EXPLOITING HISTORICAL DATA AND DIVERSE GERMPLASM TO INCREASE MAIZE GRAIN YIELD IN TEXAS

A Dissertation

by

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ABSTRACT

The U.S. is the largest maize producer in the world with a production of 300 million tons in 2012. Approximately 86% of the maize production is focused on the Midwestern states. The rest of the production is focused in the Southern states, where Texas is the largest maize producer. Grain yield in Texas ranges from 18 tons/ha in the irrigated production zones to 3 tons/ha in the dryland production zones. As a result, grain yield has increased slowly because of the poor production in the non-irrigated acres. Methods to improve the grain yield in Texas is to breed for maize varieties adapted to Texas growing conditions, including mapping genes that can be incorporated into germplasm through marker assisted selection. This dissertation includes two separate projects that exploit historical data and maize diversity to increase grain yield in Texas.

For the first project, a large dataset collected by Texas AgriLife program was analyzed to elucidate past trends and future hints on how to improve maize yield within Texas. This study confirmed previous reports that the rate of increase for grain yield in Texas is less than the rate observed in the Midwestern US.

For the second project, a candidate gene and whole genome association mapping analysis was performed for drought and aflatoxin resistance in maize. In order to do so, maize inbred lines from a diversity panel were testcrossed to isogenic versions of Tx714. The hybrids were evaluated under irrigated and non-irrigated conditions. The irrigated trials were inoculated with *Aspergillus flavus* and the aflatoxin level was quantified. This study found that the gene *ZmLOX4* was associated with days to silk, and the gene

ZmLOX5 gene was associated with plant and ear height. In addition, this study identified 13 QTL variants for grain yield, plant height, days to anthesis and days to silk. Furthermore, this study shows that diverse maize inbred lines can make hybrids that out yield commercial hybrids under heat and drought stress. Therefore, there are useful genes present in these diverse lines that can be exploited in maize breeding programs.

DEDICATION

I dedicate this dissertation to my wife and kids who were always very patient and supportive in the toughest moments during graduate school. To my parents who always foster my passion and love for studying and who always reinforce the core values of my formation.

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CHAPTER I

INTRODUCTION

Maize is the most important crop in the world with a world production of 800 million tons in 2010 (FAOSTAT) [Verified on May 8/2013]. The United States is the major producer of maize with a production of 320 million tons in 2011 having an estimated value in the market of 77 billion dollars (National Agricultural Statistical Service [NASS], 2013). Maize is currently grown on approximately 33 million hectares of land, with the majority in the Midwest region. The rest of the production is in Southern states, where Texas is the largest maize producer, and 13th in the U.S. (NASS, 2012). The maize production in Texas was eight million tons in 2010 with a farm gate value of 1.5 billion dollars (NASS, 2012).

Different studies have showed that average grain yield has steadily increased over years in the U.S. and Midwestern states. In contrast, the average grain yield has remained steady in the state of Texas. One of the reasons for this lack of improvement in grain yield is the use of unadapted germplasm and dryland locations with inadequate rainfall. In addition, major obstacles for maize producers in Texas are drought stress and aflatoxin accumulation. Methods to improve the grain yield in Texas is to breed for maize varieties adapted to Texas growing condition, including mapping genes that increase resistant to drought tolerance and aflatoxin contamination, which can be incorporated into germplasm through marker assisted selection. In order to address these problems, two separate projects are included in this dissertation.

For the first project, a multi-environment trial (MET) dataset collected by the Texas AgriLife Crop Testing Program from 2000 to 2010 was analyzed. The Texas Agrilife Crop Testing program is an extension program that has been testing hybrids for almost fifty years. In this program hybrids submitted from different companies are evaluated every year to provide relevant information to farmers in the region. Like testing programs in many states, these METs are conducted to provide unbiased information to growers about the best currently available varieties for their area. Approximately 1,500 to 2,200 datapoints are generated each growing season and important agronomic traits, including days to silk, plant and ear height, lodging, plant population, moisture, test weight, and grain yield are collected. The Texas Agrilife Corn Performance Trials represent a large historical dataset with broad sampling of genotypes and environments that allow direct investigation of many questions that cannot be addressed by one of few years of data (DeLacy et al., 1996a,b). The major goal of this project is to elucidate past trends and future hints on how to improve maize yield within Texas using this MET dataset collected from 2000 to 2010.

For the second project, a candidate gene and whole genome association mapping study for drought and aflatoxin resistance in maize was performed. The first major objective of this project is to evaluate the effect of natural alleles of *ZmLOX4* and *ZmLOX5* on conferring tolerance to drought and resistance to aflatoxin accumulation, respectively. Two different hypotheses were tested under this goal. The first hypothesis is that genetic background impacts the effects of mutant *LOX* alleles. The second

hypothesis is that different alleles at *ZmLOX4* and *ZmLOX5* will have different impacts on drought tolerance and aflatoxin resistance respectively. These hypotheses are supported by previous research that showed that the deletion of the *ZmLOX4* gene results in increase seedling and mature plant drought tolerance. On the other hand, knock-out mutants of the *ZmLOX5* gene in different genetic backgrounds accumulated 5-fold less aflatoxin when infected with *Aspergillus flavus*. The second major goal for this project is to map genomic regions that confer drought tolerance, aflatoxin resistance and influence important agronomic traits such as flowering time, plant and ear height, test weight and grain yield in a testcrossed association mapping panel. The hypothesis for this goal is that different regions in the maize genome mediate quantitative variation for these traits.

CHAPTER II

LITERATURE REVIEW

Maize production, breeding and the Texas AgriLife program

The United States produced 320 million tons of maize in 2011 with an estimated market value of 77 billion dollars (National Agricultural Statistical Service [NASS], 2012). Maize is currently grown on approximately 33 million hectares of land, with the majority in the Midwest region. A series of field studies that compared successful hybrids released by Pioneer Hi-Bred International in the Midwest since 1930s - the so-called "ERA hybrids studies", showed that grain yield has steadily increased over years (Duvick, 1984, 2001; Duvick and Cooper, 2003, Duvick et al., 2004; Crosbie et al., 2008). This trend in maize yield increase in the U.S. is further corroborated by United States Department of Agriculture (USDA) data collected since 1900s (NASS, 2012). Yield increase in maize has been largely attributed to genetic gain, which accounts for approximately 50%, and improvement in crop management practices are attributed to the other half (Russell, 1991; Duvick, 1992). These studies provided evidence that yield increases were caused by breeding for plants better adapted to stress and capable of production under high planting densities (Duvick, 1977, 1984; 1992, 2001; Duvick and Cassman, 1999; Duvick and Copper, 2003; Duvick et al., 2004). A number of traits were also associated with greater biomass accumulation and enhanced plant growth in historical U.S. yields including: number of ears per 100 plants, small tassel size, reduced anthesis-silking interval (ASI) and stay-green (Duvick, 1977, 1984; Duvick and Cooper,

2003; Duvick et al., 2004). Recent studies based on simulations and empirical data has identified that the modification of the harvest index and root structure has a greater direct effect than canopy modification in explaining yield increase and its interaction with plant density (Hammer et al., 2009).

Both the public and private sector played a major role in historical yield increases in maize. Maize varieties were bred and released by land-grant universities, state agricultural experimental stations, and other public agencies in the early 1930. This role evolved with the consolidation and widespread use of hybrid seed during the 1950 and the approbation of the plant protection and variety act in the 1970 (Knudson and Pray, 1991; Frey, 1996; Fuglie, 2000,2008; Duvick, 2001; Fernandez-Cornejo, 2004; Alston and Venner, 2002;). The industry investment in maize research has increased fourfold since the 1970s, and it is now estimated that 80% is focused on major corn producing states in the Midwest (Frey, 1996; Fuglie, 2000,2008; Schimmelpfennig et al., 2004).

In contrast, seed industry investment in R&D within the southern states which include Texas, has not been significant compared to the Midwestern states. This is believed to be because of the smaller market share that those states represent (NASS, 2012). As a consequence, there are fewer commercial maize breeding programs developing inbreds adapted to specific growing conditions in Southern U.S. and Texas. To develop new varieties for Texas inbreds are generally test crossed elsewhere and the resulting hybrids are evaluated for two to three years in Texas before commercialization. As a likely result, the use of unadapted germplasm has increased major constraints to maize production in Texas and the Southeastern US, specifically aflatoxin contamination

and drought stress (Horne et al., 1991; Brown et al., 1999; Widstrom et al., 2003; Williams et al., 2003; Betran et al., 2005; Mayfield et al., 2011). Texas is the largest maize producer of the southern states, and 12th in the U.S. (NASS, 2012). The maize production in Texas was 8 million ton in 2010 with a farm gate value in the market of 1.5 billion dollars (NASS, 2012). Only 86 out of 254 counties produce maize in Texas, but these occupy a wide geographical range, in turn making each production zone unique in its precipitation, wind and solar irradiation patterns, types of soils, and agronomic practices. Little work has been conducted to quantify these different environments so far.

The Texas A&M Agrilife (formally Texas Agricultural Experiment Station) maize program began in 1927, and it was complemented by the corn performance trials, which began in 1969. The extension based Crop Testing Program, housed at Texas A&M University, specifically began to test elite hybrids from different companies to provide the most relevant evaluations to regional farmers. Like testing programs in many states, these MET's are conducted to provide unbiased information to growers about the best currently available varieties for their area. The Texas AgriLife Crop Testing Program annually evaluates approximately 100 to 200 commercial maize entries in Texas Corn Performance Trials every year over 9 to 12 different locations believed to be most representative of the growing production areas of Texas. The goals of these studies are immediate and no retrospective study has yet been conducted. Approximately 1,500 to 2,200 datapoints are generated each growing season and important agronomic traits, including days to silk, plant and ear height, lodging, plant population, moisture, test weight, and yield are collected.

Drought stress, physiological effects and hormones involved in drought acclimation

The majority of the regions where maize is grown in Texas are dryland locations that depend on rainfall to get moisture for the crop. A highly variable inter-annual precipitation in the state increases the change of drought episodes during flowering and milky stage, which are the most sensitive to drought effects. This is further worsened by the fact that the majority of hybrids that are grown in the state of Texas were not developed for Texas environments. Drought stress is a complex trait that involves thousands of genes and different physiological responses (Cooper et al., 2009). Breeding for drought is challenging because residual error variance in drought trials is greater than well-watered trials, which decrease the statistical power to detect significant differences between the hybrids tested. Therefore, it is important to control field error variation and water input to ensure the survivability of the plants in the field. Drought stress is first sensed by the root, which in turn triggers several physiological and acclimation responses that are regulated by different phytohormones. The duration of the response depends on the severity of the stress and adaption of the plants to the drought conditions. The goal of this section is to describe the different physiological responses to drought and the role of different phytohormones involved in the drought response. The first part is dedicated to one of the well-known effects of drought stress in plants: lipid peroxidation and Reactive Oxygen Species (ROS) production (Munné-Bosch, 2005; Shalata and Tal, 1998; Yan et al., 2007; Cruz de Carvalho, 2008). This section is followed by the role of major phytohormones in drought stress.

Drought stress and lipid peroxidation

Lipid peroxidation occurs during free radical damage to cell membranes under drought, cold or high salinity stress (Shalata and Tal, 1998; DaCosta and Huang, 2007; Hara et al., 2003; Türkan et al., 2005;; Anjum et al., 2012;). Lipid peroxidation induces changes in the lipid composition of the membrane, which affect the structural and functional properties of cell membranes (Smirnoff, 1993). Lipid peroxidation is caused by the accumulation of reactive oxygen species (ROS) during abiotic stress. Singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydrogen peroxide(H_2O_2) and the hydroxyl radical ($OH^\cdot$) are the principal ROS in plants (Cruz de Carvalho, 2008). ROS plays a dual role in abiotic stress response, based on the concentration, they can participate in the stress-signaling pathways, which contributes to the acclimation and stress responses. On the other hand, if ROS accumulation reaches high levels, they can initiate an oxidative burst that will lead to cell death (Cruz de Carvalho, 2008). One of the principal enzymes in lipid peroxidation are lipoxygenases (LOX). These family of proteins regulate the dioxygenation of polyunsaturated fatty acids containing a cis, cis-1,4-pentadiene backbone (Feussner and Wasternack, 2002; Porta and Rocha-Sosa, 2002; Liavonchanka and Feussner, 2006; Christensen and Kolomiets, 2011). The primary product of this reaction is hydroperoxy fatty acids, which are highly reactive compounds toxic to cells. These intermediaries are rapidly metabolized into jasmonates, conjugate dienoic acids, and volatile aldehydes such as malondialdehyde (MDA) (Blée, 2002).

Production of ROS species during drought stress

and scavenging mechanisms

One of the first organs that sense the limitation in water supply is the root system. Under water stress, abscisic acid (ABA) accumulates in the roots and aboveground parts of the plant (Sauter et al., 2001; Ren et al., 2007). One of the well recognized roles of ABA in drought stress is the induction of stomata closure. This water-saving strategy has a direct impact on photosynthesis and ROS production (Smirnoff, 1993; Cruz de Carvalho, 2008; Türkan et al., 2005). Depending on the extent and duration of stomatal closure, the water balance in the leaves and fixation of carbon dioxide (CO_2) is affected. The lack of CO_2 fixation reduce the regeneration of $NADP^+$ by the Calvin cycle, which provoke a major leakage of electrons from the photosynthetic electron transport chain to O_2 by the Mehler reaction, generating superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (Smirnoff 1993).

ROS are produced naturally by the chloroplast during photosynthesis and photorespiration. The mitochondrial electron transport is also responsible for ROS generation. Plants have developed scavenging systems to protect cells against lipid peroxidation and cellular damage. ROS are increased significantly under drought stress, which represent a risk to the chloroplast and thylakoid membranes because of their high content in polyunsaturated fatty acids (Smirnoff, 1993). The risk that lipid peroxides represent to the cell membrane is evidenced by studies showing a positive relationship between the concentration of MDA and reduced electron transport capacity. Singlet

oxygen and hydroxyl radical are the most toxic ROS and these are kept under minimal production under normal conditions because of their capacity to oxidize lipids, DNA and RNA. Under drought conditions, the real threat to the thylakoids membranes is the production of the hydroxyl radical by the Mehler reaction through "iron-catalized" reduction of (H_2O_2) by the superoxide dismutase (SOD) and ascorbate. It has reported that wheat under drought stress experienced a 50% in the electron leakage to O_2 under water stress (Biehler and Fock, 1996).

Plants are natural producers of ROS and have developed scavenging systems that protect the cells against oxidative damage. The major enzymes of this scavenging system are SOD, metabolites and enzymes of the ascorbate-glutathione cycle, and catalase. Different studies have given contradictory evidence regarding the role of each of this systems in scavenging ROS; however, it is well established a positive relation between the level of induction of these antioxidant systems and the degree of drought resistance (Quartacci and Navari-Izzo, 1992; Pastori and Trippi, 1993; Moussa and Abdel-Aziz, 2008; Sofo et al., 2004; Torres-Franklin et al., 2008; Kakumanu et al., 2012; Pal et al., 2012). Similarly, studies have shown that enzymes from the glutathione metabolism, the glutathione-S-transferase (GST) and glutathione peroxidase (GPX) are induced under drought stress. GPX has the capacity to scavenge H_2O_2 and lipid hydroperoxides, which further protect the cellular membranes under drought stress (Sasaki-Sekimoto et al., 2005; Torres-Franklin et al., 2008). Antioxidants also play a role under drought stress in scavenging ROS and preventing membrane damage. Alpha-tocopherol has been shown to accumulate under drought stress. This antioxidant has the ability to scavenge lipid

peroxyl radicals, hybroxyl radicals and singlet oxygen, which are the most dangerous ROS to the cell (Munné-Bosch, 2005). Other strategy of the plant during drought stress is shifting the production of H_2O_2 to the peroxisomes to prevent formation of hydroxyl radicals by the Mehler reaction. This is accomplished by the increase in the photorespiration rate by the rubisco enzyme, which produces glycolate. Glycolate is transported to peroxisomes, where it is oxidized to produce glycine, a precursor of the glutathione, and H_2O_2 (Cruz de Carvalho, 2008).

Role of lipoxygenases in drought stress

The role of lipoxygenases (*LOXs)* in abiotic stress is still unclear. A study on olive trees, which are considered drought tolerant, shows an accumulation of MDA and *LOXs* when leaf water content (LWC) and net photosynthetic rate is reduced in stressed trees. This paper hypothesized that *LOX* generate high levels of lipid peroxidation that could produce lipid derivates that serve as secondary messengers. These secondary messengers may initiate the response to desiccation in the plant. Additionally, the hydroperoxy fatty acids metabolism can also generate jasmonic acid that has been shown to reduce plant growth during the drought stress conditions (Sofo et al., 2004).

Role of jasmonic acid, ethylene and ABA in drought stress

Plants are sessile organisms that have evolved different mechanisms to respond to drought stress. Under water-deficit conditions, the root, the first organ in the plant that senses the water deficit, signals the production of ABA and ethylene in the roots and

above ground parts of the plants. The abscisic acid (ABA) phytohormone has been intensively study because of its well-known role in plant acclimation under drought stress (Wang et al., 2008; Sirichandra et al., 2009; Cutler et al., 2010; Kim et al., 2010; Wilkinson and Davies, 2010). Plants can tolerate drought by different mechanisms such as stomata closure, osmotic regulation, reduction of plant growth rates, increases in root extension rates, leaf senescence, and increase in antioxidant activity (Wilkinson and Davies, 2010; Wang et al., 2008; Skirycz et al., 2011; Rengel et al., 2012). The response to drought in plants involves stress signaling pathways that are interconnected and complex. These signaling pathways are ABA-dependent and ABA-independent. Other pathways that are involved in these mechanisms are ethylene and derivates of the octadecanoid pathway responsible for jasmonate (JA) biosynthesis (Golldack et al., 2011). In this section, the role of ABA, ethylene and jasmonate methyl-jasmonate (MeJA) is discussed (Anjum et al., 2011).

The phytohormone ABA plays an essential role in drought response

The phytohormone ABA was discovered in the 1960, shown to act as a long-distance signaling hormone between the root and the shoot. ABA synthesis and accumulation is started in the roots. Most of the enzymes of the biosynthetic ABA pathway has been characterized and it has been determined that 9-*cis*-epoxycarotenoid dioxygenase (NCED) is a key enzyme in the ABA biosynthesis (Tan et al., 1997; Liotenberg et al., 1999; Ren et al., 2007). Accumulation of ABA in the roots and leaves triggers downstream responses that adapt the plant to the drought stress in an ABA-

dependent manner. A well-known effect of ABA accumulation is the concentration of cytosolic Ca^{2+} inducing stomata closure by efflux of anions and potassium ions (LeNoble et al., 2004; Ren et al., 2007; Hubbard et al., 2010; Wilkinson and Davies, 2010). Several signaling pathways, receptors and molecules participate in the ABA-mediated changes in efflux and uptake of ions and anions. Recently, researchers have focused on NO and H_2O_2 roles in affecting potassium uptake and the efflux channels in the guard cells (Hetherington and Woodward, 2003; Yan et al., 2007; Sirichandra et al., 2009; Kim et al., 2010; Wilkinson and Davies, 2010). Different studies have reported different roles of ABA in promoting or inhibiting shoot growth. Some studies suggest that ABA induces the inhibition of leaf growth to reduce water loss by transpiration (Parent et al., 2009; Thompson et al., 2007). In contrast, other studies report that ABA promotes shoot growth by suppressing ethylene-mediated growth under drought stress (LeNoble et al., 2004). These inconsistencies could be explained if ABA participates directly or indirectly in shoot growth by interacting with other important signaling pathways (Wilkinson and Davies, 2010). Thompson et al., (2007) investigated the effect of over-expressing two enzymes that are important for the biosynthesis of ABA in tomato plants under drought stress. One of the major findings of this study was that ABA increases the root hydraulic conductivity, which in turn, increases the water movement from the root to the shoot.

Ethylene reduces growth under drought stress

Ethylene plays an important role in plants under drought response. Ethylene synthesis begins in root in response to water deficit. Similar to ABA, ethylene is a long range phytohormone that travels from roots to shoots. Water deficit triggers in the roots the biosynthesis of ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) that is transported via xylem sap to shoot where the enzyme ACC oxidase (ACO) synthesizes ethylene in shoot (Gagne et al., 2004; Young et al., 2004; Manavella et al., 2006; Wilkinson and Davies, 2010). Different studies using mutant and transgenic plants have shed light on the role of ethylene during drought stress. One of the most relevant studies was the work done by Gagne et al., (2004) that shows that ethylene-insensitive Arabidopsis mutants exhibited improved leaf growth. ACC synthase mutants also reveal a possible role of ethylene in decreasing photosynthetic ability under drought conditions (Young et al., 2004). Further molecular characterization has shed light on the mechanism involved in growth suppression induced by ethylene. Skirycz et al., (2011) reported that the cell cycle progression is inhibited by a cyclin-dependent kinase A. This observation was further corroborated by transcriptome data, which shows that ethylene biosynthesis and signaling genes are upregulated during drought stress (Skirycz et al., 2011). More supporting evidence for the role of ethylene in drought stress was published by Manavella et al., (2006), which characterized the transcription factor *Hahb-4* in sunflower. *Hahb-4* is a member of the subfamily I of HD-Zip proteins that is regulated transcriptionally during water stress. This study showed that overexpression of this transcription factor repressed the biosynthesis of ethylene. Consequently, transgenic

plants that overexpressed *Hahb-4* delay leaf senescence, which allow sunflower to maintain photosynthetic activity for a longer time under drought conditions. These results suggest that *Hahb-4* is involved in the regulation of ethylene-related genes (Manavella et al., 2006).

Role of methyl jasmonate and jasmonic acid in drought tolerance

The jasmonic acid (JA) and MeJA have been extensively studied. Their function in plant development and response in biotic stresses is well-established (Blée, 2002; Creelman and Mullet, 1997). By contrast, the role of JAs during drought stress suggests different roles. A molecular and physiological analysis of drought stress in *Arabidopsis* revealed that JAs are involved in stomata closure in early drought responses and homeostasis of plant cell in late drought response, which contribute to yield under drought stress (Suhita et al., 2004; Harb et al., 2010). Furthermore, other studies have shown that JA synthesis and antioxidant activity increases under drought (Sasaki-Sekimoto et al., 2005). Similar results were obtained in study in soybeans, the authors demonstrated that the exogenous application of MeJA increase the activity of antioxidant enzymes such as superoxide dismutase (SOD), peroxidases (POD) and catalase (CAT). This study also showed that the concentration of proline, which is known for its contribution to maintaining osmotic balance, was increased in soybeans as well (Anjum et al., 2011). Other studies have found conflicting results with the possible role of JAs in sustaining yield under drought conditions. A study in rice and peanuts found that the application of exogenous Jas decreased yield (Kim et al., 2009a,b).

Aspergillus flavus and aflatoxin contamination

Aspergillus flavus infects high-oil content crops such as peanuts, nuts, cotton and maize (Amaike and Keller, 2011; Brown et al., 1999; Kelley et al., 2009;). The fungus produces and contaminates the seed with aflatoxin, which is a secondary metabolite that is carcinogenic and highly regulated. Other *Aspergillus* species are known as opportunistic fungi that infect immunosuppressed individuals causing aspergilliosis (Gerson et al., 1984; Lin et al., 2001). The sexual stage of the fungus was recently described and named *Petromyces flavus*. *Petromyces* forms cleistothecia inside the sclerotia, which provide protection for adverse environmental conditions (Varga et al., 2000; Horn et al., 2009; Varga et al., 2011). It was also reported that different strains belong to different vegetable compatibility groups, which would increase sexual diversity (Kwon-Chung and Sugui, 2009).

Conidia (spores) or sclerotia are found in the soil, where the fungus overwinters in the crop debris. Sclerotia germinate and develop conidia that are dispersed by the wind or rain splash. Spore dispersion by rain-splash is the main pathway of infecting cotton and peanuts (Payne, 1998; Zuber and Lillehoj, 1979; Amaike and Keller, 2011; Windham et al., 2005). Spore dispersion by wind is the important method to infect nuts and maize. The conidium colonizes maize kernels through silk channel. Hot and dry conditions favor the fungus infection. Different strains of *A. flavus* exist, strains L and S produced aflatoxin B1 and B2, but only strain S is capable of produce aflatoxin G1 and G2. The first report of aflatoxicosis occurred in the early 1960 when thousands of

turkeys died as a consequence of eating grain contaminated with aflatoxin (Zuber and Lillehoj, 1979; Amaike and Keller, 2011; Abbas et al., 2002; Betran et al., 2002; Betran et al., 2005; Menkir et al., 2006). The most dangerous aflatoxin is the B1, which has been extensively studied, and it has been directly linked to liver cancer. Aflatoxin is metabolized in aflatoxin B1 epoxides and B1-exo-epoxides that intercalate between the bases of DNA of the tumor suppressor protein p53 causing a mutation in the 249 codon (AGG to AGT, R249S) (Liu and Massey, 1992; Bressac et al., 1991; Hollstein et al., 1991; Hsu et al., 1991; Macé et al., 1997; Guo et al., 2003). This mutation is often found in patient with Hepatocellular carcinoma (HCC), and it is a landmark of consumption of grain contaminated with aflatoxin (Liu and Massey, 1992; Bressac et al., 1991; Hollstein et al., 1991; Hsu et al., 1991; Macé et al., 1997; Guo et al., 2003).

Aflatoxin is highly regulated in the U.S. and other countries. Quantities as low as 20 pbb are allowed in grain for human consumption and 5 pbb in grain for dairy cattle (Shephard, 2003; Gilbert and Vargas, 2003; Robens and Cardwell, 2003; Campbell et al., 2003). Economical losses caused by aflatoxin range in the billions of dollars every year in the world and Southern states. Approximately 4 million dollars in losses were reported for grain contaminated with Aflatoxin in Texas in 2011 (RMA-USDA). There are pre-harvest and post-harvest mechanisms of controlling *A. flavus* infection. The use of atoxigenic strains of *A. flavus* is a pre-harvest control and it is currently commercialized under the brand of Afla-Guard by Syngenta originally developed for peanuts, and, AF36, originally developed by the USDA for cotton by the USDA for cotton (Bruns, 2003; Widstrom et al., 2003). Other mechanisms of control are to find

resistance genes that decrease the *A. flavus* infection and aflatoxin accumulation (Windham and Williams, 2002; Widstrom et al., 2003; Williams et al., 2003; Betran et al., 2005; Mayfield et al., 2011;). Different QTLs have been reported for aflatoxin resistance as well as (Mayfield et al., 2012) different lines with better resistance have been released. Post-harvest control includes an appropriate control of grain aeration and temperature during storage (Bruns, 2003; Robens and Cardwell, 2003).

Association mapping and QTL mapping

A quantitative trait locus (QTL) is a region in the genome that is associated with a quantitative trait. A QTL can be identified via linkage or linkage disequilibrium mapping. To identify a QTL via linkage mapping, it is necessary to have a trait with contrasting phenotypes, polymorphic markers and to generate a population (backcross, F2, recombinant inbred lines, etc) from a bi-parental cross. A major advantage of bi-parental QTL mapping is that rare alleles are enriched (Kearsey and Farquhar, 1998; Collard et al., 2005; Flint-Garcia et al., 2005; Cooper et al., 2009; Eeuwijk et al., 2010). However, the major drawbacks of this approach is the low QTL resolution, which sometimes can be of several cM (million base pairs), the evaluation of the QTLs in just two genetic backgrounds and the low number of allele numbers that can be screened. Sibling mating, which consist of several generations of random mating before inbreeding, have been used to increase recombination rate and QTL resolution (Liu et al., 1996; Lee et al., 2002). An alternative method is association mapping that is based on linkage disequilibrium and has potential to greatly increase QTL resolution, and the

evaluation of the QTLs across multiple genetic backgrounds. Linkage disequilibrium mapping is the non-random association of multiple alleles in different loci caused (Flint-Garcia et al., 2003, 2005; Gupta et al., 2005; Yan et al., 2009; Chen et al., 2012). Major advantages of association mapping are that existing inbred lines and breeding populations can be directly used and multiples alleles per locus can be evaluated. One of the major drawbacks of the method is that when the diversity panels and populations exhibit high levels of population substructure and diverse levels of familial relatedness among individuals, spurious associations can occur (Balding, 2006; Neale and Savolainen, 2004; Flint-Garcia et al., 2005; Gupta et al., 2005; Yu et al., 2006; Kang et al., 2008; Atwell et al., 2010; Brachi et al., 2010; Pritchard et al., 2000a,b; Quesada et al., 2010; Varshney et al., 2012).

Other major statistical issue for genome wide association mapping (GWAS) analysis is the appropriate threshold level to declare an association significant due to the multiple testing from tens of thousands to millions of markers. Several approaches have been proposed to account for multiple testing; one of them is the Bonferroni correction, which corrects the experiment-wise error rate (EWER). The Bonferroni correction divides the alpha value into the total number of multiple tests. When a large number of markers are used, Bonferroni correction can be too conservative and lead to type II error. Other issue with the Bonferroni correction is the assumption of independence and no correlation between the multiple tests, which can be problematic since different locations in the genome can have different levels of linkage disequilibrium.

Other statistic that has been widely used in GWAS is the false discovery rate that detects the false associations (Benjamini and Hochberg, 1995; Reiner et al., 2003; Moskvina et al., 2008). FDR can be a good option when many of the null hypotheses are expected to be false (e.g. microarray analysis). However, the consequences of false negatives can significantly impact the results of association mapping since it is expected that few associations are true.

Other major issue with this statistical method is that it assumes independence between the multiple tests. Another approach proposed by Cheverud et a., (2001) circumvent the issue of independence by calculating the effect number of tests (M_{eff}). The M_{eff} is calculated from the eigenvalues obtained in a principal components analysis. Principal components analysis is a dimensionality reduction technique that calculates the eigenvalues and eigenvector from a matrix (Cheverud, 2001; Han et al., 2009). The obtained principal components (PCs) are linear combinations of the original variable that are orthogonal to the other PCs and independent. A modified approach of this method proposed by Gao et al., (2008) calculates the pair-wise composite linkage disequilibrium matrix (Gao et al., 2008, 2010, 2011). The number of PCs required to explain 99.5% of the variation in the dataset is used to calculate the Bonferroni correction. This approach has been shown to perform well in genome wide association analysis with dense marker data (Cheverud, 2001; Gao, 2008,2010,2011).

CHAPTER III

A MULTI-ENVIRONMENT TRIAL ANALYSIS SHOWS A SLIGHT GRAIN YIELD IMPROVEMENT IN MAIZE IN TEXAS*

Introduction

The United States produced 320 million tons of maize in 2011 with an estimated value in the market of 77 billion dollars (National Agricultural Statistical Service [NASS], 2012). U.S. maize is currently grown in approximately 33 million hectares of land, with the majority in the Midwest region. A series of field studies that compared successful hybrids released by Pioneer Hi-Bred International in the Midwest since 1930s - the so-called "ERA hybrids studies", showed that grain yield from genetic improvement has steadily increased over years at a rate of 0.077 ton/ha (Duvick, 1984, 2001; Duvick and Copper 2003; Duvick et al., 2004; Crosbie et al., 2008).This trend of maize yield increase in the U.S. is further corroborated by United States Department of Agriculture (USDA) data collected since 1900 (NASS, 2012). Yield increase in maize has been largely attributed to genetic gain, which accounts for approximately 50%, and improvement in crop agronomic management practices are attributed to the other half (Russell, 1991; Duvick, 1992). These studies evidenced that yield increases were caused by breeding for plants better adapted to stress and capable of production under higher

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planting densities (Duvick, 1977, 1984, 2001; Duvick and Cassman, 1999; Duvick and Cooper 2003; Duvick et al., 2004). A number of traits were also associated with greater biomass accumulation and enhanced plant growth in historical U.S. yields including: number of ears per 100 plants, smaller tassel size, reduced anthesis-silking interval (ASI) and increased stay-green (Duvick, 1977; Duvick, 1984; Duvick et al., 2003; Duvick et al., 2004). Recent studies based on simulations and empirical data have identified that the modification of the harvest index and root structure has a greater direct effect than canopy modification in explaining yield increase and its interaction with plant density (Hammer et al., 2009).

Both the public and private sector played a major role in historical yield increases in maize. Maize varieties were bred and released by land-grant universities, state agricultural experimental stations, and other public agencies in the early 1930s. This role evolved with the consolidation and widespread use of hybrid seed during the 1950s and the approbation of the plant protection and variety act in the 1970s that lead to an increase in the investment in research and development by the private sector (Frey, 1996; Fuglie, 2000; Duvick, 2001; Knudson and Pray, 1991; Huffman and Evenson, 1992; Alston and Venner, 2002; Fernandez-Cornejo, 2004). Private sector investment in maize research has increased fourfold since the 1970s, and it is now estimated that 80% is focused on major corn producing states in the Midwest (Frey, 1996; Fuglie, 2000; Schimmelpfennig et al., 2004).

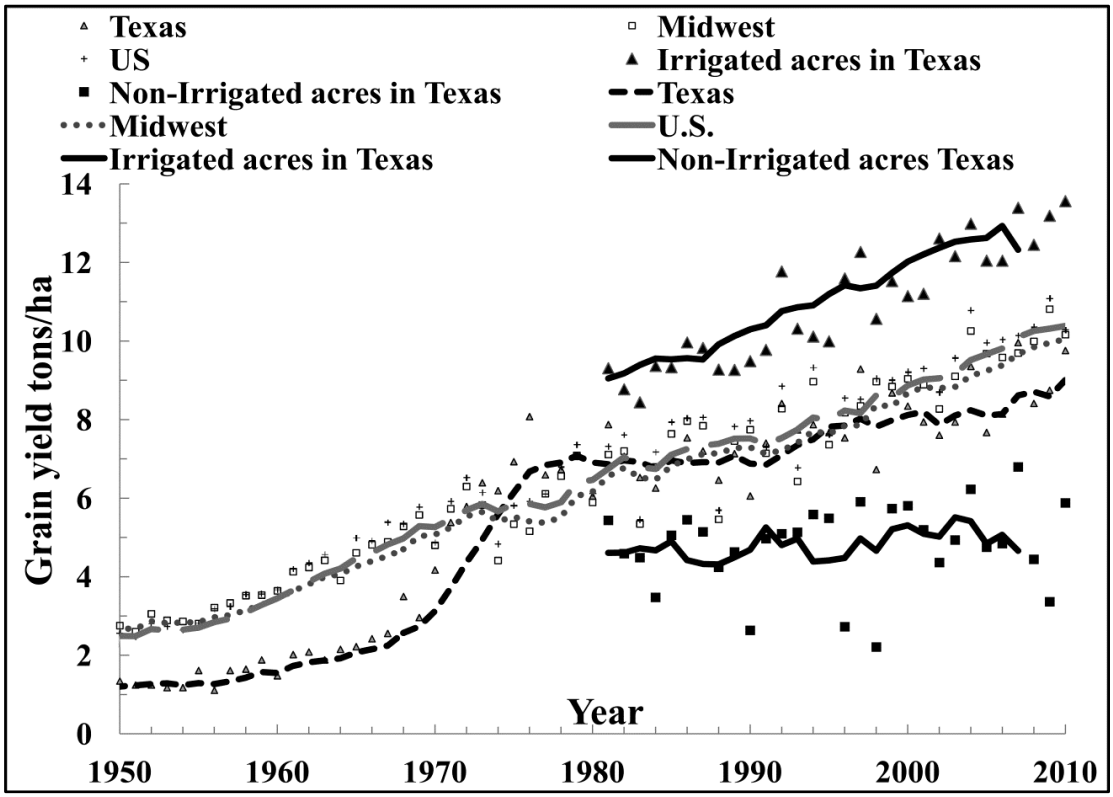
In contrast, seed industry investment in R&D within the southern states which include Texas, have not increased significantly compared to Midwestern states. This is

believed to be because of the smaller market share that those states represent (NASS, 2013). As a consequence, there are few commercial maize breeding programs developing inbreds adapted to specific growing conditions in Southern U.S. To develop new varieties for Texas, inbreds are generally bred and test-crossed elsewhere and the resulting hybrids are evaluated for two to three years in Texas before commercialization. As a likely result, the use of unadapted germplasm has increased major constraints to maize production in Texas and the Southeastern U.S., specifically aflatoxin contamination and drought stress (Payne, 1998; Robens and Cardwell, 2003; Horne et al., 1991; Brown et al., 1999; Widstrom et al., 2003; Williams et al., 2003; Betran et al., 2005; Mayfield et al., 2011). Texas is the largest maize producer of the southern states, and 12th in the U.S. (NASS, 2013). Maize production in Texas was eight million tons in 2010 with a farm gate value in the market of 1.5 billion dollars. County level average maize grain yield data (ton/ha) collected by the NASS service (data available in http://www.nass.usda.gov/) showed that grain yield in the state of Texas has not kept the same pace as the rest of the Midwest states (Figure 3.1). When grain yield data for county level are separated into irrigated and non-irrigated counties, it is evident that the lack of improvement is caused primarily by the dryland maize producing countries, which exhibit greater inter-annual variation (Figure 3.1).

Only 86 out of 254 counties produce maize in Texas, but these occupy a wide geographical range, in turn making each production zone unique in its precipitation, temperature, wind and solar irradiation patterns, types of soils, and agronomy practices. Little work has been conducted to quantify these different environments so far. The

Texas AgriLife (formally Texas Agricultural Experiment Station) maize program began in 1927, and it was complemented by the Texas AgriLife Corn Performance Trials, which began in 1969. These extension based trials are run by the Crop Testing Program housed at Texas A&M University, specifically to test elite hybrids from different companies to provide the most relevant evaluations to regional farmers. Like testing programs in many states, these METs are conducted to provide unbiased information to growers about the best currently available varieties for their area.

Figure 3.1 Five year moving average yields across irrigated and non-irrigated acres in Texas, combined acres in the state of Texas, the Midwest and the entire U.S. Data in these categories were obtained from the USDA (NASS, 2012).



The Texas AgriLife Corn Performance Trials annually evaluate approximately 100 to 200 commercial maize entries every year over 9 to 12 different locations believed to be most representative of the major growing production areas of Texas. The goals of these studies are immediate and no retrospective study has yet been conducted. Approximately 1,500 to 2,200 datapoints are generated each growing season and data are collected for important agronomic traits, including days to silk, plant and ear height, lodging, plant population, grain moisture, test weight, and yield.

The Texas AgriLife Corn Performance Trials historical dataset allows direct investigation of many questions that cannot be addressed by one or a few years of data. In addition, the testing of commercial hybrids in crop's target population of environments represents a measure of hybrid adaptation and performance (DeLacy et al., 1996a,b). The authors believed that by compiling and investigating this data, a number of objectives to elucidate past trends and future hints on how to improve maize yield within Texas would be identified. The goals of this study were: (1) to determine which sources of variation are most important for the MET in the Texas AgriLife Corn Performance Trials; (2) to estimate the reliability of METs when the number of locations, years and replications are increased from which the design of the trials could be modified to increase precision; (3) to elucidate changes and trends in yield potential across the state of Texas over the last eleven years, and to determine which hybrids have been highest yielding; and (4) to investigate the correlation between physiological and agronomic measurements with yield across Texas environments to suggest directions for future improvement.

Materials and methods

Corn performance trial dataset

A total of 11 years (from 2000 to 2010) was investigated from the Texas AgriLife Corn Performance Trials. Testing locations for these corn performance trials are distributed in different maize production areas and cover a large geographical area (Figure 3.2). Trials located in South, Central and East Texas are planted from late February to late March. In contrast, trials in the High Plains are planted from late April through early May. Yield trials are harvested in late July for trials located in South, East and Central Texas. Trials located in the High Plains are harvested from the middle of October to early November. Locations in the High Plains are fully irrigated using center pivot systems, while locations in East, Central and South Texas, when irrigation is available, generally use a furrow irrigation system. Several dryland locations (no supplemental irrigation is available) are included in this dataset (Table 3.1). For Medina county and Williamson county, the test site was changed over the 10 year period. Castroville (CA) and Hondo (HO) are both located in Medina county (Figure 3.2).

Nearly all entries were elite hybrids that were currently commercialized for different maize production areas in Texas or adjacent states (from all major companies). A few entries were experimental hybrids that have at least one inbred developed by maize breeders from Texas AgriLife Research (Texas A&M University and Lubbock research station), these were generally included for data collection and the goal was not commercialization. Within years, not all hybrids were grown in all locations because of

the perceived differences between maize production areas and because not all hybrids are commercially recommended in all areas (Table 3.2 and 3.3).

Figure 3.2. Maize production counties in Texas and testing locations used in the Texas AgriLife Corn Performance Trials from 2000 to 2010. The maize producing counties are highlighted in a grayscale. Dark represent high grain yield, light gray represent low grain yield as obtained from the USDA data (NASS, 2012). Texas AgriLife Corn Performance Trial locations include BA (Bardwell), CA (Castroville), CC (Corpus Christi), CS (College Station), DA (Dalhart), DU (Dumas), GR (Granger), HO (Hondo), HW (Halfway), LE (Leonard), PR (Prosper), SL (Springlake), TH (Thrall), TY (Tynan), WE (Weslaco), and WH (Wharton).

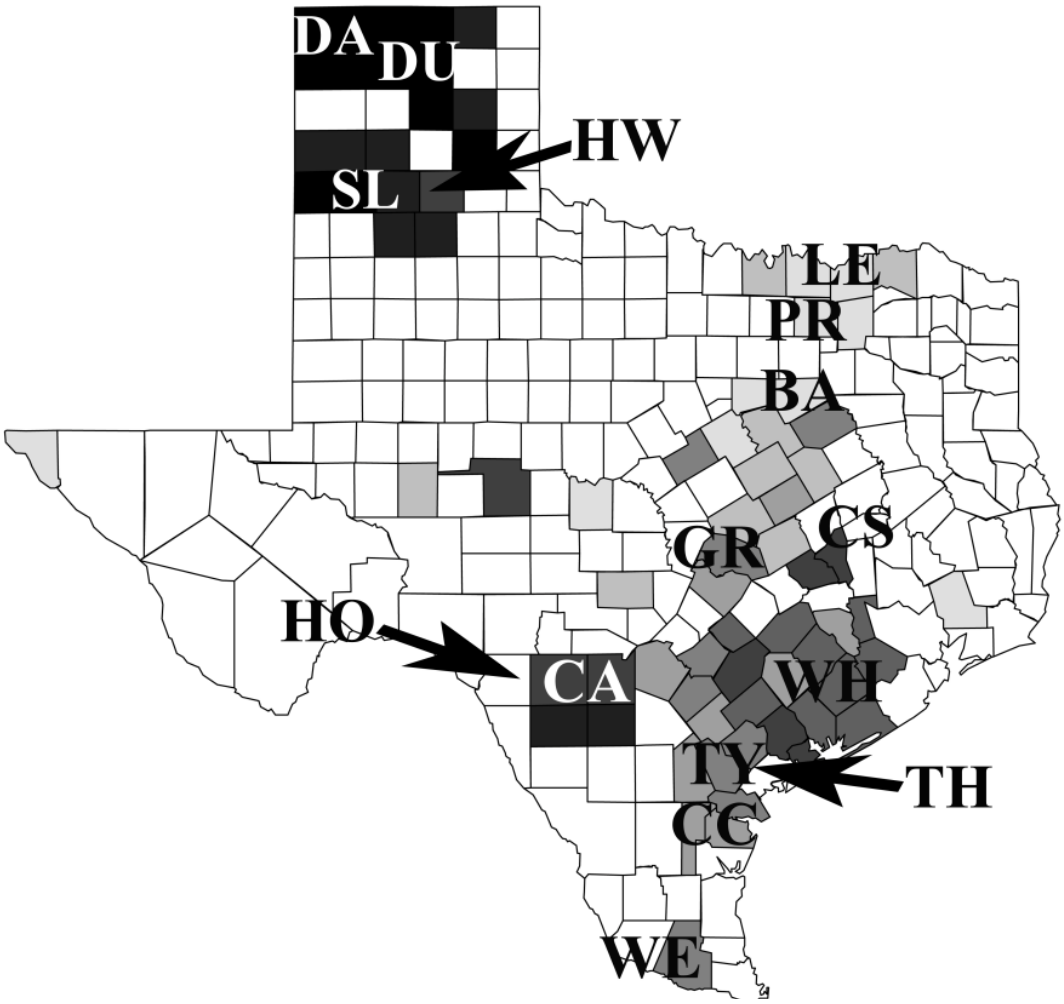


Table 3.1. Characteristics of the different locations in the Texas AgriLife Corn Performance Trials from 2000 to 2010 . RW (row width) is the width in (cm) between rows. Plant population (Ppop) per hectare was calculated based on the average for all years within each location. The planting and harvesting date cover the range where the crop was grown through all years when the locations was used. P. irrigated (partial irrigation through furrow irrigation), IRRI (crop irrigated through central pivot system). Crops abbreviation: MZ (maize), CO (cotton), CUC (cucumber), FA (fallow), SC (sugarcane), SRG (sorghum), SUN (sunflower), SYB (soybean), and WHT (wheat).

Location	County	Soil type	Planting date	Harvesting date	Ppop	Irrigation	RW	Crop rotation
Rest of Texas, dryland locations								
BA	Ellis	Houston black clay	Middle March	Late August	56,871	None	76.2	CO, SYB, WHT
CC	Nueces	Victory clay	Late February	Late July	49,814	None	96.5	CO, FA, SRG
GR	Williamson	Houston clay	Early March	Early September	50,975	None	96.5	MZ, SRG
LE	Fannin	Houston black clay	Early April	Early September	43,814	None	76.2	MZ
PR	Collin	Houston black clay	Middle March	Early September	48,141	None	76.2	FA, SRG, WHT
TH	Williamson	Burleson clay	Late March	Late August	51,398	None	96.5	CO
TY	Bee	Victory clay loam	Late February	Middle August	65,900	IRRI	76.2	CO
WH	Wharton	Lake Charles clay loam	Early March	Middle August	57,328	None	101.6	MZ, CO
Rest of Texas, non dryland locations								
CA	Medina	Castroville clay loam	Early March	Late August	62,159	IRRI	91.4	MZ, WHT
CS	Brazos	Ships clay loam	Late February	Middle of August	67,509	P. Irrigation	76.2	MZ, SRG, CO
HO	Medina	Montell clay	Early March	Late August	59,313	IRRI	91.4	MZ, CUC, WHT
WE	Hidalgo	Raymondville clay loam	Middle February	Middle July	58,383	P. Irrigation	101.6	CO, SC
High Plains								
DA	Dallam	Dally sandy loam	Late April	Middle November	74,512	IRRI	76.2	WHT, SUN
DU	Moore	Sherman silty clay loam	Late April	Middle October	73,425	IRRI	76.2	MZ, CO, SRG, WHT
HW	Hale	Pullman clay loam	Late April	Late September	59,992	P. Irrigation	101.6	CTN
SL	Lamb	Olton sandy loam	Late April	Early October	65,802	IRRI	101.6	CTN

Table 3.2. Number of hybrids common across locations for grain yield (ton/ha) and other traits in the Texas AgriLife Corn Performance Trials for 2000 to 2010 (diagonal are total hybrids evaluated in individual locations). Number of locations and datapoints for grain yield per location is given in the table.

Locations	BA	CA	CC	CS	DA	DU	GR	HO	HW	LE	PR	SL	TH	TY	WE	WH
BA	**311**															
CA	73	**111**														
CC	82	37	**115**													
CS	177	68	77	**264**												
DA	68	11	24	60	**177**											
DU	84	26	31	73	134	**239**										
GR	155	72	64	136	41	57	**237**									
HO	100	12	46	110	44	39	59	**142**								
HW	7	6	3	7	3	19	6	0	**21**							
LE	26	0	8	22	9	11	5	18	0	**32**						
PR	164	58	53	117	47	58	129	57	7	7	**216**					
SL	26	25	9	26	5	55	28	0	17	0	22	**87**				
TH	45	0	27	42	19	18	4	36	0	6	3	0	**50**			
TY	65	8	54	72	30	28	30	67	0	17	27	0	35	**91**		
WE	123	55	71	141	39	51	82	92	6	18	76	16	31	58	**251**	
WH	218	68	92	199	59	77	142	114	7	25	142	26	46	78	147	**312**
Grain yield datapoints	1729	588	516	1452	945	1389	1276	718	104	94	1197	432	192	426	1465	1724

Locations are abbreviated using: BA (Bardwell), CA (Castroville), CC (Corpus Christi), CS (College Station), DA (Dalhart), DU (Dumas), GR (Granger), HO (Hondo), HW (Halfway), LE (Leonard), PR (Prosper), SL (Springlake), TH (Thrall), TY (Tynan), WE (Weslaco), and WH (Wharton).

Table 3.3. Number of hybrids common across years for grain yield (ton/ha). The hybrids were tested in the Texas AgriLife Corn Performance Trials for 2000 to 2010 (diagonal are total hybrids evaluated in individual years).

Years	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
2000	**84**										
2001	23	**89**									
2002	14	27	**80**								
2003	10	15	27	**88**							
2004	6	7	8	19	**104**						
2005	3	3	4	9	25	**128**					
2006	2	2	2	6	19	39	**134**				
2007	2	2	2	5	11	21	42	**121**			
2008	0	0	0	0	4	10	19	26	**121**		
2009	0	0	0	0	0	1	3	5	21	**97**	
2010	0	0	0	0	0	2	2	2	7	23	**94**
Datapoints	1268	1129	888	947	1135	1457	1599	1570	1327	1127	1800
Mean grain yield (ton/ha)	8.92	7.96	9.44	8.90	10.94	8.60	8.28	11.59	8.33	7.85	9.65

Number of locations, datapoints and arithmetic means for grain yield per year is given in the table. Across the entire dataset, the average grain yield was 9.17 ton/ha

Across years, the data were unbalanced and no single hybrid was grown in all years (Table 3.3). All trials were laid out in a randomized complete block design (RCBD) with four replications and 20-45 entries per trial. Plots were harvested using a John Deere 3300 combine equipped with an HM-1000B Grain Gauge (Harvestmaster, Logan, UT). Although data are available for years before 2000, these samples were hand harvested and they were believed to be incomparable to combine collected data. For grain yield, a total of 14,568 datapoints were collected, and approximately the same number were collected for plant height, ear height, days to silk, lodging, number of plants per plot / plant population, test weight and grain moisture (Table 3.4).

Table 3.4. Summary of raw data collected for the Texas AgriLife Corn Performance Trials from 2000 to 2010 across all locations. A total of 107 different trials, 11 years for each trait with four reps per location.

Trait	Datapoints	Min	Mean	Max	SD
Days to silk	13,020	55	72.88	92	5.9
Plant height (cm)	13,923	134.62	241.55	355.6	31
Ear height (cm)	13,915	27.94	91.58	180.34	19.8
Plant density (plants/ha)	14,057	10,252	59,492	106,443	4,349
Lodging (% lodged plants /plot)	13,714	0	4.1	85	9.85
Test weight (kg/hl)	14,199	58.91	74.19	84.48	2.93
Grain moisture (%)	14,346	6.9	13.23	33.7	2.83
Yield (ton/ha)	14,247	0.81	9.18	20.7	3.64

Number of datapoints, minimum (min), arithmetic mean (mean), maximum (max), and standard deviation (SD)

Plant height was measured as the number of centimetres from ground to top of the tassel. Ear height was measured as the number of centimetres from ground to the bottom ear node. Days to silk were measured by 50% of the plants in a plot showing any silks. Plant population was expressed as plants per hectare, calculated from number of plants in a harvested plot multiplied by a hectare conversion factor. Lodging was calculated as the percentage of plants per plot that were lodged or broken below the ear. These counts were made at the time of harvest. Grain moisture was determined at harvest with the HM-1000B Grain Gauge mounted on the plot combine. Moisture was expressed as percentage of weight. Test weight was determined at harvest with the HM-1000B Grain Gauge and was expressed as kg/hl. Yield values determined at harvest with the HM-1000B Grain Gauge were corrected to 15.5% moisture and expressed as ton/ha. All raw data have been posted at http://maizeandgenetics.tamu.edu/CTP/CTP.html

Principal component analysis of Texas county level yield data

A principal component analysis (PCA) was performed using the raw means per county level from 2000 to 2010 for grain yield data (NASS, 2012) to determine the relationship between maize producing counties based solely on yield data (ton/ha). The NASS data are freely and publicly available from http://www.nass.usda.gov/ . Counties that had from 8 to 10 missing datapoints were discarded from this analysis. The matrix of years-by-counties was centered by columns through subtraction of the county mean (mean centered) and normalized by dividing by the standard deviation. The Bayesian PCA (bPCA) approach, originally developed for analyzing missing microarray data (Oba et al., 2003) was used because of the amount of missing observations which this method tolerates (Oba et al., 2003). This method combines an expectation maximization (EM) approach together with a Bayesian model to calculate the likelihood for a reconstructed value.

Statistical analysis

The analysis of MET data was complicated by the endemic unbalanced nature as a result of changing entries, locations, checks and missing plots or locations. Many different methods have been devised for dealing with imbalanced METs. The general linear mixed model using the residual maximal likelihood (REML) approach provides a powerful method to analyze any linear model with or without covariates (Patterson and Thompson, 1975; Gilmour et al., 1995; Smith et al., 2001, 2005; Gilmour et al., 2009). A one step linear mixed model that accommodates different residual error variance per

environment was used to analyze the Texas AgriLife Corn Performance Trials (Alison et al., 2001; Smith et al., 2005). The phenotypic observation y_{ijkl} on hybrid i in replicate j of location k and year l was modeled as:

$$Y_{ijkl} = \mu + e_k + y_l + e*y_{kl} + g_i + (r/e/y)_{jkl} + (g*y)_{il} + (g*e)_{ik} + (g*e*y)_{ikl} + \varepsilon_{ijkl} \quad (3.1)$$

where, μ is the grand mean; e_k is the fixed effect of location k, y_l is the fixed effect of year l, $(ey)_{kl}$ is the fixed effect of the interaction between year l and location k; g_i is the random effect of hybrid i and is ~ NID (0, σ^2_g), i= 1, . . .,847; $(r/e/y)_{jkl}$ is the random effect of replication j nested in location k and year l and is ~ NID (0, σ^2_r), j= 1, . . .4; $(gy)_{il}$ is the random effect of the interaction between hybrid i and year l and is ~ NID (0, σ^2_{gy}); $(ge)_{ik}$ is the random effect of the interaction between hybrid i and location k and is NID (0, σ^2_{ge}); $(gey)_{ikl}$ is the random effect of the interaction between hybrid i in location k and year l and is NID (0, σ^2_{gey}) and ε_{ijkl} is the random residual effect for hybrid i in the replication j of location k and year l (experimental error) and is NID (0, $\sigma^2_{\varepsilon(lxk)}$). The components of variance and standard error of random terms were estimated using REML (Patterson and Thompson, 1975); the best linear unbiased estimators (BLUEs) and best linear unbiased predictors (BLUPs) were computed using the sparse average information algorithm as implemented in ASREML (Gilmour et al., 1995, 2009).

Repeatability was estimated as follows (Rasmusson and Lambert, 1961):

$$H = \frac{\sigma^2_g}{\sigma^2_g + \sigma^2_{ge}/e + \sigma^2_{gy}/y + \sigma^2_{gye}/ey + \sigma^2_e/rey} \quad (3.2)$$

Using the estimated variance components for hybrids (σ^2_g), hybrid-by-location interaction (σ^2_{ge}), hybrid- by-year interaction (σ^2_{gy}); hybrid-by-location-by-year interaction (σ^2_{gey}) and residual variance (σ^2_ε) obtained from the linear mixed model described in Eq. (3.1). The effect of different combinations of locations and years on hybrid-mean repeatability was evaluated by substituting numerical values in Eq. (3.2) and plotting.

Relationship between the different traits and maize yield

The relationships between grain yield, days to silk, plant and ear height, test weight and grain moisture were evaluated using PCA. Because of the different scales of measurement, the matrix of hybrids-by traits was centered by columns through subtraction of the trait mean (mean centered) and normalized by dividing by the standard deviation. The principal components of the squared Euclidean distance matrices of hybrids by traits were calculated by single value decomposition and biplots (Gabriel, 1971) of the first two PCs using the R statistical package (R Development Core Team, 2013). The Pearson correlation between grain yield and plant and ear height, plant

population, lodging, test weight, and grain moisture were calculated separately for the High Plains and the rest of Texas testing locations.

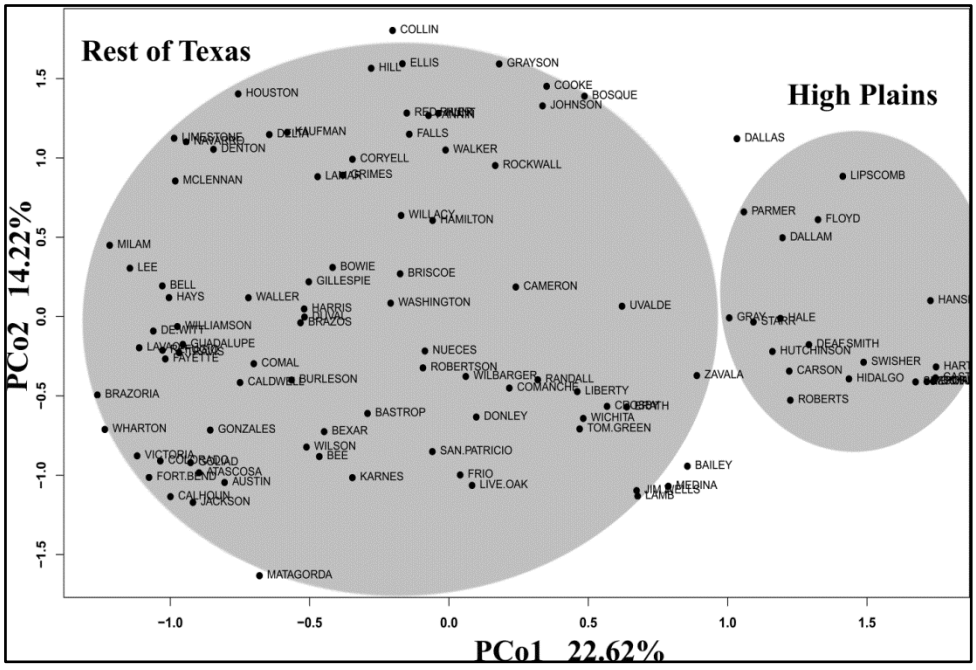
Results and discussion

Maize production in Texas

Plotting the county level average maize yield data (ton/ha) obtained from the USDA-NASS (http://www.nass.usda.gov/) by year evidenced that the lack of improvement for the average grain yield for the state of Texas is largely a product of the non-irrigated counties which experience significant inter-annual variation (Figure 3.1). It is noticeable that for the non-irrigated counties grain yield improved with huge and widening gaps between the best and worst years. Breeding for drought is complex because of the variation on timing of moisture stress, temperature, solar irradiation, wind, relative humidity and other environmental conditions. USDA county level yield data contained 1155 observations of counties average yield, acreage and production. There were 212 missing observations in corn producing counties, likely from a lack of production in these counties in specific years accounting for 20% of the data. Major production zones were also determined from USDA-NASS maize yield data per county from the last 11 years and the first two principal components accounted for 36.84 % of the variation. Despite this data having been mean-centered and normalized, the first principal component divides the counties based approximately on grain yield; counties above the mean are counties that have irrigation and have higher yields than the average grain yield for Texas. Plotting the first two principal components, neighboring counties

tended to group together. It was evident that production counties located in the High Plains form a group (Figure 3.3). The northern High Plains region has the highest maize yields in Texas; this zone is characterized by high daytime temperatures, low humidity and high evaporation rates and demands a large supplemental irrigation to achieve maximum yields (Colaizzi et al., 2009; Kapanigowda et al., 2010; Allen et al., 2011).

Figure 3.3. Bayesian principal component analysis of maize grain yield (ton/ha) per county in Texas from 2000 to 2010.Two major production areas are recognized by plotting the scores of the first two principal components, the High Plains and rest of Texas. All data are publically available from USDA website (NASS, 2012). Data were centered and standardized to unit variance before analysis.



Additional counties with higher values of the first principal component are located in the South and some areas in Central Texas, where supplemental irrigation could be available. The counties of Uvalde and Zavala were plotted close to the High

Plains and are located on the highly productive area of the Winter Garden located west from San Antonio. Growers in these counties can use water for irrigation from the Edwards aquifer. The counties that had the lowest yields are located in Central Texas and the Coastal Bend. The precipitation pattern among the years investigated was highly variable and counties located in the Coastal Bend generally could not use water for irrigation because of high salinity. The second principal component corresponds weakly to a north (higher) south (lower) trend but no other pattern was recognized. Based on the plot of the first two principal components, the rest of Texas counties cannot be further separated based on the PCA scores. Consequently, it was decided to divide the maize production zones in Texas in two main areas: the High Plains and the rest of Texas (Figure 3.3).

Analysis of MET data from the Texas AgriLife Corn Performance Trials

Since the year 2000, the Texas AgriLife Corn Performance Trials have had 107 yield trials in 16 locations testing a total of 847 different maize hybrids representative of Texas growing conditions. The summary statistics for the 107 trials, which include the minimum, mean, maximum, and standard deviation calculated from the original data exhibited a large amount of variation across years, hybrids, and locations (Table 3.4). When the trials were divided into locations from the High Plains, and rest of Texas, it was evident that yield, plant and ear height, and plant population means were all much higher for the High Plains (Table 3.5).

The residual variance obtained after fitting the linear mixed model (Eq. 3.1) was different between locations nested in years for grain yield, plant and ear height (data not shown). The three factor hybrid-by-location-by-year interaction was the largest component of variance of the G x E term for grain yield, plant height, ear height, days to silk, grain moisture and test weight (Table 3.6). The magnitude of the variation explained by the hybrid (g) component for all four traits ranged from 29.19% (grain yield) to 64.3% (grain moisture).

Comparing the magnitude of the genotype by enviornment (G x E; same as the hybrid by enviornment in maize) component of variance to other studies, it is evident that studies in other crops have also reported the three factor hybrid-by-location-by-year interaction as the largest component of variance for G x E. Basford and Cooper, (1998) showed that the three factor genotype-by-location-by-year interaction was the largest component of variation for the wheat MET trials data from two Australian wheat databases. Cooper et al., (1996) summarized different studies that documented the magnitude of the G x E interactions in Queensland. Both the two factor genotype-by-location and three factor genotype-by-location-by-year interactions appear to be the largest component of variance in separate MET studies.

Similar results were reported for MET trials in sorghum and rice (Cooper et al., 1999; Chapman et al., 2000; DeLacy et al., 2010a,b). Smith et al., (2001) reported that the four factor interaction genotype-by-region-by-location-by-year was the largest component of variance for the wheat MET dataset collected for the South of Australia.

Table 3.5. Summary of the Texas AgriLife Corn Performance Trials data for the different traits from 2000 to 2010 separated for the High Plains and the rest of Texas.

	High Plains			Rest of Texas		
Trait	**Min**	**Mean**	**Max**	**Min**	**Mean**	**Max**
Days to silk	63	75.66	90	55	72.39	92
Plant height (cm)	215.9	280.59	355.6	134.6	234.7	330.2
Ear height (cm)	73.66	117.97	180.34	27.94	86.96	157.5
Plant density (plants/ha)	31,862	73,595	106,443	10,252	57,072	92,271
Lodging (% lodged plants/plot)	0%	3%	56%	0%	4.30%	85%
Test weight (kg/hl)	64.61	74.43	81.18	58.91	74.14	84.48
Grain moisture (%)	10.4	17.33	33.7	6.9	12.44	22.3
Yield (ton/ha)	4.56	14.45	20.7	0.81	8.14	18.29

Number of missing datapoints, minimum (min), average (mean), maximum (max), and standard deviation (SD).

Other studies that did not partition the G x E interaction into different components (genotype-by-location, genotype-by-year, and genotype-by-location-by-year interactions) still evidenced that the ratio between magnitude of the G x E variance and genotypic variance is greater, which indicated strong G x E interaction for MET data collected for maize (Chapman, 2008; Chapman et al., 1997), quinoa (Bertero et al., 2004) and sunflower (de la Vega et al., 2007a;b). One possibility for a lower magnitude of the genotype-by-location interaction in our study was the use of elite and commercial hybrids that have been tested extensively by private industry for broad adaptation in different locations before commercial release. This in turn implies that these hybrids should be found to exhibit a wide adaptation, high yield mean and decreased variability (Cooper et al., 2008).

Table 3.6. Estimated variance components (±standard errors) and percentage of variation explained for grain yield, plant height, ear height, days to silk, test weight and grain moisture. These were derived from the locations (e), hybrids (g), replications (not reported), years (y), experimental error (ε), and the interactions of the entire dataset. The percentage of variation explained for each term was calculated by dividing the amount of variation explained by the sum of all variance components in the linear mixed model.

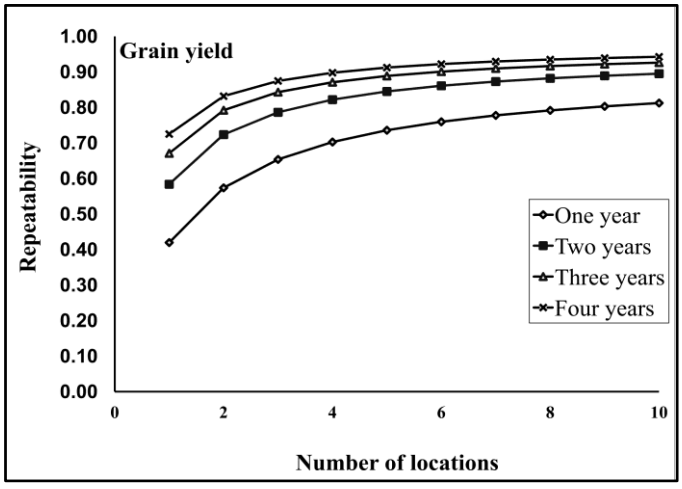
	Grain yield (ton/ha)		**Plant height (cm)**		**Ear height (cm)**		**Days to silk**		**Grain moisture (%)**		**Test weight (kg/hl)**	
σ^2_g	0.51 ± 0.04	29.19 %	93.33 ± 5.95	45%	61.85 ± 3.89	46%	2.76 ± 0.17	58%	1.15 ± 0.07	64.3%	2.43 ± 0.15	58%
σ^2_{ge}	0.02 ± 0.02	1.32 %	3.72 ± 2.52	1.8%	2.53 ± 0.85	1.89%	0.07 ± 0.03	1.4%	0.07 ± 0.01	4.13%	0.09 ± 0.03	2%
σ^2_{gy}	0.05 ± 0.01	2.98 %	5.57 ± 4.11	2.7%	2.91 ± 0.80	2.18%	0.1 ± 0.03	3%	0.01 ± 0.01	0.37%	0.08 ± 0.03	2%
σ^2_{gey}	0.21 ± 0.02	11.95%	10.47 ± 6.41	5%	5.24 ± 0.96	3.92%	0.31 ± 0.03	7%	0.19 ± 0.01	10.5%	0.48 ± 0.04	12%
σ^2_{ε} [a]	0.83 ± 0.12	48. 14%	86.19 ± 12.04	42%	58.73 ± 0.96	43.8%	1.36 ± 0.19	29%	0.37 ± 0.05	21%	1.09 ± 0.16	26%

[a] Variance components and standard errors were averaged from the residual error obtained for each of the 107 trials

Hybrid-mean repeatability in the Texas AgriLife Corn Performance Trials

Hybrid-mean repeatability is important because indicates the extent of testing necessary to maximize genetic gain. Hybrid-mean repeatability was estimated from the components of variance estimated using the linear mixed model (Eq. 3.1) (Figure 3.4). Based on these estimates, the repeatability for one year and one environment was 0.41 for two replications, and for four years and one environment was 0.72 for two replications. The Texas AgriLife Corn Performance Trials always use four replications and also suggest companies enter their hybrids for three years and multiple locations.

Figure 3.4. Predicted hybrid-mean repeatability for grain yield (ton/ha) in the maize production areas in Texas as the number of testing locations and years are changed using two replications. The repeatability was calculated based on the components of variance given in Table 3.6 and estimated using the mixed model and Eq. (3.2) from Texas AgriLife Corn Performance Trial data between 2000 and 2010.



Based on the trends from these results, testing the hybrids in two years and two replications for the Texas AgriLife Corn Performance Trials would give a nearly adequate estimate of performance. However, increasing the number of different

environments and the number of years is advisable since the largest component of variation for the G x E interaction was the three factor hybrid-by-location-by-year interaction. This hybrid-mean repeatability was similar to those reported in studies of other crops. In sunflower, the yield repeatability has been estimated as 0.63-0.77 for one and three replicates for 10 trials (de la Vega et al., 2007a,b; DeLacy et al., 2010a,b). For stover yield of dryland Rabi sorghum (DeLacy et al., 2010a,b), the estimated repeatability was approximately 0.7 for 15-20 trials, two years and two replicates per trial.

Changes in grain yield in Texas since 2000

A major motivation of this study was to confirm the trend observed in the USDA county level data (Figure 3.1) that Texas grain yield has remained steady over 20 years for dryland counties. The BLUPs for the hybrid grain yield based on the linear mixed model (Eq. 3.1) and all environments were regressed against the first year of testing. The estimated trend-line slope was 0.0078 ton/ha (Figure 3.5). Regressing the BLUPs for the top five hybrids against the first year of testing increased the trend-line slope to 0.0189 ton/ha (Figure 3.6). Both of these values are smaller than the 0.077 ton/ha reported by Duvick et al., (2004). Based on these results, it can be concluded that genetic increases in grain yield have not nearly kept the same pace than the average of the U.S. and Midwest. In fact, the hybrids with the highest BLUPs were first tested in 2002 and 2006 (Table 3.7), which corroborates the trend observed in the USDA data. In order to discard that the differences in grain yield between the High Plains and the rest of Texas

testing locations were causing this effect, an analysis was performed separately for the High Plains and the rest of Texas. The same pattern was observed for the rest of Texas testing locations (results not shown). In contrast, the estimated trend-line slope obtained for the High Plains testing locations by regressing the BLUPs of all hybrids tested against the first year of testing was of 0.0305 ton/ha (Figure 3.7). This value is about 40% of the increase reported by other authors (Duvick et al., 2004). Interestingly, when the BLUPs for the top five hybrids for the High Plains were regressed against the first year of testing, the trend-line slope was 0.0088 ton/ha (Figure 3.8).

Figure 3.5. Genotypic BLUPs for grain yield for all the hybrids tested in the Texas AgriLife Corn Performance Trials from 2000 to 2010. The BLUPs were obtained from the linear mixed model described in Eq. (3.1). The BLUPs for the commercial hybrids tested in 107 trials across all Texas testing locations from 2000 to 2010 were regressed against the first year of testing.

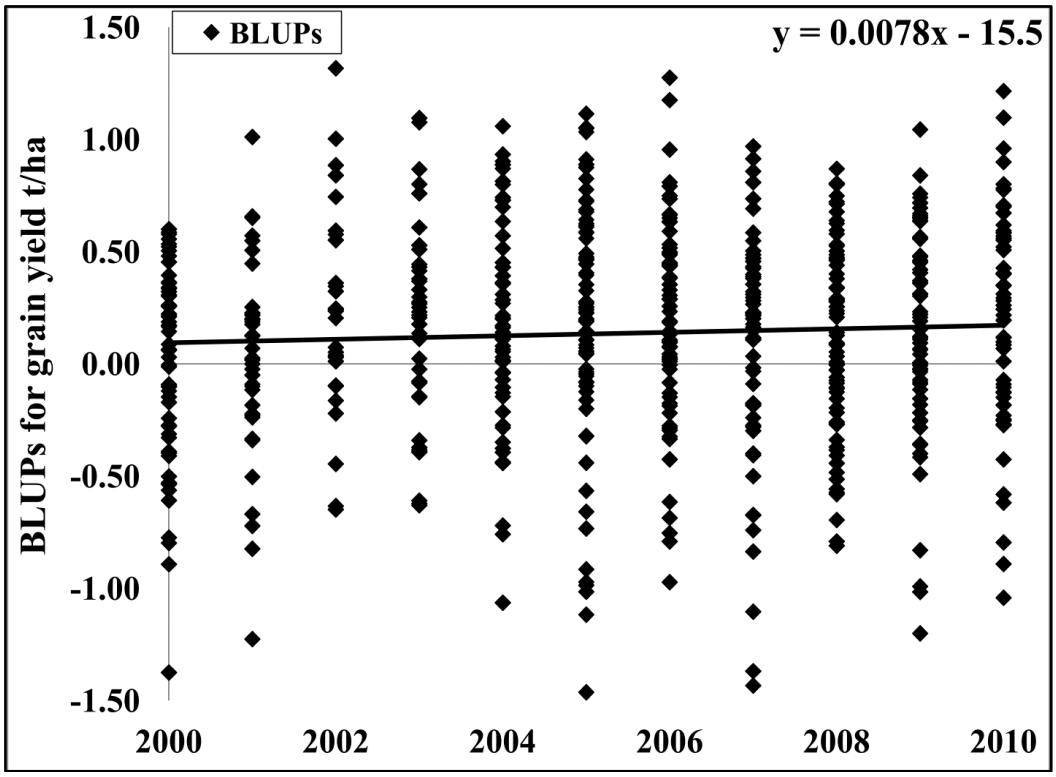
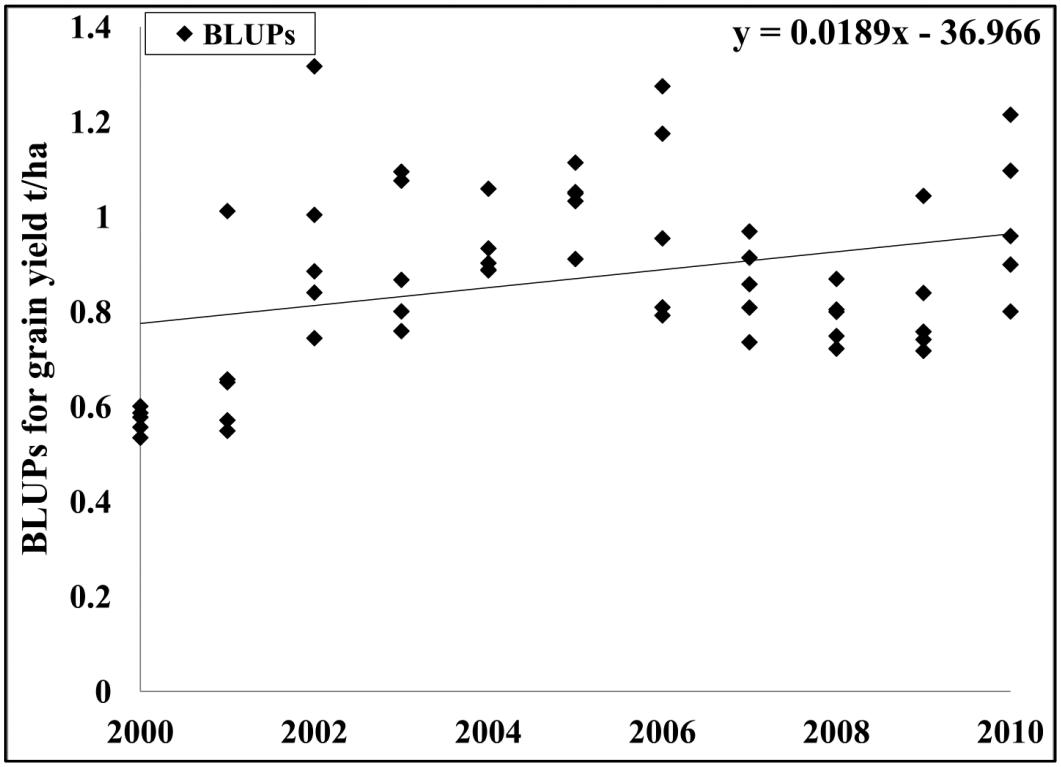


Figure 3.6. Genotypic BLUPs for grain yield for the top five hybrids per year tested in the Texas AgriLife Corn Performance trials from 2000 to 2010. The BLUPs were obtained using the linear mixed model described in Eq. (3.1). The BLUPs for the top five commercial hybrids per year tested in 107 trials across all Texas testing locations from 2000 to 2010 were regressed against the first year of testing.



This value was similar to the value estimated when the BLUPs from the combined analysis were regressed against the first year of testing (Eq. (3.1)) (0.0078 ton/ha). The best linear unbiased estimators (BLUEs) for grain yield for the year (fixed) effect obtained using the linear mixed model (Eq. (3.1) were plotted against year to ensure that the variation was not partitioned into this term of the model. Significant inter-annual variation was observed. This variation is in part explained by the drought episodes that occurred during the growing seasons of specific years (http://droughtmonitor.unl.edu/dmtabs_archive.htm).

Table 3.7. BLUPs and standard error (SE) for the worst and best 10 hybrids respectively tested in the Texas AgriLife Corn Performance Trials from 2000 to 2010. The number of trials tested is over all years and locations.

Hybrid	BLUP (ton/ha)	SE	First year of testing	Number trials tested
RX718RRYG	-1.662	0.532	2004	1
Cazador	-1.584	0.47	2009	1
TRX01601X	-1.572	0.433	2010	2
WWFH07	-1.463	0.453	2005	1
TG891W	-1.433	0.445	2007	1
5660	-1.375	0.468	2000	2
W2612	-1.369	0.458	2007	1
F76.225	-1.225	0.434	2001	3
DonAbel	-1.201	0.470	2009	1
NF246	1.052	0.339	2005	4
8292YG1	1.059	0.254	2004	10
x676.26RRBT	1.076	0.341	2003	5
CXO3415	1.095	0.491	2003	2
Exp942117	1.097	0.398	2010	2
TV26B34	1.114	0.372	2005	3
CXO5819	1.175	0.48	2006	2
DKC6469GENVT3P	1.215	0.537	2010	1
6361RB	1.275	0.2467	2006	10
5202B	1.317	0.4419	2002	3

The years of 2006, 2008, and 2009 had the lowest BLUEs for the year effect of grain yield, and corresponded to years limited by drought. Those years with the lowest grain yield BLUEs for the year effect also grouped together when the loadings of the two

principals components from the bPCA were plotted (results not shown). These loadings reveal the relationships and influence of the different years for grain yield and reinforced results shown in Figure 3.9. Previous studies have used the best historical hybrids, across time, to estimate genetic gain (Duvick, 1977, 1984, 1992; Duvick and Cassman, 1999; Duvick and Cooper, 2003; Duvick et al., 2004). A major drawback of those studies is the fact that agronomic practices have changed through time and the common testing environments are not representative of the target environments the hybrids were developed for.

Some authors have suggested that MET data provides an opportunity to gain historical perspectives since this expands the number of hybrids and represents the target environments with relevant agronomic practices (de la Vega et al., 2007a,b). A major limitation of using MET data across years is that there may not be sufficient checks or controls to ensure data compatibility across years or locations. The MET data collected in the Texas AgriLife Corn Performance Trials have common checks between many years and locations to ensure data compatibility (Tables 3.2 and 3.3). The inbred lines used in the commercialized Texas hybrids over the last 11 years have not been specifically bred for Texas growing conditions, and are likely not directly related, so it would not be appropriate to refer to genetic gain in the traditional sense. However, by regressing the hybrid's BLUPs against the first year of testing, it is possible to estimate the yield increases of commercial relevant material through time.

Figure 3.7. Genotypic BLUPs for grain yield for all the hybrids tested in the High Plains in the Texas AgriLife Corn Performance trials from 2000 to 2010. The hybrid effect was obtained using the linear mixed model described in Eq. (3.1). The BLUPs for the commercial hybrids tested in 18 trials across the High Plains locations of the Texas AgriLife Corn Performance Trials from 2000 to 2010 were regressed against the first year of testing.

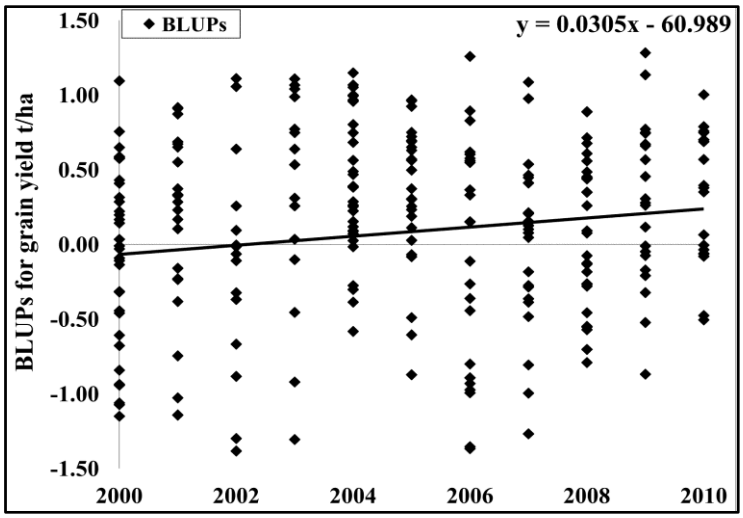


Figure 3.8. Genotypic BLUPs for grain yield for the top five hybrids per year tested in the High Plains in the Texas AgriLife Corn Performance trials from 2000 to 2010. The hybrid effect was obtained using the linear mixed model described in Eq. (3.1). The BLUPs for the top five commercial hybrids per year tested in 18 trials across the High Plains locations of the Texas AgriLife Corn Performance Trials from 2000 to 2010 were regressed against the first year of testing.

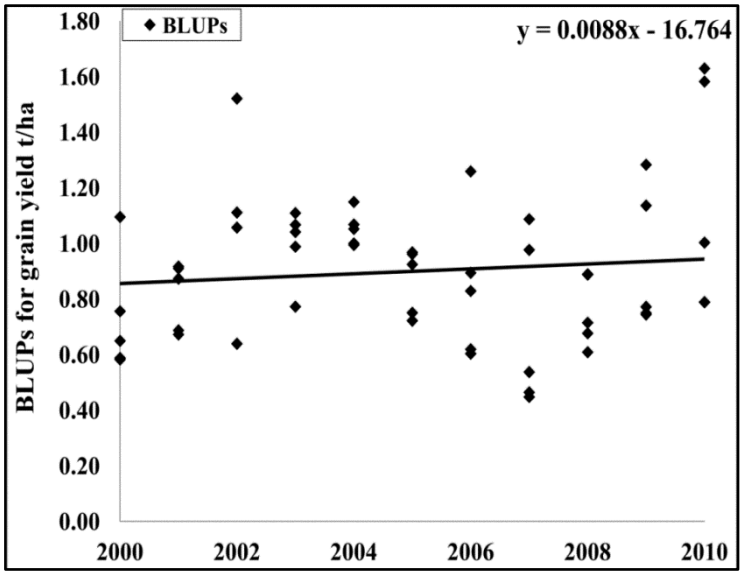
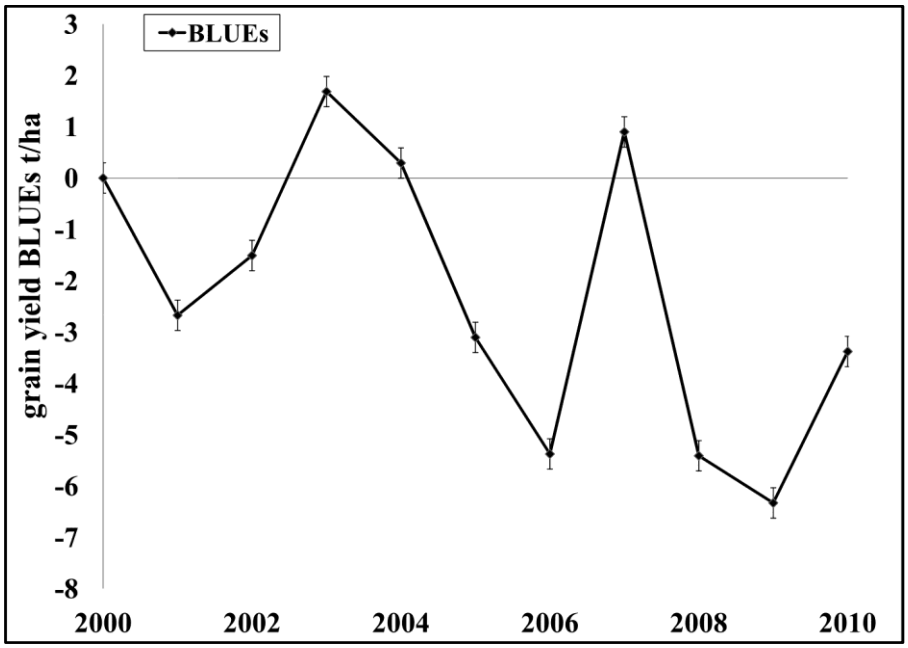


Figure 3.9. BLUEs of grain yield for the year effect. The BLUEs were obtained using the linear mixed model described in Eq. (3.1) of the Texas AgriLife Corn Performance Trials from 2000 to 2010. Error bar are the standard error for the BLUEs



Overall, USDA/NASS data suggest that grain yield has been steadily increasing for irrigated maize acres in the High Plains, while remaining stable for dryland and partially-irrigated acres (Figure 3.1). In contrast, this MET analysis suggests that yield has not increased at the same pace as the Midwest for the last 11 years anywhere in Texas. Because the majority of irrigated maize is located on the High Plains; the yield increases observed in the county level data for the USDA/NASS data could be the result of improved agronomics or increased use of water resources. The High Plain irrigated hectares have depended on the Ogalla aquifer for irrigation. A growing concern is that water has been used at a rate exceeding replacement and it is expected that those resources will be more limited in the future. This suggests a need for maize breeding

programs to develop lines and hybrids that are adapted to the specific growing conditions in Texas and are heat and drought tolerant as well as water use efficient. It is interesting that grain yield increases when the grain yield for all the hybrids is regressed against the first years of testing for the High Plains trials, but, is generally similar to the rest of Texas when only the top five hybrids are regressed (trend-line slope of 0.0088 ton/ha). The differences between the trend-line slopes could be a result of improvement of the average available commercial hybrids with little genetic improvement in the best commercial hybrid

Other possible reason for the slower improvement of grain yield in Texas is that plant densities in the different maize producing counties are lower and have not increased as fast as in the Midwest (Table 3.5). Duvick et al., (2004) clearly demonstrated that maize grain yield increased in the Midwest by breeding for plants capable of producing under higher densities and stress conditions. In order to increase plant densities, it is necessary to further increase plant stress tolerance to already stressful Texas growing conditions. Breeding for heat and drought tolerance will be essential to increase plant density in all maize growing counties. This further strengthens the argument that the primary explanation for the lack of grain yield gains for the state of Texas is the lack of line and hybrid development programs under Texas conditions. Another explanation, such as changing crop rotations, could be possible because maize may be grown after cotton in different maize producing counties depending on yearly commodity pricing. Cotton is well-known by its dehydrating capabilities with respect to residual soil moisture; nonetheless, this analysis cannot determine the effect of rotating

maize after cotton based on the data collected. Additional alternative explanations such as a change in the amount of irrigated acres and shifting production areas do not appear to be valid based on NASS data.

Relation between grain yield, plant height, ear height and days to silk

This dataset allowed a comparison between measured agronomic traits and yield which suggests future ideotype targets of plant breeding for Texas environments. The first two components of the biplots of hybrid performance values (BLUPs) estimated over all locations and years retained 81% of the variation (Figure 3.10). The biplots provided a visual representation of the relations between four traits. Grain yield was more positively correlated to plant height than to ear height and days to silk. Grain yield was not correlated with grain moisture. The longest vector was for grain yield, which meant that it was the best criteria to discriminate between hybrids. Separately, using the Pearson correlations (Table 3.8) grain yield was found to be positively correlated with plant and ear height, plant population, test weight, and grain moisture. This effect was more pronounced in the rest of Texas region then in the High Plains. Within the rest of Texas region, this correlation is higher for the locations that are grown in dryland. For ear height, positive correlations were observed with grain yield in dryland locations, the combined data and all the locations except the High Plains. A likely explanation for lower correlations between yield with plant and ear height in irrigated locations is that they are under better growing conditions, so traits or factors other than grain moisture were more important to achieve higher yields. Grain yield was slightly positively

correlated with days to silk; however, for the High Plains the correlation was slightly negative suggesting longer season hybrids could have value in the rest of Texas while shorter season hybrids might be beneficial in the High Plains. Lodging was negatively correlated (~15 to 21%) with grain yield in all different environments as would be expected.

Figure 3.10. Biplot for 648 unique hybrids and six traits. Grain yield (yield), plant height (PH) ear height (EH), days to silk (DTS), test weight (TestWt) and grain moisture (moist) derived from the BLUPs calculated from 2000 to 2010 Texas AgriLife Corn Performance Trials using the linear mixed model described in Eq. (3.1).All data were centered and standardized to unit variance.

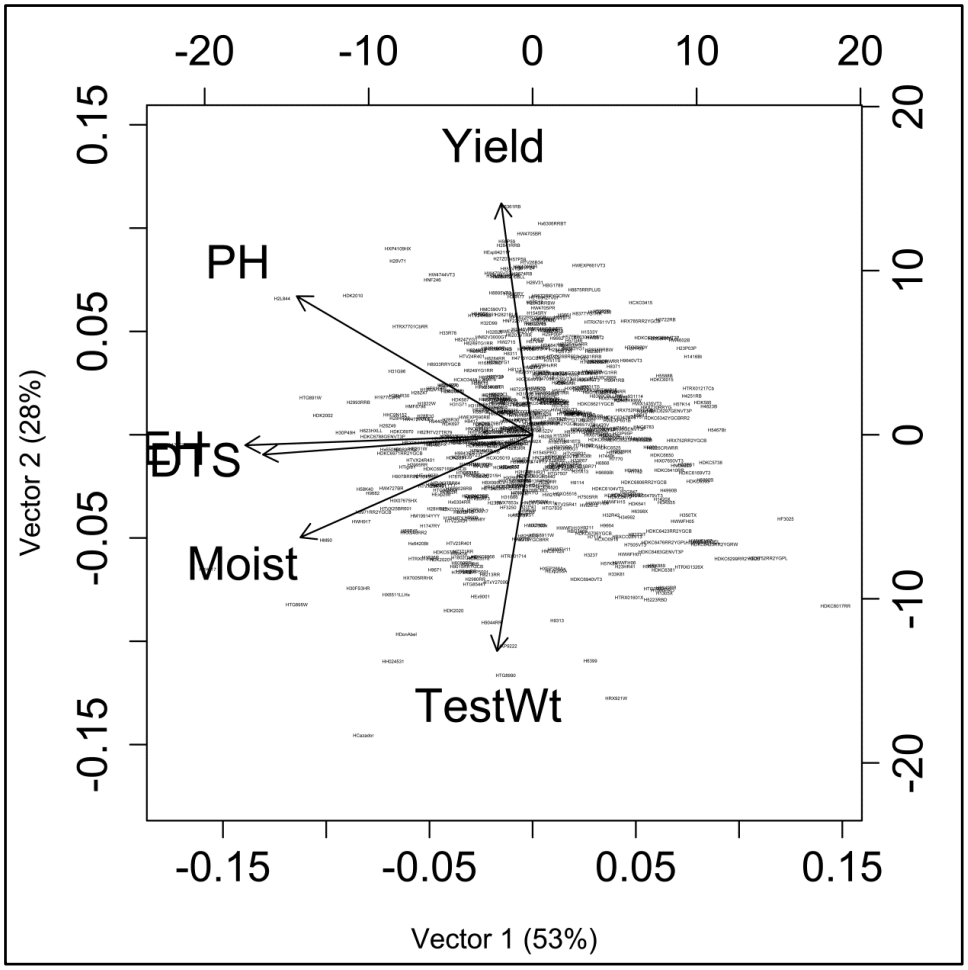


Table 3.8. Pearson correlation estimates for the 2000 to 2010 calculated from the raw data collected for Texas AgriLife Corn Performance Trials between grain yield and other important agronomic traits. Separation between the High Plains and the rest of Texas including irrigated and dryland locations show distinctly different patterns. Further separation of only dryland locations within the rest of Texas region shows similar patterns to including irrigation in these regions.

		Grain yield (ton/ha)		
Trait	**Texas**	**High Plains**	**Rest of Texas**	**Dryland**
Plant height (cm)	0.61***	0.19***	0.46***	0.45***
Ear height (cm)	0.56***	0.03NS	0.40***	0.35***
Days to silk	0.13***	-0.25***	0.05***	-0.08***
Plant density (plants/ ha)	0.66***	0.44***	0.51***	0.36***
Lodging (% plants/ plot)	-0.16***	-0.24***	-0.15***	-0.21***
Moisture (%)	0.55***	0.04*	0.28***	0.30***
Test weight (kg/hl)	0.33***	0.04NS	0.45***	0.50***

*P <0.05; **P <0.01; ***P <0.001; NS: non-significant.

The positive correlation between grain yield with both plant and ear height has been reported in different studies (Liu and Wiatrak, 2011; Sreckov et al., 2011; Yin et al., 2011). Ear height was reported to be correlated with grain yield. However, the regression analysis evidenced that plant and ear height explained from 6 to 8% of the variation for grain yield under well-watered and well-fertilized conditions (Liu and Wiatrak, 2011). In contrast, Yin et al., 2011 found that plant height is related with grain yield in late stages of growing in different production systems; these authors even proposed to use plant height to predict maize yields. This basic agronomic trait data in our study is in agreement with a hypothesis that selecting taller plant ideotypes with higher ear heights would improve yields in Texas as long as lodging does not become an issue.

CHAPTER IV

CANDIDATE GENE AND WHOLE GENOME ASSOCIATION STUDY FOR DROUGHT AND AFLATOXIN RESISTANCE IN MAIZE IN A SUB-TROPICAL ENVIRONMENT

Introduction

Maize is one of the three most important crops of the world with rice and wheat. World production in 2011 was of 883 million tons (http://faostat.fao.org [verified 6 May 2013]). Maize is the most important crop in the United States (U.S) with a production of 301 million tons of maize in 2012 with an estimated value in the market of 77.4 billion dollars (National Agricultural Statistical Service [NASS] 2013). The most important and highest yielding production areas are in the temperate Midwestern U.S. and other temperate regions throughout the world, which is where the majority of investment in maize breeding is focused (Frey, 1996; Fuglie, 2000; Schimmelpfennig et al., 2004). Other important but lower yielding regions, such as the sub-tropics, experience different challenges to maize production. These production zones are hotter and drier, among the two greatest challenges for maize production are drought stress and aflatoxin contamination. Sub-tropical maize production in the U.S. Southern states, account for approximately 9% of the production of maize in the U.S. (NASS, 2013). Texas is the largest producer of maize in the Southern states, summers in Texas are hot and dry, and there is a strong inter-annual precipitation variation across the state (Barrero et al. 2013). Severe drought episodes have occurred in the last ten years; therefore, major constraints

for maize production in Texas are heat and drought stress, and aflatoxin contamination (Payne, 1998; Horne et al., 1991;Brown et al., 1999; Robens and Cardwell, 2003; Widstrom et al., 2003; Williams et al., 2003; Betran et al., 2005; Mayfield et al., 2011; Smith, 2011). Consequently, Texas provides deal environmental conditions to do research in drought tolerance and aflatoxin contamination.

Breeding for drought is important because agriculture is the major use of surface and ground water in the U.S. It has been estimated that water usage in agriculture for the Western states accounts for up to 90% of the total water used in these states (USDA-ERS [Economic research service, verified May 6-2013]). The use restrictions and competition for water by growing urban areas will make drought stress even more common in irrigated agriculture. Additionally, drought episodes are likely to increase in the Midwestern U.S., because of a stronger inter-annual variation in precipitation and temperature attributable to a changing climate (Rosenzweig et al., 2002; Wuebbles and Hayhoe, 2004). Drought tolerance is difficult to quantify and improve. It is clearly quantitative and complex and regulated by thousands of genes (Campos et al., 2004; Kakumanu et al., 2012; Rengel et al., 2012). In addition the impact of drought stress depends on the severity, onset and length of the stress. Maize is most sensitive to drought stress during flowering and milky stages (Bolaños and Edmeades, 1996; Bänziger et al., 2000; Campos et al., 2004). Drought stress in maize causes reduction in plant height, leaf rolling, early senescence, kernel abortion and barren ears (Bolaños and Edmeades, 1996; Bänziger et al., 2000; Campos et al., 2004).

Drought episodes are often followed by pre-harvest aflatoxin contamination. Aflatoxin is a carcinogenic mycotoxin, produced by the fungus *Aspergillus flavus,* which thrives under hot and dry stressful conditions. Aflatoxin is federally regulated at 20 ng g^{-1}, and is believed to cause over $200 million dollars of economic losses in the Southern U.S. each year (Abbas et al., 2002; Windham and Williams, 2002; Williams et al., 2003; Betran et al., 2005). Aflatoxin susceptibility in plants is a highly complex trait and no complete source of resistance is known for maize (Mayfield et al.2012). Adding to the complexity of this pathogen, both colonization and aflatoxin production appears to have strong host-by-pathogen interaction (Scheidegger and Payne, 2003; Kelley et al., 2009; Amaike and Keller, 2011; Christensen and Kolomiets, 2011). As a consequence, breeding for aflatoxin resistance is a complex challenge. Despite this complexity a number of breeding lines and germplasm with improved aflatoxin resistance have been released. The lines Mp313E, Mp715, and Mp420 (Scott and Zummo, 1990; Williams and Windham, 2012) were derived from the tropical maize race Tuxpeño after several cycles of selection. The maize inbred lines Tx736, Tx739, Tx740, and Tx772 (Llorente et al., 2004; Mayfield et al., 2012) were selected by pedigree selection from Argentinean and Bolivian lines. Other sources of resistance such as the line GT603 were selected from temperate elite hybrids from the 1970s (Guo et al., 2011). This diverse germplasm has already been used in QTL linkage mapping studies to identify the loci responsible for conferring resistance (Abbas et al., 2002; Paul et al., 2003; Widstrom et al., 2003; Brooks et al., 2005; Alwala et al., 2008; Warburton et al., 2010; Xiang et al., 2010; Willcox et al., 2013). Different QTLs for aflatoxin resistance have been reported for

chromosomes one, three, four, five, and nine (Paul et al., 2003, Warburton et al., 2010; Mayfield et al., 2012). These findings evidence that diverse germplasm has been the primary source for resistance to different plant and ear rot diseases (Scott and Zummo, 1990; Wisser et al., 2006; Mayfield et al., 2012; Williams and Windham, 2012). In addition, it also indicates that aflatoxin resistance is heritable. Diverse and tropical maize germplasm has also demonstrated potential to outyield commercial hybrids and exhibit improved drought tolerance or unique traits unavailable in commercial temperate hybrids when testcrossed to elite temperate lines (Carson et al., 2006; Nelson et al., 2006; Whitehead et al., 2006; Nelson and Goodman, 2008; Flint-Garcia et al., 2009; Ortiz et al., 2010). However, tropical and diverse germplasm can also have many undesirable traits, such as delayed flowering time/ photoperiod sensitivity and dry down, lower yield, poor stalks which makes it challenging to use in lines per se (Nelson et al., 2006; Whitehead et al., 2006; Nelson and Goodman, 2008).

To identify new sources of diverse genetic variation to deal with heat and drought stress and aflatoxin contamination outside of the elite Midwestern germplasm (the so called exPVP's – Nelson et al. 2008) diverse germplasm should be investigated. Different sources of diverse germplasm previously characterized for the maize research community include the 282 maize association panel (Flint-Garcia et al., 2005; Flint-Garcia et al., 2009; McMullen et al., 2009). The 282 maize association panel includes several lines that originated from the dent maize commercial lines in the 1970 and 1980 (Mikel and Dudley, 2006). The identification of these favorable alleles in diverse germplasm will allow targeted genetic improvement of current germplasm and the

incorporation of those alleles into elite material. In addition, diverse germplasm with differences in phenotype and high density of polymorphisms in the genome facilitates linkage mapping and association studies (Yan et al., 2011). Despite that there has been a number of bi-parental linkage QTL mapping studies on aflatoxin and drought, this approach finds many loci specific to a couple genetic backgrounds, but not to maize as a whole. By contrast, association studies using diverse germplasm can identify important alleles or test candidate genes that have effects across diverse genetic backgrounds.

Previous research showed that two maize lipoxygenases mutants *zmlox4-3::mu* and *zmlox5-3::mu* exhibit greater drought tolerance and aflatoxin resistance, respectively in a few inbred lines (Park et al., 2010). However, these mutants have not been validated across multiple genetic backgrounds. In order to do so, a previous study characterized the genetic diversity of *zmLOX4* and *zmLOX5* in an assembled association panel, which include 200 lines out of the 282 maize association panel, to identify polymorphisms that can be used in a candidate gene association study (De la Fuente et al, 2013). *ZmLOX4* and *ZmLOX5* genes belong to the lipoxygenase family, which are ubiquitous enzymes present in animals, fungi and plants. The lipoxygenases are non-heme iron-containing dioxygenases that catalyzes the oxygenation of poly-unsaturated fatty acids (PUFAs) (Feussner and Wasternack, 2002; Porta and Rocha-Sosa, 2002; Liavonchanka and Feussner, 2006). The intermediaries are involved in multiple responses in plants that range from green leaf volatile production, plant development and plant defense responses to biotic and abiotic stresses, which suggest likely candidates (Blée, 2002;

Mosblech et al., 2009). *ZmLOX4* and *ZmLOX5* genes share 95% sequence homology but have different expression patterns in the root and shoot respectively (Park et al., 2010).

Based on the hypothesis that *ZmLOX4* and *ZmLOX5* genes conditioned drought tolerance and aflatoxin resistance respectively, this study testcrossed isogenic lines having these knock-outs in the same genetic background to a 400 lines assembled diversity panel to determine the effects of native variation at these two *LOX* genes and other loci for a variety of traits. Specifically, the goals of this study were: 1) to evaluate the effect of natural alleles of *ZmLOX4* and *ZmLOX5* on conferring phenotypic variation in drought tolerance, aflatoxin resistance and other important agronomic traits using a candidate gene association study; 2) to estimate the yield potential, aflatoxin resistance, and agronomic abilities of these diverse lines in hybrid combination; and 3) to identify other genomic regions that confer these phenotypes in hybrids grown in a Southern sub-tropical environment using a genome wide association study (GWAS).

Materials and methods

Phenotypic and genotypic data collection

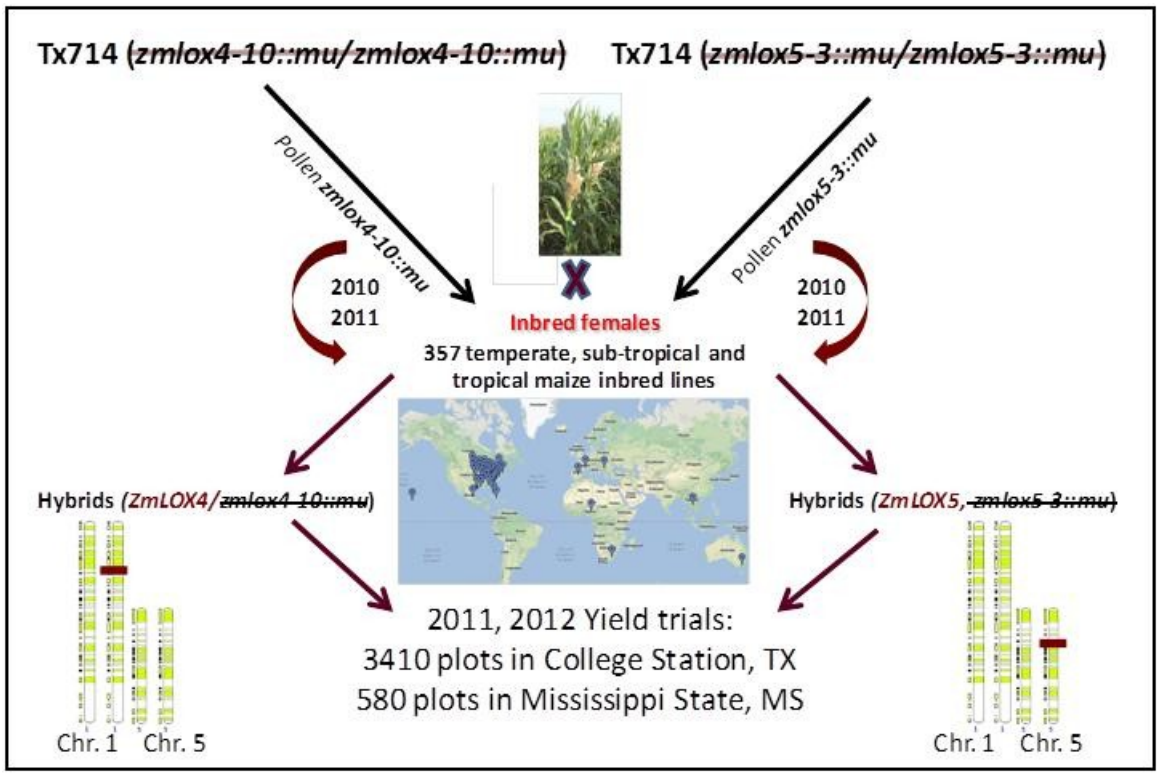
A temperate focused panel comprising USDA Flint-Garcia/Buckler/Goodman panel (300 lines, Flint-Garcia et al., 2005) and a diverse Southern subtropical focused panel Williams/Warburton panel (100 new lines plus 302 lines from Flint-Garcia et al., 2005) (Warburton et al., In review) was assembled for a total of 400 lines (De La Fuente et al., 2013). These 400 lines were crossed to two *LOX* family knock-out-mutant isogenic lines in the Tx714 (Betrán et al., 2004) background (*Tx714zmlox4-*

8::Mu/zmlox4-8::Mu and *Tx714zmlox5-3::Mu/zmlox5-3::Mu*) in a summer and fall nursery in College Station, TX and Weslaco, TX, respectively in both 2010 and 2011 (Figure 4.1).

The hybrids (*ZmLOX4/zmlox4-8::Mu*) were evaluated in two replicates in a randomized complete block (RCBD) design separately under irrigated and non-irrigated treatments with commercial checks randomly assigned. Irrigation was provided using furrow irrigation. Separate hybrids (*ZmLOX5/zmlox5-3::Mu*) were evaluated in two replicates under irrigated conditions and inoculated with *A. flavus* isolate NRRL 3357 (Wicklow et al., 1998) using a modified colonized kernel technique. In the colonized kernel technique, maize kernels are autoclaved and inoculated with *A. flavus* spores. The maize kernels are incubated 24 to 36 hour to promote *A. flavus* growth and sporulation. Infected kernels are placed on the soil surface between treatment rows when the maize hybrids reach mid-silk stage (Windham et al., 2003; Betran et al., 2005;). The hybrids were grown in College Station in 2011 (CS11) and 2012 (CS12) in one row plots 7.92 meters long and 76.2 centimetres wide, and measured for all reported traits. The target plant population was 75000 plants/ha and the soil type was a ships clay loam. Combination of year and treatment were designated trials and the following coding system was adopted in this research: The irrigated trials were inoculated with *A. flavus* and coded LIYT (lipoxygenase yield trials) and the non-irrigated trials were coded as DYTL (drought yield trials). An additional location was evaluated in two replicates at Mississippi State, MS in 2012 (MS12) only for aflatoxin accumulation and days to silk.

Hybrids in this location were inoculated using the side needle method (Windham et al., 2003, 2005).

Figure 4.1. Crossing scheme for hybrid generation. Two isogenic lines with the *zmLOX4* and *zmLOX5* genes mutated were crossed to 357 inbred lines from a diversity panel. The hybrids have only one native and functional copy for the *zmLOX4* or *zmLOX5* allele.



Plant height was measured from the ground to top of the tassel; ear height was measured to the bottom ear node. Days to silk and days to anthesis was measured by 50% of the plants in a plot showing silks or pollen shed respectively. Anthesis silking interval (ASI) was calculated using the difference between days to silk and days to anthesis. Because of the severe drought in 2011 Texas, the CS11-DYTL non-irrigated

trial was completely hand-harvested. In contrast, for the non-irrigated trial in CS12-DYTL and the irrigated trials in CS11-LIYT and CS12-LIYT, 10 ears were hand harvested skipping the first five plants in the plot and then hand harvesting every other plant; the rest of the plot was harvested using a John Deere 3300 combine with a HM-1000B Grain Gauge (Harvermaster, Logan, Utah) from which plot weight, moisture and test weight were obtained. For the MS12-LIYT irrigated trial only the 10 inoculated ears were hand harvested and processed for aflatoxin content. Hand harvested ears from each hybrid in CS were photographed and phenotyped for disease, percentage of kernel abortion and pollination. 500-kernel weight was determined after shelling. All yields were adjusted to 15.5% moisture as determined from the combine at harvest or for hand harvested ears after shelling using a Dickey-John mini GAC plus® portable moisture tester. Moisture was expressed as percentage of weight. Aflatoxin content was determined by the Vicam Aflatest (Vicam, Watertown, MA) following standard procedures (Brooks et al., 2005; Warburton et al., 2010; Mayfield et al., 2011). Aflatoxin values were transformed using the transformation (Log10 [aflatoxin + 10]) to improve normality and constant variance.

Evaluation of the ZmLOX4 *and* ZmLOX5 *alleles and treatment effects*

The isogenic hybrids (*ZmLOX4/zmlox4-8::Mu* and *ZmLOX5/zmlox5-3::Mu*) of each inbred were grown side by side under irrigated conditions in the CS trials to decrease environmental variation. A single analysis to determine the effect of the *LOX* alleles and the treatment effects (irrigated vs non-irrigated) was not implemented

because the experiment was unbalanced, and the *ZmLOX5/zmlox5-3::Mu* hybrids were grown only under irrigated conditions. The significance of the *zmLOX4* and *zmLOX5* gene for the different traits collected was estimated using the general linear model:

$$Y_{ijkl} = \mu + y_k + LOX_l + LOX{*}y_{kl} + g_i + (r/LOX/y)_{jkl} + (g{*}LOX)_{il} + (g{*}y)_{ik} + (g{*}LOX{*}y)_{ikl} + \varepsilon_{ijkl} \quad (4.1)$$

Where Y_{ijkl} is the response for hybrid *i* in replicate *j* in year *k* and *LOX l*; where μ is the general mean; y_k is the fixed effect for year; LOX_l is the fixed effect for *LOX l;* $LOX{*}y_{kl}$ is the fixed effect of the interaction between *LOX l* and year *k;* g_i is the hybrid fixed effect; $(g{*}LOX)_{il}$ is the fixed effect of the interaction between hybrid *i* and *LOX l*; $(g{*}y)_{ik}$ is the fixed effect of the interaction between hybrid *i* and year *k*; $(g{*}LOX{*}y)_{ikl}$ is the fixed effect of the interaction between hybrid *i*, year *k* and *LOX l;* $(r/LOX/y)_{jkl}$ is the random effect of replication *j* nested in *LOX l* and year *k*; ε_{ijkl} and is the random residual effect for hybrid *i* in replicate *j* in year *k* and *LOX l* and is NID (0, $\sigma^2_{\varepsilon(lxk)}$).

Based on finding non-significance of the *LOX* term, data from the hybrids were combined (or analyzed separately if significant) to evaluate the treatment effect (drought vs irrigated) for the different traits. An initial linear model that fit all the terms as random effects was fit to estimate the magnitude of the variance components.

The significance of the non-irrigated and irrigated treatments (Trt) was estimated using the general linear model:

$$Y_{ikl} = \mu + y_k + trt_l + trt*y_{kl} + g_i + (r/trt/y)_{jkl} + (g*trt)_{il} + (g*y)_{ik} + (g*trt*y)_{ikl} + \varepsilon_{ijkl} \qquad (4.2)$$

Where Y_{ikl} is the response for hybrid i in replicate j in year k and treatment l; where μ is the general mean; y_k is the fixed effect for year k; trt_l is the fixed effect for treatment l; $trt*y_{kl}$ is the fixed effect of the interaction between treatment l and year k; g_i is the hybrid fixed effect; $(g*trt)_{il}$ is the fixed effect of the interaction between hybrid i and treatment l; $(g*y)_{ik}$ is the fixed effect of the interaction between hybrid i and year k; $(g*trt*y)_{ikl}$ is the fixed effect of the interaction between hybrid i, year k and treatment l; $(r/trt/y)_{jkl}$ is the random effect of replication j nested in treatment l and year k; ε_{ijkl} and is the random residual effect for hybrid i in replicate j in year k and treatment l and is NID $(0, \sigma^2_{\varepsilon(lxk)})$. The significance of the effects for Eq. (4.2) was tested using the Type 3 Test of fixed effects as implemented in PROC MIXED in SAS 9.3 (SAS Institute, INC., Cary, NC, USA).

Phenotypic analysis for the GWAS study

The different hybrids within each treatment were laid out using a randomized complete block (RCBD) design with commercial checks randomly assigned to different plots in the trial. The commercial checks were used to adjust for field spatial variation and to estimate the residual variance, but were excluded for estimating variance

components and Best Linear Unbiased Predictors (BLUPs) for the GWAS. A combined multi-environment trial (MET) analysis was performed considering three different models. For the first one, an RCBD model was fit. The phenotypic observation y_{ijk} on hybrid *i* in replicate *j* of trial *k* was modeled as:

$$Y_{ik} = \mu + e_k + g_i + (r/e)_{jk} + (g*e)_{ik} + \varepsilon_{ijkl} \quad (4.3)$$

where, μ is the grand mean; e_k is the fixed effect of trial *k;* g_i is the random effect of hybrid *i* and is ~ NID (0, σ^2_g), i= 1, . . .,g; $(r/e)_{jk}$ is the random effect of replication *j* nested in environment *k* and is ~ NID (0, σ^2_r), *r= 1,2*; $(ge)_{ik}$ is the random effect of the interaction between hybrid *i* and trial *k* and is NID (0, σ^2_{ge}), and ε_{ijk} is the random residual effect for hybrid *i* in the replication *j* of trial *k* and is NID (0, σ^2_ε). This model was further expanded to account for field spatial variation by fitting a two-dimensional AR1 x AR1 terms for the row and column effects for the different traits (Cullis and Gleeson, 1991; Gilmour et al., 1997; Cullis et al., 1998; Gilmour et al., 2009).

A third model assumes a genetic variance-covariance (VCOV) matrix based on an unstructured model for the random genetic effects where a specific variance was fit for each trial and a specific covariance was fit for each pair of trials. The phenotypic observation *yik* on hybrid *i* of trial *k* was modeled as:

$$Y_{ik} = \mu + e_k + (gge)_{ik} + \varepsilon_{ijkl} \quad (4.4)$$

where, μ is the grand mean; e_k is the fixed effect of trial *k; gge*$_i$ represents the hybrid main effect together with the genetic environmental interaction (GEI) for hybrid *i* in trial *k*. This model was enhanced by including the significant terms for autoregressive terms for row and column effects. All three different models were fit using restricted maximal likelihood (REML) (Patterson and Thompson, 1975) in ASREML v3.0 (Gilmour et al., 2009). Heritability (h^2) was calculated: $h^2=1-\frac{PEV}{2x\,\sigma^2}$ (Cullis et al., 2006; Oakey et al., 2006). The predicted error variance (PEV) is calculated as the square of the standard error of the (BLUPs), and it is used to determine the accuracy of the predictions.

Genetic diversity, population structure and estimation of kinship matrix

The exon 5 for *ZmLOX5* and *ZmLOX4* was sequenced using the Big Dye terminator method in a previous study (De La Fuente et al., 2013). Briefly, SNPs with a minor allele frequency (MAF) > 0.10 were extracted and used for the candidate gene association mapping analysis. Additional genomewide genotype data from 213 lines from the sub-tropical diverse panel was obtained from the USDA-ARS Corn Host Plant Resistance Research Unit (Mississippi State, MS) (Warburton et al. in Press) using the Genotype By Sequence (GBS) method (Elshire et al., 2011). Genotype data for 133 lines from a temperate panel was extracted from the maize diversity panel of 282 inbred lines available in Panzea (Flint-Garcia et al., 2005). These genotypes were identical to Warburton et al. (in review) with the additional lines obtained from the 282 association

mapping panel (Flint-garcia et al., 2005). SNPs with a minor allele frequency (MAF) greater than 25% and a low missing data rate (<7.5%) were extracted to perform the genetic diversity and structure analysis (total 1999 SNPS). The genetic distance was calculated using Nei's genetic distance (Nei, 1972) using the software PowerMarker. A principal coordinate analysis (PCoA) was then carried out using the prcomp function in R (R Development Core Team, 2013).

Population structure was determined using the software Structure v2.93 (Pritchard et al., 2000). The number of subpopulations was estimated from five independent runs having 5 x 10^5 burn-in and sampling iterations, the number of subpopulations varied between 1 and 15. The ancestry model allowed for population admixture and correlated allele frequencies. The optimum *K* was estimated using the ad hoc statistic ΔK, which is based on the rate of change in the log probability of the data between successive *K* values (Evanno et al., 2005). Based on the estimated k determined, a run of 5 x 10^6 burn-in and sample iterations was used. A kinship coefficient estimation matrix was created using the VanRaden algorithm as implemented in the software GAPIT (Lipka et al., 2012).

Candidate gene and whole genome association mapping analysis

Four sets of phenotypic observations were used for the association mapping to ensure that the findings were robust and to identify QTL variants for specific environments; these included 1) the entry mean for the different traits collected for all trials, 2) the BLUPs for the combined MET analysis in Eq. (4.3), 3) the BLUPs for the

MET analysis in Eq. (4.3) including the rows and columns effects for the spatial analysis and 4) the BLUPs from GEI analysis in Eq. (4.4). Population structure and relatedness were taken into account in the linear mixed model as described by Yu et al., (2006). The association mapping analysis was done using the compressed mixed linear mixed model (CMLM) and the P3D method, as implemented in GAPIT (Kang et al., 2008; Zhang et al., 2010; Lipka et al., 2012). SNPs with a MAF< 0.05 were discarded for the association mapping analysis as they are known to cause false positives (Myles et al., 2009). The p-value was corrected for multiple testing using the modified method proposed by Gao et al., (2008). This approach calculates the M_{eff}, which is the effective number of independent test to correct for (i.e. Bonferroni correction), by estimating the pair-wise composite LD matrix (Gao et al., 2008; Gao et al., 2010; Gao, 2011). The number of PCs required explaining 99.5% of the variation in the dataset was used to calculate the Bonferroni correction. The M_{eff} calculated for this study was 49030 independent tests, which is equivalent to 1.01×10^{6} or 5.99 (-log10[p]). This study also use the false discovery rate (FDR) to correct for multiple testing for all the different analysis and traits in this study (Benjamini and Hochberg, 1995).

Results and discussion

The results of this chapter are divided into two major sections. The first section addresses the phenotypic results obtained for the *ZmLOX4/zmlox4-8::Mu* and *ZmLOX5/zmlox5-3::Mu* hybrids, which were grown side by side under irrigated conditions. This part is followed by the results obtained for the candidate gene

association study using the different alleles found by De La Fuente et al., 2013. The second section includes the results for grain yield and aflatoxin trials under irrigated and non-irrigated conditions, as well as, the performance of the different hybrids from the testcrosses between Tx714 and the different inbred lines that composed the diversity panel. The third section includes the results obtained for GWAS.

Phenotypic results and candidate gene association study for *zmLOX4* and *zmLOX5*

Effect of ZmLOX4/zmlox4-8::Mu *and* ZmLOX5/zmlox5-3::Mu *hybrids*

The 250 *ZmLOX4/zmlox4-8::Mu* and *ZmLOX5/zmlox5-3::Mu* hybrids grown side by side in CS11 and CS12, exhibited slight differences in summary statistics for grain yield, days to silk and anthesis, plant and ear height (Table 4.1). These traits were all slightly lower for the and *ZmLOX5/zmlox5-3::Mu* hybrids. The mean for aflatoxin content for the *ZmLOX5/zmlox5-3::Mu* hybrids was ~ 224 ng g^{-1} lower than the *ZmLOX4/zmlox4-8::Mu* hybrids (Table 4.1). Based on the type 3 test of fixed effects results days to anthesis, days to silk and aflatoxin content are statistically significantly different between the *ZmLOX4/zmlox4-8::Mu* and *ZmLOX5/zmlox5-3::Mu* but plant and ear height were not (Table 4.2).

Most traits were highly correlated between different traits within *ZmLOX4/zmlox4-8::Mu* and *ZmLOX5/zmlox5-3::Mu* hybrids (Tables 4.3). Grain yield, 500-kernel weight and height were negatively correlated with aflatoxin content in *ZmLOX4/zmlox4-8::Mu* and all traits were negatively correlated in *ZmLOX5/zmlox5-3::Mu* hybrids and the magnitude of the correlation was greater (Table 4.3).

The correlation between plant height and aflatoxin level evidenced that shorter plants exhibited greater aflatoxin contamination in these trials, which may also be partially explained by the colonized kernel technique since infected maize kernels are placed on the soil between the plots where shorter plants are likely more exposed to the spores of *A. flavus*.

Table 4.1. Summary of the raw data for the *zmLOX4/zmLOX4-10::mu* and the *zmLOX5/zmLOX5-3::mu* hybrids across all trials. Arithmetic means (mean) and their respective standard deviation, minimum (min), and maximum (max).

	***zmLOX4/zmLOX4-10*::mu hybrids**			***zmLOX5/zmLOX5-3::mu* Hybrids**		
Variable	**Mean** ± S.D.	**Min**	**Max**	**Mean** ± S.D.	**Min**	**Max**
Days to anthesis	62.8 ± 5.2	49	74	61.4 ±5.1	49	73
Days to silk	63.5 ± 5.5	50	77	62 ± 5.5	49	78
Anthesis silking interval (days)	0.7 ± 1	-3	5	0.6 ± 1	-2	7
Plant height (cm)	234.5 ± 33.2	165.10	304.80	231.5 ± 32	160.02	292.10
Ear height (cm)	98.1 ± 23.3	48.26	152.40	96.4 ± 21.8	48.26	162.56
Moisture (%)	12.6 ± 2.2	5.80	21.30	12.4 ± 2	4.70	25.40
Weight 500 kernels (gr)	115.4 ± 25.5	31.10	166.75	113.6 ± 23.5	57.20	171
Yield (ton/ha)	6.9 ± 2.9	0.45	14.64	5.9 ± 2.5	0.43	13.40
Aflatoxin (ng g^{-1})	671.6 ± 579.4	0	3,200	447 ± 649.8	0	3,100
Plot weight combined harvested (kg)	3.1 ± 1.5	0.08	8.38	2.5 ±1.3	0.01	6.39
10 ears weight (kg)	1 ± 0.3	0.12	2.17	0.9 ± 0.3	0.15	3.30
Ear Count	10.2 ± 1	7	31	10.5 ± 2.6	1	44
Pollination (%)	93.8 ± 5.7	60	100	93.2 ± 7.4	10	100
Abortion (%)	2.4 ± 4.6	0	35	2.9 ± 6	0	55
***A. Flavus* colonization (%)**	5.2 ± 11	0	100	6.4 ± 11.9	0	85
Number of kernel rows	15.6 ± 1.5	12	20.67	15.1 ± 1.6	12	30.33

Table 4.2. Estimated F-value for the Type 3 test of fixed effect calculated from the general linear model in Eq. (4.1) to test the effect of *ZmLox* loci.

Fixed effect	Days to anthesis	Days to silk	ASI	Plant height (cm)	Ear height (cm)	500-kernel weight (gr)	Grain yield (ton/ha)	Aflatoxin (ng g^{-1})
Hybrid	42.9***	37.15***	4.23***	13.2***	10.9***	7.4***	4.84***	3.16***
LOX	84.4***	76.56***	3.29NS	2.11NS	2.89NS	0.34NS	2.73NS	35.38*
Year	3142.36***	2902.2***	133.33***	838.89***	1647.6***	158.79***	50.14***	NE
LOX ***Year**	0.36NS	0.5NS	2.63NS	0.82NS	2.83NS	0.29NS	0.43NS	NE
Hybrid* *LOX*	0.96NS	0.83NS	0.81NS	0.63NS	0.6NS	0.7NS	0.74NS	0.99NS
Hybrid*Year	3.26***	3.07***	1.57***	2.5***	1.54***	2.58***	2.05***	NE
Hybrid* *LOX* ***Year**	0.87NS	0.98NS	0.94NS	0.59NS	0.61NS	0.55NS	0.58NS	NE

*P <0.05; **P <0.01; ***P <0.001; NS: non-significant; NE: non-estimable

Table 4.3. Pearson correlation coefficient estimates for the different traits collected for the *zmLOX4/zmLOX4-10::mu* and the *zmLOX5/zmLOX5-3::mu* hybrids from raw data of all locations. The lower half diagonal correspond to the Pearson correlations for the different traits for the *zmLOX4/zmLOX4-10::mu* hybrids. The upper diagonal corresponds to the Pearson correlations for the different traits for the *zmLOX5/zmLOX5-3::mu.*

Hybrids	***zmLOX5/zmLOX5-3::mu* hybrids**									
***zmLOX4/zmLOX4-10::mu* hybrids**		**Days to anthesis**	**Days to silk**	**ASI**	**Plant height (cm)**	**Ear height (cm)**	**Moisture (%)**	**500-kernel weight (gr)**	**Grain yield (ton/ha)**	**Aflatoxin (ng g^{-1})**
	Days to anthesis	.	0.98***	0.29***	-0.57***	-0.54***	-0.45***	-0.69***	-0.47***	0.58***
	Days to silk	0.98***	.	0.46***	-0.58***	-0.55***	-0.44***	-0.70***	-0.49***	0.58***
	ASI	0.16***	0.35***	.	-0.25***	-0.28***	-0.13***	-0.31***	-0.28***	0.24***
	Plant height (cm)	-0.63***	-0.63***	-0.19***	.	0.91***	0.43***	0.71***	0.73***	-0.67***
	Ear height (cm)	-0.58***	-0.59***	-0.24***	0.91***	.	0.44***	0.64***	0.65***	-0.64***
	Moisture (%)	-0.54***	-0.54***	-0.15***	0.55***	0.53***	.	0.40***	0.29***	-0.40***
	500-kernel weight (gr)	-0.68***	-0.70***	-0.23***	0.78***	0.71***	0.51***	.	0.71***	-0.61***
	Grain yield (ton/ha)	-0.55***	-0.57***	-0.23***	0.79***	0.72***	0.43***	0.77***	.	-0.58***
	Aflatoxin (ng g^{-1})	-0.03NS	-0.06NS	-0.06NS	-0.38***	-0.29***	-0.02NS	-0.29***	-0.38***	.

*P <0.05; **P <0.01; ***P <0.001; NS: non-significant.

The correlation between days to silk and aflatoxin level was slightly negative for the *ZmLOX4/zmlox4-8::Mu* hybrids, but it was not significant. Authors have had conflicting findings on the correlations between flowering time and aflatoxin. Betran et al., (2002, 2005) found a negative correlation between flowering (days to silk) and aflatoxin content in two different experiments. By contrast, Mayfield et al., (2011) reported a positive correlation between days to silk and aflatoxin level. This indicates that the relationship between days to silk and aflatoxin level varies with the year, environment and population and likely is a result of the shifting inoculum and weather conditions day to day.

Candidate gene association analysis for zmLOX4 *and* zmLOX5

Although novel variation was found in this panel at the *ZmLOX4* and *ZmLOX5* genes (De la Fuente et al. 2013), only a few alleles were at high enough frequency to be formally tested in association mapping. The threshold after adjusting for multiple testing for *zmLOX4* was 2 (-log10[p]) and the threshold for *zmLOX5* was 2.44. For this candidate gene association analysis the phenotypic observation was obtained separately for the *ZmLOX4/zmlox4-8::Mu* and *ZmLOX5/zmlox5-3::Mu* hybrids for the different trials. *Zmlox4* was not significantly associated with grain yield for any of the trials. However, *zmLOX4* was associated with days to anthesis and days to silk (Table 4.4). The effect ranges from 0.6 to 0.7 days and the percentage of phenotypic variation ranges from 2.3 to 2.8% for days to anthesis and days to silk. The association between *zmLOX4* and days to anthesis is not surprising since it has been reported that the gene is expressed

in the root and the shoot apical meristems (Park et al., 2010; De La Fuente et al., 2013). Based on the tissue-specific expression of the *zmLOX4* transcript that corresponds to GRMZM2G109056 in the maize B73 reference genome version 2 (Maize B73 RefGen_v2, available at www.maizegdb.com/), it is observed that the gene is highly expressed in the primary root, the coleoptiles and the shoot apical meristems (SAM) in V1. The expression decreases in the SAM as the plant growths. The gene is also highly expressed during flowering in the cob and outer husk, as well, as there is an intermediate expression in the silks, the pericarp and the embryo (Winter et al., 2007; Sekhon et al., 2011).

Table 4.4. Candidate gene association analysis results for *zmLOX4* for days to anthesis and days to silk. Significant markers associated after correcting for multiple testing (SNP), their MAF, number of lines with the SNP tested, p value of the association (-log10 [p]), allele estimated effect, percentage of variation explained by marker (R^2), and the assigned name of the QTL in this study.

SNP	CHR	MAF	Log10	Effect	R2 (%)
Days to silk					
CS11-LIYT[a] (average from raw data)					
S1_264224380	1	0.31	2.04	0.6	2.3
CS12-LIYT[a] (average from raw data)					
S1_264224380	1	0.31	2.33	0.7	2.8
Days to anthesis					
CS11-LIYT[a] (average from raw data)					
S1_264224380	1	0.31	2.05	0.6	2.3
CS12-LIYT[a] (average from raw data)					
S1_264224380	1	0.31	2.34	0.7	2.8

[a]Phenotypic observation for the candidate gene association analysis was the average for days to anthesis or days to silk for the *zmLOX4/zmLOX4-10::mu* hybrids.

The *zmLOX5* gene was not significantly associated with aflatoxin level as hypothesized; however, it was significantly associated with grain yield, plant and ear height (Table 4.5). The association between *zmLOX5* was only detected in the irrigated CS11-LIYT trial. By contrast, the association between *zmLOX5* and plant and ear height was detected in both the irrigated CS11-LIYT and CS12-LIYT trials. The effect for grain yield was 0.4 ton/ha explaining 2.6% of the phenotypic variation. The effect for plant height ranged from 3.5 to 5.7 centimetres explaining 2.2 to 3.1% of phenotypic variation ranges. The effect for ear height ranged from 2.6 to 3.5 centimetres and explained 2 to 2.8% of phenotypic variation.

zmLOX5 is expressed in the aboveground parts of the plants especially in the silks (Park et al., 2010). The expression of the *zmLOX5* transcript that correspond to GRMZM2G102760 in the Maize B73 RefGen_v2 genome, further reveal that the gene is expressed in the SAM from V1 to V5 and the internodes from V5 to V9, which supports an effect on plant height as evidenced by the candidate gene association mapping analysis.

The differences in flowering time between the *ZmLOX4/zmlox4-8::Mu* and *ZmLOX5/zmlox5-3::Mu* observed in table 4.2 was further corroborated by the candidate gene association study evidenced that *zmLOX4* alleles play a role on the flowering time. By contrast, the candidate gene association study showed that the *zmLOX5* allele was not associated with aflatoxin level, but it is associated with plant and ear height. This result contrasts with unpublished data that have shown that the *zmlox5* mutants exhibited lower

levels of aflatoxin contamination. A possible explanation for this result is the fact that the different *ZmLOX5* alleles were evaluated across multiple genetic backgrounds.

Table 4.5. Candidate gene association analysis results for *zmLOX5* gene for plant and ear height. Significant markers associated after correcting for multiple testing (SNP), their MAF, number of lines with the SNP tested, p value of the association [-log10 (p)], allele estimated effect, percentage of variation explained by marker (R^2), and the assigned name of the QTL in this study.

Grain yield (ton/ha)					
SNP	**CHR**	**MAF**	**Log10**	**Effect**	**R2 (%)**
CS11-LIYT (average from raw data)					
S5_12289748	5	0.32	2.68	0.42	2.6
Plant height (cm)					
CS11-LIYT (average from raw data)					
S5_12289534	5	0.26	2.81	4.7	2.8
CS11-LIYT-US[a]					
S5_12289534	5	0.26	4.12	3.9	3.1
S5_12289504	5	0.28	3.6	3.5	2.6
CS12-LIYT (average from raw data)					
S5_12289963	5	0.28	2.99	5.1	2.2
S5_12289534	5	0.27	3.36	5.7	2.5
SNP	**CHR**	**MAF**	**Log10**	**Effect**	**R2 (%)**
CS12-LIYT-US[a]					
S5_12289534	5	0.26	4	5.5	2.8
S5_12289504	5	0.28	3.7	5.1	2.6
Ear height (cm)					
CS11-LIYT (average from raw data)					
S5_12289534	5	0.29	2.58	3.5	2.7
CS11-LIYT-US[a]					
S5_12289534	5	0.26	3.58	3.1	2.8
CS12-DYTL-US[a]					
S5_12289534	5	0.26	2.93	2.6	2.1
CS12-LIYT-US[a]					
S5_12289534	5	0.26	3.24	3.2	2.3

[a]The phenotypic observation used was the BLUP for the hybrid effect obtained using Eq. (4.4).

Yield and aflatoxin trials and potential of the diverse lines in hybrid combinations

Drought and irrigated trials

The mean difference in grain yield between the non-irrigated trials: CS11-DYTL and CS12-DYTL was plus 4.19 ton/ha (Table 4.6). A similar result was observed for grain yield between the irrigated trials for CS11-LIYT and CS12-LITY. The difference between the non-irrigated trial CS11-DYTL and the irrigated CS11-LIYT trial was of 2.56 ton/ha and between the non-irrigated trial CS12-DYTL and the irrigated CS12-LIYT trial was of 1.78 ton/ha (Table 4.6). Therefore difference between drought and irrigated trials within year was smaller than the difference between years for the same treatment. The type 3 tests of fixed effects for the general linear model in Eq. (4.2) showed that the year and the treatment effect were significant for all the traits collected (Table 4.7).

A modified model from Eq. (4.2) fitting all the effects as a random, showed that the largest magnitude of variation was for the year effect for all traits in addition to yield (results not shown). Differences between treatments (drought vs irrigated) within year and differences between years within treatments were observed for all traits collected (Table 4.6). Plant height was shorter in the non-irrigated DYTL trial, which is expected for pre-flowering drought stress (Bänziger et al., 2000). The flowering time window (silk, anthesis and ASI) was the longest for the non-irrigated trials CS11-DYTL, which totaled 32 days.

Table 4.6. Summary of phenotypic data collected for this study across the two non-irrigated and irrigated trials.

	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
Trait	**CS11-DYTL**			**CS12-DYTL**			**CS11-LIYT**			**CS12-LIYT**		
Days to anthesis	70 ± 2.9	51	84	59.3 ± 2.6	48	63	67.1 ± 2.9	56	74	58.1 ± 2.8	49	63
Days to silk	71.6 ± 3.1	59	85	61 ± 2.9	49	66	68.2 ± 3.1	57	78	58.5 ± 2.9	49	65
Anthesis silking interval	1.6 ± 1.4	-1	8	1.7 ± 1.2	-2	9	1.1 ± 1	-2	7	0.4 ± 0.9	-3	4
Plant height (cm)	184.5 ± 15.3	116.8	221	236.6 ± 18.5	165.1	281.9	199.1 ± 17.2	139.7	243.8	255.3 ± 20.2	193	304.8
Ear height (cm)	72.8 ± 11.9	35.6	99.1	92.2 ± 12.9	50.8	137.2	74.7 ± 12.5	35.6	119.4	109.4 ± 16	50.8	162.6
Moisture (%)	10.1 ± 1	7.9	14.1	10 ± 0.8	7.5	12.8	11 ± 1.9	4.7	20.2	13 ± 1.8	6.6	25.4
Weight 500 kernels (gr)	78.6 ± 11.7	48.5	120.2	108.4 ± 17.9	57.8	152.8	95 ± 19.9	11.2	166.5	130.1 ± 14.6	73.5	171
Yield (ton/ha)	1.9 ± 1	0	5	6.1 ± 2.2	0.9	13.4	4.5 ± 2	0.4	10.9	7.9 ± 2.4	1.2	16.1
Aflatoxin (ng g−1)	.	.	.	.	.	.	877.1 ± 676.7	0	3,200	79.1 ± 133	0	990
Pollination (%)	95.9 ± 5	50	100	86.2 ± 9.6	5	95	96.1 ± 4.2	60	100	91.5 ± 7	10	98
Abortion (%)	7.6 ± 6.1	0	45	12.1 ± 8.9	0	65	1.4 ± 3.4	0	30	4 ± 6.3	0	55
A. Flavus colonization (%)	18.4 ± 13.6	0	100	0.3 ± 1.5	0	20	13.9 ± 14.1	0	100	0.3 ± 1.3	0	16
Number of kernel rows	14.1 ± 1.4	11.3	20.7	15.9 ± 1.5	12	20.7	15.3 ± 1.5	12	30.3	15.5 ± 1.5	12	20.7

Arithmetic mean (mean) and their respective standard deviation, minimum (min), and maximum (max). CS11-DYTL College Station non-irrigated 2011, CS11-LIYT College Station irrigated 2011, CS12-DYTL College Station non-irrigated 2012, CS12-LIYT College Station irrigated 2012.

Table 4.7. Estimated F-value for the Type 3 test of fixed effect calculated from the general linear model in Eq. (4.2). Treatment (Trt) is full irrigation vs. drought irrigation.

Fixed effect	Days to anthesis	Days to silk	ASI	Plant height (cm)	Ear height (cm)	500-kernel weight (gr)	Grain yield (ton/ha)
Hybrid	49.07***	36.12***	5.68***	16.93***	13.33***	11.51***	7.09***
Trt	156.99***	328.63***	170.48***	63.86***	4.5NS	19.77***	27.94***
Year	8805.74***	6297.48***	20.65**	723.97***	63.04***	36.97***	57.94***
Trt*Year	59.11***	18.53**	21**	0.39NS	2.11NS	0.01NS	1.23NS
Hybrid*Trt	2.12***	1.72***	1.47***	1.42***	1.28***	1.98***	1.05NS
Hybrid*Year	4.13***	3.06***	1.76***	2.46***	2.12***	2.12***	1.96***
Hybrid*Trt*Year	2.01***	1.82***	1.57***	1.31**	1.40***	1.52***	1.19*

*P <0.05; **P <0.01; ***P <0.001; NS: non-significant; NE: non-estimable

This was expected since silk elongation is delayed by water stress, and significant effort has been devoted to improve hybrids capable of silking under drought stress conditions (Bolaños and Edmeades, 1996; Chapman and Edmeades, 1999; Campos et al., 2004). Flint et al., (2005) reported a flowering time window of 40 days for the maize inbred lines for the 282 maize association panel and the same window was observed on the 400 lines when grown in College Station in 2009 (data not shown) .

The average, maximum and minimum for aflatoxin levels were lower for the irrigated CS12 trials (Table 4.6) than CS11 trials though the same inoculum and inoculation technique were used. 2011 was one of the hottest and dries growing seasons in College Station and much more so than 2012, which likely influenced the average and maximum value observed for aflatoxin contamination between the years. *A. flavus* infection and colonization strongly depend on the environmental conditions, both hot and dry conditions and any forms of stress increase both the fungal colonization and

aflatoxin contamination (Payne and Widstrom, 1992; Paul et al., 2003; Betran et al., 2005; Cotty and Jaime-Garcia, 2007; Warburton et al., 2010; Amaike and Keller, 2011; Mayfield et al., 2011).

Variance component estimates and heritability

The heritability estimates for the non-irrigated and irrigated trials for grain yield ranged from 0.61 to 0.83 (Table 4.8). The lowest heritability, in the non-irrigated trial during the extreme drought of 2011 was expected and heritability estimates are expected to decrease under stressed trials (Bänziger and Lafitte, 1997; Banziger et al., 1999; Chapman and Edmeades, 1999; Bänziger et al., 2000; Badu-Apraku et al., 2004).

Heritability estimates ranging from 0.64 to 0.82 for heat stress trials, and 0.47 to 0.8 under drought stress have reported for maize in a MET study that evaluated maize inbred lines to identify heat and drought stress sources (Cairns et al., 2013). Within each environment heritability estimates for aflatoxin level ranged from 0.67 to 0.83 for both the transformed and raw aflatoxin level data (Table 4.8). However across environments a much lower heritability estimate 0.59 was obtained (Table 4.9). Transformation of aflatoxin is a standard procedure used before QTL mapping (Paul et al., 2003; Warburton et al., 2010; Mayfield et al., 2011) and is needed because the data are often skewed. In addition, data transformation in this study increased the heritability in some environments, but decreased it in others.

Table 4.8. Estimates of the hybrid (σ^2_g), replicate (σ^2_r), error (σ^2_ε) variances and their respective standard errors for each trial for grain yield (ton/ha) and aflatoxin (ng g^{-1}). The estimates were obtained using a linear mixed model that fit hybrids and replicate as a random effect and commercial checks as a fixed effect

Effect	College Station		College Station	Mississippi	College Station	Mississippi
	Irrigated	Non-irrigated	Irrigated		Irrigated	
	Grain yield (ton/ha)		Aflatoxin (ng g−1)		(log10 [aflatoxin + 10])	
	2011					
σ^2_g	299.9 ± 41.3	59.3 ± 16.6	233,845 ± 36,311.3	NA[a]	0.05 ± 0.009	NA[a]
σ^2_r	536.2 ± 385.8	20.5 ± 22.8	3,008.1 ± 4359.6	NA[a]	0.001 ± 0.001	NA[a]
σ^2_ε	534.5 ± 28.9	187.1 ± 17.1	264,382 ± 22,235.6	NA[a]	0.08 ± 0.006	NA[a]
h^2	0.73	0.61	0.81	NA[a]	0.77	NA[a]
	2012					
σ^2_g	767.9 ± 81.3	732.8 ± 83.9	296.7 ± 309	652,827 ± 87,276.3	0.07 ± 0.02	0.118 ± 0.02
σ^2_r	7.6 ± 10.7	28.6 ± 34	3,173. 8 ± 837.4	10,654.5 ± 18,058.5	0.004 ± 0.004	0.002 ± 0.004
σ^2_ε	703.6 ± 32.8	562.5 ± 50	11,612 ± 815.4	617,598 ± 51,338.2	0.2 ± 0.01	0.181 ± 0.015
h^2	0.83	0.80	0.67	0.83	0.70	0.70

[a] Data was not collected for this trait for this trial

Across all environments heritability estimates ranged from 0.59 (aflatoxin) to 0.98 for the different traits collected (Table 4.9), based on the components of variance estimated using the MET model from Eq. (4.3). Heritability estimates for grain yield, test weight and ASI ranged from 0.82 to 0.87. The lowest heritability estimate was for aflatoxin, which was 0.59 for the raw data and 0.70 for the (log10 (aflatoxin + 10)) transformation (Table 4.9). These heritability estimates are considerable higher than estimates reported by previous studies (Walker and White, 2001; Campbell et al., 2003; Warburton et al., 2009; Mayfield et al., 2011).

Table 4.9. Estimates of the hybrid (σ^2_g), hybrid-by-environment (σ^2_{ge}), error (σ^2_ε) variances, and their respective standard errors. The variance components were estimated using the combined MET analysis in Eq. (4.3) without spatial adjustment for the traits with the highest heritability estimates.

Effect	Days to anthesis	Days to silk	ASI	Plant height (cm)	Ear height (cm)	500-kernel weight (gr)	Grain yield (ton/ha)	Aflatoxin (ng g^{-1})	Log10 (aflatoxin (ng g^{-1}))
σ^2_g	7.8 ± 0. 5	8.5 ± 0.5	0.3 ± 0.04	228.5 ± 19.2	107.3 ± 9.2	122.1 ± 11.5	0.08 ± 0.008	47465 ± 17197.5	0.04 ± 0.008
σ^2_{ge}	0.3 ± 0.03	0.4 ± 0.05	0.2 ± 0.03	32.2 ± 3.6	13.8 ± 2.1	42.5 ± 4.2	0.02 ± 0.003	238610 ± 24273.7	0.04 ± 0.008
σ^2_ε	0. 7 ± 0.02	0.9 ± 0.03	0.7 ± 0.02	91.7 ± 2.8	63.2 ± 1.9	94.2 ± 2.8	0.1 ± 0.003	273774 ± 12332.2	0.2 ± 0.007
h^2	0.98	0.97	0.82	0.93	0.92	0.88	0.87	0.59	0.70

Heritability estimates for aflatoxin that range from 0.20 to 0.42 have been reported for different bi-parental QTL studies using different parents and QTL validation studies in different testcrosses in maize (Walker and White, 2001; Brooks et al., 2005; Warburton et al., 2009; Warburton et al., 2010; Mayfield et al., 2011). The differences observed in the heritability estimates for grain yield and aflatoxin level between this study and others are partially explained by the high genetic variation present in our study. It is also explained largely on the 2011 environment which had extremely high stress throughout the field. Based on the heritability estimates observed, it can be concluded that genetic variation is present in this panel to perform an association analysis.

Spatial analysis

The number of hybrids investigated in the experiment resulted in large tests. Spatial analysis has proven useful in identifying and reducing error in large field studies when checks are replicated throughout the field (Gilmour et al., 1997; Cullis et al., 1991, 1998). The analysis showed that field spatial variation was most important for the trials in CS11 for grain yield (results not shown). This would be expected with the extreme drought in 2011 which brings out the highest level of field variation. For other traits such as plant, ear height, days to anthesis, days to silk, and 500 kernel weight field spatial variation was observed. By contrast, field spatial variation was not observed for aflatoxin contamination.

Genetic correlation across different environments

The modeling of the variance-covariance structure for GEI analysis using Eq. (4.4) showed that the lowest genetic correlation for grain yield was 0.46, which correspond to the genetic correlation between the non-irrigated trial in CS11-DYTL and the irrigated trial in CS12-LIYT (Table 4.10) (van Eeuwijk et al., 2010). By contrast, the genetic correlation between other trials for grain yield ranged from 0.70 to 0.94. The highest genetic correlation was for the trials grown in CS12 (Table 4.10). Based on these results, it was concluded that hybrid ranking between years and trials was consistent. This result is similar to other QTL studies in sorghum and wheat (Mathews et al., 2008; Sabadin et al., 2012). The lowest genetic correlation for aflatoxin level was between the irrigated trial in CS12-LIYT and MS12-LIYT (Table 4.11).

Table 4.10. Variance-covariance unstructured matrix for grain yield (ton/ha) for four trials grown in College Station in 2011 and 2012. The diagonal represents the genetic variance for each trial. The elements off the diagonal in the lower half of the matrix are the specific genetic covariance per each pair of trials. The elements off the diagonal in the upper half of the matrix (shaded in gray) represent the specific genetic correlation for each pair of trials.

Environment	CS11-DYTL	CS11-LIYT	CS12-DYTL	CS12-LIYT
CS11_DYTL	0.06	0.76	0.70	0.63
CS11_LIYT	0.06	0.09	0.95	0.84
CS12_DYTL	0.07	0.11	0.15	0.94
CS12_LIYT	0.05	0.08	0.12	0.10

CS11-DYTL College Station non-irrigated 2011, CS11-LIYT College Station well watered 2011, CS12-DYTL College Station non-irrigated 2012, CS12-LIYT College Station well watered 2012.

Table 4.11. Variance-covariance unstructured matrix for aflatoxin level (ng g^{-1}) for the College Station 2011 and 2012 trials and Mississippi 2012 trial. The diagonal represent the genetic variance for each trial. The elements off the diagonal in the lower half of the matrix are the specific genetic covariance for each pair of trials. The elements off the diagonal in the upper half of the matrix (shaded in gray) represent the specific genetic correlation for each pair of trials.

Environment	CS11-LIYT	CS12-LIYT	MS12-LIYT
CS11_LIYT	0.02	0.77	0.60
CS12_LIYT	0.04	0.10	0.46
MS12_LIYT	0.03	0.05	0.13

CS11-LIYT College Station well watered 2011, CS12-LIYT College Station well watered 2012, MS12-LIYT Mississippi well watered 2012.

Best performing hybrids

There were multiple hybrids that performed as well as or better than the elite commercial checks used in this study for grain yield. This study evidenced that diverse material has the potential to outyield elite commercial hybrids under water stress. Under the severe drought that experienced Texas in 2011, neither of the commercial checks was in the top five hybrids for the CS11-DYTL trial. For the CS11-LIYT and CS12-DYTL trials only one of the commercial checks was in the top five hybrids. In contrast, the top two hybrids for the CS12-LIYT, a "good environment" were commercial checks (Table 4.12). For aflatoxin resistance none of the testcrosses accumulated less aflatoxin than the commercial checks in any environment (results not shown), likely because the tester used, Tx714, is known to have high aflatoxin susceptibility (Betrán et al., 2004). However, it was observed that some of the testcrosses exhibited decreased aflatoxin

susceptibility for the lines bred in tropical and sub-tropical areas. These results indicate the presence of favorable genes for stress tolerance and *Aspergillus* ear rot disease resistance in diverse material. The presence of favorable alleles in diverse germplasm has been previously reported by different authors (Betrán et al., 2003; Nelson et al., 2006; Whitehead et al., 2006; Nelson and Goodman, 2008; Flint-Garcia et al., 2009; Ortiz et al., 2010).

Table 4.12. BLUPs for the best 15 grain yield (GY) hybrids of the MET analysis using Eq. (4.3) without spatial adjustment and for each trial using Eq. (4.4). Eq. (4.4) was expanded to include AR1 x AR1 terms for row and column spatial effects. Four commercial checks were used in this study.

MET analysis		CS11-DYTL		CS11-LIYT		CS12-DYTL		CS12-LIYT	
Line	**GY (ton/ha)**	**Line**	**GY (ton/ha)**	**Line**	**GY (ton/ha)**	**Line**	**GY (ton/ha)**	**Line**	**GY (ton/ha)**
CML381	2.80	**AAP244**	1.17	**AMY07222**	2.45	**AMY07222**	3.52	**check 3**	3.49
NC334	2.78	**NC358**	1.15	**CML381**	2.11	**CML381**	3.33	**check 4**	3.31
AMY07222	2.65	**check 3**	0.94	**CML254**	2.06	**NC334**	3.13	**CML264**	3.26
NC370	2.56	**AAP102**	0.94	**check 4**	1.97	**CML264**	2.98	**CML115**	3.23
CML115	2.42	**CML108**	0.92	**CML264**	1.96	**CML115**	2.96	**CML45**	3.20
CML264	2.42	**AMY07222**	0.90	**CML115**	1.94	**B14A**	2.95	**NC370**	3.20
CML45	2.37	**AAP281**	0.89	**AAP122**	1.92	**check 4**	2.88	**Tzi11**	3.13
Check 3	2.33	**CML348**	0.87	**B14A**	1.88	**CML254**	2.87	**NC334**	3.09
Check 4	2.31	**AM407004**	0.84	**Tzi11**	1.88	**Tzi11**	2.86	**AMY07222**	2.92
Tzi11	2.22	**NC408**	0.84	**CML108**	1.87	**CML9**	2.78	**SC357**	2.82
Check 2	2.17	**check 2**	0.81	**CML432**	1.77	**CML5**	2.64	**CML9**	2.75
CML5	2.07	**AAP122**	0.77	**NC318**	1.74	**NC370**	2.61	**CML381**	2.74
NC366	1.97	**CML423**	0.76	**CML9**	1.72	**SC357**	2.56	**NC322**	2.68
AAP242	1.95	**CML381**	0.75	**check 3**	1.72	**NC318**	2.52	**B14A**	2.62
NC322	1.86	**AAP242**	0.74	**check 2**	1.71	**CML45**	2.52	**Mp707**	2.58

CS11-DYTL College Station non-irrigated 2011, CS11-LIYT College Station irrigated 2011, CS12-DYTL College Station non-irrigated 2012, CS12-LIYT College Station irrigated 2012.

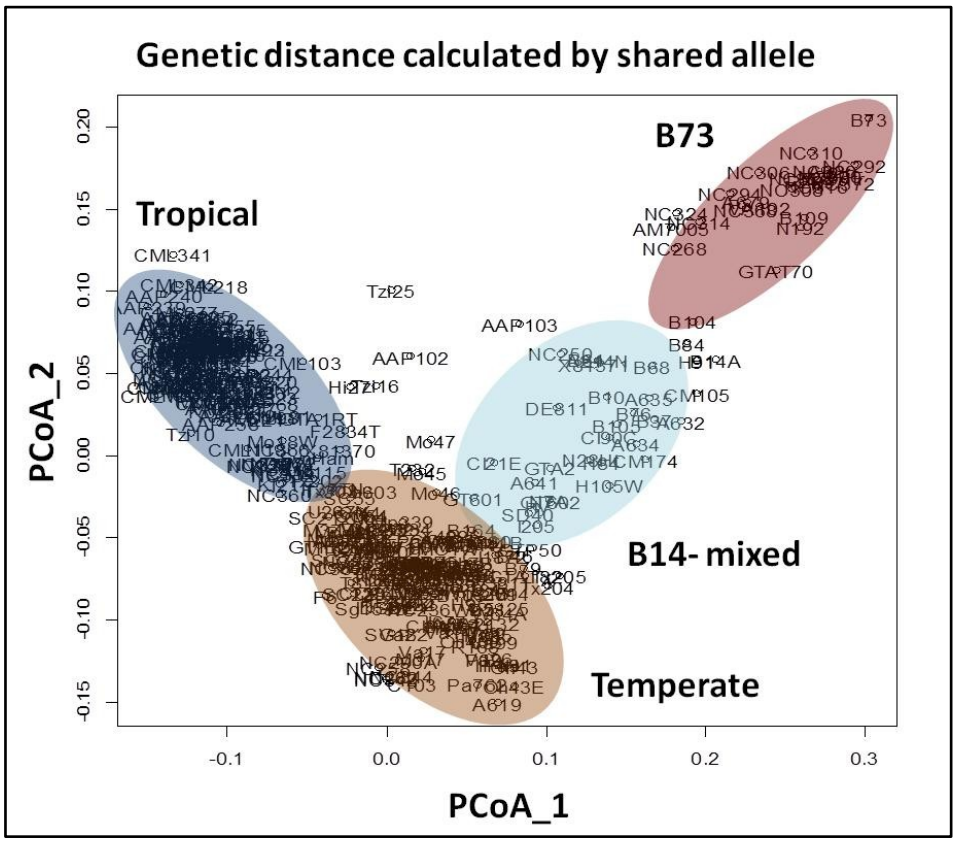
Genome wide association study for drought tolerance, aflatoxin resistance and other important agronomic traits

Genetic diversity, population structure and estimation of kinship matrix

Genetic diversity analysis between the 346 inbred lines from this diversity panel that have genotypic data available evidenced that the majority of the lines are not related to each other, only 0.23% of the pairs of entries exhibit a genetic distance less than 0.2. between all pairs of lines the mean and median for the genetic distance was 0.50 and 0.49, respectively. 55% of the entry pairs exhibit a genetic distance of less than 0.5 and 97% of the entry pairs exhibit a genetic distance less than 0.6 suggesting most of the lines are equally distantly related. Using Structure (Pritchard et al. 2000), a preliminary analysis to estimate the optimum K (number of populations) evidenced that the rate of change in the log probability, as measured by the ad hoc statistic ΔK was K=4. It was determined that this diversity panel consisted of four different clusters that correspond to: Tropical, temperate, B73 and a mixed group. Although it is normally uncommon that the Northern U.S./B14 stiff stalk lines (Mikel and Dudley, 2006) would cluster together with non-stiff stalk lines (Mo17 related lines) this is likely because this Northern germplasm is poorly represented in the hybrids used in this study. In contrast, the B73 group is well-defined and it is formed by a few lines. Structure analysis of the 300 lines that compose the diversity panel found six clusters that correspond to B73, temperate, Lancaster/C103, NorthernUS/B14, SC76, tropical, and a mixed group (Warburton et al., 2013 under review). This demonstrates how important the sample choice is in the Bayesian clustering methods of Structure.

The genetic distance pairwise matrix was visualized using a PCoA and four general clusters were identified based on additional knowledge about the germplasm (Figure 4.2). One well-defined cluster that corresponds to lines closely related to B73 was observed. There are other three clusters that are interconnected between and correspond to tropical and temperate lines. These first two PCoA eigenvectors explained 0.13% and 0.14% respectively. To explain 80% of the variation, at least 100 PCoA eigenvectors are required, which further corroborated the weak relatedness between the inbred lines from this panel.

Figure 4.2. PCoA eigenvector plot of maize inbred lines that composed the diversity panel in this study. Nei's (1972) genetic dissimilarly matrix was calculated from 1999 SNP markers.



GWAS analysis for grain yield and 500-kernel weight

GWAS analysis was performed on raw data and on spatially adjusted data to ensure results were robust across analysis methods. Five quantitative trait variants (QTV) for grain yield on chromosomes two, seven, nine and ten were identified (Figure 4.3). The allelic effects for the different QTV ranged from 0.14 to 0.59 ton/ha and the amount of phenotypic variation ranged from 3 to 5% (Table 4.13). QTV1, QTV2, and QTV3 were detected under both irrigated and non-irrigated conditions in 2011 and 2012 (Table 4.13). QTV1 has the strongest effect in the non-irrigated trial in 2012. These QTV were also detected in the MET analysis that adjusted for field spatial variation (Figure 4.3). No QTV were detected in the non-irrigated trial in 2011, likely due to low overall heritability. QTV4 was only detected in the well-irrigated trial in 2012 and QTV5 was only detected through MET analysis that adjusted for field spatial variation (Table 4.13). Several linkage studies have reported multiple linkage mapping QTLs for grain yield but to our knowledge this has not yet been examined using association approaches in testcrosses (Ajmone-Marsan et al., 1994; Beavis et al., 1994; Veldboom and Lee, 1994; Ajmone-Marsan et al., 1995; Austin and Lee, 1996; Veldboom and Lee, 1996a,b; Ajmone-Marsan et al., 2001; Schaeffer et al., 2006). These studies identified one to five linkage QTLs for grain yield explaining 20 to 35% of phenotypic variation for all QTLs together.

QTV1 found in this study for grain yield is located in bin 2.03. No QTLs associated with grain yield has been reported in this bin by other authors and this SNP is in the abph1 - aberrant phyllotaxy1- gene (Maize B73 RefGen_v2available at

www.maizegdb.com/). *abph1* is expressed in the shoot apical meristems and the gene mutation alters the regular arrangement of leaves and flowers (Lee et al., 2009). Despite LD decaying rapidly in this area of the genome, further investigation needs to be done to confirm that this SNP is indeed associated with the abph1 locus. QTV2 is located in bin 7.04, the allelic effect ranges from 0.14 to 0.42 and the percentage of explained phenotypic variation ranges from 4.5 to 5% (Table 4.13).

Figure 4.3. GWAS results for grain yield (ton/ha) using spatial adjustment. The phenotypic observation was the BLUPs for the MET analysis, which includes AR1 x AR1 terms to adjust for column and row effects. The red line represents the threshold value after correcting for multiple testing using the M_{eff}. The arrows point significant SNPs detected by the FDR statistics

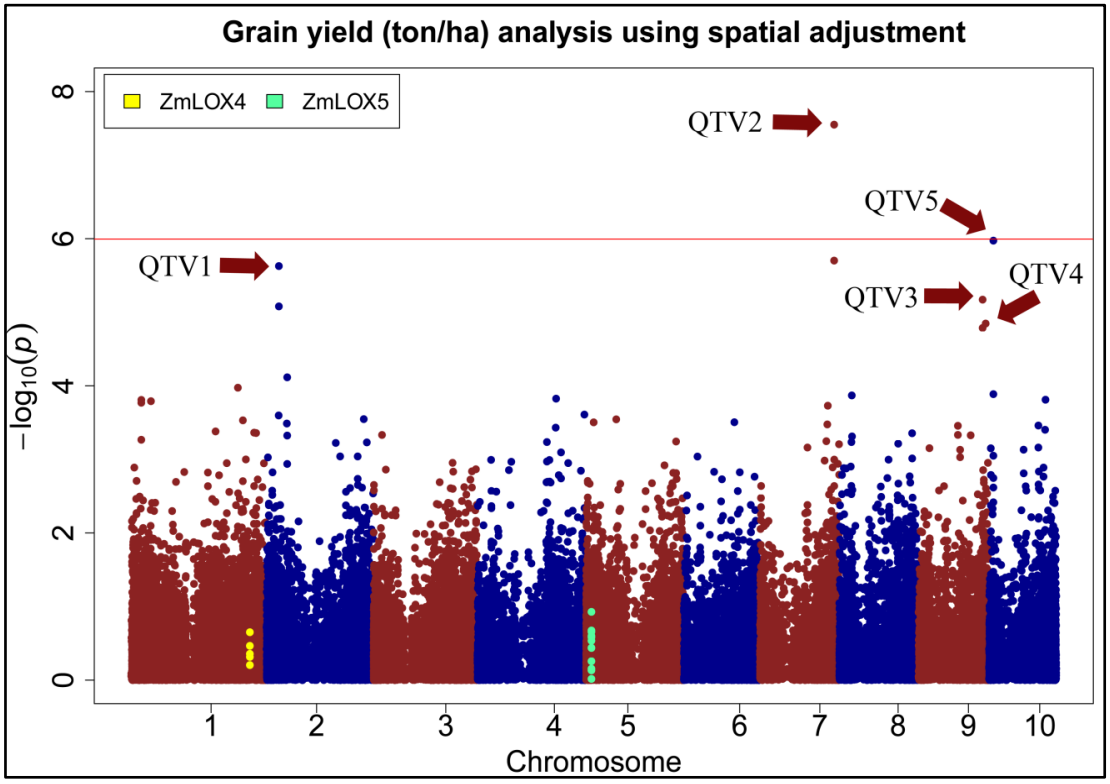


Table 4.13. GWAS results for grain yield (ton/ha). Significant markers associated after correcting for multiple testing (SNP), their MAF, FDR adjusted p value of association, p value of the association [-log10 (p)], allele estimated effect, percentage of variation explained by marker (R^2), and the assigned name of the QTV in this study.

SNP	CHR	MAF	FDR_adjusted_P	Log10	Effect	R^2 (%)	QTV
CS11-LIYT-US[a]							
S7_164955163	7	0.08	0.0003	8.25	0.37	4.9	QTV2
S9_142746374	9	0.26	0.0147	6.32	0.28	3.6	QTV3
S9_142746338	9	0.27	0.0197	6.02	0.28	3.4	QTV3
S2_27482479	2	0.22	0.0585	5.42	0.26	3	QTV1
CS12-DYTL (average from raw data)							
S2_27482431	2	0.24	0.0212	6.21	0.59	4.7	QTV1
S2_27482479	2	0.22	0.0212	6.16	0.59	4.7	QTV1
CS12-DYTL-US[a]							
S7_164955163	7	0.08	0.0005	8.11	0.42	5	QTV2
S9_142746374	9	0.26	0.0083	6.57	0.33	3.9	QTV3
S2_27482479	2	0.22	0.0133	6.08	0.31	3.6	QTV1
S9_142746338	9	0.27	0.0133	6	0.32	3.5	QTV3
S2_27482431	2	0.24	0.0133	5.97	0.31	3.5	QTV1
CS12-LIYT-US[a]							
S7_164955163	7	0.08	0.0028	7.35	0.14	4.9	QTV2
S2_27482431	2	0.24	0.0457	5.69	0.28	3.7	QTV1
S2_27482479	2	0.22	0.0457	5.63	0.28	3.6	QTV1
S9_142746374	9	0.26	0.0457	5.46	0.28	3.5	QTV3
S9_149545863	9	0.06	0.0457	5.43	0.37	3.5	QTV4
Spatial analysis[b]							
SNP	**CHR**	**MAF**	**FDR_adjusted_P**	**Log10**	**Effect**	**R^2 (%)**	**QTV**
S7_164955163	7	0.08	0.0017	7.6	0.35	4.5	QTV2
S10_10246117	10	0.24	0.0326	6	0.26	3.4	QTV5
S7_164954968	7	0.09	0.0361	5.7	0.32	3.2	QTV2
S2_27482479	2	0.22	0.0361	5.6	0.25	3.2	QTV1

[a] The phenotypic observation used for the GWAS was the BLUP for the hybrid effect obtained using model in Eq. (4.4).

[b] The phenotypic observation used for the GWAS was the BLUP for the hybrid effect obtained using the MET model in Eq. (3.3) including AR1 ⊗ AR1 terms for row and column effects.

In addition to yield, QTV2 was also detected for plant height, days to anthesis and days to silk, suggesting a pleiotropic effect on multiple traits (Table 4.14). In a recent meta-analysis of Texas commercial yield trial data, the R^2 of plant height and grain yield was determined to be 0.61 suggesting the importance of robust tall plants under Southern stress (Barrero et al. 2013). In order to further address this question an association analysis was performed including plant height as a covariate in Eq. (4.3) and (4.4). The QTV2 variant was no longer significant for grain yield providing an additional line of evidence that this QTV has a pleiotropic effect variant on grain yield (Figure 4.4).

Figure 4.4. GWAS results for grain yield (ton/ha) using spatial adjustment and a covariate for plant height. The phenotypic observation was the BLUPs for the MET analysis, which includes AR1 x AR1 terms to adjust for column and row effects. In addition, this model includes a centered and standardized covariance for plant height. The red line represents the threshold value after correcting for multiple testing using the $M_{eff.}$ The arrows point significant SNPs detected by the FDR statistics

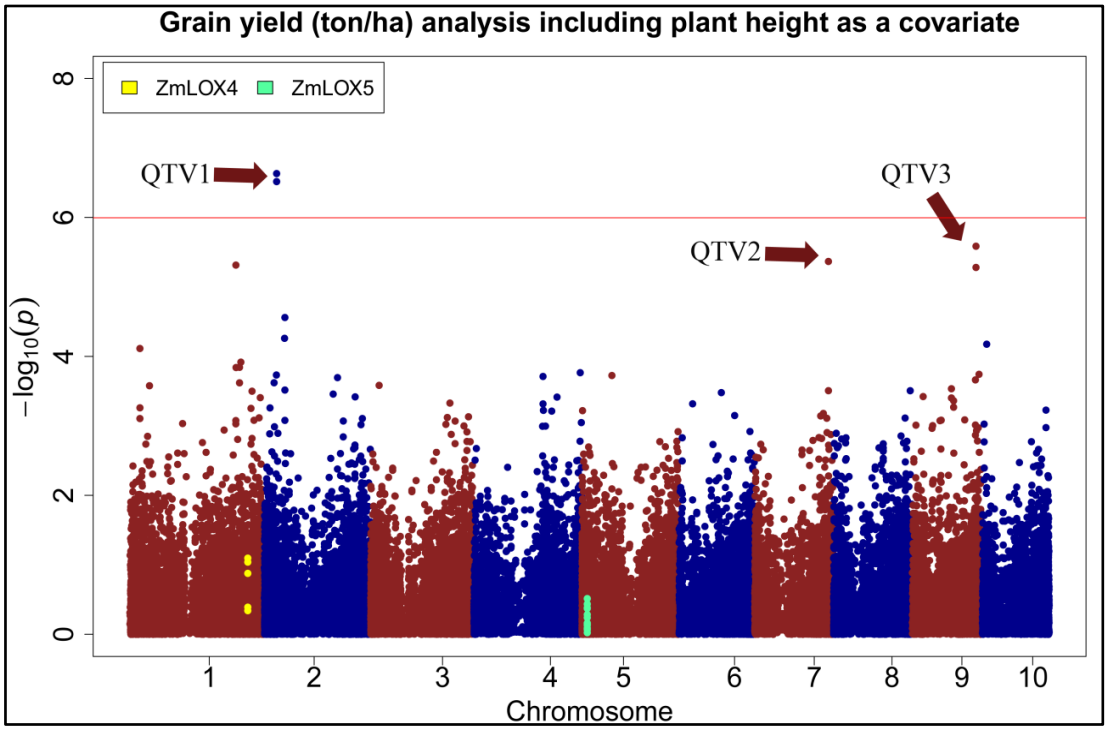


Table 4.14. Summary of the most promising QTV variants found in this study. SNP position test, QTV name, bin, chromosome, SNP_1 allele one present, SNP_2 allele two, description of the translated protein motif, plausible transcript as reported in the B73**.**

SNP	QTV variant	Bin	Chr.	Allele 1	Allele 2	Description	Plausible transcript
Grain yield (ton/ha)							
S2_27482431	QTV1	2.03	2	A	C*	PUT-2-171a-Zea_mays-13770	GRMZM2G035688
S7_164955163	QTV2	7.04	7	A	C*	Protein unknown function	GRMZM2G009320
S9_142746374	QTV3	9.06	9	A	G*	Clp amino terminal domain	GRMZM2G150598
S9_149545863	QTV4	9.07	9	C	T*	unknown motif	GRMZM5G864133
S10_10246117	QTV5	10.02	10	G*	T	unknown motif	GRMZM2G475197
Plant height (cm)							
S7_164955163	QTV2	7.04	7	A	C*	Protein unknown function	GRMZM2G009320
S3_168307280	QTV6	3.05	3	A*	C	Chromatin assembly factor I	GRMZM2G096458
Ear height (cm)							
S2_34433893	QTV7	2..04	2	C	T*	Proton-dependent oligopeptide transporter	GRMZM2G138731
S4_62573339	QTV8	4.05	4	C*	G*	four cysteine-rich zinc finger protein	GRMZM2G153722
S4_173817044	QTV9	4.07	4	C	T*	Promoter region	GRMZM2G549279
S4_173996901	QTV10	4.07	4	A	G*	Promoter region	GRMZM2G010755
Days to anthesis or days to silk							
S7_164955163	QTV2	7.04	7	A	C*	Protein unknown function	GRMZM2G009320
S8_131176630	QTV11	8.05	8	C*	T	Protein tyrosine kinase	GRMZM2G120839
S4_173817044	QTV9	4.07	4	C	T*	Promoter region	GRMZM2G549279
S8_123509373	QTV12	8.05	8	C	G*	Protein unknown function	GRMZM2G479987
S3_1775697	QTV13	3.02	3	A	C*	Epsin N-terminal homology (Domain)	GRMZM2G123499

*****Allele with the positive effect

Based on these results, the transcript for QTL2 variant was investigated. Austin and Lee (1996) found a QTL for grain yield in the same bin that explain 3.9% of the phenotypic variation; however, the sparse map makes it impossible to know the exact location. Recent studies using a 1000 F4:5 maize testcross progenies developed by Pioneer Hi-Bred in 1995, found a QTL in the same chromosome bin across seven environments in the corn belt (Schön et al., 2004; Boer et al., 2007). To identify potential candidate genes for QTV2, the LD was investigated and found to decay within 0.2 kilobases (kp) upstream and showed no LD with the closest marker 225 (kb) downstream. Different authors have reported that LD decay rapidly in maize around 1 to 10 kb, and specifically for chromosome seven linkage disequilibrium has varied from 2 to 5 kb depending on the region and the germplasm (Remington et al., 2001; Gore et al., 2009; Yan et al., 2009; Yan et al., 2011; Chia et al., 2012). Assuming that the LD follows the same pattern observed for the upstream area from QTV2, a plausible transcript corresponds to a protein with an unknown function (Table 4.14) that is expressed in the V1 (vegetative stage 1) (Maize eFP browser) (Winter et al., 2007; Sekhon et al., 2011).

QTV3 is located in bin 9.06, the allelic effect ranges from 0.28 to 0.33 ton/ha and the percentage of explained phenotypic variation ranges from 3.5 to 3.9% (Table 4.13). No QTLs have been reported in bin 9.06 for grain yield to our knowledge. LD nearby the QTV3 variant is higher than average, which is consistent to previous reports for chromosome nine (Remington et al., 2001; Yan et al., 2009; Yan et al., 2011). Upstream from the QTV3 variant there are six transcripts that exhibited a high level of LD between

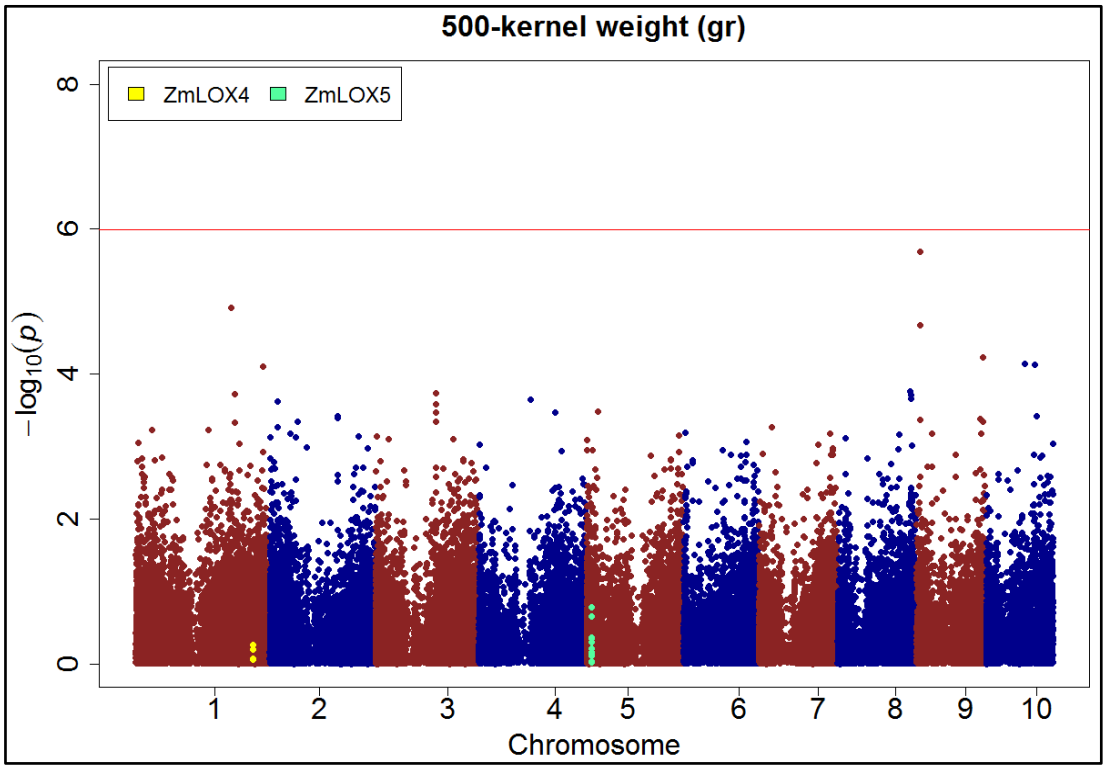
them. Downstream from the QTV3 variant, there are two additional transcripts (GRMZM2G150594 GRMZM2G573775). QTV4 (bin 9.07), and QTL5 (bin 10.02) have no previously reported QTL, and correspond to genes of unknown function (Table 4.14) This study did not find a significant QTV for 500-kernel weight after stringent corrections for multiple testing. However, a skyscraper near significance was consistently observed on bin 9.01 (Figure 4.5) across different trials and both of the MET analysis (Table 4.15).

Table 4.15. GWAS results for 500-kernel weight (gr.). Significant markers associated after correcting for multiple testing (SNP), their MAF, FDR adjusted p value of association, p value of the association (-log10 (p)), allele estimated effect, percentage of variation explained by marker (R^2), and the assigned name of the QTV in this study.

SNP	CHR	MAF	FDR_adjusted_P	Log10	Effect	R^2 (%)
CS11-LIYT-US[a]						
S9_8416672	9	0.45	0.2483	5.39	2.3	4.1
CS12-DYTL-US[a]						
S9_8416672	9	0.45	0.4963	5.09	3.6	4
CS12-LIYT-US[a]						
S9_8416672	9	0.45	0.4382	5.15	2.7	3.8
RCBD[b]						
S9_8416672	9	0.45	0.1929	5.41	2.4	4.2
SNP	**CHR**	**MAF**	**FDR_adjusted_P**	**Log10**	**Effect**	**R^2 (%)**
Spatial[c]						
S9_8416672	9	0.45	0.1248	5.69	2.6	4.3

[a] The phenotypic observation used for the GWAS was the BLUP for the hybrid effect obtained using model in Eq. (4.4).
[b] The phenotypic observation used for the GWAS was the BLUP for the hybrid effect from the MET model in Eq. (3.3).
[c] The phenotypic observation used for the GWAS was the BLUP for the hybrid effect obtained using the MET model in Eq. (3.3) including AR1 x AR1 terms for row and column effects.

Figure 4.5. GWAS results for 500-kernel weight (gr). The phenotypic observation was the BLUPs for the CS12-LIYT trial. The red line represents the threshold value after correcting for multiple testing using the M_{eff}



The effect estimates ranged from 2.3 to 3.6 (g per 500 kernels) and the explained 3.8 to 4.3% of the phenotypic variation. Despite that, this skyscraper was not significant after correcting for multiple testing, this SNP warrants further investigation based on the fact that other authors have reported a linkage QTL for 300 kernel weight, named q300k21, in the same bin (Goldman et al., 1993, 1994; Schaeffer et al., 2006) and because the SNP was consistently detected near significance in all analyses.

GWAS for plant and ear height

Two QTVs for plant height were detected (Figure 4.6). QTV2, was previously described for grain yield (Table 4.13 and 4.14) and several studies have reported a QTL for height at bin 7.04 (Schön et al., 1994; Veldboom and Lee, 1996a,b). QTV2 has an effect that ranges from 5.3 to 5.6 centimetres, and explained 4.6 to 5% of the phenotypic variation (Table 4.16). The other SNP, QTV6 at bin 3.05 had an effect that ranged from 3 to 3.2 centimetre and the percentage of explained 4.7 to 4.8% of the phenotypic variation (Table 4.16).

Figure 4.6. GWAS results for plant height (cm). The phenotypic observation was the BLUPs for the CS11-DTYL trial. The red line represents the threshold value after correcting for multiple testing using the Meff. The arrows point significant SNPs detected by the FDR statistics

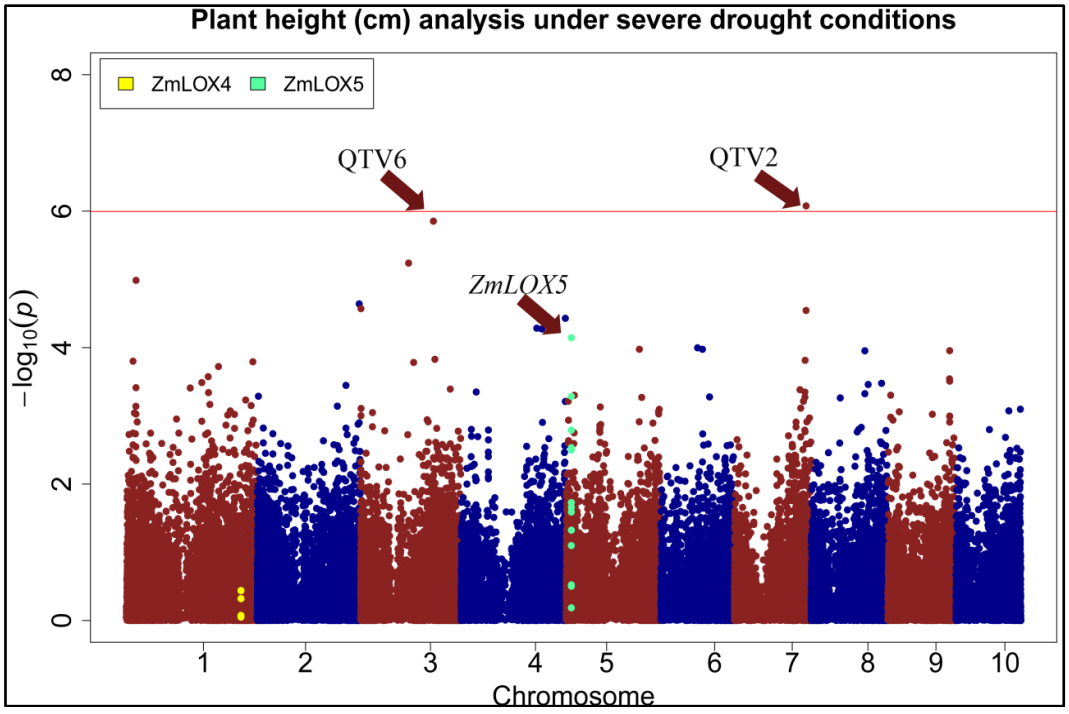


Table 4.16. GWAS results for plant and ear height (cm). Significant markers associated after correcting for multiple testing (SNP), their MAF, FDR adjusted p value of association, p value of the association (-log10 (p)), allele estimated effect, percentage of variation explained by marker (R^2), and the assigned name of the QTV in this study.

SNP	CHR	MAF	FDR_Adjusted_P	Log10	Effect	R^2 (%)	QTV
Plant height (cm)							
CS11-DYTL-US[a]							
S7_164955163	7	0.08	0.0429	6.08	5.3	5	QTV2
S3_168307280	3	0.36	0.0429	5.85	3	4.8	QTV6
CS11-LIYT-US[a]							
S3_168307280	3	0.36	0.0577	5.81	3.2	4.7	QTV6
S7_164955163	7	0.08	0.0577	5.73	5.6	4.6	QTV2
Ear height (cm)							
CS12-DYTL (average from raw data)							
S2_34433893	2	0.31	0.0391	6.2	3.9	6.3	QTV7
CS12-DYTL-US[a]							
S4_173996901	4	0.13	0.0811	5.88	3.8	4.6	QTV10
SNP	**CHR**	**MAF**	**FDR_Adjusted_P**	**Log10**	**Effect**	**R^2 (%)**	**QTV**
CS12-LIYT (average from raw data)							
S4_173817044	4	0.14	0.0789	5.69	6.5	5	QTV9
S4_62573339	4	0.41	0.0789	5.59	4.4	5	QTV8
CS12-LIYT-US[a]							
S4_173996901	4	0.13	0.1468	5.62	4.3	4.4	QTV10
RCBD[b]							
S4_173817044	4	0.13	0.362	5.23	3.8	4.2	QTV9

a The phenotypic observation used for the GWAS was the BLUP for the hybrid effect obtained using model in Eq. (4.4).
b The phenotypic observation used for the GWAS was the BLUP for the hybrid effect from the MET model in Eq. (3.3).

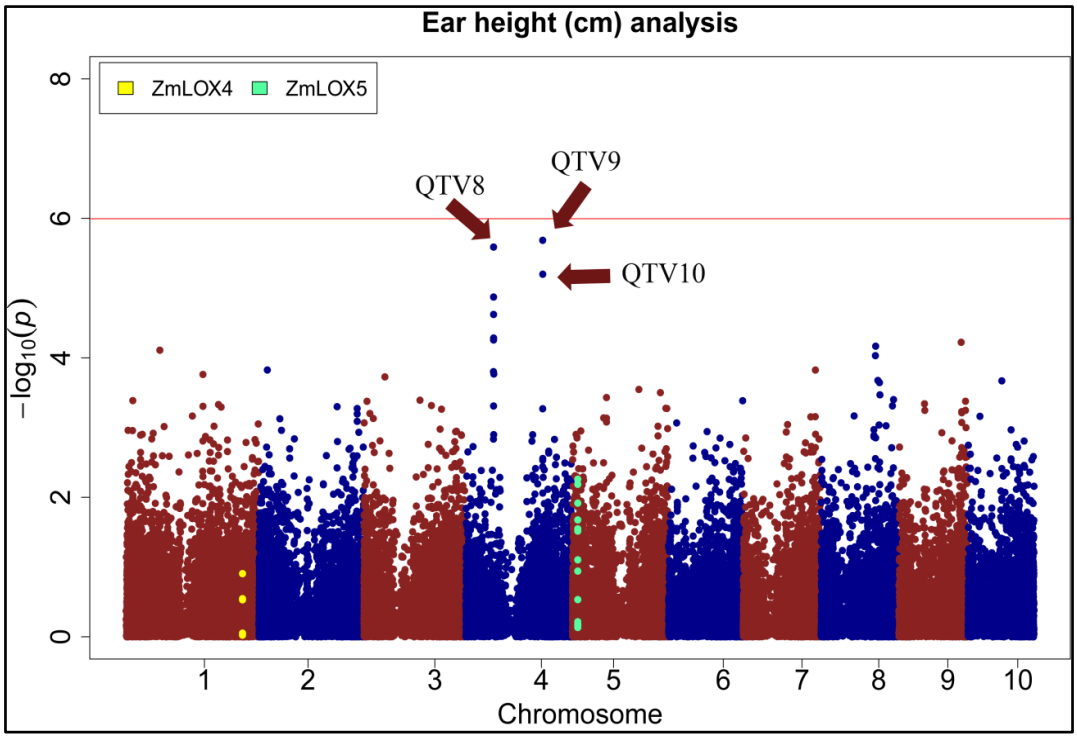
A QTL for plant height has been reported previously in bin 3.05 using a bi-parental cross between ki3 and CML139 (Bohn et al., 1997). Both QTV2 and QTV6 were detected in the non-irrigated and in the irrigated trials in 2011 (Figure 4.6).

For ear height only one significant SNP, QTL7 (bin 2.04) was detected after correcting for multiple testing and only in the non-irrigated trial in 2012, it had an estimated effect of 3.9 centimetres and explained is 6.3% of the phenotypic variation. No QTL have been previously reported for ear height in this bin to our knowledge. Three additional skyscrapers were consistently detected across different analyses and the effect was strongest in the CS12-LIYT trial (Figure 4.7). These SNPs are worthy of additional consideration despite the fact that neither of them was significant after adjusting for multiple testing. The authors decided to consider these SNPs as QTL variants based on the FDR p value, which was < 0.10 for all these SNPs (Table 4.16). The S4_62573339 SNP , which was named QTV8, is located in bin 4.05, and it has an allele effect of 4.4 centimetres and explained 5% of the phenotypic variation (Table 4.16). Although a putative transcript is located in the location of QTL8; however, the LD extends around 100 kb indicating that this transcript may not be the causal gene.

There is a strong LD in this area, which indicates that this region of the genome has been under selection since LD generally decays around 1 to 5 kb in chromosome four (Remington et al., 2001; Gore et al., 2009; Yan et al., 2009; Yan et al., 2011; Chia et al., 2012). A QTL for plant height, but not ear height has been reported in bin 4.05 by different authors (Beavis et al., 1994; Veldboom and Lee, 1994; Veldboom and Lee, 1996a,b). The S4_173817044 and S4_173996901 are located in bin 4.07; however, they

are separated 174 kb apart we decided to consider them two different QTVs. These SNPs were named QTV9 and QTV10, respectively. The effect of QTV9 ranges from 3.8 to 6.5 centimetres, and the percentage of explained phenotypic variation ranges from 4.2 to 5%. The effect of QTV10 ranges from 3.8 to 4.3 centimetres and the amount of phenotypic variation ranges from 4.4 to 4.6%. Similarly to the previous QTL for ear height, neither of them has been reported by other authors. Additionally, the LD decay around 100 bp in this area of chromosome four and based on the Maize B73 RefGen_v2 genome, these SNPs seems to be in the promoter regions of different transcripts (Table 4.14).

Figure 4.7. GWAS results for ear height (cm). The phenotypic observation was the BLUPs for the CS12-LIYT trial. Arrows point possible significant SNPs based on different analysis



GWAS for flowering time traits

QTLs for days to anthesis and days to silk (Figure 4.8) were found, with effects ranging from 0.5 to 1.8 days and the percentage of explained phenotypic variation range from 4.2 to 7.4% (Table 4.17). QTV2, previously reported for grain yield and plant height was also detected for days to anthesis and days to silk in the irrigated trial of 2011 and 2012. Buckler et al., (2009) reported three QTLs for days to anthesis (PZA03624, PZA03728, PZA-1744) and four QTLs for days to silk (PHM15501.9, PZA00986.1, PZA02722.1, PZA01044.1) on chromosome seven. However, based on the physical location and genetic maps distance none of the markers are located in bin 7.04. QTV11 (bin 8.05) was detected for both traits with effects for days to anthesis of 0.9 days to anthesis and 1 day for days to silk of 1 day both explaining 5.8% of phenotypic variation. Two different SNPs were found for QTV12 (bin 8.05), S8_123509373 and S8_123511933, separated by 2.5 kb. The effect for these QTL variant ranges from 0.5 to 0.8 and the phenotypic variation ranges from 4.6 to 4.7% (Table 4.17).

Buckler et al., (2009) reported three QTLs for days to anthesis (PZA00908.2, PZB02155.1, PZA00675.1) and three QTLs for days to silk (PHM4711.14, PZB02155.1, PHM1834.47) across eight environments for NAM panel. One of the QTL reported is located on chromosome eight in bin 8.05 (locus pzb02155), located between position 123,542,426 and position 125,974,265, which is 30 kb downstream from QTV12. LD extends for 3 kb upstream from QTV12 (results not shown); however, downstream from the QTV12, there is gap between the markers of 80 kb. As a consequence, this study cannot definitively determine if QTV12 and locus PBZ02155.1 are the same QTL or not.

QTV9 (bin 4.07) was previously reported for ear height (Table 4.16) and it has had an estimated effect that ranges ranged from 1.1 to 0.8 for days to anthesis and the percentage of explained phenotypic variation ranges from 4.6 to 4. 9%. The effect for days to silk was 1.2 days and the percentage of explained phenotypic variation is 4. 9%. No QTLs have been reported for this bin in previous studies. QTV13 has an effect of 0.6 for days to anthesis and 0.8 for days to silk. The amount of phenotypic variation explained is 4.2 for days to anthesis, and 4.3 for days to silk (Table 4.17).

Figure 4.8. GWAS results for days to anthesis and days to silk. The phenotypic observation was the BLUPs for the CS12-LIYT trial. Arrows point significant SNPs after correcting for multiple testing using FDR or effective number of independent test (M_{eff}).

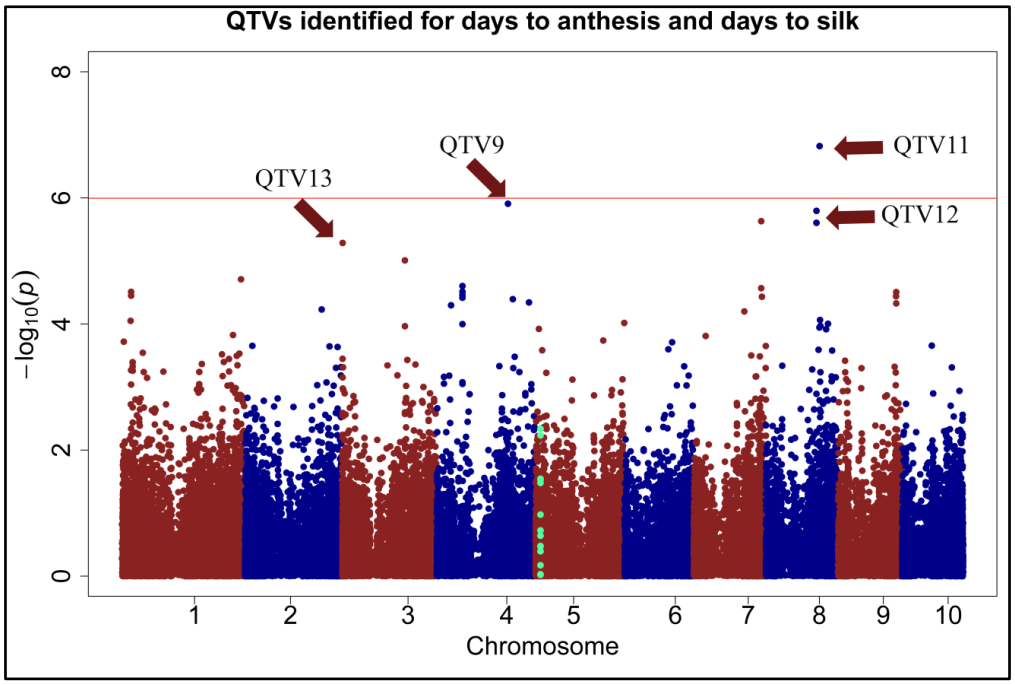


Table 4.17. GWAS results for days to anthesis and days to silk. Significant markers associated after correcting for multiple testing (SNP), their MAF, FDR adjusted p value of association, p value of the association [-log10 (p)], allele estimated effect, percentage of variation explained by marker (R^2), and the assigned name of the QTV in this study.

Days to anthesis							
SNP	**CHR**	**MAF**	**FDR_adjusted_P**	**Log10**	**Effect**	**R2 (%)**	**QTV**
CS12-DYTL[a]							
S7_164955163	7	0.07	0.0006	8.03	1.8	7.4	QTV2
CS12-LIYT[b]							
S8_131176630	8	0.26	0.0088	6.85	0.9	5.8	QTV11
S4_173817044	4	0.14	0.0314	5.91	0.8	4.9	QTV9
S8_123509373	8	0.37	0.0314	5.78	0.6	4.7	QTV12
S7_164955163	7	0.08	0.0314	5.65	1.8	4.6	QTV2
S8_123511933	8	0.38	0.0314	5.59	0.5	4.6	QTV12
S3_1775697	3	0.41	0.0571	5.25	0.6	4.2	QTV13
RCBD[a]							
S7_164955163	7	0.07	0.0026	7.37	1.6	6.4	QTV2
S8_131176630	8	0.27	0.0563	5.74	0.9	4.6	QTV11
S4_173817044	4	0.14	0.0563	5.74	1.1	4.6	QTV9
Days to silk							
CS12-DYTL[a]							
S7_164955163	7	0.07	0.0006	8.01	1.8	7.4	QTV2
CS12-LIYT[a]							
S8_131176630	8	0.26	0.0092	6.82	1	5.8	QTV11
S4_173817044	4	0.14	0.0304	5.91	1.2	4.9	QTV9
S8_123509373	8	0.37	0.0304	5.8	0.8	4.8	QTV12
S7_164955163	7	0.08	0.0304	5.63	1.3	4.6	QTV2
S8_123511933	8	0.38	0.0304	5.61	0.8	4.6	QTV12
S3_1775697	3	0.41	0.0529	5.29	0.8	4.3	QTV13
RCBD[a]							
S7_164955163	7	0.07	0.0029	7.33	1.6	6.4	QTV2

[a]Phenotypic observation for the GWAS was the average for days to anthesis or days to silk

This study did not find any significant QTL variants for ASI despite that multiple analysis we run using the average for the raw data, and the MET analysis described in Eq. (4.3) and (4.4).

Buckler et al. (2009) study was the most powerful to date for flowering time QTV detection, and many of the QTV were not shared between studies, it raises questions if this differences observed was because the use of different germplasm, different environments, or the use of a tester. If germplasm differences causes the observed differences this suggests that the hypothesis of multiple variants on common genes may play a role more in temperate than tropical germplasm. If different environments are the cause, this suggests that local testing is critical. If the use of a tester than this suggests that for the most relevance to crop improvement, only testcrossed hybrids should be used in GWAS.

GWAS for aflatoxin resistance

Although this study was primarily designed to detect natural variation for aflatoxin resistance, none of the SNPs above a MAF of 5% were found to be significant for aflatoxin accumulation in any of the environments. The GWAS did not find any significant QTLs for the transformed data after correcting for multiple testing (Figure 4.9). Three skyscrapers consistently appeared for the irrigated trials CS11-LIYT and CS12-LIYT, and the combined MET analysis (Table 4.19). SNP: S3_185272026 (bin 3.06) had an allele effect of 0.20 (ng g−1) and explained 6.06% of phenotypic variation (Table 4.18). This SNP corresponds to transcript GRMZM2G399433, which is highly

expressed in the pericarp, embryo and endosperm, the silks and the cob during flowering and post-flowering (Sekhon et al., 2011). A QTL for aflatoxin and Aspergillus ear rot resistance has been reported in bin 3.06 (Paul et al., 2003; Warburton et al., 2010; Xiang et al., 2010). Paul et al., (2003) reported a significant QTL in bin 3.06 for aflatoxin resistance. These results were further corroborated by Xing et al., 2010 who reported two QTLs: MQTL11 (molecular markers zmm16, umc165a, TB-3Li) and MQTL12 (molecular markers IDP4468, K3L, Bnlg197) in bin 3.06 associated with *Aspergillus* ear rot resistance.

Figure 4.9. GWAS results for aflatoxin (ng g−1). The phenotypic observation was the BLUPs for the CS12-LIYT trial. Arrows point skyscrapers found by this analysis that might be potential QTVs.

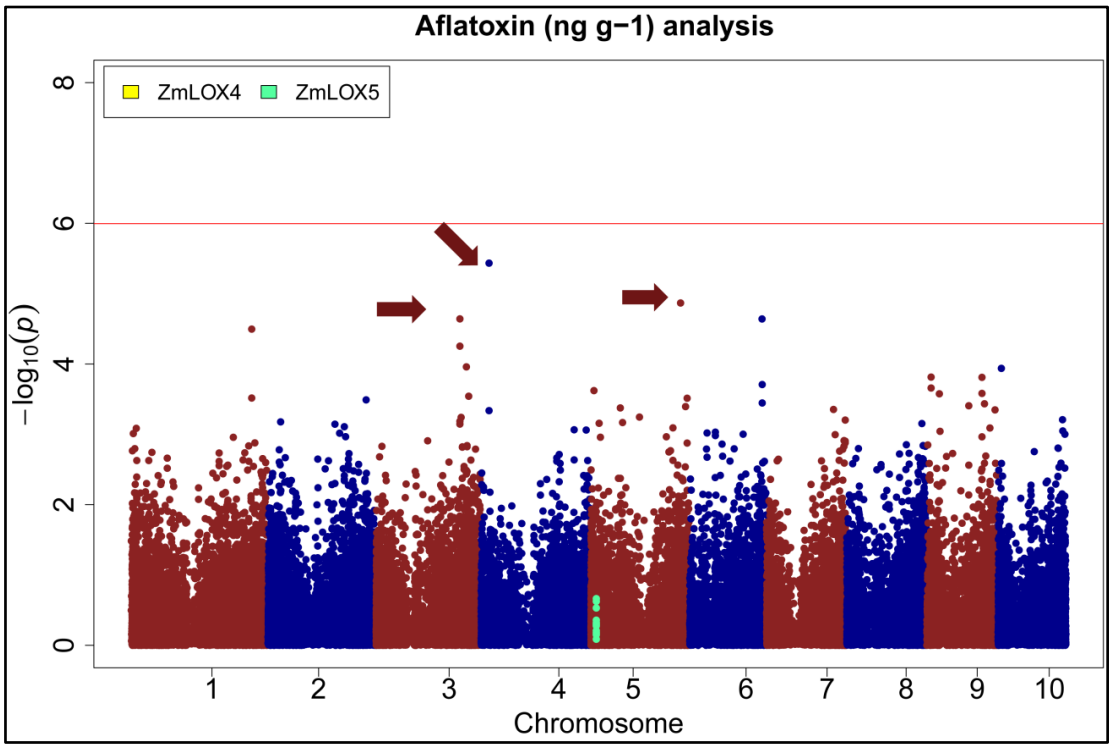


Table 4.18. GWAS results for aflatoxin level (ng g−1). Plausible significant markers associated after correcting for multiple testing (SNP), their MAF, FDR adjusted p value of association, p value of the association [-log10 (p)], allele estimated effect, percentage of variation explained by marker (R^2), and the assigned name of the QTV in this study.

SNP	CHR	MAF	FDR_adjusted_P	Log10	Effect	R2 (%)
CS11-LIYT[a] (log10 [aflatoxin + 10])						
S4_17376432	4	0.32	0.2	5.48	-0.03	5.27
S5_197707198	5	0.15	0.24	5.1	0.03	4.85
CS12-LIYT (log10 [aflatoxin + 10])						
S3_185272026	3	0.3	0.34	5.11	-0.2	6.06
CS12-LIYT[a] (log10 [aflatoxin + 10])						
S4_17376432	4	0.32	0.23	5.43	-0.07	5.69
RCBD (log10 [aflatoxin + 10])						
S5_197707198	5	0.15	0.27	5.22	0.05	4.98
S4_17376432	4	0.32	0.27	5.05	-0.04	4.79

[a] The phenotypic observation used for the GWAS was the BLUP for the hybrid effect obtained using model in Eq. (4.4).

The SNP: S3_185272026 (bin 4.03) had an allele effect ranges from 0.07 to 0.03 (ng g−1) and explained phenotypic variation ranging from 5.3 to 5.7%. The transcript GRMZM2G013546 correspond to this SNP marker and is highly expressed in the pericarp and the husk of maize (Sekhon et al., 2011). Warburton et al., (2009) reported the markers phi029 and marker umc1970. These markers were identified in a progeny between a highly susceptible line Va35 and the highly resistance line Mp313E (Brown et al., 1999; Willcox et al., 2013). Further evidence of the presence of a significant QTL in this bin comes from a recent meta-analysis study that names these markers as mqtl1 and mqtl2. After building a consensus map the study found that these markers extended from bins 4.02 to 4.04, which seems to contain six QTL for the three diseases (Mideros et al, 2013 under review). Multiple QTL for ear rot resistance have been reported in bin 4.03

for resistance to ear rot diseases such as Fusarium and Gibberella ear rot (Wisser et al., 2006; Xiang et al., 2010).

The other skyscraper, SNP: S5_197707198 (bin 5.06) corresponds to the transcript GRMZM2G057789, which is highly expressed in the silks during the R1 stage (Sekhon et al., 2011). Xiang et al., (2010) reported a QTL responsible for ear rot resistance in the same bin 5.06, and a QTL is also reported by Mideros et al., (2013 – under review).

Major findings of this study

Several candidate gene or GWAS analysis have now been performed in different plant species (Neale and Savolainen, 2004; Breseghello and Sorrells, 2006; González-Martínez et al., 2007; Murray et al., 2009; Weber et al., 2009; Atwell et al., 2010; Quesada et al., 2010; Pasam et al., 2012; Larsson et al., 2013). These studies have reported on average fewer associations than linkage mapping based studies even for genes involved in domestication events. Although many maize association mapping studies have been conducted (Wilson et al., 2004; Weber et al., 2009; Krill et al., 2010) there have been no previous reports of mapping in a hybrid testcross background. This study found 13 QTV and other potential associations which were nearly significant after correcting for ~50,000 tests; several of these QTVs have been reported by other studies suggesting association mapping in a diverse set of germplasm using testcrosses is consistent and relevant. The detection power of association mapping is affected by several factors such as the sample size, population structures, the extend of the LD, the

magnitude of the effect and the quality and density of the SNP markers used (Remington et al., 2001; Yu et al., 2006; Pongpanich et al., 2010; Yan et al., 2011). The results here clearly highlight the importance of sample size to be able to detect genes of small effect, likely the genetic basis for complex trait such as drought tolerance and aflatoxin resistance. Yan et al., (2011) reported that using a population of 500 individuals in GWAS can detect associations that explain 3% of the phenotypic variation or more. Increasing the sample size to 1500 genotypes can detect associations that explain 1% of the phenotypic variation but is not practical given the resources needed with replication. Similar results have been obtained for QTL mapping, where it has been shown that increasing the number of individuals is more efficient than increasing the number of replications (Schön et al., 2004). The importance of the sample size was well-exemplified by the results obtained for aflatoxin where no significant associations were detected after correcting for multiple testing. The experiment was highly unbalanced between the trials with only 193 hybrids shared across all three locations, and 218 hybrids were shared among the CS trials. This unbalance in the hybrids between the trials could be another reason that even with high heritability values, GWAS failed to identify associations that explain around 5% of the phenotypic variation for some traits such as ear height (Table 4.16). One of the largest challenges to detecting significance is the issue of multiple testing, an ongoing area of research.

Future work

Two different approaches can be used to exploit this germplasm and the QTVs that were detected. The first approach involve the validation of the QTV with the highest significant p values (-log10 [p]) in the inbred lines that produce the highest yielding hybrids. Near isogenic lines (NIL) could be generated for this QTV under these "elite" genetic backgrounds. These NILs would be crossed to the most elite material and the effect of these alleles should be evaluated under multiple genetic backgrounds and environments. It is expected that the effect will not be as background and context specific (environment, genetic background) as might be observed for QTL in a narrow diversity bi-parental linkage population. Depending on the magnitude of the effect and the results, a marker assisted selection could be implemented.

Other possible option to validate the effect of the QTVs that will not involve the generation of NILs is to validate the effect of the QTVs in the most genetically similar maize inbred lines that carry different alleles for the specific locus. First individuals with the "good" QTV allele should be identified and then genetic distance can be used to find partner lines with the "bad" QTV allele. Both of the maize inbred lines should be testcrossed to Tx714 and evaluated under different environments. Based on these results, the validation could be expanded to other genetic backgrounds to determine the effect of the QTV.

CHAPTER V

SUMMARY AND CONCLUSIONS

This dissertation covered two different projects that use historical datasets and maize diversity to characterize trends and find methods to increase grain yield in the state of Texas. The first project found that Texas is divided into two major growing regions: the High Plains and the rest of Texas by a number of agronomic factors. This was corroborated by the analysis of this data, where grain yield, plant height and ear height differed significantly between the High Plains and the rest of Texas. The analysis of the genotypic BLUPs showed that grain yield has not substantially increased in the last 11 years in Texas, which further corroborated the trend observed in the USDA data for Texas and specifically for dryland and partial irrigated maize producing counties. Despite USDA data showing that irrigated maize producing counties have increased similarly to other production zones in the Midwest, the genetic yield potential has generally remained stable for the last 11 years. This study demonstrates that data collected on an annual basis is valuable in retrospective meta-analyses to give insight into traits, patterns and processes that can suggest hypotheses for improved regional yield.

The second project used a maize diversity panel to identify genomic regions associated with grain yield, aflatoxin resistance and important agronomic traits. This study found that there is useful variation in diverse germplasm for aflatoxin resistance and drought tolerance. Additionally, this diverse germplasm when testcross to an elite,

although older, Texas line has the potential to out yield the commercial hybrids. The *ZmLOX4* genes were associated with days to anthesis and days to silk, while the *ZmLOX5* genes were associated with plant and ear height. This study also found 13 QTVs for grain yield, plant and ear height, days to anthesis and days to silk demonstrating the utility of GWAS. Once these QTVs are validated, they will be useful for molecular improvement of Southern maize germplasm and, if cloning is pursued, for understanding the basic biology of improvement of these traits.

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