BREEDING MAIZE FOR DROUGHT TOLERANCE: DIVERSITY CHARACTERIZATION AND LINKAGE DISEQUILIBRIUM OF MAIZE PARALOGS *ZMLOX4* AND *ZMLOX5*

A Thesis

by

GERALD NEIL DE LA FUENTE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2012

Major Subject: Plant Breeding

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Approved by:

Chair of Committee,	Seth C. Murray
Committee Members,	Mike V. Kolomiets
	Thomas S. Isakeit
Head of Department,	David D. Baltensperger

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ABSTRACT

Breeding Maize for Drought Tolerance: Diversity Characterization and Linkage Disequilibrium of Maize Paralogs *ZmLOX4* and *ZmLOX5*. (May 2012)

Gerald Neil De La Fuente, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Seth C. Murray

Maize production is limited agronomically by the availability of water and nutrients during the growing season. Of these two limiting factors, water availability is predicted to increase in importance as climate change and the growing urban landscape continue to stress limited supplies of freshwater. Historically, efforts to breed maize for water-limited environments have been extensive; especially in the areas of root architecture and flowering physiology. As progress has been made and new traits have been discovered and selected for, the different responses to drought stress at specific developmental stages of the maize plant have been selected as a whole when drought tolerance is evaluated. Herein we attempt to define the characteristics of the maize drought response during different developmental stages of the maize plant that can be altered through plant breeding. Towards breeding for drought tolerance, 400 inbred lines from a diversity panel were amplified and sequenced at the *ZmLOX4* and *ZmLOX5* loci in an effort to characterize their linkage disequilibrium and genetic diversity. Understanding these characteristics is essential for an association mapping study that

accompanies this project, searching for novel and natural allelic diversity to improve drought tolerance and aflatoxin resistance in maize.

This study is among the first to investigate genetic diversity at important gene paralogs *ZmLOX4* and *ZmLOX5* believed to be highly conserved among all Eukaryotes. We show very little genetic diversity and very low linkage disequilibrium in these genes, but also identified one natural variant line with knocked out *ZmLOX5*, a variant line missing *ZmLOX5*, and five line variants with a duplication of *ZmLOX5*. Tajima's D test suggests that both *ZmLOX4* and *ZmLOX5* have both been under neutral selection. Further investigation of haplotype data revealed that *ZmLOX12*, a member of the *ZmLOX* family, showed strong LD that extends much further than expected in maize. Linkage disequilibrium patterns at these loci of interest are crucial to quantify for future candidate gene association mapping studies. Knockout and copy number variants of *ZmLOX5*, while not a surprising find, are under further investigation for crop improvement.

DEDICATION

In memory of, Mr. Patrick Joseph Lamon, on whose family farm I discovered my love for agriculture. Sincere appreciation is extended to the family of Mr. Lamon, especially his wife Margaret, his son Mark, daughter-in-law Krista, and his grandchildren Joey, Jacob, William, and Alex, for recognizing my passion and allowing it to grow through the years. My career path was chosen, in no small part, because of the time I spent helping care for the crops on this farm.

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Finally, thanks to my parents, grandparents, and extended family for believing in me and for giving me the strength to pursue the career that I love.

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CHAPTER I

INTRODUCTION

Nutrient and water availability are the two most limiting resources in crop production (Boyer, 1982; Lea and Azevedo, 2006; Moser et al., 2006). Nutrient application has benefitted from advancements in precision agriculture techniques. Producers are now able to customize applications by specifying the blend ratio of nutrients and by precisely controlling rates of application based on data from the previous years' yield and soil sampling results (Miao, 2007). Water and irrigation, however, because of the large quantities needed, still pose problems to producers in regions that receive less than adequate rainfall, despite advances in technology and management practices (Sadler et al., 2005). In Kansas, Nebraska, and Texas, maize uses 21-38 inches of water per year depending on water supply, hybrid, maturity, and soil type (Howell et al., 1998). A 2010 production map of maize and 2011 rainfall map for counties in the United States is shown in Figure 1-1. Circled regions are those which might have suffered from water deficit in the 2011 growing season, and it is highly improbable that all the rainfall fell during the growing season. These regions historically have also had the most erratic rainfall patterns.

Despite ranking second in total land area, maize leads crop yield both globally, at 813 million metric tons, and within the United States, at 333 million metric tons (USDA-FAS, 2011). Maize production surpasses wheat by over 150 million metric tons, rice by

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This thesis follows the style of Crop Science.

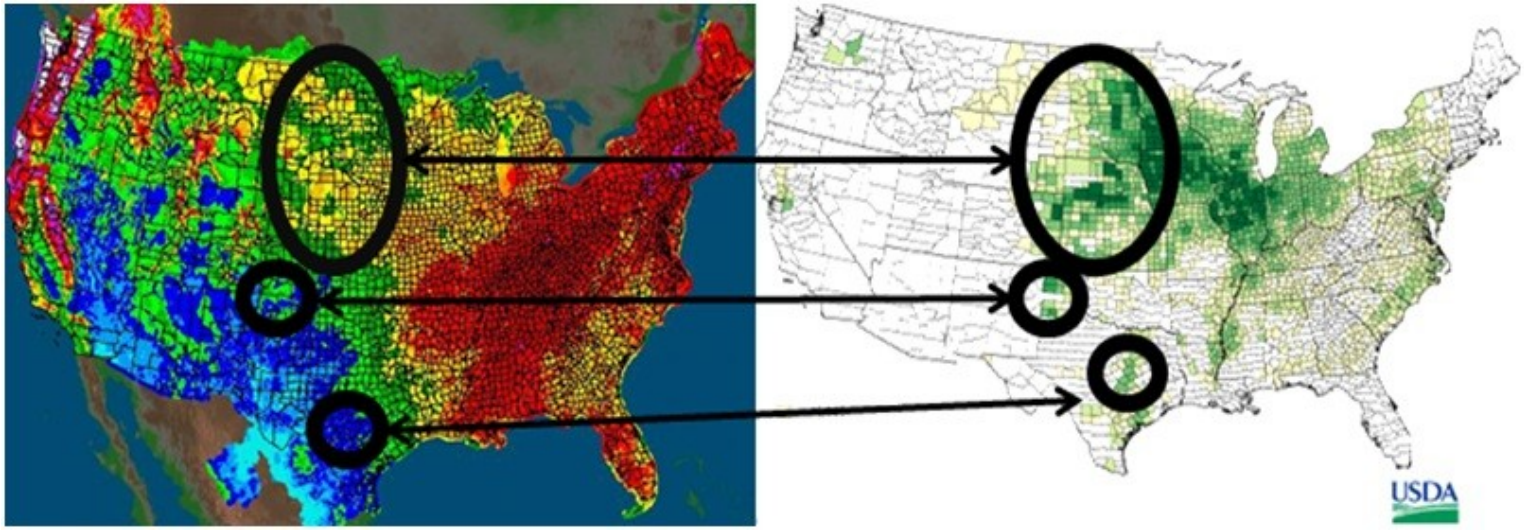

Figure 1-1. Comparison of 2011 rainfall totals and maize production. At left: distribution of rainfall across the continental United States during the 2011 water year. At right: Acreage distributions of maize across the Unites States in 2010. Circled regions are those that saw both low rainfall and large planted acreage of maize. (NWS-AHPS, 2011; USDA-NASS, 2011)

over 350 million metric tons, sorghum by over 700 million metric tons globally, and dominates U.S. cereal grain production (USDA-FAS, 2011). Therefore high yield per acre maize is critical to food security of calories, though not complete nutrition, in the U.S. and worldwide, and is often grown in rainfall-deficient areas.

A global climate change of only 1°C is estimated to have no effect on agricultural production; however, this change and the added demands of a growing and wealthier population, is expected to put a strain on water resources around the world (Hare, 2005). Water competition between urban and agricultural use will continue to increase, putting stress on maize production areas that are highly dependent upon available surface and subsurface water supplies. These increased demands will most likely force a choice between water and calories for certain areas of the world where maize production is dependent upon supplemental irrigation.

Describing drought stress can be difficult, but is important to define for crop improvement objectives. The term "drought" can carry many meanings depending on the situation in which it is used. For example, while a given region may not be in a drought over an entire year, a dry season during that year may lead to drought conditions. Here we define drought (also referred to as agricultural drought) as the time point when the amount of moisture in the soil no longer meets the needs of the crop (Mannocchi et al., 2009). Throughout the growing season, there are often periods where the soil moisture drops below the needs of the crop. Seasonal water use of the maize plant fluctuates and follows the pattern of increasing through the vegetative stages, peaking at flowering, and decreasing through grain fill and plant maturity (Figure 1-2). Yield components are

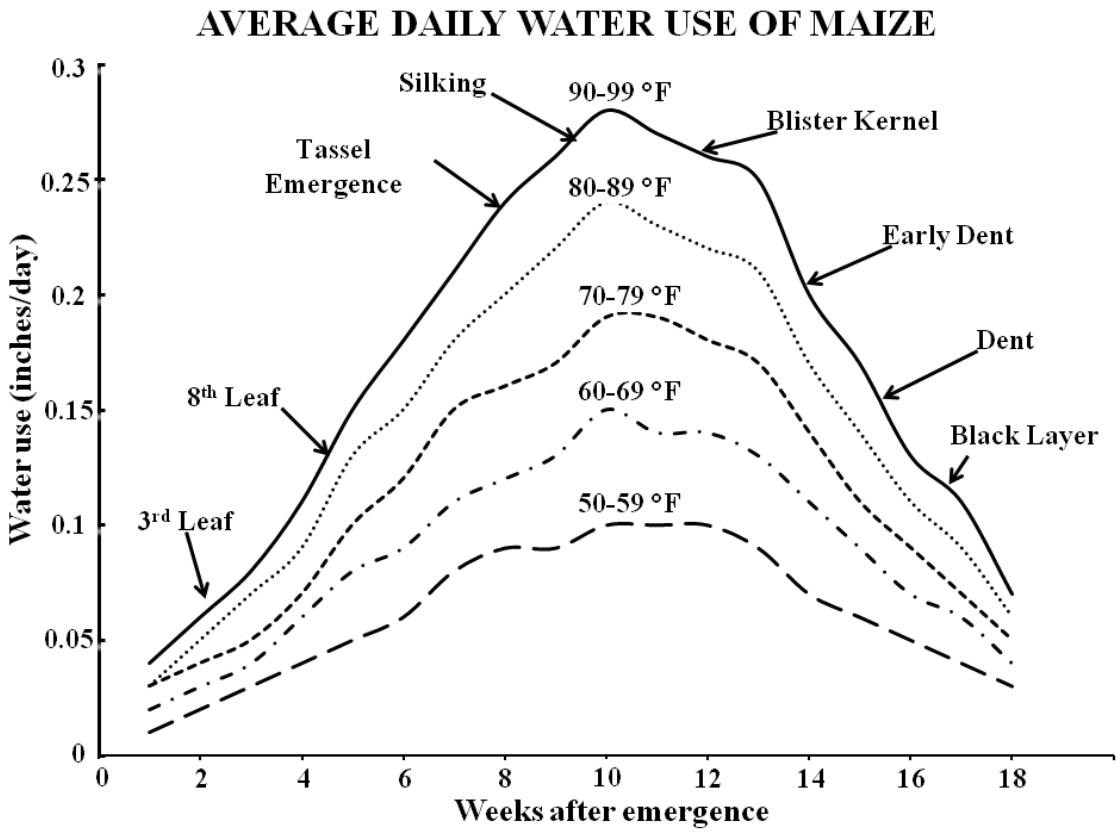

Figure 1-2. Average daily water use of maize at different temperature ranges (Killen, M. 1984; Wright, 2002). While more water is used at higher temperature ranges the same general trend is seen at all temperatures. Water use increases steadily through the vegetative stages, quickly peaks around flowering and then decreases through grain fill and up to maturity.

determined throughout the growing season and especially from V8 (eighth leaf stage) to black layer. Thus, moisture stress has a large window in which it can affect yield, so improvement of maize lines for drought stress is one of the most critical areas of cultivar development (Bruce et al., 2002).

An assortment of physiological mechanisms for coping with drought stress and classical breeding techniques have been investigated and used. Additional modern breeding methods are now being utilized to improve the ability of maize to cope with drought stress. However, the ideotype that is needed for the particular target area is not always clear from the literature, as the stages of drought tolerance are complex (Blum, 2005). In addition, even in those years where drought is not a serious problem on a large scale, producers with sandier soils that don't retain water could see localized drought symptoms, even with adequate rainfall. Because periods of drought conditions are expected to increase both locally and globally, drought tolerant lines of maize will not only aid in weathering stresses of the natural environment such as timely rainfall and the ability of the soil to hold water, but also aid with the stresses of inevitable failure of irrigation equipment such as well-head pumps and pivots.

DROUGHT TOLERANCE VS. DROUGHT AVOIDANCE

Herein we will define drought terms relevant to the discussion presented. For further discussion of terminology, the reader is directed to Levitt (1980) and a comprehensive review by Blum (2005) on drought resistance and water-use efficiency. Drought resistance across the plant kingdom is a rare phenomenon. Water is essential to plant growth and no plant can fully resist extended periods of time without water uptake. However, there are multiple mechanisms that allow plants to complete a life cycle even when the locality may suffer from drought conditions. Plants that can complete their life cycle in a short period of time allowing them to grow, for example, during the wet season exhibit 'drought escape' (Yue et al., 2006). Plants that have adapted to an

environment that follows the rainy and dry season pattern by flowering and maturing during the wet season can be classified as 'drought resistant'. In contrast, plants that can withstand periods of minimal water supply without excessive damage or loss of function are classified as 'drought tolerant'. Drought tolerance is separate from drought resistance and there are likely different physiological and genetic mechanisms involved. Drought tolerance can be subdivided into two categories: dehydration avoidance and dehydration tolerance (Levitt, 1980). Dehydration avoidance is defined as the plant's capacity to sustain high plant water status or cellular hydration under the effects of drought, while dehydration tolerance is defined as the relative capacity to sustain or conserve plant function in a dehydrated state (Levitt, 1980; Blum, 2005). Drought escape by mechanisms of early flowering is not a feasible trait to breed for as the growing season is long, and weather patterns are highly variable. Breeding for drought tolerance in the context of dehydration tolerance and avoidance must be accomplished by utilizing native physiological and morphological traits.

DROUGHT TOLERANCE VS. WATER USE EFFICIENCY

Water use efficiency (WUE) is a term that is commonly heard when discussions of crop improvement for drought conditions are discussed. However, a very important distinction needs to be made between the concepts of drought tolerance and water use efficiency. Similar to drought tolerance, the definition of water use efficiency fluctuates depending on the context. In its strictest sense water use efficiency is the reciprocal of the transpiration ratio, defined as the total quantity of water transpired over the amount of carbon dioxide used by photosynthesis (Taiz and Zeiger, 2006). In other words, how

much carbon can the plant take up per volume of water used. Therefore, WUE can be considered when drought is not present. A recent argument was made that the effective use of water and not WUE is the true target of breeding for drought tolerance (Blum, 2009). In other words, yield in crops is driven by transpiration and thus an effort should be made to extract as much water as possible to drive photosynthesis and transpiration versus using only part of the available water and distributing it within the plant in the most efficient way.

IMPROVING DROUGHT TOLERANCE IN MAIZE

Drought tolerance in maize is a complex trait and likely involves many interconnected genes and pathways, but there are genes that have shown some useful effects towards increasing drought tolerance. One of these genes is *ZmLOX4*, a member of the *Z. mays* lipoxygenase gene family and segmentally duplicated paralog to *ZmLOX5*. Molecular characterization and preliminary investigations of these two genes was done by Park et al. (2010). Through the use of transposon tagging, knockout mutant isolines of *zmlox4* and *zmlox5* were created to investigate the functions of these two genes in maize plants. Physiological, phenotypic and molecular observations were made on the knockout mutants to see what effect turning off the gene would have on normal functioning of the maize plant. Among many responses seen, knocking out *ZmLOX4* conferred drought tolerance in a seedling lab screen and knocking out *ZmLOX5* conferred a decrease in field contamination of aflatoxin, a potent carcinogen whose accumulation is usually associated with drought environments.

Using this knowledge, we hypothesized that novel and natural allelic variation could be present at the *ZmLOX4* and *ZmLOX5* loci that could be used in breeding programs for crop improvement. These alleles would likely be rare, and therefore a diverse panel of inbred lines was selected that captured the majority of the diversity found in modern maize. Based on sequence architecture and knowledge of the conserved regions of the two paralogs, the 3' end of the gene, consisting of the last exon was chosen to be sequenced. Diversity characterization and polymorphisms present would be used in future association mapping analyses.

Measurements of drought tolerance and aflatoxin resistance, and other traits of interest, would be conducted on testcross hybrids of the inbred lines of the diversity panel crossed with two variants (*zmlox4* and *zmlox5* mutant isolines) of Tx714. Hybrid combinations of Tx714 mutants with the diverse inbred lines provides a way to screen one functional copy of the *ZmLOX4* and *ZmLOX5* genes in search of new beneficial alleles.

CHAPTER II

REVIEW OF LITERATURE: BREEDING MAIZE FOR DROUGHT TOLERANCE

LEVELS OF THE MAIZE DROUGHT RESPONSE

Historically, grain yield components for maize have been established and vary among studies with respect to the type and timing of stresses. For maize, grain yield per unit land area can be broken down into four major components, each having a critical developmental stage that affects the particular yield component. Plant density or population per acre is established first during the growing season and reflects seedling drought tolerance. Ears per plant, while mostly no longer variable in elite maize hybrids, plays a role in inbred and open pollinated (OP) varieties of maize and is established in the vegetative stages of plant development. Kernel row number per ear is a major component of yield potential set by the maize plant before anthesis. Kernel number per row, across the length of the ear, is a function of several stages of development and can fluctuate with stress levels after kernel row number is established (e.g. pollination success and aborted kernels, both discussed later) (Hoeft et al., 2000). For maize, the five days prior to and following silking are the most crucial in sensitivity to water stress, along with the two weeks that follow (Shaw, 1974). Water stress must be minimal and supplemented with irrigation where possible. Simply having the capacity to irrigate, however, is not always enough. Mechanical failures in irrigation equipment or sudden decreases in the amount of water available to be used could have effects on yield. These

four major components combine to give the per acre yield that breeders and farmers strive to maximize.

Seedling Drought Tolerance

Except in unique cases, field-based drought stress during the seedling stage is rarely observed because moisture is typically adequate after the fallow season and planting into dry soil is rare. Because plant density has become one of the most important factors for yield per land area in maize, stand establishment is critical to final crop yield and reaching full yield potential (Tuberosa and Salvi, 2009; Grassini et al., 2011). Therefore, pre and post emergence drought tolerance, while normally unseen, is an important trait that saves producers money by eliminating the need for re-planting a poor stand early in the season, thereby also preventing potential losses from a late-planted crop. Simple screening techniques are commonly used such as plant recovery after re-watering in the lab setting. Measurement of this trait can be conducted as a live vs. dead plant ratio, but this method is subject to excessive measurement error (Meeks, 2010). More complicated objective techniques have been investigated such as membrane stability measurements (Blum and Evercon, 1981), but such methods are both time consuming and expensive. A novel technique variant pioneered by the International Maize and Wheat Improvement Center (CIMMYT) takes advantage of the plants recovery overnight (Banzinger et al., 1996). In this method, plants are scored for their recovery before daytime heating induces leaf rolling again. Plants that do not recover overnight are considered dead since the inability to regain turgor overnight is indicative of severe physiological failures. Correlations between this method and the

traditional method of re-irrigation and dead plant counts are high and significant. While this technique reduces phenotyping time by not having to re-irrigate, field testing allows for typically large environmental variations that can reduce heritability estimates. In an attempt to remove environmental variation, Meeks (2010) screened maize seedlings for drought tolerance in a greenhouse setting using a method that had previously been successful in cowpea (Singh et al., 1999) and cotton (Longenberger et al., 2006). The objectives of these studies, as in many phenotyping method development studies, were to create a quick and easy method to screen large numbers of lines for drought tolerance and correlate this to predictions of yield under drought. While the cowpea and cotton studies were successful in differentiating drought tolerant and drought susceptible lines, the study with maize had much lower success in predicting field-based drought tolerance, possibly due to the extreme physiological changes that accompany the transition from juvenile to adult phase (Revilla et al., 2002; Revilla et al., 2004; Chandler and Tracy, 2007; Riedeman and Tracy, 2010).

Drought During the Vegetative Stages

While not usually thought of as a part of drought adaptation and certainly not the most crucial, the vegetative stages of maize (V3-V12) are nonetheless important for obtaining maximum yield potential. As will be discussed later, photosynthate produced prior to anthesis only accounts for ~10% of that present in the mature grain (Swank et al., 1982; Simmons and Jones, 1985). This suggests that mild to moderate stress during the vegetative states will not affect kernel number directly through decreased photosynthesis. A major yield component, however, is known to be determined during

the vegetative stage of growth. The number of kernel rows is determined around the V8 stage with the initiation of the ear shoot (Hoeft et al., 2000). If moisture stress, or other stress, is present during the initiation of the ear shoot, kernel row number may decrease as a response. However, despite its quantitative inheritance, the number of kernel rows is only slightly affected by environmental stresses during development and once established kernel row number is fixed and always an even number (Emerson and Smith, 1950; Daniel, 1963). The number of kernels per row can be further modified by the plant based on moisture and other stress conditions all the way up to and after flowering. As seen in Figure 1-2, daily water use of the plant from V8 to flowering is high and increases very quickly, especially in the high temperatures the generally occur during mid-summer. The number of kernel rows has been shown to have significant additive effects and it is likely that through artificial selection over the past ~80 years that the genes controlling kernel number have become fixed in elite germplasm (Daniel, 1963; Lu et al., 2011b). Compared with other traits kernel rows are now only moderately affected by the environment in elite germplasm and likely play a small role in drought adaptation.

Maize Flowering Physiology Is Critical to Tolerate Drought

Maize has monoecious and imperfect flowers with male and female organs located on the same plant in different positions. The result of imperfect flowers are that pollen is forced to travel from the tassel (contains anthers), located at the top of the plant, to the silks (stigmas) that protrude from the developing ear shoot. This floral anatomy of maize heightens the effects that water stresses have on plant development

and ultimately yield (Westgate, 1997). The vertical distance that pollen must travel to reach the silks can range from 1-1.3 meters and pollen is generally moved by the wind, therefore the pollen and silks must be exposed to a desiccating environment. (Aylor et al., 2003).

Pollen shed is theoretically adequate to pollinate all of the kernels on the developing ear shoot. Older maize varieties can produce up to 25-50 million pollen grains (Kiesselbach, 1999; Burris, 2001) and an average-sized modern hybrid tassel produces 2-5 million (Burris, 2001) while the average number of ovaries on the shoot ranges from 750 to 1000 (Aylor et al., 2003). Thus, pollen density is far more than adequate to pollinate all of the embryos under favorable environmental conditions and pollen viability has been shown to be unaffected by water deficit (Herrero and Johnson, 1981; Hall et al., 1982; Schoper et al., 1986). For maximum yield in maize, it is essential to have successful pollination of the maximum number of kernels possible and for these kernels to subsequently reach maturity since kernel number per unit field area has been found as an important yield component (Otegui et al., 1995). One of the most obvious traits observed in maize is delay in silk emergence (silking) with respect to pollen shed (anthesis), as a result of stress. This phenomenon, termed the anthesis silking interval (ASI), has been studied extensively (Bolaños and Edmeades, 1996; Westgate, 1997).

Anthesis Silking Interval

Increased ASI has been extensively shown to have significant effects on reducing yield across a variety of cultivars, hybrids, and environments (Bolaños and Edmeades,

1996; Westgate, 1997; Chapman and Edmeades, 1999; Monneveux et al., 2006). There are several different effects as well as different responses that the ASI has to varying types and levels of stress. The ASI can take on both negative and positive values. Negative values arise from the silks emerging before pollen shed, known as protogyny and is rare, while positive values arise from the silks emerging after pollen shed, known as protandry and is much more common. Drought stress has little effect on time to pollen shed, but will increasingly delay silking, as the severity of drought stress increases at critical times (Bolaños and Edmeades, 1996). This follows the accepted dogma of Bateman's Principle that more energy is required from the female than the male to produce viable offspring (Burd, 1994). It is important to note, evidence has shown that the ASI is not an indicator of the water status of the plant, but rather, an indicator of the amount of biomass being used in the developing ear (Chapman and Edmeades, 1999; Monneveux et al., 2006). For example, when other abiotic stresses, but not drought stress, are present, the maize plant will respond by delaying silking. The delayed development of silks has been primarily linked to a decrease in silk elongation rate (Herrero and Johnson, 1981). As the plant's water status deteriorates, the silk elongation rate decreases and can sometimes reach zero, until the water status of the plant recovers. Silk elongation occurs through a four-phase process of cell division and tissue expansion that occurs along its entire length. Water deficit affects tissue expansion significantly more than cell division, leading to smaller silk cell size at the cessation of growth (Faud-Hassan et al., 2008). Thus, the silk elongation rate is highly dependent on the water status of the plant. As a consequence, drought stress in the plant affects the

silk elongation rate which, in turn, affects the ASI. Lengthening of the ASI causes floral asynchrony that can lead to bareness (loss of kernel set, leading to an incompletely pollinated ear) which greatly decreases yield. While the causes of the ASI have been well established, a significant knowledge gap exists in the understanding of the mechanism and genes regulating it (Setter et al., 2011). Further research into this relationship could provide new avenues to search for quantitative variation.

Floral asynchrony is not limited only to the loss of viable pollen. An early hypothesis for improvement of drought tolerance in maize was to use incremental or varying maturities so that the window of pollen shed was larger, in an effort to provide a pollen source for later emerging silks (Fischer et al., 1982). However, the addition of pollen to silks which have emerged later than the tassels does not improve kernel number. Silks that asynchronously flower significantly after pollen shed are permanently damaged and cannot be recovered, putting to rest the idea of including a later flowering line in drought conditions to provide a later pollen source (Westgate and Boyer, 1986; Otegui et al., 1995).

Lengthening the duration of pollen shed within a plant has not been investigated as an avenue for drought improvement to our knowledge, but variation has been observed. Minimum pollen densities for full kernel set have been determined (Uribelarrea et al., 2002; Westgate et al., 2003), but not under drought conditions. The trend for tassel size selection has been a decrease in size over time (Fischer et al., 1987), reducing the amount of pollen produced per plant. Could lengthening the duration of pollen shed effectively pollinate late emerging silks? No studies have been conducted

addressing this, but speculation based on other work cautions against this strategy. If the ASI lengthens beyond 4 days under drought stress, then silk elongation rate quickly drops to zero (Anderson et al., 2004). However, this has only been investigated in four hybrids and even within these four there existed variation for kernel set, pollen shed duration, silk growth rate, and senescence time. It may well be that quantitative variation exists for these traits that could be exploited for improvement. For the time being, however, focus should be on keeping the ASI as short as possible, decreasing bareness and thus, increasing yield. If the plant is able to synchronize its flowering, then the next obstacle that must be overcome is the receptivity of the silks under stress.

While the viability and amount of pollen shed are not affected by drought, the receptivity of the silks to pollen is affected by drought (Schoper et al. 1986; Bassetti and Westgate, 1993). Receptivity to pollen can decrease even after the pollen has germinated and begun to move down the silk. Senescence of the silk causes it to collapse around the growing pollen tube, ceasing its growth and consequently, not allowing it to fertilize the ovary. Bassetti and Westgate (1993) found that this senescence and thus, loss of receptivity, occurred only when decreases in silk water potential occurred over four days after the emergence of the first silk. When stress was induced more than four days after first silk, senescence of the silks were accelerated, leading to decreases in silk receptivity. Additionally, pollen tube growth in silks with low water potentials is slower, which allows time for the silk base to collapse and cut off the pollen from the ovary. Bassetti and Westgate (1993) argued, however, that it is unlikely in a field situation silk senescence would significantly harm yield, since

pollination from surrounding plants occurs long before any silks senesce if ASI is short. The most likely reason for a loss in kernel set in a field setting is from abortion of the developing kernel.

Post Flowering Drought Tolerance

If the sequence of events and conditions discussed so far are successful, namely, good stand and vegetative development, synchronous development of male and female floral organs, favorable pollen shed, pollen remaining viable and traveling down to the silks, and silks staying receptive and healthy enough to allow the germinated pollen to grow down to the embryo, fertilization is successful. However, kernel number is not dependent solely on successful fertilization. The plant now enters the second, longer part of the period critical for yield that is affected by water deficit, the two weeks after silking (Shaw, 1974). Kernel number at this point is no longer potential kernel number, which is a function of the number of spikelets (unfertilized embryos) in the developing ear, but rather, the number of developing kernels. As a response to environmental triggers, gene expression patterns affecting metabolism and growth now regulate the number of kernels on the ear (Hayano-Kanashiro et al., 2009; Wei et al., 2009). A spikelet that has been pollinated, is developing, has the potential to reach maturity but is allowed to die, may then be considered an aborted kernel (Hanft and Jones, 1986) and can be seen in Figure 2-1. Kernel number has been related to plant photosynthesis (Edmeades and Daynard, 1979) which can then be decreased by water deficit (Pelleschi et al., 1997). Water deficit increases until it reaches a threshold where photosynthetic rate decreases, which varies among genotypes (Pelleschi et al., 1997).

Figure 2-1. Incomplete ear tip pollinations and aborted kernels. Likely brought on by excessive exposure to drought stress both during and after pollination occurred.

Water Is Necessary for Photosynthesis, Not Kernel Filling

Artificial infusion of water-stressed plants with a liquid culture medium helps maintain kernel number, while artificial infusion with water has no beneficial effect on kernel number (Boyle et al., 1991). While intuitive, this reminds us that decreased water availability in the plant is not the direct cause of yield loss. The decrease in water availability in the plant affects photosynthesis and decreases the amount of photosynthate available to the developing ear. Kernels that developed on the water-infused stressed plants only had kernels on the basal or mid region of the ear and aborted kernels usually occur in the apical region of the ear (Boyle et al., 1991), as seen in Figure 2-1. This suggests that filled kernel number is ultimately a function of the amount of available carbohydrates (Boyle et al., 1991; McLaughlin and Boyer, 2004), which can be moved to supply the developing kernels. However, an inhibition of photosynthesis because of drought-induced stress decreases the amount of available carbohydrates and the low "sink strength" of the ear during pollination also limits the amount of nutrients moving into the ear (Lafitte and Edmeades, 1995; Zinselmeier et al., 1995; Westgate, 1997; Setter et al., 2001). Nitrate and carbohydrate tracking studies have shown that the most photosynthate is moved from the leaves to the ear sometime between 26 and 34 days after anthesis (Swank et al., 1982; Simmons and Jones, 1985), and was mostly generated in the plant after silking (Swank et al., 1982). As a consequence, reduction in photosynthesis after silking greatly reduces grain yield. A full ear of successful pollinations cannot be supported by a drought stressed plant. Thus, the plant's response is to abort the development of kernels keeping only the ones it can support with its

current available resources reducing the number of kernels on the ear which reduces yield. These responses appear to occur once the plant has no other physiological way to uptake more water and the genetic mechanism remains unknown. The key to preventing drought-induced kernel abortion, and overall drought stress, is to therefore delay, during grain fill, the time point when the amount of moisture in the soil no longer meets the needs of the crop. Maize has a number of physiological responses and traits that maximize available moisture uptake which are mostly heritable and can be used for breeding.

ROOT TRAITS INVOLVED IN DROUGHT TOLERANCE

Beneath the soil surface, out of view, is the most important organ of the maize plant when considering water deficiency. The root serves not only as the source of water uptake for the plant, but also plays a crucial role in the signaling of the drought response to the stem, leaves, and reproductive organs. It is suggested that changes in root system architecture (more compact root systems), not changes in leaf angle have allowed for the increase in plant density tolerance of maize (Hammer et al., 2009). This reinforces that root systems are a major component of maize adaptation. Roots have been called the "new frontier of drought research" (Pennisi, 2008) and represent a critical part of plant physiology that remains very difficult to explore on a large scale. Root traits to improve drought tolerance include root distribution, structure and development, root metabolic efficiency, and osmotic adjustment.

Root Distribution, Structure, and Development

Roots can exhibit beneficial traits and physiological responses to water deficit. Maize roots continue to grow even when environmental conditions inhibit the growth of shoots (Zhu et al., 2010). However, this exploration of the soil profile for moisture is metabolically costly to the plant. While intuitive, a common observation is that in maize, the rooting depth of drought tolerant lines is greater than the rooting depth of drought susceptible lines (Sharp and Davies, 1985; Bruce et al., 2002; Hund et al., 2009). When compared to temperate germplasm, tropical maize germplasm generally has root systems with less lateral root structure in the upper portion of the soil profile, but with larger and deeper roots in the lower soil profile (Hund et al., 2009). Tropical growing regions of the world, which account for about 70 million hectares of production, experience more drought and drought-like conditions than temperate growing regions (Pingali, 2001), which likely contributed to their evolution of these root systems and the temperate derived lines losing this. Deeper roots allow the plant to access deeper supplies of moisture. Larger roots allow increased water flow, explained by models of xylem water transport in which the volumetric flow rate of water through the root is proportional to the fourth power of the radius of the vessel (Taiz and Zeiger, 2006). Root distribution, however, can be costly to the maize plant, as nutrient distribution in the soil, especially non-mobile nutrients, might not follow the same pattern as water distribution, causing the plant to be starved of nutrients that are held only in the uppermost levels of the soil profile (Ho et al., 2005). Deeper exploration of the soil profile requires energy that can add up to over 50% of daily metabolic production

(Lambers et al., 2002). Therefore, decreasing the subsoil root sink demand during the reproductive period could allow better allocation of metabolites to the ear (Zhu et al., 2010).

Root development, structure, and distribution, however, should be carefully considered in the context of how environmental factors will affect them as they will have serious effects on whole plant health and yield. For example, a hybrid or variety with a root system that develops deeply in the soil profile would be poorly suited to land that is irrigated with a center pivot, as the water only penetrates the uppermost portions of the soil profile (Musick et al., 1988). The opposite is true for furrow irrigated land which soaks deeply into the soil profile and will hold water deeper for significantly longer than a pivot-irrigated field (Musick et al., 1988). An intriguing question can now be posed. Most of the literature points to studies done on seedling root structure and development (Voetberg and Sharp, 1991; Bohn 2006), since it is much easier to phenotype seedlings at a large scale as opposed to mature maize plants. So, what effect does early-season water stress have on the root system later in the season? Suppose a field is water stressed and the roots develop deeper, earlier in the season as has been documented in a previous study (Stasovski and Peterson, 1991). Does this cause the mature plants to become more drought tolerant later in the season? This environmental effect, along with known differences between juvenile and adult plants, would have serious complications on selections made at flowering. A substantial knowledge gap is present concerning the structure and distribution of mature plant roots. Imaging technologies are helping to create non-invasive techniques to examine plant roots (Bohn et al., 2006; Grift et al.,

2011; Surovy et al., 2011), however, more efforts should be made to understand the relationships between late season and early season root structure. Up to this point, responses to drought stress have all been about the search for water by roots and its uptake from areas where water is relatively abundant. However, once the plant has exhausted its ability to explore, or exploration of the soil profile yields no more moisture, genetic mechanisms in the plant signal the next response: osmotic adjustment.

Root Extraction of Soil Moisture at Low Saturation Levels

As soil moisture decreases, soil water potential decreases due to the decrease in matric potential: the attraction of water to a solid surface in the soil (Brady and Weil, 2004). In order for the plant to be able to take up water adsorbed to the soil surfaces, root cell water potential must be lower than that of the soil water to create the gradient needed to move the water, or else there is no water flow into the plant. Maize, like many other plants decreases osmotic potential by accumulating solutes in root cells lowering its water potential, allowing flow along the gradient into the plant again (Morgan, 1984; Bolaños and Edmeades, 1991; Taiz and Zeiger, 2006). Osmotic adjustment in maize is localized to the elongation zone of the root (Ogawa and Yamauchi, 2006) but has mixed findings for use in drought tolerance. Bolaños and Edmeades (1991) concluded that osmotic adjustment was poorly correlated with yield under drought across many tropical and temperate lines. However, more recently Chimenti et al. (2006) found detectable differences in yield between lines selected for both low and high capacities for osmotic adjustment. The authors posed an important question when they compared their results to those of Bolaños and Edmeades (1991): are there lines that exhibit constitutive

expression of osmotic adjustment while others are drought inducible? This suggests important distinctions between the environment for which the variety/line is being developed and the origin of material used for improvement.

Metabolic Efficiency of Maize Roots

Roots do not photosynthesize and are a plant photosynthesis sink (Kriedemann et al., 1976; Barnett and Pearce, 1983) so increasing their metabolic efficiency increases the photosynthate available elsewhere. Efforts should be made to find traits and genotypes that not only have deep roots, but have roots that are metabolically efficient in their growth (Liedgens and Richner, 2001). One trait that increases the metabolic efficiency of maize roots is an expansion of the cortical aerenchyma. Expansion decreases root metabolism and allows an increase in root growth and water uptake (Zhu et al., 2010). These aforementioned changes in root structure are also aided by other traits which occur at a molecular level. Cell wall loosening proteins such as expansons respond to drought by maintaining the elongation rate of the roots under the stress conditions, allowing the plant to continue root growth in an effort to reach soil moisture (Wu and Cosgrove, 2000). Wu and Cosgrove (2000) also demonstrated that responses that occur in the cell walls of root cells are complicated, as are techniques of measurement for the different variables in question and until easier to phenotype it will be difficult to select for.

LEAF TRAITS INVOLVED IN DROUGHT TOLERANCE

Leaves in maize are very diverse and one of the most easily observed tissues in the growing plant, increasing opportunities for rapid phenotyping. Leaf length, width,

angle, erectness, and color can be seen readily, while leaf thickness, stomate density, waxiness, and stomatal conductance can be measured using instruments. The majority of leaf traits tend to be heritable (Flint-Garcia et al., 2005; Hung et al., 2011) and since leaves are easily accessible, unlike roots, they are prime targets for measurements and speculative observations on the drought response by researchers and farmers alike.

Maize Leaf Rolling: Highly Visible, but Controversial

The most visible indicator of the water status of maize is leaf rolling, in which the leaf curls transversely along the edges (Figure 2-2). Leaf rolling has been

Figure 2-2. Leaf rolling as seen in a drought stress field experiment in College Station, TX in the summer of 2011. Observable leaf rolling is likely a function of both high incident radiation and lack of moisture, and as such is a controversial indicator of water stress.

associated with leaf conductance and decreased transpiration rates in maize and other crops such as rice (Sobrado, 1987; Kadioglu et al., 2012). The loss of leaf conductance and decrease of transpiration decreases the rate of photosynthesis, thus decreasing the productivity of the plant. Periods of water deficit cause leaf rolling in maize and help to reduce water loss where stomatal closure is incomplete (Fernandez and Castrillo, 1999). In other words, when the plant cannot close all of the stomata, water is still being lost to the environment and the capacity to roll its leaves allows the plant to reduce the continued losses. Leaf rolling also decreases the amount of radiation that is intercepted by the leaves (Kadioglu and Terzi, 2007). Transpiration rates are reduced in maize through the creation of a microclimate near the rolled leaf surface (Oppenheimer, 1960), along with the rapid reduction of effective leaf area (Clarke, 1986). This has also been shown in maize's close relative, sorghum (Matthews et al., 1990). Unlike senescence, leaf rolling is a temporary and reversible process, allowing the leaves to unroll once turgor is reestablished (Begg, 1980). One problem with using leaf rolling as a trait is that it also can appear with high incident radiation and/or heat stress and might confound estimates of drought adaptation (Cartwright et al., 2001; Kadioglu et al., 2012). No studies, to our knowledge, have definitely tested leaf rolling; developments of isogenic hybrids would be a valuable resource to test this trait.

The Leaf Cuticle and Cuticular Wax: Layers of Protection

Another conserved mechanism that maize and other higher plants have for desiccation protection is a cuticle which covers the surface of aboveground plant tissue with a thin layer of material, primarily lipids (Holloway, 1982). The cuticle plays a

fundamental protective role against loss of water especially when the stomata have already closed because of stress (Jenks and Ashworth, 1999). Furthermore, when the stomata are closed, the epidermal transpiration is inversely proportional to the cuticle's thickness or weight per unit area (DeLucia and Berlyn, 1984; Jenks et al., 1994). This inverse relationship has also been shown in maize leaves measured in the dark (when stomata are presumably closed) and suggests, along with other studies (Hajibagheri et al., 1983; DeLucia and Berlyn, 1984), that a thicker cuticle may reduce plant epidermal conductance to water vapor at all times (Ristic and Jenks, 2002). The leaf cuticle is comprised of a number of layers. The outermost layer of the cuticle, known as the epicuticular wax layer, is a mixture of long-chained hydrocarbons, esters, fatty acids, alcohols and aldehydes (Bianchi and Avato, 1984). The presence of an epicuticular wax layer has been suggested to benefit drought tolerance in many crops (Bondada et al., 1996; Luo, 2010). Unfortunately in maize, cuticular wax is not well correlated with epidermal water losses, yield, or yield stability under stress (Ristic and Jenks, 2002, Meeks et al., 2011). However, it is possible that the variables are much more complex and could involve polar pores and their distribution, and wax composition along the leaf and between the cutin meshwork and deserves future investigative work.

HORMONES PLAY AN IMPORTANT ROLE IN WHOLE PLANT PHYSIOLOGY

So far we have discussed plant organs and their response to drought in isolation. However, hormones allow the plant to coordinate physiological responses between different organs creating a new level of complexity. Phytohormones (abscisic acid

(ABA), auxins, cytokinins, jasmonates, and salicylic acid, among others), serve as the messengers between plant tissues and play an important role in the timing, duration, and type of response maize has to stress (Wang et al., 2008). Among these hormones, indole-3-acid, zeatin, gibberellin$_3$, and ABA have been the most clearly shown to affect drought, while the other hormones have not been well investigated. Because of their large effect on many traits, common and conserved, hormones may not be the best target to manipulate constitutively for drought.

Phytohormones, Primarily ABA, Are Manipulated to Cope with Drought Stress

Among known hormones ABA is one of the most important and extensively studied with respect to drought adaptation (Cutler et al., 2010). ABA is involved in the regulation of the growth of the plant and the opening and closing of the stomata, especially when the plant is experiencing periods of stress (Schroeder et al., 2003; Taiz and Zeiger, 2006; Sirichandra et al., 2009). ABA accumulation in higher plants is thought to act as an early signal for the initiation of processes involved in adaptation to drought and other environmental stresses (Hartung and Davies, 1991; Bray, 1993). ABA's effects on plants work in such a way as to reduce the amount of water lost through transpiration (e.g. closing of the stomata) and greater uptake of water through the roots (Setter, 1997). ABA has been shown to decrease the relative growth rates of leaves and increase the growth rates of roots, thereby increasing the root to shoot ratio (Sharp et al., 1994). ABA increases hydraulic conductance for water movement, thereby enhancing the transport of water from the roots to the leaves (Zhang et al., 1995).

Wilting of plants is usually accompanied by an increase in ABA levels, but correlation versus causation has not be determined (Taylor, 1991). ABA is also one of the known components in the initiation of the aforementioned process of osmotic adjustment (Ober and Sharp, 2003). A single nucleotide polymorphism (SNP) was statistically associated with ABA levels within maize silks during water stress, suggesting that the gene containing the SNP might be involved in the regulation of the ASI (Setter et al., 2011). Because of the strong effects in the initiation of the stress response, ABA has been proposed to be used as a secondary trait for selection in development of drought tolerant germplasm.

Hormones play important roles in the response to diverse exogenous and endogenous stimuli. Using hormones as a measurement of the drought response in maize could aid in selection of superior genotypes that might have otherwise been overlooked because of the lack of visible phenotypes. Unfortunately, the high phenotyping cost and temporal and environmental variability limit the screening that can be done (Mugo et al., 1999).

USING PHYSIOLOGICAL TRAITS FOR CROP IMPROVEMENT

Now that the traits and adaptive mechanisms of the maize plant have been discussed, we will revisit some of these, along with others used as selection criteria, from the perspective of crop improvement. The basic research involved in most of the aforementioned studies have been critical to the understanding of the mechanisms of drought tolerance and avoidance, however, simple understanding is not adequate for crop improvement. Many of these traits can have deleterious pleiotropic effects and their

interactions are unknown. Additionally, some traits are likely conserved across the species and we are unlikely to identify any substantial or useful genetic variation. Interdisciplinary cooperation between basic research and field breeding has and will continue to determine whether the aforementioned traits and adaptive mechanisms are beneficial as selection criteria for the improvement of maize to tolerate drought conditions.

Environments for Screening and Selection

Many different methods have been used to screen and select maize for drought traits. To our knowledge, no comprehensive study has been undertaken to look at the advantages and disadvantages of these methods, largely due to the difficulty in measuring each phenotype. It is clear that one of the most important aspects to genetic gain in selection procedures for drought tolerance is the proper management of the timing, duration, and intensity of the water stress (Bruce et al., 2002). Each treatment serves the purpose of exposing the genetic variation for the traits evaluated in such a way that the plants would experience similar conditions in a farmer's field. One of the more successful institutional examples in breeding maize for drought tolerance, as defined by citations and imitation, is the work of CIMMYT, which first initiated breeding specifically for drought tolerance in their tropical germplasm in 1975. The method described by Edmeades et al. (1987), a maize physiologist with CIMMYT, consisted of growing maize under three different controlled water regimes: no moisture stress, stress during grain filling, and full-season stress. Selections of the top 30-40% of full-sib families were made for eight cycles using the following criterion: maintenance of days to

anthesis, maintenance of yield under drought stress and a shortening of the ASI. Bruce et al. (2002) notes that in the process of this selection and subsequent reporting, CIMMYT created an ideotype that most others have used when breeding for drought tolerance: high grain yield, short ASI, low level of leaf senescence under intermediate water stress while maintaining yield stability, and small tassels and upright leaves under well-watered conditions. It is important to remember to also make observations in a favorable environment, ensuring that the yield potential of lines being moved forward in selection is relatively high. This ensures that selection does not favor plants that only perform well under drought and have no potential in favorable environments (Edmeades et al., 1994).

Selection for drought-associated traits needs to be conducted in carefully-controlled environments to minimize additional effects that may occur. For example, selecting against kernel abortion could involve providing sufficient moisture up to and including flowering and then withholding supplemental irrigation after pollination at different levels and for different durations to evaluate the ability of the lines to maintain kernel set. The question that arises at this stage of selection is what trait(s) are actually being selected for or against when kernel abortion is being evaluated. In other words, is there selection pressure exhibited on a gene, or group of genes, that directly controls kernel abortion? Or is the selection against kernel abortion simply an indirect method for selection of lines with superior alleles for other secondary traits such as improved root systems or flowering physiology because of epistatic effects and/or pleiotropic effects present? When considering environments for selection, even if irrigation could

be strictly controlled, relative maturities and days to flowering will have confounding effects on the final analyses. This could occur if the breeder decides to impose stress only at flowering and not during grain fill, and the duration of flowering time spans 20 days from the earliest line to the latest line. Table 2-1 outlines ways to manage this. It is likely that a well-established breeding program will have a target maturity for the majority of its material, but this might not always be the case especially when introgressing more exotic drought tolerant material. Relevant genetic gains are dependent upon the skill in reproducing an environment that is identical or similar to the one from the previous selection cycle and adapted to the trait being investigated. Similarly, if this selection environment is different or artificial from what the grower will experience, the gain is not useful.

Methods of Selection and Traits Utilized in the Conventional Selection of Drought Tolerant Maize

Drought can occur at many stages and with various intensities. Because of the inherently large genotype by season, genotype by location, and genotype by micro-environment interactions present, mass selection of plants within populations is not a beneficial method for making selections resistant to drought (Jackson et al., 1996) especially if flowering time differences are not taken into account (Table 2-1). For areas of the world that still grow open pollinated varieties and for organizations like CIMMYT, population improvement techniques such as reciprocal recurrent selection are popular and have been successful (Bolaños and Edmeades, 1993). These have been used to develop new populations such as the well-known Tuxpeno Sequia, DTP1, and DTP2

Table 2-1. Methods for dealing with flowering time differences in screening for drought tolerance.

	Advantages	**Disadvantages**
1) Ignore flowering time differences.	Simplest method, no additional work needed	Could be imposing stress/selection on trait of interest in material flowering at one point in time while material flowering at other times outside of stress/selection may escape causing erroneous conclusions to be drawn about the trait.
2) Measure flowering time, harvest all material at same time, and use flowering time to partition out variation.	Simple. Measurement necessary for ASI also. Easy post-harvest correction, plant and harvest all samples at the same time, consistent with traditional field management	Could still be imposing stress/selection on the wrong trait. Significant loss in statistical power. Cannot truly separate effect, but can determine how much error it is causing.
3) Stagger drought stress based on factor (i.e. maturity/ days after anthesis)	Stress is imposed at correct time for each plot which treats all material alike.	Difficult to manage and requires expensive monitoring equipment and intensive irrigation management.
4) Classify material into specific maturity groups based on target adaptation and test each group separately.	Virtually eliminates differences in material. Stress is imposed at same physiological stage across all maturities. Should put stress on same trait.	Cannot make comparisons between different maturity groups. Makes management of the program more difficult as more space/time/analysis is needed.

(Monneveux et al., 2006). From such populations, superior lines can be selected and then inbred for use as parents for hybrid combinations following the pedigree method.

Quantification of and selection for drought tolerance in maize is usually calculated and carried out by measuring decreases in yield per unit area in a drought stressed treatment plot versus a control plot, since the most economically undesirable response of maize to drought stress is a reduction in yield (Bruce et al., 2002). However, since the heritability of grain yield under drought conditions is very low, even relative to low heritability of grain yield under well-watered conditions, selecting only for grain yield is not highly beneficial (Monneveux et al., 2008). For this reason, secondary traits have become a major focus in the progress of selection. Lafitte et al. (2003) defines secondary traits as plant characteristics other than grain yield that provide additional information about how the plant performs under a given environment. A good secondary trait should be associated with grain yield under drought conditions, have genetic diversity and be highly heritable. Low cost and ease of measurement are also beneficial for secondary traits and developing these methods remains an important area for future studies. Among secondary traits ASI is likely the most commonly used. Decreases in the heritability of grain yield in water stressed environments are accompanied by increases in the heritability and genetic variance of the ASI and ears per plant (Bolaños and Edmeades, 1996). In other words, as the effect of asynchronous flowering becomes more predictable, yield becomes less predictable. Bolaños and Edmeades (1996) also describe that in order to maximize the genetic gain per cycle, the environment must be carefully managed to create a mean ASI of at least 5 days and limit

irrigation to drop the mean ears per plant below 0.7. While intuitive, in their effort to elucidate the degree of impact that the ASI has on the drought tolerance of maize, Bolaños and Edmeades (1996) concluded that progress with secondary traits is limited until grain yield under stressed environments can be established. Remember that ASI is not an indicator of the water status of the plant, but has effects that are directly related to (current or past) water stress. Based on these observations, a sensible first step when initiating breeding for drought tolerance is to shorten the ASI in order to reveal genetic diversity for other traits.

Direct Selection of Non-Reproductive Secondary Traits

As reproductive asynchrony and failure must be reduced before secondary traits that are related to the water status of the plant can be accurately selected for. Secondary traits involving leaves and roots can thus be evaluated in an effort to further improve the drought tolerance of the selected lines. Phenotypic selection of secondary traits related to plant water status such as leaf rolling, rooting depth, and leaf wax content can be measured relatively easily and for minimal cost. The effort can be made here to complete the ideotype described earlier by selecting for lines that exhibit low leaf senescence, extensive leaf rolling, and deep roots. Secondary traits involved with physiological processes can also be evaluated (e.g. ABA and osmotic adjustment). Looking at these conclusions and at the challenges presented, there seems to be a significant gap between the understanding of the physiology of the drought response and the genetic information used for selection.

GENOMICS, GENE DISCOVERY, AND MARKER ASSISTED SELECTION

While advances in genomics technology have been remarkable over the past fifteen years, the complexity of the drought response and its many pathways has made accurate phenotyping a major challenge (Tuberosa and Salvi, 2006). Failure to accurately phenotype renders a QTL study useless and QTL for very small effects or those with high genotype by environment interactions are very difficult to detect. This establishes the continual need for conventional breeding and breeders, as their skill and experience with plants is absolutely essential for the successful application of genomics to breeding for drought tolerance.

While recent QTL studies have been numerous and successful in identifying significant QTL (Lu et al., 2010; Messmer et al., 2011; Lu et al., 2011c), many are focused on the ASI (Zehr et al., 1994; Beavis et al., 1994; Ribaut et al., 1996; Liu et al., 2010) and are outside the scope of this review, but should be acknowledged. Despite the inherent environmental influences on the expression of QTL that have been linked to drought tolerance and especially ASI (Liu et al., 2010), there could be benefits from the use of marker assisted selection (MAS) which can both eliminate the need to reproduce the given environment in which the initial phenotype was seen (Ribaut et al., 1996). However, if the QTL is in trans-linkage with a QTL expressed in other environments and in the opposite direction, this approach of using MAS alone may be counter-productive.

Once the ASI has been shortened sufficiently, lines being evaluated for their drought tolerance should begin to show genetic diversity for other secondary traits that

are measureable and can be selected. Banziger et al. (2000) nicely outlines that if the genetics underlying the phenotype observed in the field cannot be properly identified, there is no way to use molecular techniques to aid in selection.

CONCLUSION

Water availability will continue to limit production of maize in the southern United States, and worldwide. Drought tolerance is a highly quantitative and complex trait involving many genes from many different pathways. Basic research should be targeted towards understanding of the pathways in an effort to find additional targets for selection. Physiology efforts should develop methods to rapidly and inexpensively quantify the importance of secondary traits. Breeding efforts should be divided and focused on the specific target adaptation, as the target environment is crucial. Pleiotropy and epistatic effects are likely to hamper efforts to create the ideal drought tolerant phenotype, as pathways involved in ear shoot development, root development, ASI, kernel abortion, and the other traits mentioned in this review are likely interconnected through common hormones or precursors. Linkage of these genes with small effects further restricts progress without impractically large populations.

Future advances in improvement of maize for drought tolerance will no doubt take multi-disciplinary cooperation and approaches. A problem outlined by Banziger et al. (2000) is that phenotypic selection for improvement of drought tolerance has not improved water use efficiency of lines due in part to the limitations of methodology used. It is also argued that in classical breeding, selection for secondary traits involving leaves and roots becomes unfruitful as the association between these secondary traits and

yield is significantly reduced over the course of continuous selection. As the fields of plant breeding and genetics move from classical phenotypic selection toward increased reliance on molecular techniques, parallel efforts to make selection more efficient and to improve the phenotypes screened and selected for will be just as important as identifying specific genes. Even with new technology, traditional field breeding techniques will still be necessary to create variability to be evaluated, to regulate the stress environment, and to search for new traits useful to screen lines for drought tolerance. The sheer complexity of the stress response and its many pathways gives hope that there are still many unexplored natural traits and genetic variation present that can be used to help create new, more robust, drought tolerant ideotypes.

CHAPTER III

GENETIC DIVERSITY AND LINKAGE DISEQUILIBRIUM OF MAIZE PARALOGS *ZMLOX4* AND *ZMLOX5*

INTRODUCTION

Lipids and their oxidized derivatives, called oxylipins, are well recognized for their role in plant reactions to stress and plant-microbe interactions (Andreou et al., 2009; Christensen and Kolomiets 2011). Lipid mediated interactions between pathogens and plants have gained increased attention, as a disruption of plant-microbe communication could provide an avenue for resistance to diseases (Gao et al., 2007). In the context of maize (*Zea mays* L.), the grain crop with the highest worldwide production (FAO, 2011), the biotic stress of drought and the abiotic stress of colonization with the fungus *Aspergillus flavus* are two sources of grain loss worldwide (Pingali, 2001). *A. flavus* produces the carcinogenic mycotoxin, aflatoxin, a highly-regulated potent liver carcinogen that causes stunting as well as chronic and acute deaths in humans and animals worldwide (Castegnaro and McGregor, 1998). While quantitative resistance to *A. flavus* has been identified and selected for in maize, no major genes for resistance have been identified and the problem remains complex (Mayfield et al. 2011). The regulation of mycotoxin production in fungi is partially mediated by genes belonging to the lipoxygenase (LOX) family (Gao and Kolomiets, 2009). LOXs are found in plant, fungal and animal kingdoms, and LOX mediated cross-kingdom interactions are hypothesized to be involved in the susceptibility of plants to fungal

invasion and subsequent production of mycotoxins (Christensen and Kolomiets 2011). However, the specific molecular signals, whether lipid or other chemicals that trigger mycotoxin production during infection in both plants and fungi are poorly understood.

LOX Functionality and Purpose

LOXs are non-heme iron-containing dioxygenases that catalyze the oxygenation of polyunsaturated fatty acids (PUFAs) (Vick and Zimmerman, 1983) which are further processed into such products as jasmonic acid (JA) and green leaf volatiles (GLVs) (Mosblech et al., 2009). Both JAs and GLVs are important plant defense signals (Arimura et al., 2010, 2011; Kim et al., 2011; Stitz et al., 2011; Zhang et al., 2011) helping to regulate and coordinate plant defense responses to stress both within the plant and between the plant and other plants or pathogens. LOXs are subdivided into two main functional groups; 9-LOXs and 13-LOXs depending on which carbon on the fatty acid chain is oxygenated. Maize LOXs (*ZmLOX*s) are similarly subdivided and currently total up to 13 different genes with varying functions, localization, regulation and induction within the plant (Nemchenko et al., 2006; Gao et al., 2008; Park et al., 2010).

ZmLOX4* and *ZmLOX5

Of the 13 *ZmLOX*s, *ZmLOX4* (located on maize chromosome 1) and *ZmLOX5* (located on maize chromosome 5) are the two most closely related paralogs when considering sequence homology, sharing 94% identity with each other, but only 40-67% with other *ZmLOX*s (Park et al. 2010). Both *ZmLOX4* and *ZmLOX5* are 9-LOXs and are not the only pair of segmentally duplicated LOXs in the *ZmLOX* family. *ZmLOX*1 and

*ZmLOX*2, *ZmLOX*7 and *ZmLOX*8, *ZmLOX*10 and *ZmLOX*11 are also closely-related paralogs that are suspected to have distinct functionality (Nemchenko et al. 2006). Despite their similarities in sequence, *ZmLOX4* and *ZmLOX5* have distinct organ-specific and stress-induced expression patterns, suggesting their differential involvement in diverse physiological processes. *ZmLOX4* is expressed mainly in the roots and the shoot apical meristem while *ZmLOX5* is expressed predominantly in the above ground organs especially the silks (Park et al. 2010). *ZmLOX5* was also found to be locally inducible while *ZmLOX4* was only slightly inducible in the leaves (Park et al. 2010). These findings of localization and expression support our hypothesis that the *ZmLOX4* locus (expressed in the roots) provides a source of quantitative variation for drought tolerance while the *ZmLOX5* locus (expressed in the silks) provides a source of quantitative variation for aflatoxin resistance.

Identification and Use of Allelic Diversity

Plant breeding programs are a delicate balance between continued improvements of important quantitative traits while still maintaining selectable variation present for these traits. Allelic diversity is an essential part of a breeding program to provide additional and alternative functional variation. However, this functional variation at particular genes of interest may be masked by genetic background, including both epistatic interactions and large effect alleles at "major genes". Therefore, when a gene of interest is found, it is logical to survey the natural variation at that gene for alleles that might provide improved functionality (Gilchrist et al., 2006). Sequencing of regions of the genome in many different varieties is a classic methodology for documenting

diversity for its use in mapping, phylogeny, and association analysis (Springer and Stupar, 2007). Functional allelic diversity may take many forms, including sequence changes, structural changes of the genome, varied levels of gene expression, and changes in epigenetics (Springer and Stupar, 2007). In maize it has been estimated that some form of polymorphism is present in the genome every 100 base pairs (bp) in any two randomly chosen maize inbreds (Tenaillon et al., 2001). . Since the maize genome is so polymorphic, association mapping studies require a very large number of markers to completely cover the entire genome or a candidate gene (a gene believed to play a role in a trait of interest) in which to test. The ultimate goal of association mapping is to statistically link or "associate" a given polymorphism to a specific observed phenotype; if proven marker assisted selection can be used to assist transfer of this polymorphism in the breeding process. This is particularly useful when the trait(s) of interest, in this case drought and aflatoxin, are highly quantitative and difficult or expensive to properly phenotype.

Linkage Disequilibrium and Association Mapping

Maize is primarily an outcrossing species, which is believed to explain the high polymorphism rate and rapid decay of linkage disequilibrium (LD) in most regions (Lu et al., 2011a). LD, the non-random association of alleles at two or more loci, has profound implications on successful and accurate association mapping and thus understanding of LD in regions of interest is of great interest (Falconer, 1996; Zhang et al., 2002; Inghelandt et al., 2011; Yan et al., 2011).

Sequencing of the maize genome has provided an invaluable resource for studies of genetic diversity and association mapping (Schnable et al., 2009). The availability of a reference sequence allows for much simpler alignment of sequence data and localization of contigs to proper regions of the genome. Another great resource that is available to the maize genetics community is the first and now second generation Maize HapMap (Gore et al., 2009). The Maize HapMap is 1.4 million single nucleotide polymorphisms (SNPs) collected on the 26 founders of the NAM population and Mo17 giving extensive coverage of the low copy portion of the genome of maize for polymorphism. The Maize HapMap has provided a step-off point for many preliminary diversity studies looking across the whole genome.

While these resources provide an excellent tool for most regions of the maize genome, it is likely not very helpful when working with paralogs in the genome as the short reads created using next generation sequencing techniques would not distinguish between two genes that share a very high identity such as *ZmLOX4* and *ZmLOX5*. For this reason terminator dye sequencing and standard protocols were using to investigate the genetic diversity at the *ZmLOX4* and *ZmLOX5* loci which are involved in important metabolic functions. Identifying genetic polymorphism in these loci is essential for using them in candidate gene association mapping studies.

MATERIALS AND METHODS

Germplasm

ZmLOX4 and *ZmLOX5* were sequenced in an association mapping panel consisting of 400 inbred lines. 300 of these lines were originally put together as an

association panel adapted to the temperate mid-west U.S. (Flint-Garcia et al., 2005), but were bred in diverse locations such as Minnesota, France, Iowa, Texas and Mexico. While there is plentiful information on the 300 Flint-Garcia et al. lines, many do not do well in the Southern U.S. and Texas; additionally they would have been unlikely to be selected for traits such as aflatoxin or drought tolerance that *ZmLOX4* and *ZmLOX5* condition. Therefore an additional 100 lines were selected to be part of an aflatoxin screening association panel adapted to the Southern U.S. bred at CIMMYT in Mexico in the Southern U.S. such as Mississippi, Georgia, Texas, and in the Germplasm Enhancement of Maize (GEM) project (Pollak, 2003). In total, these lines should represent the vast majority of diversity in elite domesticated maize, with only the rarest of alleles not included.

DNA Extraction/PCR/Sequencing

For sequencing, genomic DNA was extracted from V2 (second leaf) stage seedlings of the maize inbred lines of the association panel using the protocol as described by Zhang et al. (2005). Sequence homology of *ZmLOX4* and *ZmLOX5* is so high that gene specific primers (GSPs) had to be located in the 3' UTR of both genes to avoid amplification of both genes simultaneously during PCR reactions. Forward and reverse primer sequences are shown in Table 3-1 as well as expected amplicon size. The 3' ends of the gene, where active sites are located (Park et al. 2010), were isolated via PCR and sequenced using primers from Table 3-1. PCR reactions were carried out using the commercially available Qiagen Taq PCR Core Kit using Qiagen recommended protocols (available at http://www.qiagen.com/products/pcr/taqsystem/taqpcrcore.aspx#

Table 3-1. Gene specific PCR primers used to amplify *ZmLOX4* and *ZmLOX5* and the theoretical amplicon size produced from the reaction.

	Forward Primer	Reverse Primer	Theoretical Amplicon Size
ZmLOX4	5' – TGC CGG ACC AGT CAA GCC CCT AC – 3'	5' – CAC ACA TGA CAA CAT TAT CCA GAC G – 3'	948bp
ZmLOX5	5' – GCG GTG ATC GAG CCG TTC GTA ATC – 3'	5' – CAA GCG TGG ACT CCT CTC TC – 3'	1266bp

Tabs=t2). PCR conditions were: 1) 95°C for 5 minutes, 2) 35 cycles of 95°C for 45 seconds, 58°C annealing temperature for both *ZmLOX4* and *ZmLOX5* primers for 1 minute, and 72°C for 2 minutes, 3) 72°C for 10 minutes. Sequencing and PCR purification was carried out by DeWalch Life Technologies (http://ls.dewalch.com/, Houston, TX) using the terminator dye method. Sequences were then aligned using Sequencher 4.8 (http://www.genecodes .com, Gene Codes Corporation) and trimmed using internal trim algorithm in Sequencher 4.8. Reverse and forward sequences were combined into consensus sequences and then aligned for comparison. Availability of the whole maize genome (http://www.maizesequence.org) allowed for the use of reference sequence data. Reference sequence contigs from B73 RefGen_V2 were used as an anchor to align experimental sequences. As a source for comparison *Z. perrenis*, an ancestral species of modern maize, was used to establish the ancestral state of the polymorphism. LD was calculated using TASSEL, freely available software from the Panzea project (www.panzea.org) (Bradbury et al., 2007). Molecular genetic diversity

parameters were calculated using the aligned Sanger sequence data trimmed to equal lengths and analyzed in DNA Sequence Polymorphism (Rozas et al. 2003), freely available software (www.ub.edu/dnasp/).

Southern Blot Analysis of *ZmLOX5*

2-week old seedlings of maize inbred lines were used for extraction of genomic DNA as described by Zhang et al. (2005) and 10µg genomic DNA of each inbred line was digested with a restriction enzyme, *BamH* I, overnight at 37°C. Digested DNA was electrophoresed in a 1.0% agar gel prepared with Tris-acetate, EDTA (TAE) buffer, then transferred with 0.025 M phosphate buffer (pH 6.5) to the nylon membrane (Magna Nylon Transfer Membrane, Osmonics Inc., Minnetonka, MN, USA). The membrane with transferred DNA was cross-linked by UV Stratalinker 2400 and then hybridized in ULTRAhyb hybridization buffer (Ambion, Austin, TX, USA) with *ZmLOX5*-specific probe which is a 149bp-fragment of 3' UTR of *ZmLOX5* (Park et al., 2010). The probes were labeled using Ready-To-Go DNA Labeled Beads (GE Healthcare, UK, Limited) with ^{32}P-dCTP according to the manufacturers protocol. Blot membranes were exposed to X-ray film (Kodak, Rochester, NY, U.S.A.) in cassettes at –80°C for 3-14 days depending on the signal strength.

Restriction Digestion of *ZmLOX5* PCR Fragment

Southern Blot Analysis of *ZmLOX5* in inbred lines showed that the inbreds I29 (Ames27115), Yu796_NS (Ames27196), 4226 (NSL30904), HP301 (PI587131), CI 187-2 (Ames26138) have two *ZmLOX5* bands while other inbred lines have single or no band, indicating either these inbreds have two *ZmLOX5* genes or *ZmLOX5* genes in these

inbred lines have BamHI site in the probe region. We PCR amplified this region of *ZmLOX5* from these inbreds. Purified PCR products were digested with BamHI and the electrophoresis results showed there is no BamHI site in the probe region of *ZmLOX5* of these inbreds.

RESULTS

SNPs in *ZmLOX4* and *ZmLOX5*

The 400 lines used in this study were a combination of two separate maize association panels and are expected to represent the majority of genetic diversity in domesticated maize. Sequencing and alignment of *ZmLOX4* and *ZmLOX5* sequences to the B73_RefGen_v2 (http://www.maizesequence.org) revealed 9 SNPs in *ZmLOX4* and 14 SNPs in *ZmLOX5* as seen in Figures 3-1 and 3-2. Insertions were found in both *ZmLOX4* and *ZmLOX5* loci. The insertion in *ZmLOX4* is 5bp in length, however, it has no functional consequence as it is located in the 3' UTR and thus after the stop codon and will not encode for any amino acid changes in the protein. *ZmLOX5*, however, has a 28bp insertion that was found in the inbred line Va99 and shifts the reading frame of the protein in the C-terminus.

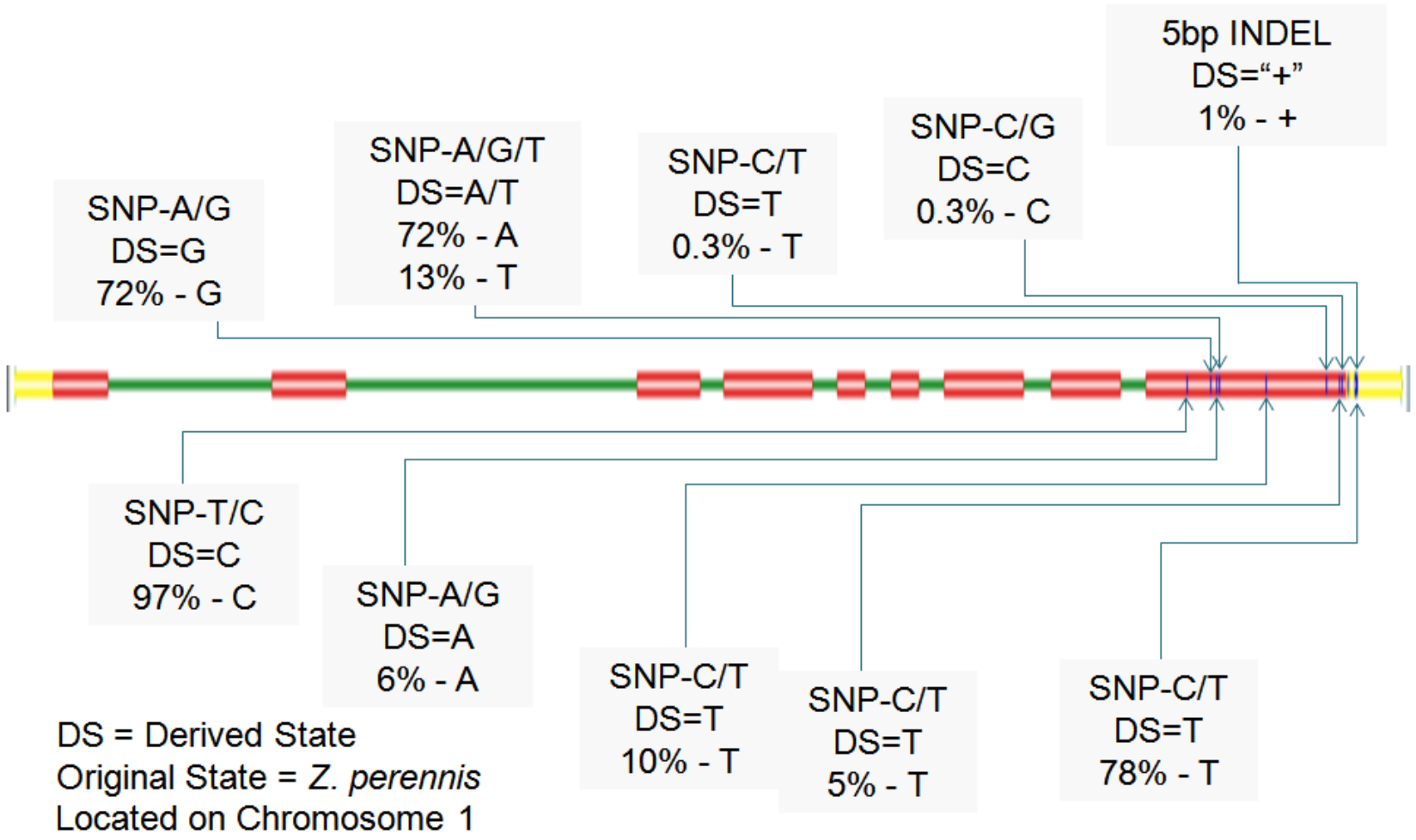

Figure 3-1. Sequence architecture of *ZmLOX4*. Location of SNPs, their derived state (DS), and percentage of the DS are shown. Red regions represent exons, yellow regions represent the UTRs, and green regions are introns.

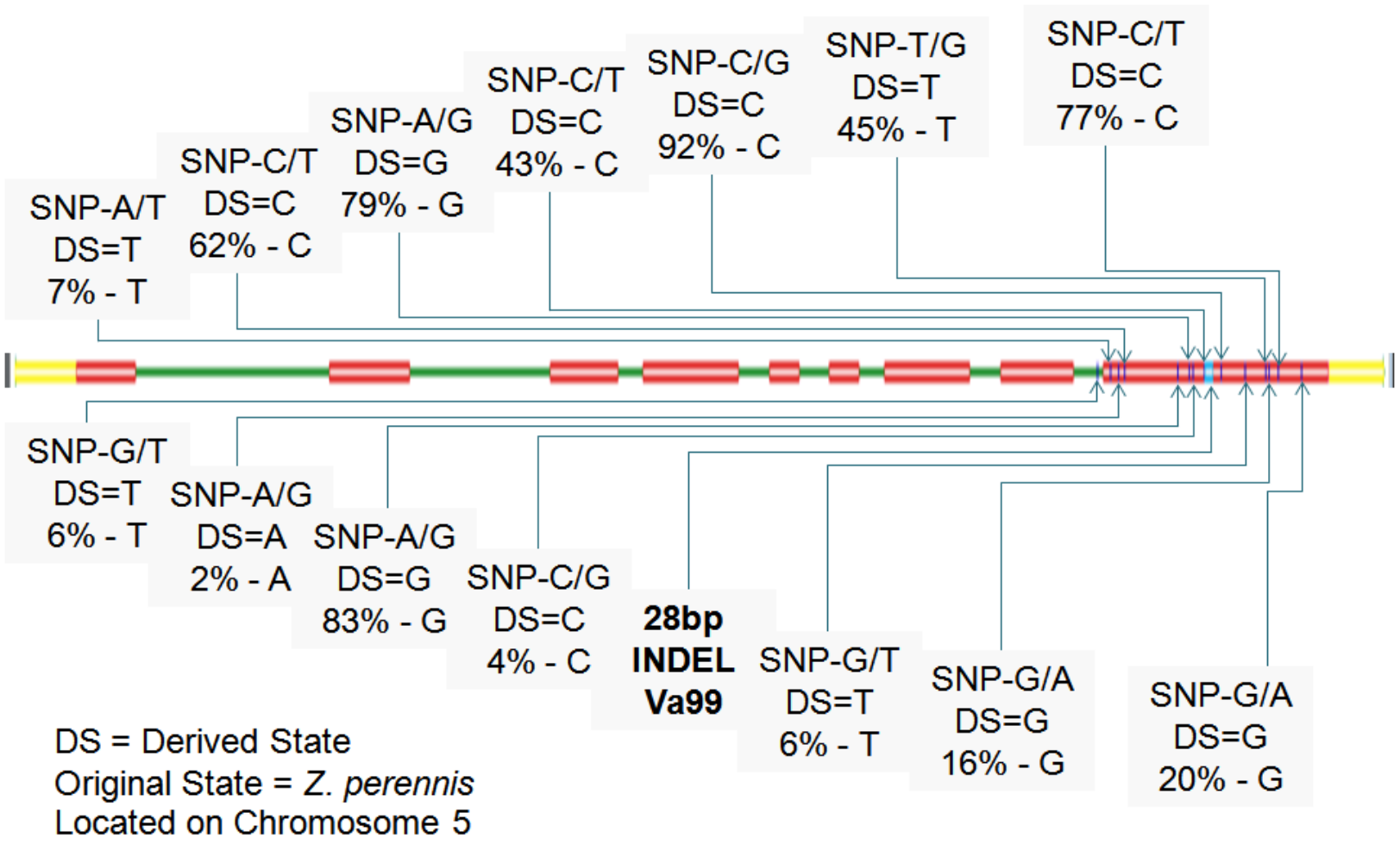

Figure 3-2: Sequence architecture of *ZmLOX5*. Location of SNPs, their derived state (DS), and percentage of the DS are shown. Red regions represent exons, yellow regions represent the UTRs, and green regions are introns.

Table 3-2. SNP locations based on B73 RefGen_v2, base pair changes, the derived state of the polymorphism, and percentage of derived state as compared to *Z. perrenis.*

SNP Location	Base Pair Change	Derived State	Derived State %
ZmLOX4			
1:264224287	T/C	C	97
1:264224380	A/G	G	72
1:264224403	A/G	A	6
1:264224414	A/G/T	A/T	72/13
1:264224594	C/T	T	10
1:264224645	C/T	T	0.3
1:264224831	C/T	T	5
1:264224915	C/G	C	0.3
1:264224941	+/-	+	1
1:264224950	C/T	T	78
ZmLOX5			
5:12289458	G/T	T	6
5:12289504	A/T	T	7
5:12289534	A/G	A	2
5:12289555	C/T	C	62
5:12289748	A/G	G	83
5:12289789	A/G	G	79
5:12289804	C/G	C	4
5:12289845	C/T	C	43
5:12289877	C/G	C	92
5:12289963	G/T	T	6
5:12290038	T/G	T	45
5:12290050	G/A	G	16
5:12290083	C/T	C	77
5:12290167	G/A	G	20

Z. perennis a perennial tetraploid teosinte considered to be an ancestral relative to maize (Doebley, 1990) was sequenced at the *ZmLOX4* and *ZmLOX5* loci to establish the ancestral/derived state of the alleles in question. Derived states and percentages are found on Figures 3-1 and 3-2; also, Table 3-2 outlines the SNPs found in the inbred

lines, their location based on B73RefGen_v2 and the percentage of the derived state that is seen in the inbred lines screened.

Presence/Absence/Duplication of *ZmLOX5*

PCR isolation and amplification of *ZmLOX4* and *ZmLOX5*, while not difficult on the small sample sizes used for primer design and genotyping of mutant alleles as part of line development, proved more difficult when genetic diversity was increased across the lines included in the association panels. *ZmLOX4* amplified much more robustly than did *ZmLOX5*. An interesting question that needed answering based on these difficulties during amplification was whether or not *ZmLOX5* was present in these lines. Of the 400 lines 50 failed to amplify and were never sequenced for *ZmLOX5*. These 50 lines were then tested for presence/absence of *ZmLOX5* using Southern blotting. Blot images (Figure 3-3) revealed that of the 50 lines tested one (CML 247 = PI595541) has no copies of *ZmLOX5* present. Five of the lines screened (I29 = Ames27115, Yu796_NS = Ames27196, 4226 = NSL30904, HP301 = PI587131, CI 187-2 = Ames26138) have two bands. To quickly rule out that there is a cut site within the probe used to specifically detect *ZmLOX5* the probe region was PCR amplified and cut. As seen in Figure 3-4, it is clear that there is only one band in all of the samples, suggesting that the four lines that showed two bands on the blot results contain two copies of *ZmLOX5*. For the remaining 46 lines it is likely that the gene specific primer used to amplify *ZmLOX5* had

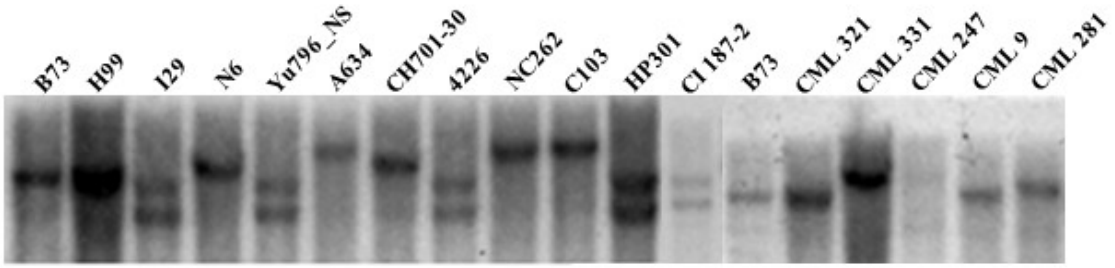

Figure 3-3. Southern blot of *ZmLOX5* in lines that did not PCR amplify. Five show double banding: Yu_796_NS, 4226, I29, HP301, and CI 187-2. One line is missing a band: CML 247. B73 is used as a control since it has been confirmed that *ZmLOX5* is present.

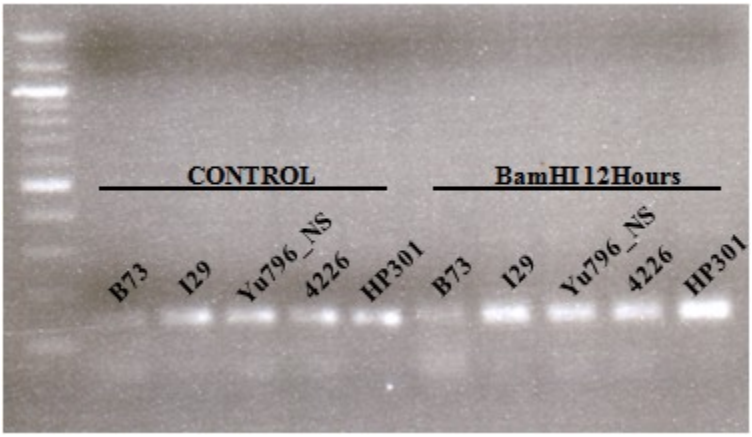

Figure 3-4. BamHI cut of *ZmLOX5* probe to check for cut site within the probe. As seen only a single band is found meaning no cut site is present.

no binding site in the 3'UTR. Contig alignments of the lines which successfully sequenced (not shown) showed high polymorphism in the 3'UTR of the gene which would not allow for the binding of the primer pair available for distinguishing *ZmLOX5* from *ZmLOX4*.

LD in *ZmLOX4* and *ZmLOX5*

LD patterns in *ZmLOX4* and *ZmLOX5* were investigated in both sequence data that was collected in this experiment and publically available data that is part of the Panzea project's HapMap Genotypes search (available at www.panzea.org). The sequencing data demonstrated that there was essentially no LD found in these genes (Figure 3-5). Of the 9 SNPs and one InDel that were described earlier for *ZmLOX4* only 6 SNPs and the InDel were considered spanning a total of 663bp across 264 lines. Each base pair of the InDel was considered and because no variants of the InDel were found across the 5bp that it spans. It shows complete LD, but was not linked to any other polymorphism. Looking at the rest of the data, the outlines of linkage are seen among the SNPs, but the block is not complete for two of the SNPs. Therefore, the final exon of *ZmLOX4* is not in complete LD and LD decays very rapidly (<100bp) in this region despite some linkage being present. *ZmLOX5* shows a similar pattern for the 14 SNPs considered across 204 lines and 709bp. The outlines of two blocks of LD can be seen, however the blocks are not complete and LD breaks down inside the final exon of *ZmLOX5* similarly to *ZmLOX4*.

The publicly available SNP call data from the Maize HapMap project was used to complement our results. LD patterns across the entire locus of *ZmLOX4* and *ZmLOX5*

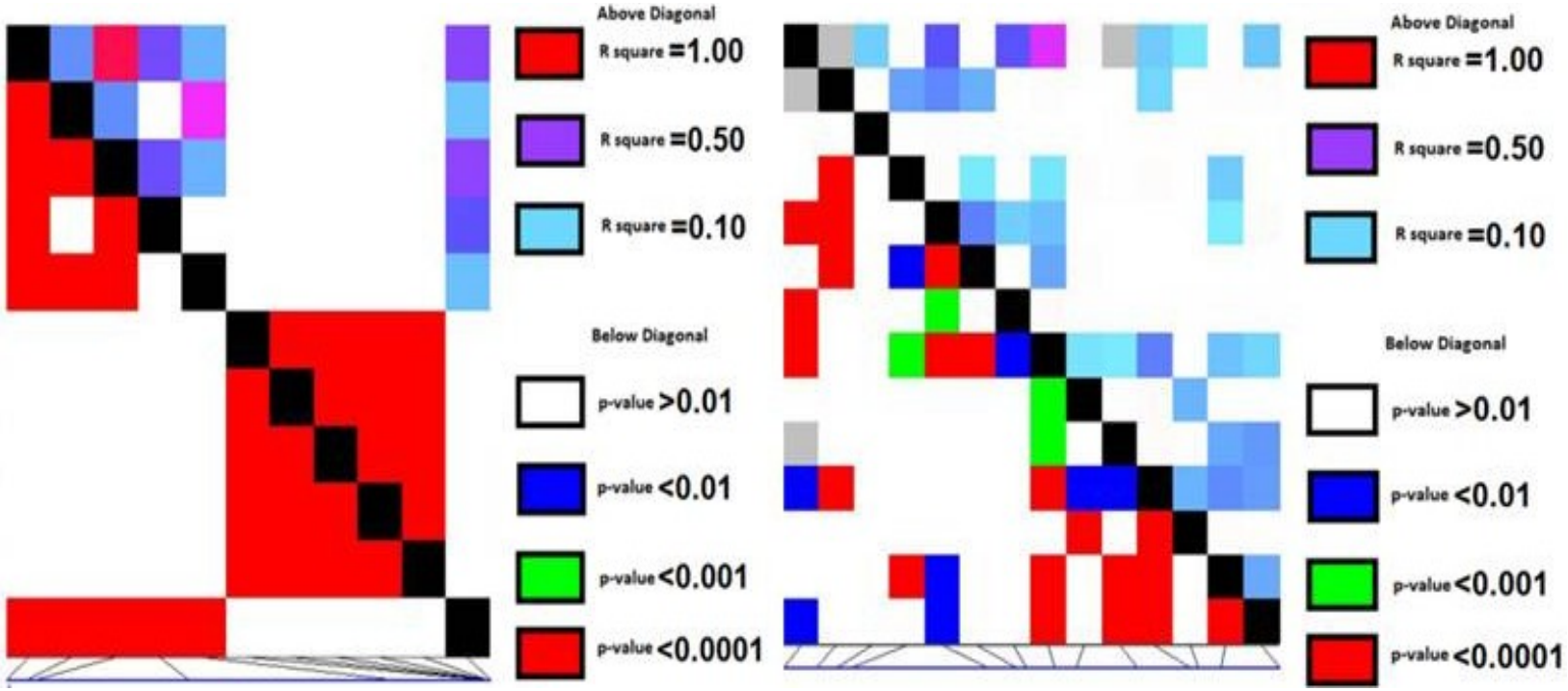

Figure 3-5. Linkage disequilibrium plots of *ZmLOX4* and *ZmLOX5* using Sanger sequence data. Linkage disequilibrium patterns in the C-terminus exon of *ZmLOX4* (left) and *ZmLOX5* (right) decays very rapidly (<100bp).

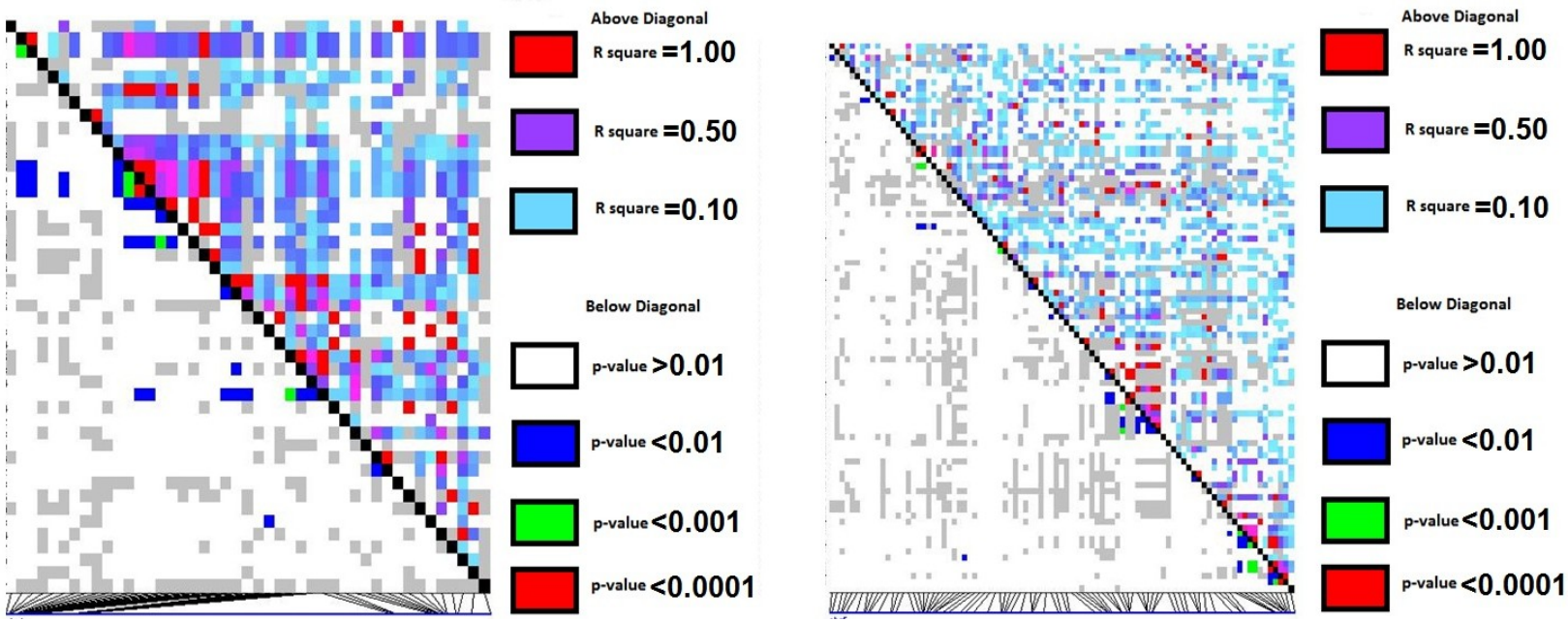

Figure 3-6. Linkage disequilibrium plots of *ZmLOX4* and *ZmLOX5* using Maize HapMap data. Linkage disequilibrium patterns across the entire genic locus of *ZmLOX4* (left) and *ZmLOX5* (right) is shown. Linkage disequilibrium decays rapidly in all regions of the gene with only small portions of linkage disequilibrium present.

reveal the same general pattern as our investigation of the part of the gene that encodes for the active site. LD decays very rapidly as seen by a lack of any large linkage blocks in either of the LD plots (Figure 3-6), but small regions of LD are present.

LD of Other Members of the *ZmLOX* Family

After observing how quickly LD decays within *ZmLOX4* and *ZmLOX5*, we wanted to compare this with LD patterns in the other members of the *ZmLOX* family (Figure 3-7). LD patterns that we saw in *ZmLOX4* and *ZmLOX5* were indeed the same for all but one of the other members of the *ZmLOX* family, namely *ZmLOX12* (Figure 3-8). As a stark contrast to the rest of the *ZmLOX* family, *ZmLOX12* shows highly significant and correlated LD across the entire locus extending >3000bp.

Genetic Diversity Measures for *ZmLOX4* and *ZmLOX5*

Using the sequence data generated, other measures of genetic diversity were considered to further characterize the two loci of interest. The first measure that was considered was nucleotide diversity which is a measure of the degree of polymorphism within a population. When looking at nucleotide diversity (π/bp) for the two genes *ZmLOX4* shows a π value of 0.00054 and *ZmLOX5* shows a pi value of 0.0053. Nucleotide diversity in *ZmLOX5* is near the lower range of reported values and *ZmLOX4* is lower than values that have been reported (Whitt et al., 2002, Tenaillon et al. 2001). However, nucleotide diversity measures have been shown to vary 16-fold and have been related to chromosome structure, LD and recombination (Buckler and Thornsberry, 2002, Tenaillon et al., 2001). Along with the nucleotide diversity measures, Tajima's D was also calculated to investigate any detectable selection on these two loci. Tajima's D

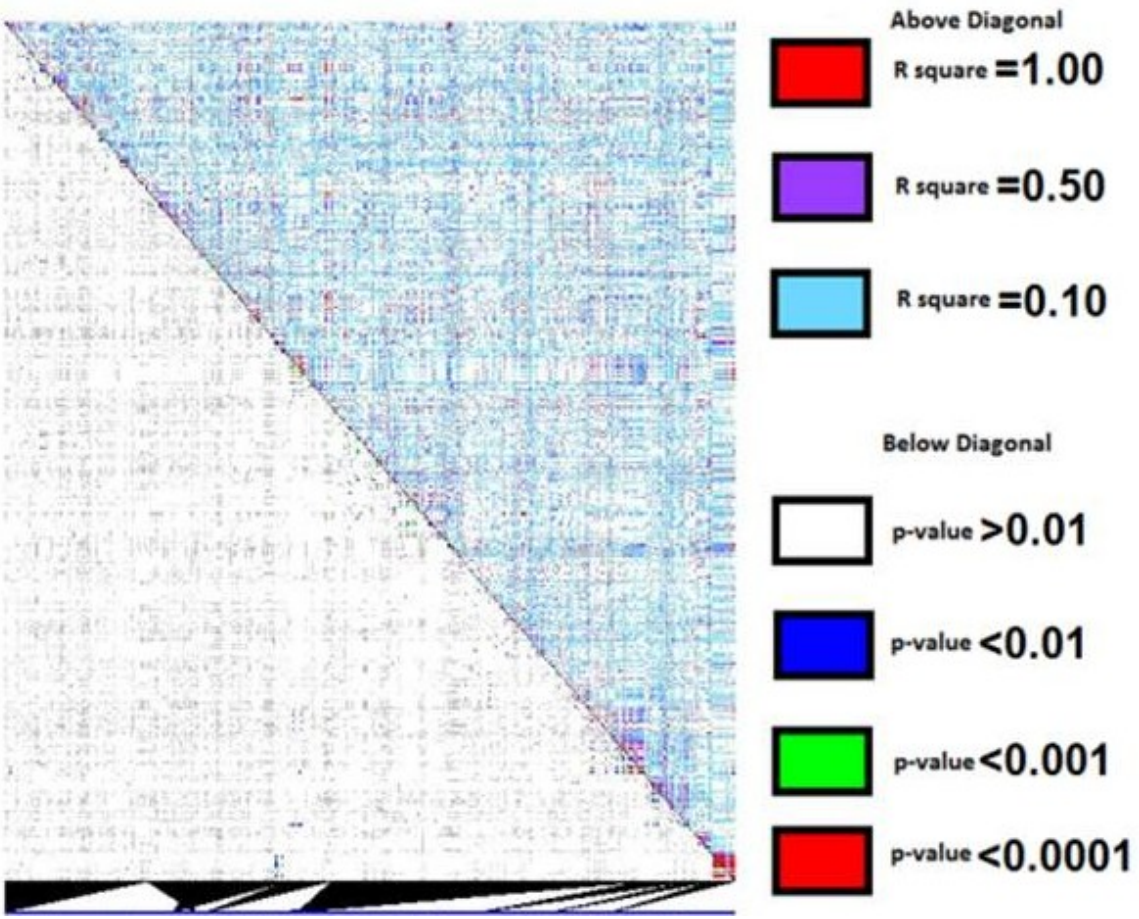

Figure 3-7: Linkage disequilibrium plot containing members of the *ZmLOX* gene family. Similar to *ZmLOX4* and *ZmLOX5*, linkage disequilibrium decays very rapidly in all loci, except *ZmLOX12* which is seen in the bottom right corner of the plot.

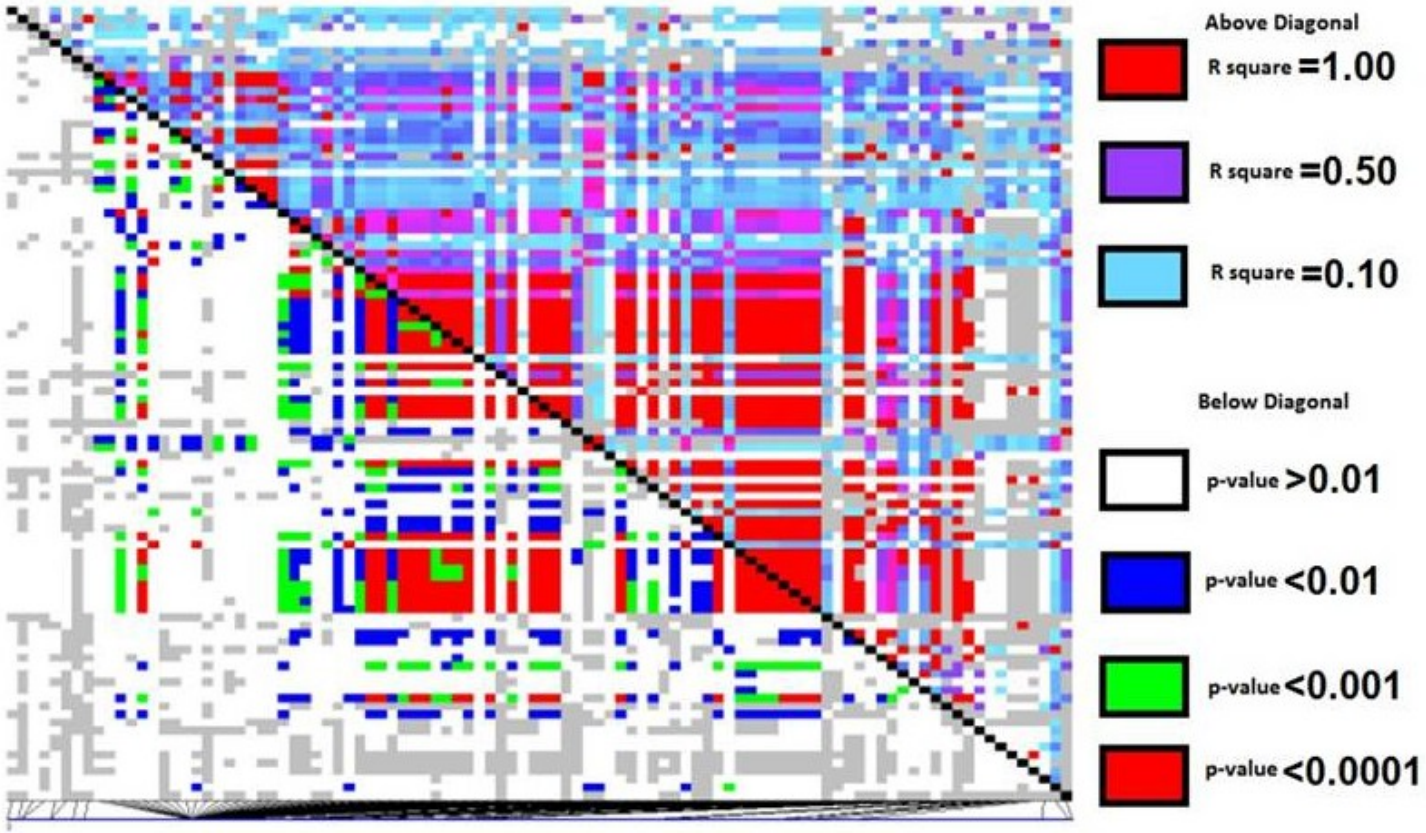

Figure 3-8. Linkage disequilibrium plot of *ZmLOX12* locus and flanking 100kb. Linkage disequilibrium extends further than expected for maize (>3000bp) and shows evidence of strong selection. Examination of the plot and gene location data for the B73 reference genome shows that the lower block in the above plot is a gene in close physical proximity to *ZmLOX12*.

test for neutrality values are -1.182 and -1.323 for *ZmLOX4* and *ZmLOX5*, respectively. Neither of these values is statistically significant from zero leading to the conclusion that the polymorphisms that are present in these two genes are selectively neutral.

DISCUSSION

LD Pattern and Conclusions Based on Population Genetics Theory

Patterns of LD present in members of the *ZmLOX* family (except for *ZmLOX12*) are extremely low compared with typical LD patterns that have been reported in maize (Remington et al. 2001; Tenaillon et al. 2001). LD in maize does decay rapidly when compared to other crops, however not as rapidly as is seen in this study. One reason is that most of the SNPs discovered along the region of *ZmLOX4* and *ZmLOX5* were rare SNPs and occurred in relatively low minor allele frequencies which makes examining LD difficult. LD along the final exons of both *ZmLOX4* and *ZmLOX5*, as seen in Figure 3-5, break down very rapidly and no LD blocks are found along the entire length of the *ZmLOX* genes (except for *ZmLOX12*) when the Panzea data is considered.

While these polymorphisms are selectively neutral and synonymous, that does not mean they are completely useless. As seen from the derived state percentages in Figures 3-1 and 3-2 and Table 3-2, frequencies of the derived state are sometimes very high, in the 70-90% range. This could be caused by three different scenarios. Intuitively, genetic drift is the first explanation for what is seen as the ancestral species likely had both variants and through drift the ancestor acquired one variant in higher percentage and modern maize acquired the other in a higher percentage. However, this could also be due to codon bias where different codons that encode for the same amino

acid are preferred because they are found in higher frequency than others (Hartl and Clark, 1997). While the high derived state frequency of some of these neutral mutations could be caused by this phenomenon it seems unlikely given the close genetic relatedness of *Z. mays* and *Z. perennis*. Another possible cause for this is that through evolution of the loci, selections on variants of interest have favored one allele over the other. The neutrality test conclusions are also in agreement with the conclusions that can be drawn from the LD plots. Along the entire length of all the *ZmLOX* loci (except *ZmLOX12*) there are polymorphisms, but none appear to be in linkage throughout the gene.

Not surprisingly, our results did not agree with HapMap data in the region of the gene we sequenced. This could be caused by a few different things such as our increased sample size and diversity, or the hypothesis stated earlier that the next generation sequencing used by the HapMap project was unable to distinguish between the two paralogs since they are so similar. When the overlapping regions are compared *ZmLOX4* shows no linkage blocks in the final exon while *ZmLOX5* shows complete linkage in the final exon. This shows that, despite being a powerful tool, there are limitations to next generation sequencing and the regions of the genome being investigated should be carefully considered. As a stark contrast Figures 3-7 and 3-8 show ten members of the *ZmLOX* family and the *ZmLOX12* locus including the 100kb on either side of *ZmLOX12*. It is evident that LD in this region is very strong and extends farther than expected in maize.

Genetic Diversity at the *ZmLOX4* and *ZmLOX5* Loci

The lack of LD that we see in these two loci is most likely due to the very low genetic diversity that is seen in the genes. There has likely been very heavy selection pressure on these genes in the past as they are important for normal physiological function. However, selection pressure on these genes has since been reduced as there is likely functional redundancy in the biochemical pathways that these genes are involved in. The evidence that supports this is that the knockout mutant lines survive and function very much like their wild-type relatives. However, local expression patterns of these genes are the difference that is seen between the mutants and wild-types. Also, the loss of a functioning copy of *ZmLOX5* in CML 247 and the putative frame-shift mutation of Va99 is found in released and successful elite inbreds, while other lines have two copies of *ZmLOX5*. As a stark contrast, *ZmLOX12* has well defined linkage blocks indicating that recent selection pressure has been very stringent around a beneficial mutation that has arisen. Further analysis of this locus shows that there is another, unknown but putative protein coding, gene in close proximity (<500bp) to *ZmLOX12*. While a conclusion cannot be made on which gene is being selected upon, there is clear evidence that selection pressure is acting on this locus creating two distinct haplotypes. Unlike many of the other *ZmLOX*s, *ZmLOX12* has no documented function, but could prove to be a future target for selection.

Presence/Absence of *ZmLOX5* and its Implications

While the finding that some lines had multiple copies of *ZmLOX5* and one was missing *ZmLOX5* was an interesting and unexpected find, it is not an uncommon

occurrence among maize lines. Non-collinearity or hemizygosity, where genetic loci can be present in one line but not in another, is becoming a common observation in maize inbred lines (Fu and Dooner, 2002). Furthermore, it has been documented among even elite maize lines that functional genes can be present in one line and absent in another (presence-absence variation) or have different numbers of copies across lines (copy number variation (Springer et al., 2009; Lai et al., 2010). This may be due to unequal crossover events, transposition, or other unknown phenomenon (Swanson-Wagner et al., 2010). Presence absence variation and copy number variation is suspected to be a causal agent to the large amount of genetic diversity seen across maize species (Springer et al., 2009), so it is not surprising to see this type of polymorphism when a diverse set of maize inbreds is screened for allelic diversity. However, what is unique is the finding that a gene that is so rigorously conserved is missing or duplicated within lines that are considered to be elite and have been used in breeding programs around the world. Whether this suggests that there may be functional redundancy in the *ZmLOX* pathways, or the line has evolved another mechanism to cope with the missing enzyme is not known. Interestingly two of the five lines confirmed to have duplicated *ZmLOX5*'s are popcorns as defined by previous subpopulation groupings (Flint-Garcia et al. 2005), and there are only nine popcorn lines out of the 400 individuals tested. The phenotypic effect that multiple copies of the *ZmLOX5* locus might have, if any, is unclear and under further investigation.

Implications for Association Mapping Studies

Causal mutations are the ideal polymorphism to use as a marker in association mapping and marker assisted breeding. If there is a marker very near the causal mutation/ functional polymorphism and LD does not decay between the mutation and the marker, then the marker could be detected with association mapping methods based on the nonfunctional marker. For this reason, it is critical to understand the patterns of LD around candidate genes. Based on the results presented in this study, it will not be possible to use LD patterns to associate a marker mutation in *ZmLOX4* or *ZmLOX5* to the phenotype. However, if there is a statistical association between a drought tolerant (*ZmLOX4*) or aflatoxin resistant (*ZmLOX5*) phenotype, then it is likely that the marker associated with the phenotype is the causal mutation. We generally find low diversity in these two genes but did identify a line with a disrupted *ZmLOX5* (Va99) and missing *ZmLOX5* (CML 247). Because of low frequency these mutations will be difficult to formally test in an association panel but linkage mapping populations are being created.

This study is among the first to investigate genetic diversity at important gene paralogs *ZmLOX4* and *ZmLOX5*. Conclusions that are drawn from this study will be directly applied to an association mapping experiment that is underway.

CHAPTER IV

SUMMARY AND FUTURE RESEARCH

Based on an extensive review of the literature that is present in Chapter II, it is clear that there are many gaps of knowledge present when the topic of breeding maize for drought tolerance is considered. Many physiological functions that control the drought response in maize are well understood, however, their application in breeding is not. Basic research efforts that are targeted toward the understanding of new physiological traits and the accurate and cost effective phenotyping of these traits is a key goal for the future of drought tolerance breeding. Of the avenues that have not fully been explored, root traits are some of the most important to consider and difficult to measure. Along with the understanding of root traits, molecular techniques applied to drought tolerance breeding should be investigated and applied where possible to aid in the selection process. Finding a new drought tolerant trait will not be an easy task as most of the major effect alleles have likely been fixed in elite lines and smaller effect alleles will need to be exploited and combined in elite lines or more diverse germplasm needs to be investigated. Many of these rare alleles are likely in tropical or exotic material which will provide a unique challenge to breeders in the future. However, candidate genes which putatively provide a beneficial and desirable phenotype are excellent targets for association mapping studies to first test new alleles which can then be bred directly into new lines. While both *ZmLOX4* and *ZmLOX5* have low genetic diversity and show no evidence of recent selection, the function of knock-out mutant versions suggests that genetic diversity at these candidate loci would be useful in an

association mapping study. The research and conclusions presented here are the necessary first steps required for the larger association mapping project that started in 2010 and will conclude in 2013.

ZMLOX4 AND *ZMLOX5* ASSOCIATION MAPPING STUDY

The ultimate goal of both this thesis project and the larger scope association mapping project, of which genetic diversity is one component, is the identification of novel, natural, and beneficial alleles that can be used for crop improvement for both drought tolerance (*ZmLOX4*) and aflatoxin resistance (*ZmLOX5*). Towards this goal, phenotyping of drought tolerance and aflatoxin resistance was carried out on testcross hybrids following the scheme shown in Figure 4-1. The 400 inbred lines that made up the diversity panel that was described in Chapter III were crossed to two variants of the inbred line Tx714. One variant of Tx714 was a homozygous mutant for a transposon tagged knockout of *zmlox4* while the other was a homozygous mutant for *zmlox5*. I used pollen bulked individually in both the *zmlox4* and *zmlox5* inbreds to pollinate the 400 inbreds of the diversity panel. In some cases, because of flowering time differences in the 400 lines, which ranged from 55-90 days, Tx714 was used as a female parent. While time and labor constraints did not allow for it, proper characterization of the diversity panel inbreds for flowering time would have made pollinations easier. Delay plantings could have then been properly calculated to account for flowering time differences between the inbred lines of the diversity panel and the Tx714 pollinator.

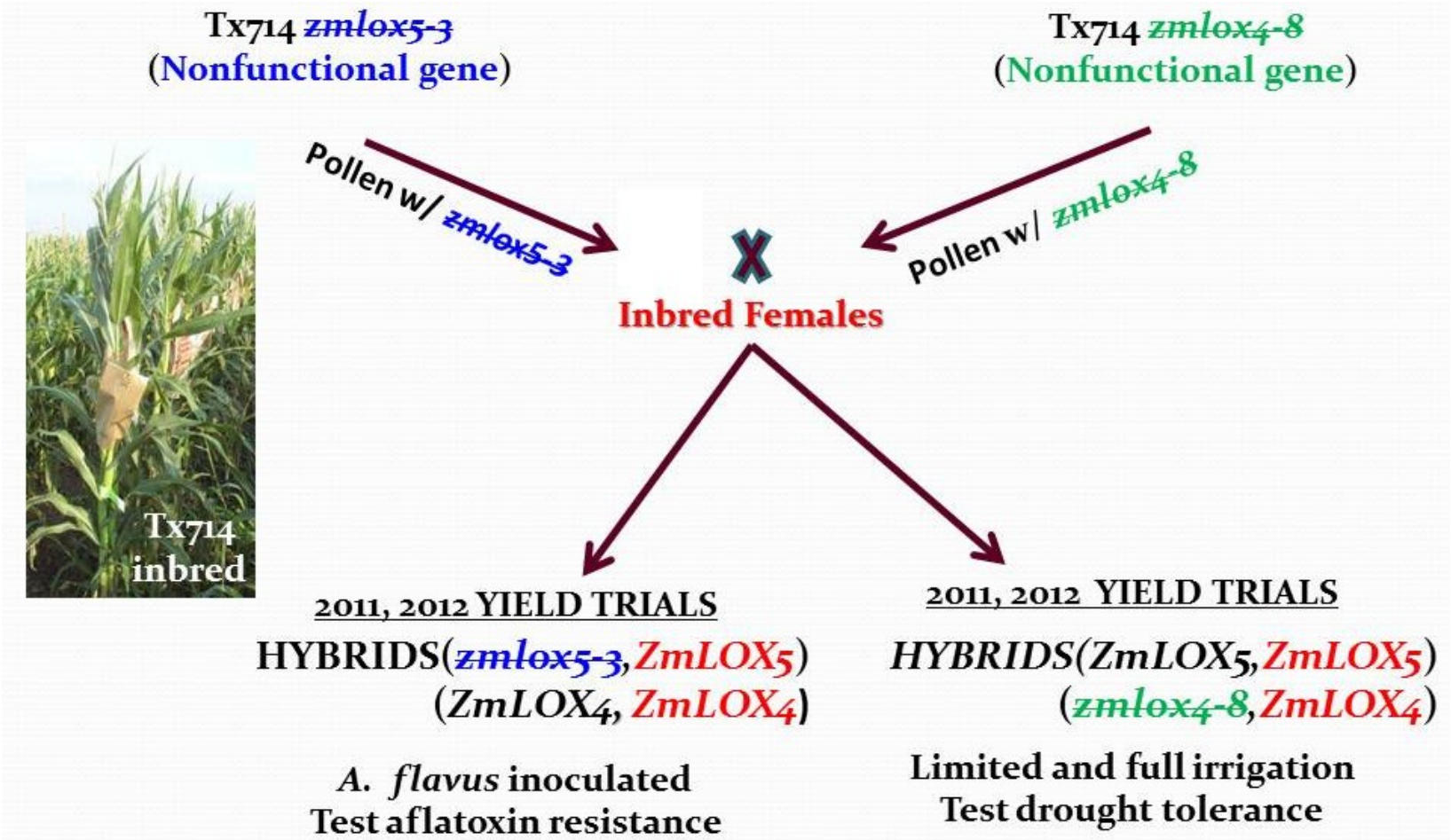

Figure 4-1. Crossing scheme for generation of testcross hybrids to be phenotyped for association mapping study.

Initial seed production of these testcross hybrids was done in College Station, TX and Weslaco, TX in the summer and fall of 2010, respectively. Hybrid seed produced for each set had one functioning natural copy of *ZmLOX4* or *ZmLOX5*. This functioning natural copy is what was evaluated in yield trial in 2011 and will be repeated in 2012 for two years of phenotypic data for the association analysis. *ZmLOX4* hybrids were evaluated with two replications in each of two treatments: well watered and water stressed. The *ZmLOX5* mutants were evaluated under full irrigation and were ground kernel inoculated with the aflatoxin producing fungus *Aspergillus flavus*. The severe heat and drought of 2011 provided an ideal stress environment to evaluate these hybrids. Preliminary analysis of the 2011 yield trial data showed a good range of separation for yield between the well watered and water stressed treatments and between the measured aflatoxin values. While a separation of yield values was not surprising as the drought was severe in 2011, the separation of aflatoxin values was better than expected, especially using the ground kernel inoculation technique. I attribute this to the fact that there was a rainfall event right after the first inoculation, and that I inoculated a second time while the soil was still moist and during a rainfall event providing an excellent environment for fungal growth. Based on the excellent data that we collected on aflatoxin values, I conclude that the ground kernel inoculation method works best with a moist environment and two inoculations spaced approximately one week apart providing spores throughout the flowering window.

Another aspect of the project that is still in progress was the backcross introgression of *zmlox4* and *zmlox5* mutant alleles into elite lines of the TAMU corn

breeding program. Depending on their backcross stage, some of these lines will be crossed with a tester line and evaluated in replicated yield trails to understand the effect of fully knocking out the *ZmLOX4* and *ZmLOX5* loci. The lines with multiple copies and missing copies of *ZmLOX5* are also under further investigation for crop improvement. There has also been some preliminary analysis of haplotype data for the *ZmLOX12* locus and the two distinct haplotypes have been identified. Lines from each of the two haplotype groups will be grown and the opposite haplotype will be introgressed into these lines for future evaluation. Running the molecular markers on this marker based backcross selection project I learned a great deal about optimization of PCR reactions and how to manage large amounts of genotype data across a large number of samples and years. This part of the project was one of the most difficult as each sample required two PCR reactions of genotyping to distinguish between the wild-type, heterozygote, and homozygote mutant. This process could be made much more efficient though the design of primers that could be run simultaneously eliminating the need for two reactions. Also, tissue sampling techniques need to be improved to avoid cross contamination between sample wells that causes genotyping errors.

Sequence analysis data from this thesis, along with phenotype data I helped collect, will be directly applied to the final association analysis of the testcross hybrids that were generated. Along with the sequence data from this project, these 400 lines have recently been re-sequenced for 1.5 million SNPs. These two sets of sequence data will be utilized in the final analysis, revealing new alleles that can be used for maize improvement of drought tolerance and aflatoxin resistance.

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VITA

Gerald Neil De La Fuente received his Bachelor of Science degree in Plant and Environmental Soil Science from Texas A&M University at College Station, TX in December of 2010. He entered the Plant Breeding program at Texas A&M University in January of 2011, working on corn breeding. His research interests include, breeding corn for adaptation to drought environments, aflatoxin resistance, and breeding corn for sub-tropical adaptation. He will be moving to Iowa State University in July of 2012 to start his Ph.D. program under Dr. Thomas Lubberstedt working with doubled haploids in corn.

Mr. De La Fuente can be reached at Iowa State University, Agronomy Department, 2101 Agronomy Hall, Ames, IA 50011-1010. His e-mail address is gerald.delafuente@gmail.com.