.71	0.4783	-0.03	0.33	-0.08	0.9350
M162W	0.27	0.39	0.70	0.4866	0.17	0.32	0.55	0.5829
Mo17	1.25	0.32	3.97	0.0001***	0.45	0.32	1.42	0.1572
NC250	2.66	0.45	5.87	<.0001***	2.12	0.39	5.49	<.0001**
NC350	1.16	0.39	3.00	0.0032***	0.89	0.32	2.80	0.0057**
Oh43	1.32	0.32	4.18	<.0001***	0.85	0.32	2.69	0.0080**
Va35	1.24	0.32	3.93	0.0001***	-0.15	0.32	-0.48	0.6349
Va35rhm	1.77	0.31	5.79	<.0001***	2.34	0.32	7.41	<.0001**

*** Significant at 1%

** Significant at 5%

* Significant at 10%

§early flowering

Table 4. 2 Yield-related traits mean values from the pilot study on isogenic lines. Yield-related traits mean values for resistant F_{2:3} families (carrying introgression 3B or 6A), for susceptible F_{2:3} families (lacking introgressions), mean difference of isogenic lines with and without introgressions 3B and 6A, Mean Confidence Interval at 0.5% (95%CI), and statistical significance of the difference. 3B F_{2:3} families values were obtained in 2010 only. 2009, 2010 and over both years values are presented for 6A F_{2:3} families.

Effect	Trait	Resistant Mean	Susceptible Mean	Difference Mean	95%CI Mean	p-value
3B	50K wt	11.5g	10.3g	1.2g	0.8g-1.5g	<0.0001***
	g/ear	82.5g	73.7g	8.8g	4.2g-13.4g	0.0004***
6A 2009	50K wt	11.3g	10.6g	0.65g	0.37g - 0.93g	<0.0001***
	g/ear	80g	77.3g	2.7g	-2.3g - 7.7g	0.2804
6A 2010	50K wt	10.6g	9.7g	0.9g	0.65g - 1.2g	<0.0001***
	g/ear	54.9g	47.9g	7g	2.3g - 11.7g	0.004***
6A total	50K wt	10.9g	10.1g	0.8g	0.6g - 1g	<0.0001***
	g/ear	67g	62g	5g	1.6g - 8.3g	0.0046***

*** Significant at 1%

** Significant at 5%

* Significant at 10%

Table 4.3 Covariance parameters estimates and type 3 tests of significance of fixed effects for sAUDPC values related to the effects of introgressions 3B and 6A on isohybrid comparisons under SLB pressure. Means are presented in a scale of 1-9, on which 9 is a highly resistant plant and 1 is a highly susceptible, or dead.

sAUDPC	
Covariance Parameter	Estimate
Environment	0.3894
rep(Environment)	0
rep*Tester(Environment)	0.09279
Residual	0.1573

sAUDPC				
Effect	Num DF	Den DF	F Value	Pr > F
Tester	9	225	61.90	<.0001***
Introgression	2	427	196.84	<.0001***
Tester*Introgression	18	426	12.63	<.0001***

Num DF = Numerator degrees of freedom
Den DF = Denominator degrees of freedom
*** Significant at 1%
** Significant at 5%
* Significant at 10%

Table 4.4 Least-square means for sAUDPC related to the effect of tester and introgressions. Estimate of the effect for SLB resistance on isohybrid comparisons with and without introgression 3B and 6A. Mean estimates are presented in a scale of 1-9, on which 9 is a highly resistant plant and 1 is a highly susceptible, or dead.

Effect	Tester	Introgression	Estimate
Tester	B97		6.1834
Tester	CML333		7.1505
Tester	H95		7.5440
Tester	H95rhm		8.0277
Tester	Mo17		6.7466
Tester	NC250		6.7838
Tester	NC350		8.1424
Tester	Oh43		7.2671
Tester	Va35		6.9991
Tester	Va35rhm		7.8045
Introgression		B73	6.8452
Introgression		B73-3B	7.5927
Introgression		B73-6A	7.3569
Tester*Introgression	B97	B73	6.0938
Tester*Introgression	B97	B73-3B	6.2229
Tester*Introgression	B97	B73-6A	6.2337
Tester*Introgression	CML333	B73	6.8287
Tester*Introgression	CML333	B73-3B	7.5864
Tester*Introgression	CML333	B73-6A	7.0365
Tester*Introgression	H95	B73	7.0205
Tester*Introgression	H95	B73-3B	7.9176
Tester*Introgression	H95	B73-6A	7.6939
Tester*Introgression	H95rhm	B73	7.4418
Tester*Introgression	H95rhm	B73-3B	8.1330
Tester*Introgression	H95rhm	B73-6A	8.5083
Tester*Introgression	Mo17	B73	6.3830
Tester*Introgression	Mo17	B73-3B	7.3613
Tester*Introgression	Mo17	B73-6A	6.4955
Tester*Introgression	NC250	B73	6.1125
Tester*Introgression	NC250	B73-3B	7.1440
Tester*Introgression	NC250	B73-6A	7.0948
Tester*Introgression	NC350	B73	7.9295
Tester*Introgression	NC350	B73-3B	8.4703
Tester*Introgression	NC350	B73-6A	8.0274
Tester*Introgression	Oh43	B73	7.0141
Tester*Introgression	Oh43	B73-3B	7.6314
Tester*Introgression	Oh43	B73-6A	7.1559
Tester*Introgression	Va35	B73	6.6972
Tester*Introgression	Va35	B73-3B	7.4108
Tester*Introgression	Va35	B73-6A	6.8892
Tester*Introgression	Va35rhm	B73	6.9309
Tester*Introgression	Va35rhm	B73-3B	8.0490
Tester*Introgression	Va35rhm	B73-6A	8.4337

Table 4.5 Least-squares mean differences for sAUDPC values related to the effect of SLB on isohybrid comparisons with and without introgressions across all testers and per tester. Means are presented in a scale of 1-9, on which 9 is a highly resistant plant and 1 is a highly susceptible, or dead.

Effect	Tester	Introgression	Tester	Introgression	Estimate	Pr > t
Introgression		B73		B73-3B	-0.75	<.0001***
Introgression		B73		B73-6A	-0.51	<.0001***
Introgression		B73-3B		B73-6A	0.24	<.0001***
Tester*Introgression	B97	B73	B97	B73-3B	-0.1292	0.2598
Tester*Introgression	B97	B73	B97	B73-6A	-0.1399	0.2223
Tester*Introgression	B97	B73-3B	B97	B73-6A	-0.01076	0.9251
Tester*Introgression	CML333	B73	CML333	B73-3B	-0.7578	<.0001***
Tester*Introgression	CML333	B73	CML333	B73-6A	-0.2078	0.0741*
Tester*Introgression	CML333	B73-3B	CML333	B73-6A	0.55	<.0001***
Tester*Introgression	H95	B73	H95	B73-3B	-0.8971	<.0001***
Tester*Introgression	H95	B73	H95	B73-6A	-0.6734	<.0001***
Tester*Introgression	H95	B73-3B	H95	B73-6A	0.2237	0.1036
Tester*Introgression	H95rhm	B73	H95rhm	B73-3B	-0.6912	<.0001***
Tester*Introgression	H95rhm	B73	H95rhm	B73-6A	-1.0666	<.0001***
Tester*Introgression	H95rhm	B73-3B	H95rhm	B73-6A	-0.3754	0.0015***
Tester*Introgression	Mo17	B73	Mo17	B73-3B	-0.9783	<.0001***
Tester*Introgression	Mo17	B73	Mo17	B73-6A	-0.1125	0.3263
Tester*Introgression	Mo17	B73-3B	Mo17	B73-6A	0.8658	<.0001***
Tester*Introgression	NC250	B73	NC250	B73-3B	-1.0315	<.0001***
Tester*Introgression	NC250	B73	NC250	B73-6A	-0.9823	<.0001***
Tester*Introgression	NC250	B73-3B	NC250	B73-6A	0.04922	0.6931
Tester*Introgression	NC350	B73	NC350	B73-3B	-0.5408	<.0001***
Tester*Introgression	NC350	B73	NC350	B73-6A	-0.09792	0.3928
Tester*Introgression	NC350	B73-3B	NC350	B73-6A	0.4429	0.0005***
Tester*Introgression	Oh43	B73	Oh43	B73-3B	-0.6173	<.0001***
Tester*Introgression	Oh43	B73	Oh43	B73-6A	-0.1418	0.2363
Tester*Introgression	Oh43	B73-3B	Oh43	B73-6A	0.4756	0.0001***
Tester*Introgression	Va35	B73	Va35	B73-3B	-0.7135	<.0001***
Tester*Introgression	Va35	B73	Va35	B73-6A	-0.192	0.0942*
Tester*Introgression	Va35	B73-3B	Va35	B73-6A	0.5215	<.0001***
Tester*Introgression	Va35rhm	B73	Va35rhm	B73-3B	-1.1181	<.0001***
Tester*Introgression	Va35rhm	B73	Va35rhm	B73-6A	-1.5028	<.0001***
Tester*Introgression	Va35rhm	B73-3B	Va35rhm	B73-6A	-0.3847	0.001***

*** Significant at 1%

** Significant at 5%

* Significant at 10%

Table 4.6 Covariance Parameters Estimates and Type 3 tests of significance of fixed effects for yield to the effects of introgression 6A and 3B on isohybrid comparisons with and without disease pressure. Yield measurements were standardized based on a stand of 40 plants at 14.5% grain moisture and then scaled up to t/ha according to the plot size and planting density. Stand counts (ST) was used as a covariate for yield.

Covariance Parameters Estimates	
Covariance Parameter	Estimate
Environment	961.08
Environment *Inoculation	46.6965
rep(Environment *Inoculation)	71.7057
Environment *Tester	40.3711
rep*Tester(Environment *Inoculation)	126.67
Environment *Tester*Introgression	3.9180
Residual	184.85

Type 3 tests of significance of fixed effects				
Effect	Num DF	Den DF	F Value	Pr > F
ST	1	551	218.68	<.0001***
Inoculation (inoculated vs. sprayed)	1	5.01	0.93	0.3802
Tester	9	45.1	3.28	0.0038***
Inoculation*Tester	9	364	1.07	0.3871
Introgression	2	92.7	1.07	0.3485
Inoculation*Introgression	2	756	4.19	0.0155**
Tester* Introgression	18	91.6	0.76	0.7365
Inoculation*Tester* Introgression	18	753	0.84	0.6492

*** Significant at 1%

** Significant at 5%

* Significant at 10%

Table 4.7 Least-squares mean contrasts for yield related to the effects of the presence or absence of SLB on isogenic hybrids comparisons with and without introgressions 3B or 6A. Yield estimate is the difference between isohybrids without any introgression (B73) and isohybrids carrying either 3B or 6A introgression. Yield mean difference estimate is presented in t/ha¹.

Effect	Inoculation	Tester	Introgression Difference		Estimate	DF	t Value	Pr > t
Inoculation*Introgression	Inoculated		B73	B73-3B	-0.19	292	-2.25	0.0249**
Inoculation*Introgression	Inoculated		B73	B73-6A	-0.06	277	-0.68	0.4985
Inoculation*Introgression	sprayed		B73	B73-3B	0.13	293	1.57	0.1186
Inoculation*Introgression	sprayed		B73	B73-6A	0.17	280	2.08	0.0381**
Inoculation*Tester*Introgression	Inoculated	B97	B73	B73-3B	-0.13	268	-0.51	0.6081
Inoculation*Tester*Introgression	Inoculated	B97	B73	B73-6A	0.14	258	0.55	0.5814
Inoculation*Tester*Introgression	Inoculated	CML333	B73	B73-3B	-0.15	287	-0.58	0.5639
Inoculation*Tester*Introgression	Inoculated	CML333	B73	B73-6A	0.34	268	1.34	0.1802
Inoculation*Tester*Introgression	Inoculated	H95	B73	B73-3B	-0.01	343	-0.02	0.9858
Inoculation*Tester*Introgression	Inoculated	H95	B73	B73-6A	0.53	299	2.01	0.0456**
Inoculation*Tester*Introgression	Inoculated	H95rhm	B73	B73-3B	-0.10	318	-0.37	0.7153
Inoculation*Tester*Introgression	Inoculated	H95rhm	B73	B73-6A	-0.34	317	-1.26	0.2094
Inoculation*Tester*Introgression	Inoculated	Mo17	B73	B73-3B	-0.34	269	-1.35	0.1786
Inoculation*Tester*Introgression	Inoculated	Mo17	B73	B73-6A	0.02	260	0.09	0.9288
Inoculation*Tester*Introgression	Inoculated	NC250	B73	B73-3B	-0.32	271	-1.13	0.258
Inoculation*Tester*Introgression	Inoculated	NC250	B73	B73-6A	-0.53	261	-1.98	0.0483**
Inoculation*Tester*Introgression	Inoculated	NC350	B73	B73-3B	-0.20	310	-0.72	0.4727
Inoculation*Tester*Introgression	Inoculated	NC350	B73	B73-6A	-0.22	258	-0.88	0.3787
Inoculation*Tester*Introgression	Inoculated	Oh43	B73	B73-3B	-0.02	297	-0.08	0.9337
Inoculation*Tester*Introgression	Inoculated	Oh43	B73	B73-6A	0.07	303	0.27	0.7881
Inoculation*Tester*Introgression	Inoculated	Va35	B73	B73-3B	-0.07	277	-0.27	0.7864
Inoculation*Tester*Introgression	Inoculated	Va35	B73	B73-6A	0.01	278	0.02	0.984

Effect	Inoculation	Tester	Introgression Difference		Estimate	DF	t Value	Pr > t
Inoculation*Tester*Introgression	Inoculated	Va35rhm	B73	B73-3B	0.03	280	-2.25	0.0254**
Inoculation*Tester*Introgression	Inoculated	Va35rhm	B73	B73-6A	-0.57	258	-2.31	0.0216**
Inoculation*Tester*Introgression	sprayed	B97	B73	B73-3B	-0.57	291	0.11	0.9137
Inoculation*Tester*Introgression	sprayed	B97	B73	B73-6A	0.03	278	-0.1	0.9232
Inoculation*Tester*Introgression	sprayed	CML333	B73	B73-3B	-0.02	288	1.73	0.0851*
Inoculation*Tester*Introgression	sprayed	CML333	B73	B73-6A	0.45	268	0.87	0.3826
Inoculation*Tester*Introgression	sprayed	H95	B73	B73-3B	0.22	294	0.51	0.6138
Inoculation*Tester*Introgression	sprayed	H95	B73	B73-6A	0.14	275	1.46	0.1467
Inoculation*Tester*Introgression	sprayed	H95rhm	B73	B73-3B	0.37	301	1.12	0.2626
Inoculation*Tester*Introgression	sprayed	H95rhm	B73	B73-6A	0.31	297	0.84	0.4024
Inoculation*Tester*Introgression	sprayed	Mo17	B73	B73-3B	0.23	295	0.41	0.6816
Inoculation*Tester*Introgression	sprayed	Mo17	B73	B73-6A	0.11	280	0.63	0.5277
Inoculation*Tester*Introgression	sprayed	NC250	B73	B73-3B	0.16	293	0.9	0.367
Inoculation*Tester*Introgression	sprayed	NC250	B73	B73-6A	0.25	277	1.64	0.1028
Inoculation*Tester*Introgression	sprayed	NC350	B73	B73-3B	0.44	272	-0.82	0.411
Inoculation*Tester*Introgression	sprayed	NC350	B73	B73-6A	-0.21	259	-0.38	0.7048
Inoculation*Tester*Introgression	sprayed	Oh43	B73	B73-3B	-0.09	348	0.17	0.8685
Inoculation*Tester*Introgression	sprayed	Oh43	B73	B73-6A	0.05	343	0.59	0.5544
Inoculation*Tester*Introgression	sprayed	Va35	B73	B73-3B	0.17	280	0.13	0.8956
Inoculation*Tester*Introgression	sprayed	Va35	B73	B73-6A	0.03	280	0.8	0.4227
Inoculation*Tester*Introgression	sprayed	Va35rhm	B73	B73-3B	0.21	276	0.66	0.5093
Inoculation*Tester*Introgression	sprayed	Va35rhm	B73	B73-6A	0.17	258	0.14	0.8895

*** Significant at 1%

** Significant at 5%

* Significant at 10%

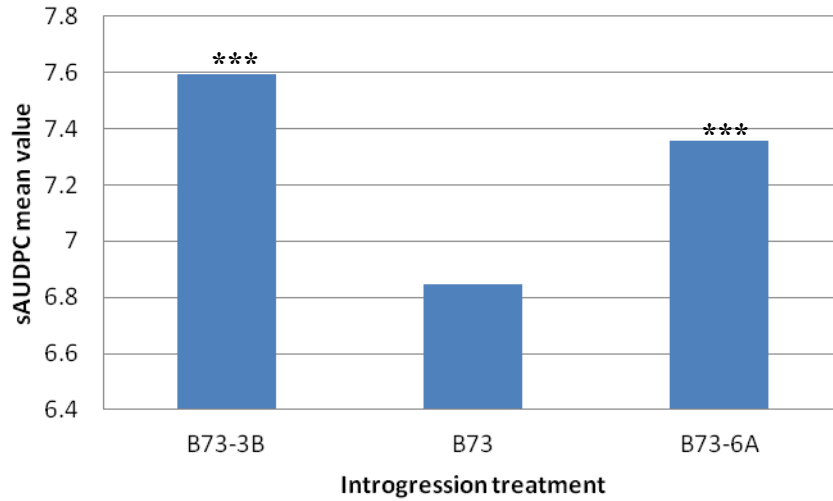


Figure 4.1 sAUDPC mean values across testers in the disease evaluation of isogenic hybrids. Effect of carrying introgression 3B or 6A across all tester treatments is presented as mean sAUDPC values. Means are presented in a scale of 1-9, on which 9 is a highly resistant plant and 1 is a highly susceptible, or dead. Significance is based on comparisons against the control B73-background isohybrids (No introgression).

*** Significant at 1%

** Significant at 5%

* Significant at 10%

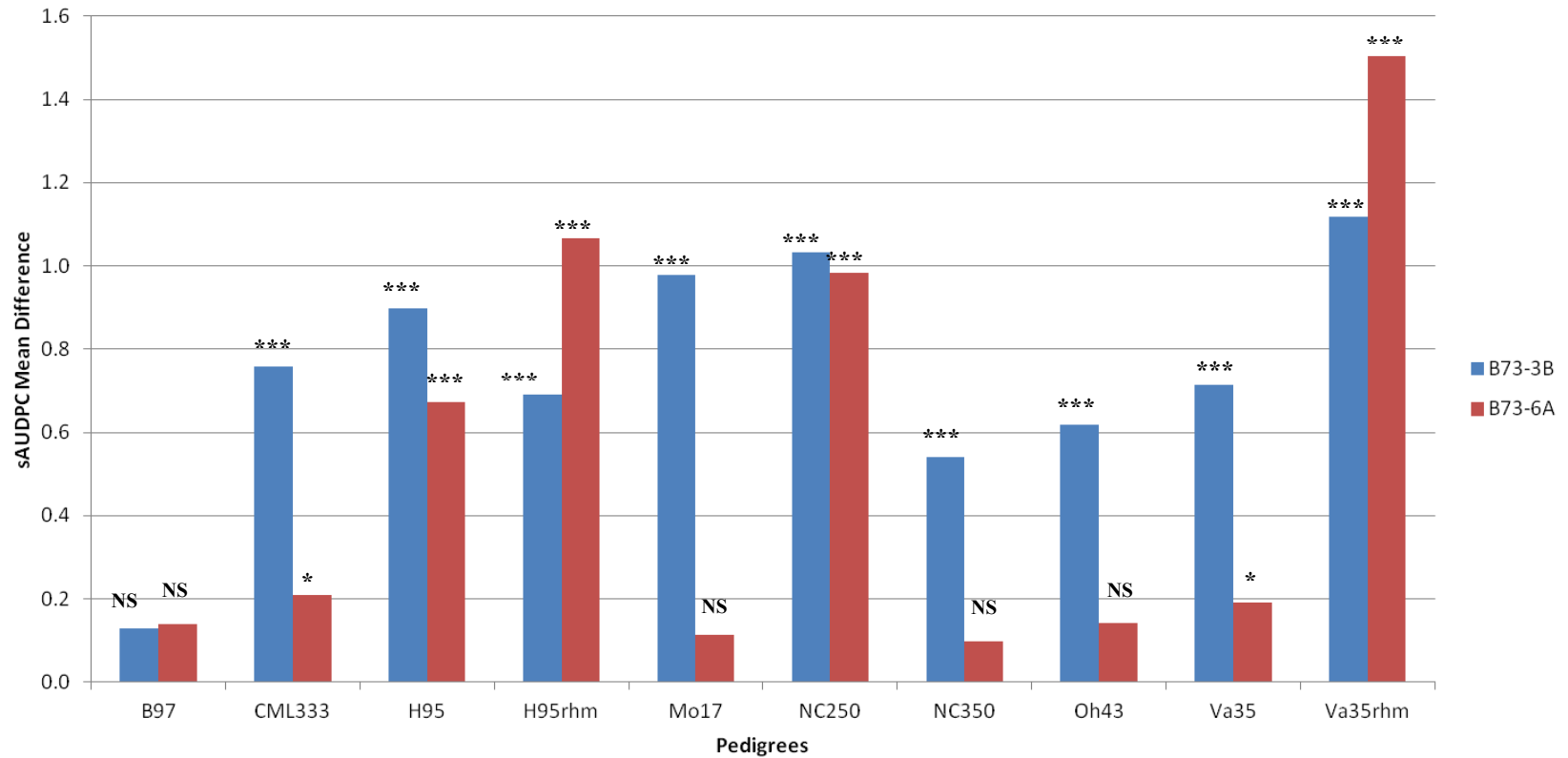


Figure 4.2 Effect of different tester haplotypes on SLB resistance on isogenic hybrid comparisons with and without introgressions 3B and 6A. sAUDPC mean differences are presented in a scale of 1-9, on which 9 is a highly resistant plant and 1 is a highly susceptible, or dead. Effect are presented as the sAUDPC mean difference between isohybrids carrying introgressions 3B or 6A and isohybrids with no introgression (B73 background hybrid control).

*** Significant at 1%

** Significant at 5%

* Significant at 10%

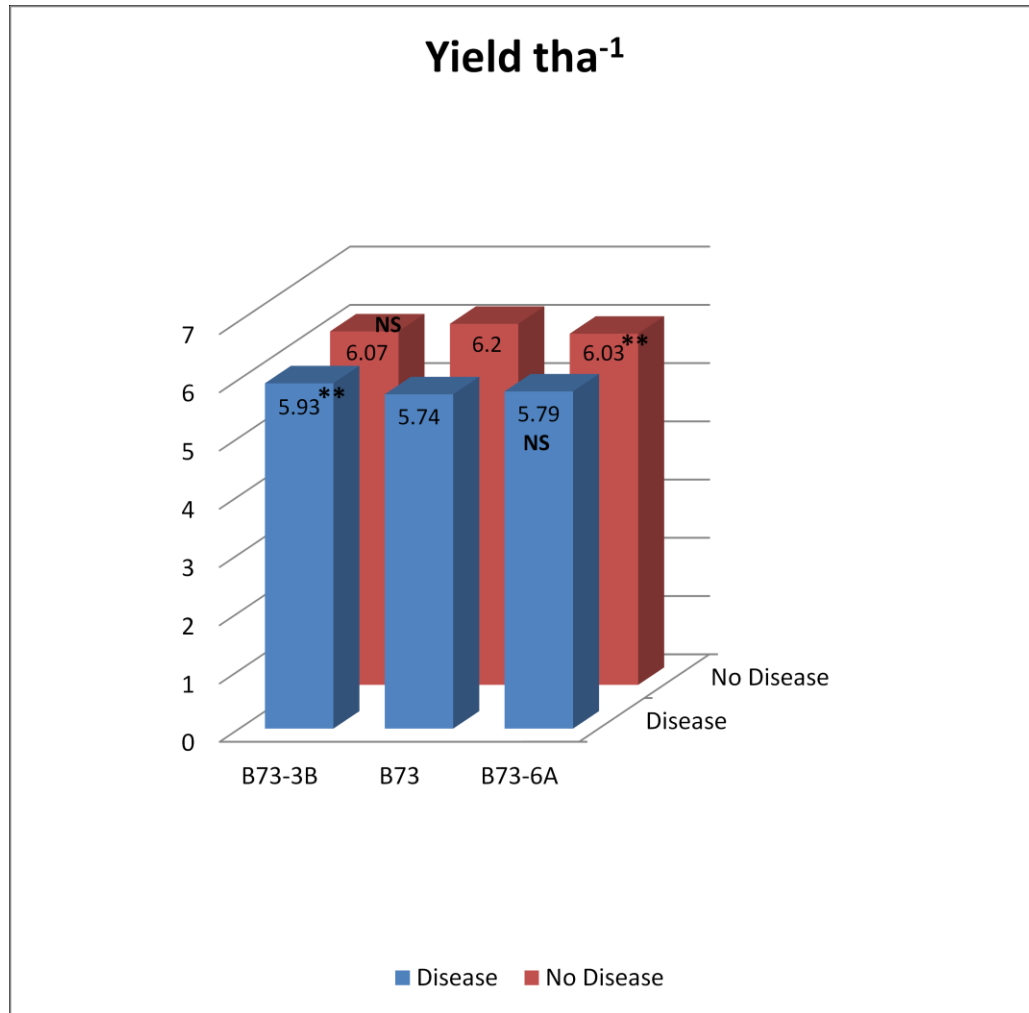


Figure 4.3 LS Mean yield estimates of isogenic hybrids with and without introgression 3B or 6A across testers under presence and absence of SLB. Effect of carrying introgression 3B or 6A on yield in t.ha⁻¹ across all tester treatments under adequate SLB disease pressure (inoculated) and no SLB (sprayed) treatments. Mean estimates for yield were standardized based on a stand of 40 plants at 14.5% grain moisture and then scaled up to bushels/acre according to the plot size and planting density. Significance is based on comparisons against the control B73-background isohybrids (No introgression).

** Significant at 5%

NS Non-significant

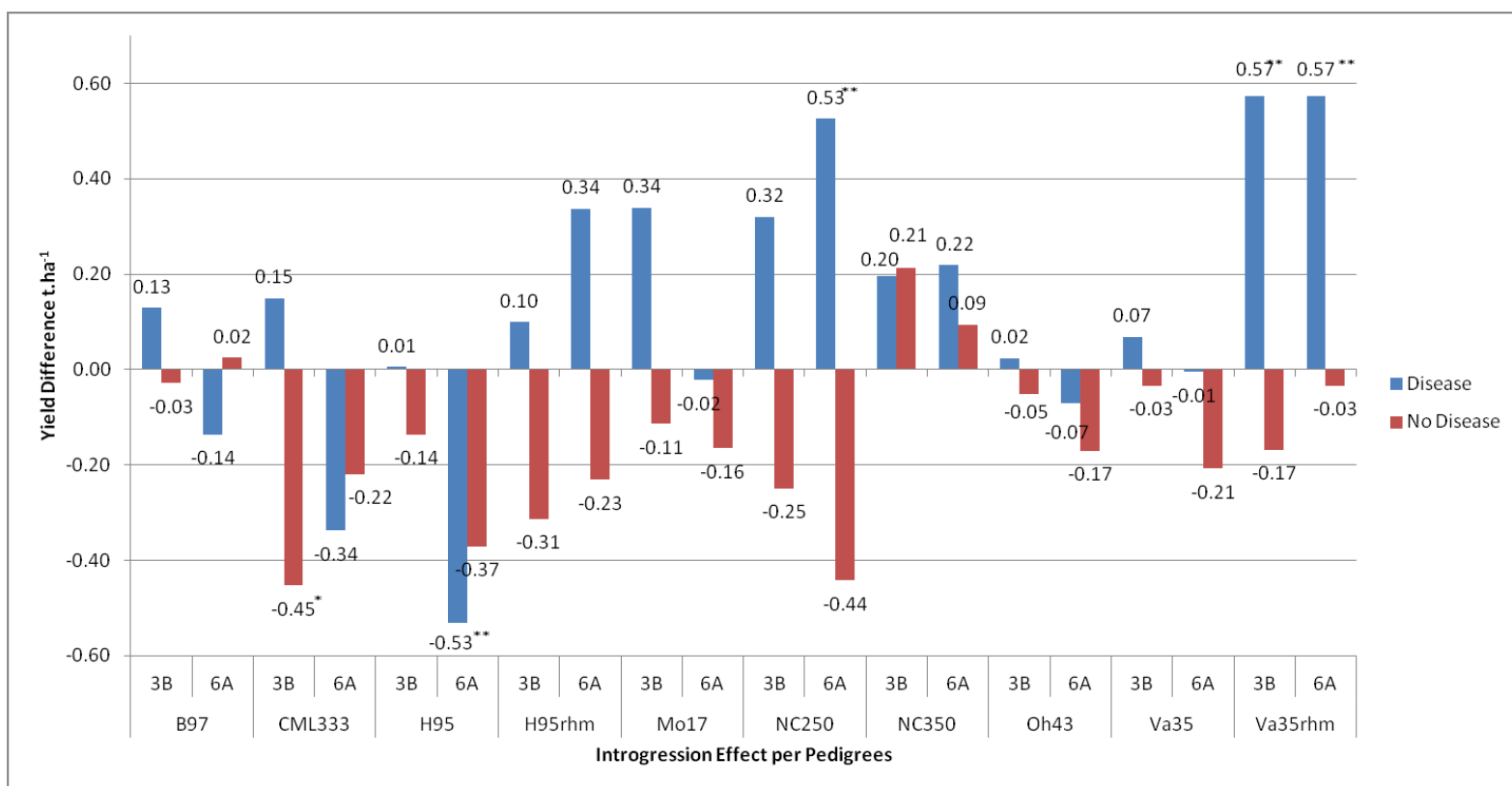


Figure 4.4 Effect of different tester haplotypes on yield on isogenic hybrid comparisons with and without introgressions 3B or 6A under SLB presence (inoculated) and absence (sprayed). Yield mean differences are presented in t.ha⁻¹. Effects are presented as the yield mean difference between isohybrids carrying introgressions 3B or 6A and isohybrids with no introgression (B73 background hybrid control). ** Significant at 5% * Significant at 10%. All other s are non significant.

APPENDIX

Supplementary Material for Chapter IV

Table S4.1 All LS mean contrasts for sAUDPC values related to the effect of SLB on isohybrid comparisons with and without introgressions. Means are presented in a scale of 1-9, on which 9 is a highly resistant plant and 1 is a highly susceptible, or dead.

Effect	Tester	Introgression	Tester	Introgression	Estimate	Pr > t
Introgression		B73		B73-3B	-0.75	<.0001***
Introgression		B73		B73-6A	-0.51	<.0001***
Introgression		B73-3B		B73-6A	0.24	<.0001***
Tester	B97		CML333		-0.97	<.0001***
Tester	B97		H95		-1.36	<.0001***
Tester	B97		H95rhm		-1.84	<.0001***
Tester	B97		Mo17		-0.56	<.0001***
Tester	B97		NC250		-0.60	<.0001***
Tester	B97		NC350		-1.96	<.0001***
Tester	B97		Oh43		-1.08	<.0001***
Tester	B97		Va35		-0.82	<.0001***
Tester	B97		Va35rhm		-1.62	<.0001***
Tester	CML333		H95		-0.39	0.0007***
Tester	CML333		H95rhm		-0.88	<.0001***
Tester	CML333		Mo17		0.40	0.0003***
Tester	CML333		NC250		0.37	0.0014***
Tester	CML333		NC350		-0.99	<.0001***
Tester	CML333		Oh43		-0.12	0.2991
Tester	CML333		Va35		0.15	0.1722
Tester	CML333		Va35rhm		-0.65	<.0001***
Tester	H95		H95rhm		-0.48	<.0001***
Tester	H95		Mo17		0.80	<.0001***
Tester	H95		NC250		0.76	<.0001***
Tester	H95		NC350		-0.60	<.0001***
Tester	H95		Oh43		0.28	0.0168**
Tester	H95		Va35		0.54	<.0001***
Tester	H95		Va35rhm		-0.26	0.0229**
Tester	H95rhm		Mo17		1.28	<.0001***
Tester	H95rhm		NC250		1.24	<.0001***
Tester	H95rhm		NC350		-0.11	0.3103
Tester	H95rhm		Oh43		0.76	<.0001***

Effect	Tester	Introgression	Tester	Introgression	Estimate	Pr > t
Tester	H95rhm		Va35		1.03	<.0001***
Tester	H95rhm		Va35rhm		0.22	0.0468**
Tester	Mo17		NC250		-0.04	0.7428
Tester	Mo17		NC350		-1.40	<.0001***
Tester	Mo17		Oh43		-0.52	<.0001***
Tester	Mo17		Va35		-0.25	0.0229**
Tester	Mo17		Va35rhm		-1.06	<.0001***
Tester	NC250		NC350		-1.36	<.0001***
Tester	NC250		Oh43		-0.48	<.0001***
Tester	NC250		Va35		-0.22	0.0579*
Tester	NC250		Va35rhm		-1.02	<.0001***
Tester	NC350		Oh43		0.88	<.0001***
Tester	NC350		Va35		1.14	<.0001***
Tester	NC350		Va35rhm		0.34	0.0028***
Tester	Oh43		Va35		0.27	0.017**
Tester	Oh43		Va35rhm		-0.54	<.0001***
Tester	Va35		Va35rhm		-0.81	<.0001***
Tester*Introgression	B97	B73	B97	B73-3B	-0.1292	0.2598
Tester*Introgression	B97	B73	B97	B73-6A	-0.1399	0.2223
Tester*Introgression	B97	B73-3B	B97	B73-6A	-0.01076	0.9251
Tester*Introgression	CML333	B73	CML333	B73-3B	-0.7578	<.0001***
Tester*Introgression	CML333	B73	CML333	B73-6A	-0.2078	0.0741*
Tester*Introgression	CML333	B73-3B	CML333	B73-6A	0.55	<.0001***
Tester*Introgression	H95	B73	H95	B73-3B	-0.8971	<.0001***
Tester*Introgression	H95	B73	H95	B73-6A	-0.6734	<.0001***
Tester*Introgression	H95	B73-3B	H95	B73-6A	0.2237	0.1036
Tester*Introgression	H95rhm	B73	H95rhm	B73-3B	-0.6912	<.0001***
Tester*Introgression	H95rhm	B73	H95rhm	B73-6A	-1.0666	<.0001***
Tester*Introgression	H95rhm	B73-3B	H95rhm	B73-6A	-0.3754	0.0015***
Tester*Introgression	Mo17	B73	Mo17	B73-3B	-0.9783	<.0001***
Tester*Introgression	Mo17	B73	Mo17	B73-6A	-0.1125	0.3263
Tester*Introgression	Mo17	B73-3B	Mo17	B73-6A	0.8658	<.0001***
Tester*Introgression	NC250	B73	NC250	B73-3B	-1.0315	<.0001***
Tester*Introgression	NC250	B73	NC250	B73-6A	-0.9823	<.0001***
Tester*Introgression	NC250	B73-3B	NC250	B73-6A	0.04922	0.6931
Tester*Introgression	NC350	B73	NC350	B73-3B	-0.5408	<.0001***
Tester*Introgression	NC350	B73	NC350	B73-6A	-0.09792	0.3928
Tester*Introgression	NC350	B73-3B	NC350	B73-6A	0.4429	0.0005***
Tester*Introgression	Oh43	B73	Oh43	B73-3B	-0.6173	<.0001***

Effect	Tester	Introgression	Tester	Introgression	Estimate	Pr > t
Tester*Introgression	Oh43	B73	Oh43	B73-6A	-0.1418	0.2363
Tester*Introgression	Oh43	B73-3B	Oh43	B73-6A	0.4756	0.0001***
Tester*Introgression	Va35	B73	Va35	B73-3B	-0.7135	<.0001***
Tester*Introgression	Va35	B73	Va35	B73-6A	-0.192	0.0942*
Tester*Introgression	Va35	B73-3B	Va35	B73-6A	0.5215	<.0001***
Tester*Introgression	Va35rhm	B73	Va35rhm	B73-3B	-1.1181	<.0001***
Tester*Introgression	Va35rhm	B73	Va35rhm	B73-6A	-1.5028	<.0001***
Tester*Introgression	Va35rhm	B73-3B	Va35rhm	B73-6A	-0.3847	0.001***

*** Significant at 1%

** Significant at 5%

* Significant at 10%

Table S4.2 Type 3 tests of significance of fixed effects for all agronomic traits related to the effects of introgression 6A and 3B on isohybrid comparisons with and without disease pressure. DTS and DTA estimated as the number of days from planting to when half of the plants in the plot were silking and shedding pollen, respectively. Plant height was measured to the height of the flag leaf, and ear height was measured at the node from which the ear emerged. Percentage of erect plants was calculated by subtracting number of lodged plants at harvest from the total stand count, and subsequently dividing by the total stand. Yield measurements were standardized based on a stand of 40 plants at 14.5% grain moisture and then scaled up to bushels/acre according to the plot size and planting density. Stand counts (ST) was used as a covariate for yield, plant height and ear height traits.

Yield bu/A				
Effect	Num DF	Den DF	F Value	Pr > F
ST	1	551	218.68	<.0001***
Inoculation (inoculated vs. sprayed)	1	5.01	0.93	0.3802
Tester	9	45.1	3.28	0.0038***
Inoculation*Tester	9	364	1.07	0.3871
Introgression	2	92.7	1.07	0.3485
Inoculation*Introgression	2	756	4.19	0.0155**
Tester* Introgression	18	91.6	0.76	0.7365
Inoculation*Tester* Introgression	18	753	0.84	0.6492

Plant Height				
Effect	Num DF	Den DF	F Value	Pr > F
ST	1	1021	0.00	0.9507
Inoculation (inoculated vs. sprayed)	1	5	0.06	0.8158
Tester	9	45.1	25.27	<.0001***
Inoculation*Tester	9	370	0.56	0.8278
Introgression	2	9.56	1.17	0.3505
Inoculation*Introgression	2	846	0.86	0.4215
Tester* Introgression	18	846	1.04	0.4158
Inoculation*Tester* Introgression	18	845	0.58	0.9128

Ear Height				
Effect	Num DF	Den DF	F Value	Pr > F
ST	1	1066	5.67	0.0174**
Inoculation (inoculated vs. sprayed)	1	5	0.09	0.7729
Tester	9	45.1	51.50	<.0001***
Inoculation*Tester	9	369	1.54	0.1319
Introgression	2	8.79	1.31	0.3170
Inoculation*Introgression	2	846	0.34	0.7085
Tester* Introgression	18	846	2.24	0.0023***
Inoculation*Tester* Introgression	18	845	1.09	0.3599

DTS				
Effect	Num DF	Den DF	F Value	Pr > F
Inoculation (inoculated vs. sprayed)	1	13.5	3.65	0.0774*
Tester	9	9.22	5.75	0.0073***
Inoculation*Tester	9	82.7	1.12	0.3600
Introgression	2	34.5	0.07	0.9314
Inoculation*Introgression	2	34.7	1.06	0.3588
Tester* Introgression	18	34.3	1.40	0.1916
Inoculation*Tester* Introgression	18	34.5	0.98	0.5035

DTA				
Effect	Num DF	Den DF	F Value	Pr > F
Inoculation (inoculated vs. sprayed)	1	15	1.77	0.2038
Tester	9	18.6	16.15	<.0001***
Inoculation*Tester	9	18.6	0.80	0.6231
Introgression	2	4.29	0.22	0.8108
Inoculation*Introgression	2	4.29	0.25	0.7921
Tester* Introgression	18	249	1.09	0.3612
Inoculation*Tester* Introgression	18	249	0.63	0.8720

Moisture Percentage				
Effect	Num DF	Den DF	F Value	Pr > F
Inoculation (inoculated vs. sprayed)	1	5	2.65	0.1643
Tester	9	45.3	24.31	<.0001***
Inoculation*Tester	9	40.6	1.35	0.2407
Introgression	2	9.74	3.01	0.0962*
Inoculation*Introgression	2	745	2.14	0.1189
Tester* Introgression	18	89.6	1.56	0.0880*
Inoculation*Tester* Introgression	18	742	1.20	0.2576

Percentage of Erect Plants				
Effect	Num DF	Den DF	F Value	Pr > F
Inoculation (inoculated vs. sprayed)	1	43.6	2.93	0.0943*
Tester	9	45.1	2.18	0.0413**
Inoculation*Tester	9	46.5	1.06	0.4072
Introgression	2	24	0.50	0.6132
Inoculation*Introgression	2	15.5	0.23	0.7989
Tester* Introgression	18	85.2	1.31	0.2021
Inoculation*Tester* Introgression	18	741	2.76	0.0001***

Num DF = Numerator degrees of freedom

Den DF = Denominator degrees of freedom

*** Significant at 1%

** Significant at 5%

* Significant at 10%

Table S4.3 Covariance Parameter Estimates for the different agronomic traits tested in this study. DTS and DTA estimated as the number of days from planting to when half of the plants in the plot were silking and shedding pollen, respectively. Plant height was measured to the height of the flag leaf, and ear height was measured at the node from which the ear emerged. Percentage of erect plants was calculated by subtracting number of lodged plants at harvest from the total stand count, and subsequently dividing by the total stand. Yield measurements were standardized based on a stand of 40 plants at 14.5% grain moisture and then scaled up to bushels/acre according to the plot size and planting density. Stand counts (ST) was used as a covariate for yield, plant height and ear height traits.

Yield bu/A	
Covariance Parameter	Estimate
Environment	961.08
Environment *Inoculation	46.6965
rep(Environment *Inoculation)	71.7057
Environment *Tester	40.3711
rep*Tester(Environment *Inoculation)	126.67
Environment *Tester*Introgression	3.9180
Residual	184.85

Plant Height	
Covariance Parameter	Estimate
Environment	0.04084
Environment *Inoculation	0.004288
rep(Environment *Inoculation)	0.002755
Environment *Tester	0.001706
rep*Tester(Environment *Inoculation)	0.003565
Environment * Introgression	0.000050
Residual	0.004778

Ear Height	
Covariance Parameter	Estimate
Environment	0.01733
Environment *Inoculation	0.001961
rep(Environment *Inoculation)	0.000824
Environment *Tester	0.000834
rep*Tester(Environment *Inoculation)	0.001539
Environment * Introgression	0.000025
Residual	0.003130

DTS	
Covariance Parameter	Estimate
Environment	0.5702
rep(Environment *Inoculation)	0.3824
Environment *Tester	0.3248
rep*Tester(Environment *Inoculation)	0.6984
Environment *Inoculation*Tester* Introgression	0.04145
Residual	1.0613

DTA	
Covariance Parameter	Estimate
Environment	0.4977
rep(Environment *Inoculation)	0.3848
Environment *Inoculation*Tester	0.1450
rep*Tester(Environment *Inoculation)	0.6225
Environment *Inoculation* Introgression	0.006729
Residual	1.1696

Moisture Percentage	
Covariance Parameter	Estimate
Environment	0.000361
Environment *Inoculation	0.000042
rep(Environment *Inoculation)	0.000012
Environment *Tester	0.000035
Environment * Inoculation *Tester	6.454E-6
rep*Tester(Environment *Inoculation)	9.23E-6
Environment * Introgression	1.662E-6
Environment *Tester* Introgression	5.47E-6
Residual	0.000068

Percentage of Erect Plants	
Covariance Parameter	Estimate
Environment	0.01652
rep(Environment *Inoculation)	0.002626
Environment *Tester	0.001462
Environment * Inoculation *Tester	0.000594
rep*Tester(Environment *Inoculation)	0.006585
Environment *Inoculation*Introgression	0.000166
Environment *Tester*Introgression	0.000474
Residual	0.007941

Table S4.4 Least-Square Means of yield and other agronomic traits related to the effect of tester and introgressions.

Estimate of the effect for SLB resistance on isohybrid comparisons with and without introgression 3B and 6A. Mean estimates for yield were standardized based on a stand of 40 plants at 14.5% grain moisture and then scaled up to bushels/acre or t/ha according to the plot size and planting density. Moisture content is presented as percentage (Mois%). Plant height (PHT) was measured to the height of the flag leaf, and ear height (EHT) was measured at the node from which the ear emerged. PHT and EHT are presented in meters. Percentage of erect plants (PE%) was calculated by subtracting number of lodged plants at harvest from the total stand count, and subsequently dividing by the total stand. DTS and DTA estimated as the number of days from planting to when half of the plants in the plot were silking and shedding pollen, respectively.

Effect	Inoculation	Tester	Introgres.	Yield (Tha ⁻¹)	Mois%	PHT(m)	EHT(m)	PE%	DTS	DTA
Tester		B97		5.865	17.3	2.09	1.09	90.0	73.7	70.7
Tester		CML333		5.975	21.0	2.21	1.28	78.9	75.0	73.6
Tester		H95		5.752	17.6	2.00	1.00	84.4	73.6	71.7
Tester		H95rhm		5.413	17.4	2.01	1.01	85.3	74.2	72.1
Tester		Mo17		6.540	16.6	2.01	1.04	86.4	72.4	69.8
Tester		NC250		5.648	18.9	1.83	0.90	85.4	72.4	69.7
Tester		NC350		6.522	19.5	2.14	1.09	83.4	74.4	72.7
Tester		Oh43		5.685	17.2	2.00	1.00	82.7	73.6	70.6
Tester		Va35		6.065	19.0	2.01	1.04	89.1	71.8	70.7
Tester		Va35rhm		6.105	18.8	2.00	1.01	88.6	71.6	70.3
Inoculation*Introgression	Inoculated		B73	5.735	17.9	2.02	1.04	83.3	73.7	71.4
Inoculation*Introgression	Inoculated		B73-3B	5.925	17.9	2.03	1.05	83.8	73.6	71.5
Inoculation*Introgression	Inoculated		B73-6A	5.790	18.2	2.02	1.04	84.5	73.5	71.4
Inoculation*Introgression	sprayed		B73	6.199	18.7	2.03	1.05	87.1	72.9	70.8
Inoculation*Introgression	sprayed		B73-3B	6.066	18.5	2.04	1.05	86.5	72.9	70.9
Inoculation*Introgression	sprayed		B73-6A	6.027	18.7	2.04	1.05	87.4	73.0	71.0

Table S4.5 Spearman correlation. Rank correlations of sAUDPC values and least-squares mean contrasts for yield under disease and no disease environments for both introgressions 3B or 6A.

Score	3B		6A	
	Rho	Prob > r	Rho	Prob > r
Disease	0.50	0.1497	0.39	0.26
No Disease	-0.59	0.0739	-0.60	0.0667