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# Characterization of Lipoxygenase (LOX) Gene Family and SNP Validation in Relation to Aflatoxin Resistance in Maize (Zea Mays L.)

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# Characterization of lipoxygenase (LOX) gene family and SNP validation in relation to aflatoxin resistance in maize (Zea Mays L.)

By

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Characterization of lipoxygenase (LOX) gene family and SNP validation in relation to aflatoxin resistance in maize (Zea Mays L.)

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An efficient approach to combat the accumulation of aflatoxin is the development of germplasm resistant to infection and spread of *A. flavus*. in maize, one of the most important cereal grains in the world. Lipoxygenases (LOXs) are a group enzymes that catalyze oxygenation of polyunsaturated fatty acids (PUFAs). LOX derived oxilipins play critical roles in plant defense against pathogens such as *A. flavus*. The objectives of this study were to report sequence diversity and expression patterns for all LOX genes, and map their effect on aflatoxin accumulation via linkage and association mapping. Genes GRMZM2G102760 (ZmLOX 5) and GRMZM2G104843 (ZmLOX 8) fell under previously published QTL in one of four mapping populations and appear to have a measurable effect on the reduction of aflatoxin in maize grains. The association mapping result shows 19 of the total 215 SNPs found within the sequence of the ZmLOXs were associated with reduced aflatoxin levels.

Key words: Maize, aflatoxin, Lipoxygenase, *A. flavus*.

DEDICATION

I dedicate this thesis to the glory of God, my parents Paul Olatunbosun and Olusola Alaba Ogunola, my sisters Oluwaseyi Bello, Oluwafunmilayo Odusoga and Opeoluwa Olamitoke. I thank you all for your support during the course of my program.

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LIST OF ABBREVIATIONS

LOX: Lipoxygenase.

ZmLOX: Zea Mays Lipoxygenase.

USDA: United States Department of Agriculture.

FDA: Food and Drug Administration.

QTL: Quantitative Trait Loci.

GWAS: Genome Wide Association Studies

PUFA: Polyunsaturated fatty acids.

9-HPOTE/ 9-LOX: 9S Hydroperoxyoctadecadienoic acid

13- HPOTE/ 13-LOX: 13S Hydroperoxyoctadecadienoic acid

HPL: Hydroperoxide lyase

AOS: Allene oxide synthase

JA: Jasmonic acid

NIL: Near isogenic lines

Tb1: Teosinte branched 1

FAOSTAT: Food and Agricultural Organization Statistics

NCGA: National Corn Growers Association.

CYA: Czapek yeast extract

CPA: Cyclopiazonic acid

AFB1: Aflatoxin B1

AFB2: Aflatoxin B2

AFG1: Aflatoxin G1

AFG2: Aflatoxin G2

UV: Ultraviolet

MAS: Marker assisted selection

SSR: Simple sequence repeats

AFLP: Amplified fragment polymorphisms

RFLP: Restriction length fragment polymorphisms

DArT: Diversity array technology

SNP: Single nucleotide polymorphisms

BC: Backcross

maizeGDB: Maize genetics and genomic database

TASSEL: Trait analysis by association, evolution and linkage software

GLM: General linear model

MLM: Mixed linear model

LA: Linoleic Acid

GLV: Green leaf volatiles

VOC: Volatile organic compounds

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# CHAPTER I

# INTRODUCTION

The most important group of cultivated crop plants in the world is cereals, of which maize (*Zea Mays*, L) is the most widely grown, with 791.6 million tons of maize produced in the year 2008 (USDA 2009). In different processed forms, maize is the staple food for a large number of people in the developing world, providing significant amounts of nutrients, in particular calories and protein. However, maize is often contaminated with aflatoxin produced by the fungus *Aspergillus flavus* (Castells et al. 2007). Globally, corn kernels infected by toxigenic fungus, including two *Aspergillus* species, pose a serious health threat to humans and animals because aflatoxins are carcinogenic and hepatotoxic. Economically, aflatoxin contamination poses a problem for farmers, as contaminated grains are not marketable. The U.S Food and Drug Administration (FDA) prohibits interstate commerce of grains that have an aflatoxin concentration equal to or greater than 20ng/g (Brown et al. 2003), and other countries have similar limits. The selection of germplasm that is resistant to either *Aspergillus flavus* or the production of its toxic metabolite aflatoxin has great potential to reduce the problems and risks posed by infected corn grains, but the highly quantitative nature of the trait makes it difficult to transfer the resistance from resistant lines to new elite inbred parents and hybrid cultivars. Quantitative trait loci (QTL) mapping studies have identified several potential QTL for reduced aflatoxin accumulation or *Aspergillus flavus* and /or ear rot resistance (Widstrom

et al.; 2003). In addition, previous Genome Wide Association Studies (GWAS) have identified many smaller genomic regions associated with a reduction in aflatoxin levels in maize (Warburton et al., 2015). Identification of loci for aflatoxin accumulation reduction found in resistant lines and the discovery of molecular markers linked to the genes or QTLs would also help to speed up the transfer of resistance from the resistant donor line to the elite cultivars (Warburton et al. 2010).

*Aspergillus flavus* is found mostly on decaying plant materials in the soil, but can also infect living plant tissues that has been stressed (i.e. by drought). It is also found in plant products, especially in oil rich seeds such as corn, cotton and peanuts. *Aspergillus. flavus* also produces a secondary metabolite known as aflatoxin, which is the most potent carcinogen known, it is hepatotoxic, and known to reduce immune system function and juvenile development in humans and many animal species (Geiser et al. 2000). *Aspergillus flavus,* Penicillium puberulum and *Aspergillus parasiticus* are the three major species of fungi that produces aflatoxin (Austwick and Elphick 1964). Aflatoxins are one of the mycotoxins regulated by the United States Food and Drug Administration (FDA). Restrictions on aflatoxin infected maize has resulted in losses of millions of dollars yearly by farmers in the United States (Robens and Cardwell, 2005). Contamination of agricultural commodities by aflatoxin also pose a serious health effect on humans and animals. Due to the economic losses and the health threat posed by *Aspergillus flavus* and aflatoxins, researchers have been trying to find a solution to reduce aflatoxin contamination of maize by implementing several strategies.

Considering all the various methods and strategies that have been employed to reduce aflatoxin accumulation in maize, host plant resistance is one of the most effective

and promising long term solution to aflatoxin accumulation. This methodology is the easiest for farmers to implement, since all the needed technology is already encapsulated into the seed. Many resistant lines have been identified, including Mp313E, Mp715, and Mp717 (Williams et al. 2006); however, these tend to lack the attributes and characteristics of acceptable commercial cultivars such as early maturity and high yield. They are thus not currently used as parents of commercial cultivars, and transferring the resistance into commercial cultivars has proven difficult due to the highly quantitative nature of the resistance (Hamblin and White 2000; Warburton et al. 2011). The trait involves multiple genes interacting together and their additive effect makes the plant more resistant.

Lipid peroxidation is common to all biological systems, appears in developmentally-regulated processes, and as a response to environmental changes. (Andrew et al.et al. 2009). Lipoxygenase (LOX), the enzyme responsible for lipid peroxidation, is ubiquitous in all eukaryotes and a number of bacteria. (Andreou et al.et al. 2009; oliw, 2002). Lipoxygenases are non-heme iron-containing fatty acid dioxygenases that catalyze the peroxidation of polyunsaturated fatty acids (PUFA) such as linoleic acid, α-linolenic acid and arachidonic acid (Acosta et al.et al., 2009) to form fatty acid hydroperoxide. Lipoxygenase reactions may also initiate the synthesis of a signaling molecule or be involved in inducing structural or metabolic changes in the cell (Brash 1999). The metabolism of PUFA via a LOX catalyzed step as well as alternative and subsequent reactions are collectively included in the Oxilipins pathway. Products derived from lipid peroxidation (called Oxilipins) are produced by Lipoxygenase pathways and are the most understood plant Oxilipins. Lipoxygenase pathways are region

specific and the dioxygenation of the substrates such as linoleic acid (18:3) and linolenic acid (18:3) to from (9S) hydroperoxyoctadecadienoic acid (9-HPOTE) or (13S) hydroperoxyoctadecadienoic acid (13- HPOTE) depends solely on the regions (the carbon where the molecular oxygen is added). The 9- and 13- HPOTEs are then used as substrates for the seven branches of LOX pathways which includes the peroxygenase, divinyl ether synthase, reductase, epoxy alcohol synthase, Hydroperoxide lyase (HPL), Allene oxide synthase (AOS) and LOX reactions (Feussner and Wasternack, 2003).

Lox and the products of the LOX pathway are involved in various biological processes such as seed germination (Feussner et al.et al.; 2001), sex determination (Acosta et al.et al.; 2009), and fruit ripening (Chen et al.et al.; 2004). Research has also shown that the Lox pathways produces a compound known as oxilipins e.g Jasmonic acid (JA) which are involved in the regulation of stress induced gene expression (Howe and Schilmiller; 2002) due to variety of biotic and abiotic stresses, and also, based on the effects of LOX products, a physiological function for LOXs has been proposed for growth and development (Rosahl, 1996). The LOX products are not formed prior to infection but are formed de novo when the plant is exposed to mechanical injury and or herbivore or pathogen attack (Howe and Schilmiller, 2002, Croft et al, 1990 and Keppler & Novacky, 1987). Mycotoxin production in fungi is partially regulated by the genes belonging to the lipoxygenase family and has been hypothesized to play an important role in the susceptibility of plants to fungal invasion (Fuente et al; 2013).

Based on the global importance of maize, the health and economic damage of aflatoxin contamination, and the possible mitigating effects of the Lox genes on aflatoxin production, the main objectives of this study are: to identify genes with lipoxygenase

activity by a search of online databases and published literature; to characterize these genes based on published or new sequence and expression data; to use genetic linkage and disequilibrium mapping to map candidate genes in one association panel and up to four QTL mapping populations using linked markers and to determine the phenotypic effect each gene has on aflatoxin accumulation resistance (if any). In addition, new Near Isogenic Line genetic mapping populations will be created to validate previously identified regions of the genome associated with aflatoxin accumulation resistance.

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CHAPTER II

LITERATURE REVIEW

## Maize (Zea Mays L)

Maize is one of the leading crops in the world (Figure 2.2) and is a critical food, fuel and fiber source, and used to extract other industrial components for plastics, paints, glues, pharmaceuticals, etc. Due to the importance of maize to food security and in industry and easy growth characteristics, it has been a model for plant geneticists as well (Tenaillon and Charcosset 2011). Maize was domesticated from of the wild Mexican grass known as teosinte (Figure 2.1). The major difference between teosinte and maize is that teosinte typically has multiple long branches with tassels and grains at their tips whereas maize processes a single stalk tipped by a tassel and one or a few short branches tipped by an ear. Genetic analysis has determined that a single gene, teosinte branched 1 (tb1), largely controls this difference (Doebley et al.; 1995).

Figure 2.1 Teosinte, the wild mexican grass believed to be the progenitor of maize (Tenaillon and Charcosset 2011).

Archeological and genetic evidence places the time of maize domestication at 9000 BP (Matsuoka et al., 2002). Maize cobs morphologically similar to modern ears have been observed to date back to 6250BP from Guila Naquitz (Piperno and Flanner 2001) and 5500BP from the Tehuacan valley (Long et al. 1989) in central Mexico. Maize was first recorded in Europe in 1493BP when it was introduced by Columbus, and from there it was taken to the Vatican where it was painted in frescoes near Rome around 1517BP (Janick and Caneva 2005). Today, maize (*Zea Mays* L), rice (*Oryza sativa*) and wheat (*Triticum æstivum*) are the world's main staple crops and maize is the 2nd largest harvested crop by area planted (FAOSTAT 2009). The United States is the world's largest producer of maize followed by China, The European Union, Brazil, and Mexico (NCGA 2011), while worldwide maize exports is led by the United States, Argentina and Brazil (NCGA 2011). In the United States, maize is mainly used for animal feed and

residual (38.7%), fuel/ethanol (36.5%), export (14.5%), and high fructose corn syrup (3.8%) (NCGA 2011).

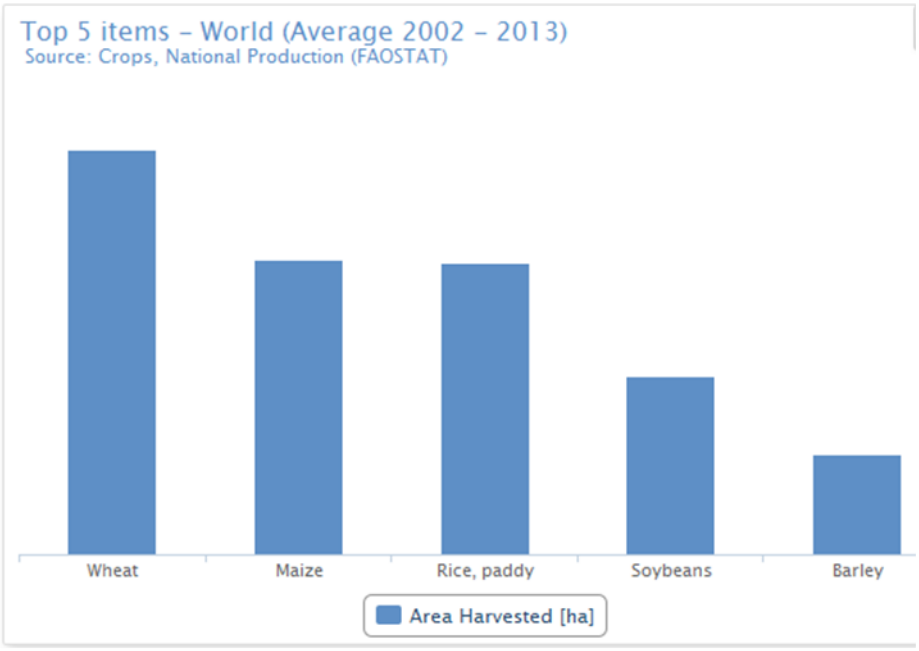


Figure 2.2 A graph showing the five highest produced (ha) crops in the world. http://faostat.fao.org/default.aspx

**Aspergillus flavus.**

*Aspergillus* is a large genus of fungi which has significant detrimental impact economically, ecologically, and medically. Species in this genus are abundant and widely distributed in the soil, water, air, and in plants (Klich 2002). During warm, dry periods, several of the aspergilla increase rapidly in association with crop plants (Cotty et al.; 1994). *Aspergillus flavus* is an anamorphic genus consisting of about 250 recognized species. It is characterized by a distinctive spore-bearing structure, the aspergillum (Figure 2.3) and in culture, *Aspergillus flavus* is characterized by fast-growing yellow-

green colonies, usually 65-70mm in diameter after 7 days growth in the dark at 25ºC on Czapek yeast extract (CYA) and it grows well at 37ºC (Klich 2007).

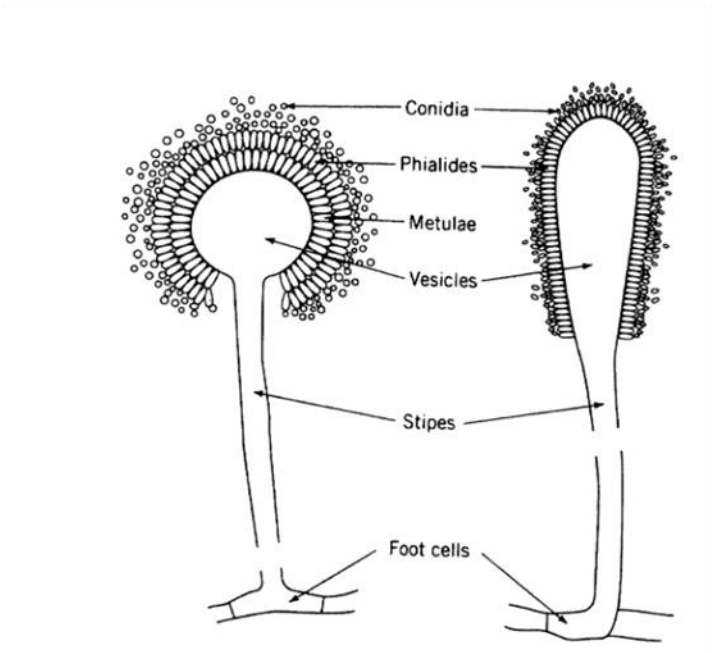


Figure 2.3 Conidiophores of *Aspergillus* (Klich, 2007)

It has been possible to isolate *Aspergillus flavus* from all of the major biomes, although it is isolated relatively more frequently in warm temperate zones (latitude 26-35º) than in tropical or cooler temperate zones, and is quite uncommon in latitudes above 45º (Klich, 2002b; Manabe and Tsuruta, 1978). There are clear interactions between agriculture and aflatoxins produced by the fungi in the *Aspergillus flavus* group. Some consequences of these interactions are obvious while some others are virtually unexplored (Cotty et al.; 1994). *Aspergillus. flavus* has a broad host range as an opportunistic pathogen/saprobe and infects many economically important crops which

can then become contaminated with aflatoxins; these include corn, cotton, peanut, and many other tree nuts (Sweany et al. 2011). *Aspergillus flavus* is also a human pathogen (Horn 2009) that has become increasingly important because immunosuppressed individuals are susceptible to infections by these fungi

**Aflatoxins**

The discovery and first characterization of aflatoxin occurred in the early 1960's when more than 100,000 turkeys died in England after consuming mold contaminated peanut meal (Blout, 1961 and Goldblatt, 1969). Aflatoxins are a group of secondary metabolites produces by *Aspergillus flavus* and *Aspergillus parasiticus* that can be recognized by the yellow-green or gray-green colored growth on corn kernels, respectively. Aflatoxin B1 is the most potent naturally formed carcinogen known (Squire 1981). Aflatoxins can be detected either on corn still growing in the field or in storage after the corn has been harvested. The risk of aflatoxin contamination is higher when moldy grains are damaged, providing easy entry of the growing fungus into the kernel. Aflatoxin contamination levels are highest during hot, dry summers compared to cool and/or wet summers.

In addition to aflatoxins, *Aspergillus flavus* also produces unrelated mycotoxins known as cyclopiazonic acid (CPA) an indol-tetramic acid that targets the liver, kidneys and gastrointestinal tracts of animals (Table 2.1)

Table 2.1 Secondary metabolites produced by different species of *aspergillus*.

| Species | Aflatoxins | Other secondary metabolites |
|---|---|---|
| *Aspergillus avenaceus* | | Avenaciolide |
| *Aspergillus bombycis* | B,G | Kojic acid |
| *Aspergillus caelatus* | | Kojic acid, aspergillic acid and cyclopiazonic acid |
| *Aspergillus flavus* | B,G | kojic acid, nominine, paspaline, paspaliline |
| *Aspergillus lanosus* | | Griseofluvin, kojic acid, met I |
| *Aspergillus leporis* | | Antibiotic Y, kojic acid, leporine, pseurotin |
| *Aspergillus nominus* | B,G | Aspergillic acid, kojic acid, nominine, pseurotin, tenuazonic acid |
| *Aspergillus oryzae* | | Cyclopiazonic acid, kojic acid |
| *Aspergillus parasiticus* | B,G | Aspergillic acid, kojic acid, parasiticol, parasiticolide A |
| *Aspergillus pseudotamarii* | B | Cyclopiazonic acid, kojic acid |
| *Aspergillus sojae* | | Kojic acid |
| *Aspergillus tamarii* | | Cyclopiazonic acid, fumigaclavine A, kojic acid |
| *Petromycesalliaceus* | | nominine, ochratoxin A and B, paspaline. |

Reproduced from Scheidegger and Payne 2005

Aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) are the four major types of aflatoxins produced by *Aspergillus* fungi (Table 2.2) and the names are derived from the fluorescence they produce under ultraviolet (UV) light (which is blue or green). In addition, aflatoxin M1 and M2 are found in milk following consumption of feed contaminated with aflatoxin by milk producing animals or lactating mothers (Richard and Payne 2002). Oil seed crops such as maize, cotton and tree nuts are very susceptible to aflatoxin accumulation because most of these crops are grown in the latitude where *Aspergillus flavus* thrive and possibly due to the carbon utilization pattern of *Aspergillus flavus* (Klich 2007). The high oil content of the grains and embryos of these seeds are a very good medium for growth of the fungus.

Table 2.2 Physical data of aflatoxin

| Aflatoxin | Molecular formula | Molecular weight | Melting point | $[\alpha]D^{23}$ |
|---|---|---|---|---|
| $B_1$ | $C_{17}H_{12}O_6$ | 312 | 268-269* | -559 |
| $B_2$ | $C_{17}H_{14}O_6$ | 314 | 286-289* | -492 |
| $G_1$ | $C_{17}H_{12}O_7$ | 328 | 244-246* | -533 |
| $G_2$ | $C_{17}H_{14}O_7$ | 330 | 237-240* | -473 |

*Decomposes (Wogan 1966)

**Aflatoxins in Maize**

Due to the essential role played by maize in feeding the world, it is important to treat any pathogen that affects maize production, consumption and byproduct utility seriously. Infection of maize ears by *A. flavus* is very difficult to predict by farmers (Smart et al. 1990), and it causes ear rot and aflatoxin contamination, with their economic and health burdens. *Aspergillus flavus* as an opportunistic pathogen has limited direct pathogenic abilities, but specific environmental conditions increase the fungi's ability to infect, rot ears, and cause aflatoxin contamination. The two most important factors are drought stress and high temperatures (Payne 1998).

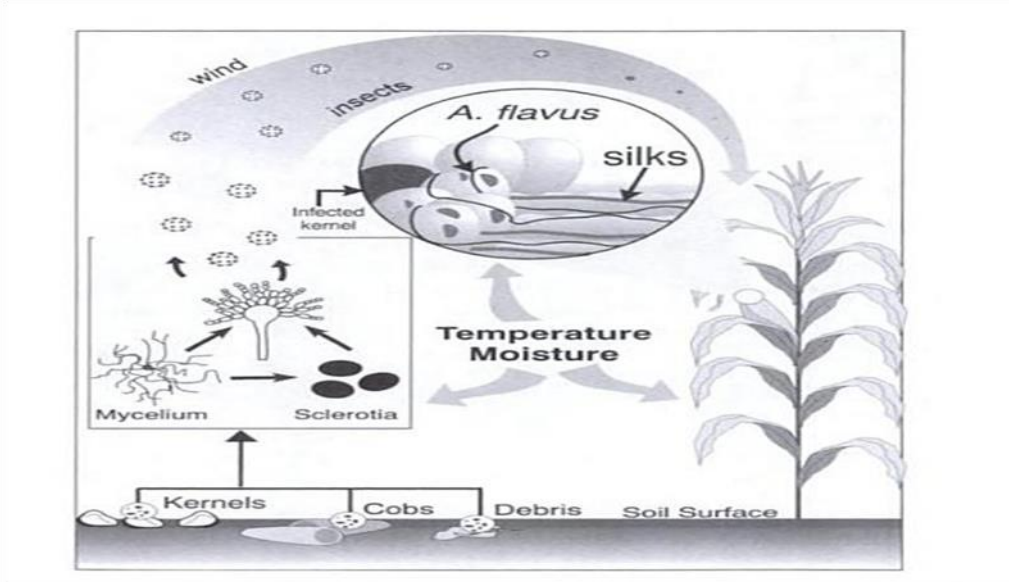


Figure 2.4 Diagram showing the life cycle of *Aspergillus flavus* and also the routes of colonization in maize. (Payne 1998).

Maize kernel colonization by *Aspergillus flavus* occurs at various stages of the plant's life when the spores are brought to the kernel surface either by insects or by the wind (Figure 2.4). Infection of the kernels generally occurs later in the ear development (Payne 1998). Preventive strategies such as stopping the infection process, control of environmental factors to minimize fungal growth, and pre- and post-harvest crop management strategies can be utilized by maize farmers to minimize the level of aflatoxin contamination in maize since to date, it is not practically possible to stop it altogether (Hell and Mutegi 2011). The most effective biological control for both pre- and post-harvest control of aflatoxin contamination is through the application of competitive non-toxigenic strains of *A. flavus* which competes with the natural toxigenic strains which can bring about 70-90% reduction of contamination (Yin et al; 2008).

Although many of the abiotic factors (heat, drought, and nutrient deficiency) that influence *Aspergillus flavus* infections cannot be prevented, pre-harvest crop management control such as earlier planting dates, irrigation of fields, proper fertilization (Jones et al. 1980; Rodriguez-del-Bosque 1996; Bruns 2003; Guo et al. 2005; Abbas et al. 2009) and other cultural practices such as weed control, low planting densities, application of fungicides and tillage (Jones et al. 1981b; Payne et al. 1986; Bruns 2003) have been shown to lower drought stress and also reduce aflatoxin accumulation in maize. Farmers employ many practices during the harvest to help reduce aflatoxin accumulation post-harvest such as a timely harvest to reduce further fungal growth, insect damage after harvest, or kernel breakage due to over drying (Hell et al. 2008). After harvesting, farmers dry corn to a moisture content of < 14%, reduce further fungal growth and subsequent aflatoxin accumulation in maize grain (Bruns 2003; Hell et al. 2008).

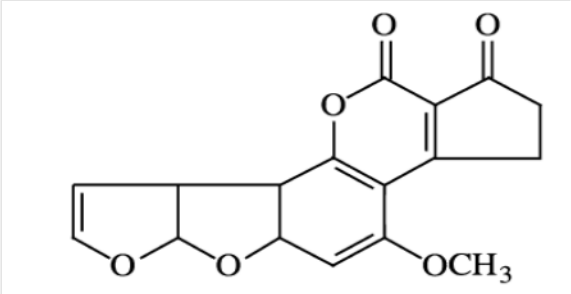


Figure 2.5 The structure of aflatoxin B1 (Klich 2007)

**Aflatoxin and Health**

Mycotoxins contaminate the diet of a large proportion of the world's population, especially in many low income countries where maize is a staple food (Council for

Agricultural Science and Technology (2003). It has been estimated that 25% of the world's crop are affected by mold or fungal growth (Mannon and Johnson 1985). *Aspergillus flavus* and *Aspergillus parasiticus* produce the secondary metabolite aflatoxin B1, (Figure 2.5) which is a carcinogenic substance that poses serious health hazards to both humans and animals. Figure 2.5. shows the structure of aflatoxin B1. Aflatoxins contaminate a variety of staple foods, particularly maize, peanuts or groundnuts and other cereals and nuts in low income countries (Williams et al.; 2004). Consumption of high amounts of aflatoxin is known to cause aflatoxicosis, symptoms of which include hemorrhaging, acute liver damage, edema, problems with nutrient uptake, and possibly death. Chronic exposure to low levels of aflatoxin will in addition cause immunosuppression, cancer, developmental problems in growth, and other pathological conditions.

In addition to health concerns, aflatoxins also have various other economic impacts on animals, such as reduced productivity, immune suppression which leads to increased incidence of other diseases, and chronic damage to vital organs. Aflatoxins cause decreased milk production in cattle, decreased egg production in poultry, and liver damage to animals. Young animals of various species are the most susceptible to aflatoxins, and nursing animals will also be affected when exposed to aflatoxin and aflatoxin metabolites which are secreted in milk. The economic impact of aflatoxin contamination in industrial nations is straightforward because the impact is mostly market-related as all commodities that contain aflatoxin above the regulation threshold (Table 2.3) for human or animal feed must be discarded (Wu et al., 2008). However, in

less developed countries, the estimation of the economic losses is more complex because the health-related costs are higher than the market-related costs (Williams 2008).

Table 2.3 Guidelines for aflatoxin levels by the U.S. Food and Drug Administration

| **Aflatoxin Level (parts per billion)** | **Commodities & Species** |
|---|---|
| 20 ppb | For corn, peanut products, cottonseed meal and other animal feeds and feed ingredients intended for dairy animals; for animal species or uses not specified below, or when the intended use is not known. |
| 20 ppb | For corn, peanut products and other animal feeds and feed ingredients, but excluding cottonseed meal, intended for immature animals. |
| 100 ppb | For corn and peanut products intended for breeding beef cattle, breeding swine or mature poultry (e.g. laying hens). |
| 200 ppb | For corn and peanut products intended for finishing swine (100 pounds or more). |
| 300 ppb | For cottonseed meal intended for beef cattle, swine or poultry (regardless of age or breeding status). |
| 300 ppb | For corn and peanut products intended for finishing beef cattle (i.e., feedlot cattle). |

http://agriculture.mo.gov/plants/feed/aflatoxin.php.

**Preventing Aflatoxin Contamination**

Biological and environmental factors such as insects, diseases, weeds and drought that can directly cause plant stress also contribute to the process of infection by *Aspergillus flavus* and thus have been the subject of much research effort (Widstrom et al; 2003). Control of environmental factors through various measures has been practiced to prevent and control fungal penetration, fungal growth and ultimately aflatoxin production and accumulation. Aflatoxin accumulation can increase 10-fold within 3 days when harvested grains are stored in a high moisture environment (Hell et al. 2008, Kaaya and Kyamuhangire, 2006); thus, post-harvest management practices such as keeping harvested grains at a safe moisture level (10-13%) can reduce aflatoxin accumulation

post-harvest. This problem is more compounded in Africa due to excessive heat, high humidity and insect and rodent damage, conditions that are favorable for the development, germination, and proliferation of *Aspergillus flavus* spores (Hell et al., 2008). Disinfecting measures such as smoking is a common practice carried out by about 4 - 12% of farmers in Nigeria to preserve their grains, and this practice was found to correlate with reduces aflatoxin accumulation in the farmers' stores (Udoh et al. 2000). Also, it has been established that nixtamalization, a maize preparation process involving soaking and cooking the maize grain in alkaline solution usually limewater is effective for reducing aflatoxin contamination by 75-90% (Albores et al, 2002).

Preventive measures not including breeding for resistant lines include good cultural practices, harvesting at the optimum stage of maturity, rapid drying after harvesting and chemical control (Lisker and Lillehoj, 1991). Other cultural practices involving tillage systems and crop rotation can affect soil inoculum availability and root/soil interface (alleviating stress during later plant development) and preventing the inoculum buildup (Jones 1987). Conventional methods of plant disease control, such as fungicide use, has proved ineffective in controlling *Aspergillus flavus* infection of corn when employed at a concentration that are both cost effective and environmentally safe (Bhatnagar et al.; 1993). Widstrom et al. (2003) focused on the identification of the most important and effective environmental factors that influence aflatoxin accumulation in corn and these include temperature, rainfall, relative humidity (Net evaporation) and soil type. The authors further stressed crop management factors including planting date, irrigation, tillage, fertilization, weed control and fungal competition (Table 2.4). Host plant resistance with the ultimate goal of developing resistant germplasm by plant

breeders is, in fact, the most effective, efficient and dependable tool that we have in the long term arsenal to protect corn from pre- and post-harvest infection and aflatoxin contamination process (Widstrom, 1992; Zuber, 1997).

Table 2.4 Managemental practices to manage aflatoxin resistance (Abass et al.; 2009).

| Strategy | Method | Rationale |
|---|---|---|
| Avoidance | Early planting, supplemental irrigation, short season hybrids | Reduce heat and moisture stress |
| Fertility management | Provide adequate nutrition | N- deficiency corn more susceptible |
| Insecticide application | Appropriate timing of application to control insect damage to ears | Insects responsible for enhanced ingress into grains |
| Bt Hybrids | Hybrids engineered with resistance to ear- damaging insects | Insects responsible for penetration into grains |
| Natural resistance to insects | Breeding and selecting hybrids for resistance | |
| Biological control | Use of non-toxigenic isolates of *A. flavus* | Competitive displacement of toxigenic isolates |
| Fungicides | Control phylosphere fungi | Reduce inoculum density |
| Soil management | Incorporation of crop residue | Reduce inoculum density |

http://faostat.fao.org/default.aspx

**Host Plant Resistance**

Breeding for resistance to aflatoxin accumulation is one of the most efficient and effective ways of reducing *Aspergillus flavus* infection and aflatoxin accumulation in maize (Paul et al. 2003). It is also a preventive measure that would be an excellent remedy without the need for additional inputs beyond the seeds; thus, commercial hybrids will ultimately save farmers money that would have been lost to aflatoxin accumulation pre- and post-harvest. Germplasm screening studies have been extensively used to identify a number of maize lines associated with lower grain aflatoxin levels (Thompson et al. 1984, Windham and William 1998). This method, however, is not

easily implemented largely due to the difficulty in finding elite lines that have high yield and good agronomic performance and that are also resistant to aflatoxin accumulation in multiple environments (Clements and white 2004) while conventional selection has helped to create inbred maize lines that are resistant to aflatoxin accumulation, it is hard to transfer aflatoxin resistance from a resistant donor line into an elite favorable commercial cultivar due to the highly quantitative nature of the trait. This means that a lot of genes are working together to make the plant resistant to aflatoxin accumulation therefore and due to recombinations that happens within these genes, it will be hard to transfer all the necessary genes into another germplasm(Stoloff and Lillehoj 1981, William et al. 2008). It should be noted that, many new strategies that may be used someday to enhance host plant resistance involving biotechnology are currently being explored (Brown et al. 2003; Warburton and Williams, 2014). These new strategies include the identification of quantitative trait loci (QTL) and related markers for marker assisted selection (Warburton et al. 2009) and the identification of resistance-associated proteins through proteomics and gene expression studies, and biochemical marker identification (Bhatnagar et al. 2008). The main purpose of the maize proteome mapping is to help in identifying and classifying functional gene products that aids in making the plants resistance to aflatoxin accumulation (Pechanova; 2013).

As generations of backcrossing with phenotypic selection are advanced, the resistance is often lost, so either the backcrossing must be terminated before the generation of a new inbred line with all the characteristics of the elite line, or the resistance of the new inbred line is lower than the resistance of the original donor.

One method to increase the efficiency of selection of desirable traits is known as marker assisted selection (MAS, Lande and Thompson 1989). Genetic or molecular markers that are linked to genes or quantitative trait loci (QTL) that are associated with the desired trait can be used to develop improved cultivars by selecting specific chromosomal regions within the maize genome (Bernardo, 2012); in this case, those that contain the gene(s) that contribute to aflatoxin accumulation resistance. The detection of these regions in the progeny of breeding crosses will help to validate that resistance was transfered into future commercial elite cultivars. Molecular markers associated with important traits in maize are becoming increasingly available and this has given rise to the incorporation of marker assisted selection into many maize breeding programs such as drought tolerance and protein quality (Gao et al. 2008). There has been an aggressive use of molecular markers in studying quantitative traits because the cost of various marker systems such as simple sequence repeats (SSR), amplified fragment polymorphisms (AFLP; Vos et al. 1995), and diversity array technology (DArT; Kilian et al. 2005), and more recently, sequence based markers such as single nucleotide polymorphisms (SNP), have decreased in most crop species (Burrow and Blake, 1998; Bhattramakki and Rafalski, 2001). In addition, there has been the development of computer software and statistical tools that can be used for the implementation of this marker assisted selection procedure (Bernardo 2008).

**Molecular Markers in Breeding Programs**

Marker Assisted Selection (MAS) schemes include marker assisted backcrossing and pyramiding are used to increase the effectiveness and efficiency of selecting for a particular trait while backcrossing or intermating one or a few genes controlling the

selected traits into an adapted cultivar (Collard and Mackill 2007). Markers can use to transfer identified QTLs into elite cultivars via marker assisted backcrossing and also be used to pyramid resistant QTLs from one or more donor lines (Warburton et al., 2010). Marker assisted backcrossing is generally used to move a single trait into a breeding line or cultivar. Marker assisted pyramiding is mostly applied to combining multiple genes for disease resistance for the development of a stable disease resistance since pathogens are likely to overcome single gene host resistance (Shanti et al. 2001, Kloppers and Pretorius 1997). Genomic selection is used to select for desired traits when the genes controlling these traits are unknown. Using MAS in early breeding generations has a tremendous advantage because plants with undesirable genes can be eliminated in the early stages, which ultimately leads to reduced labor costs and allow breeders to focus more on the important lines with the desirable alleles in subsequent generations (Collard and Mackill 2007). MAS can be combined with phenotypic screening (Moreau et al. 2004) and thus has an advantage over phenotypic screening or MAS alone in order to maximize genetic gain (Lande and Thompson 1990).

**Quantitative trait loci (QTL) mapping and Association mapping**

Quantitative traits have been the major focus of genetic studies for over a century because most traits important to plant breeding, ecology, human and animal health, etc., are associated with a quantitative inheritance. Until recently, the study of quantitative traits was based only on statistical techniques with limited knowledge as to the number and the location of the genes involved in controlling the trait (Kearsey and Farquhar 1998). Despite the large number of publications on QTL mapping studies of different

quantitative traits, only little has been reported to show the successful integration of the QTLs in breeding programs (St. Clair 2010).

The nature of the variation associated with the trait may be an indication that the trait is controlled by a few genes with large effects or by many genes each with smaller cummulative effects (Bernardo 2008). To identify QTL for a particular trait, a linkage map is constructed using a segregating population, such as $F_2$, $F_3$ or backcross (BC) population, most often derived from a bi-parental cross (Collard et al. 2005). The parents generally differ in the trait of interest. Previous reviews have shown that QTL mapping studies are usually able to detect 3 to 5 QTL for each trait, although ranges of 1 to more than 10 have been reported (Kearsey and Farquhar 1998). Detection of genes or QTLs influencing a trait is possible due to genetic linkage analysis based on the principle of genetic recombination during meiosis (Tanksley 1993). Exploiting QTL requires the genetic mapping of linked markers and genes; results of the mapping can lead to markers for selection (Bernardo 2008) and a better understanding of the genetic architecture of the trait, including the number of genes and their mode of expression, interaction, and inheritance (Beckmann and Soller 1986). In addition to the number of QTL that contribute measurably to the trait, QTL output also gives information about the magnitude and the gene action for each marker and QTL in each environment measured (Mackay 2001). Multiple studies have been carried out to find QTL that are associated with aflatoxin resistance and dozens have been identified in maize lines that are resistant to aflatoxins (Widstrom et al., 2003), although only a handful of these have a larger effect (Mideros et al., 2013).

Genetic markers that are polymorphic between the two parents are used to genotype the segregating population to create the linkage map (Young 2000). Past QTL studies have used AFLP (amplified fragment length polymorphisms), SSR (simple sequence repeats), RFLP (restriction length fragment polymorphisms), SNPs (single nucleotide polymorphism) and other markers for this purpose (Francia et al. 2005; Rafalski 2002; Robertson et al. 2005), and many maize molecular markers are available in the public online maize database (maizeGDB.org). Good molecular markers for QTL identification and marker assisted selection must be reliable and tightly linked to the targeted loci (< 5cM genetic distance). The visual output from the software after the creation of the linkage map shows the specific location of the markers on the chromosome and the distance between the markers (Collard et al. 2005), and this map, plus phenotypes of all the individuals in the mapping population, are combined to perform the QTL analysis. The association mapping involves the genetic characterization of the relatedness of over 282 diverse inbred lines (kinship), their diverse genetic makeup and also substructure analysis of the lines (Warburton et al; 2012). TASSEL software (Trait analysis by association, evolution and linkage, Bradbury et al, 2007) was used for aflatoxin association mapping for each of the candidate genes. It employs two models (the general linear model (GLM) and the mixed linear model (MLM)) to determine association between sequence polymorphisms and aflatoxin levels within the aflatoxin association mapping panel according to Elshire et al (2011).

**Near Isogenic Lines (NILs)**

Near Isogenic Lines (NILs) are homozygous plant lines that are identical to each other except at one genomic region or QTL of interest, and are useful for studying the

phenotypes associated with any specific locus (Dorweiler et al. 1993). They are a tool for detecting linkages and gene action (epistasis) that exist between quantitative trait loci (QTLs) and the markers used to map the QTLs to their specific locations on the chromosome (Pea et al. 2013). The integration of a QTL into near isogenic materials is one effective way for the identification, validation, and subsequent incorporation of the QTL into new breeding lines in a breeding program (Kaeppler, 1987). The use of NILs to verify and fine map QTLs has been successful in maize (Graham et al., 1997), rice (Yu et al., 1991) and tomato (Brouwer and St Clair 2004). QTL mapping with populations of about 300 individuals (the usual size of a mapping population) has a precision of only 10-20cM. For a detailed study of a QTL, development Near Isogenic Lines (NILs) is one method that is useful in resolving the map position of a QTL because it differs only for markers that are linked to the QTL of interest (Patterson et al. 1990; Kaeppler et al. 1993). NILs that are different in the QTL of interest are also useful for studying the different phenotypes associated with any specific locus (Dorweiler et al. 1993).

NILs to verify markers that reduce aflatoxin levels in maize are created with initial crosses between a line that is resistant to aflatoxin accumulation (donor line) and a line that is susceptible (recurrent line) to get the first progeny ($F_1$) that has 50% of the resistant parent and 50% of the susceptible parent. Several generations of backcrosses are usually the best method to introgess the region that contains the allele of the QTL from a donor genome into the recipient genome. The $F_1$ generation is then backcrossed to the susceptible parent to get the second generation of progeny that has 75% of the susceptible parent and 25% of the resistant parent with the aim of transferring the resistance present in the donor parent into the new progeny (but only genes for resistance). The process is

continued for 4 to 8 generations after which a progeny between ~93% - 98% of the susceptible parents, but containing a few genes from the resistant parent is achieved. Marker assisted selection is used in each generation after the $F_1$ to keep track of the progeny that still have the allele of the QTL from the donor line after each generation of backcrossing; otherwise, within a few generations it would surely be lost (Tuinstra et al. 1997). Despite the large number of QTLs that has been identified in various experiments and published in the literature, the use of these QTL to develop elite cultivars is very rare (Szalma et al., 2007) because breeders are unsure that the QTL and the markers linked to them will be useful in new genetic backgrounds or expressed in new environments. The use of NILs to validate previously identified QTL can help boost the confidence of breeders attempting to introgress the QTL into elite germplasm for the improvement of this germplasm for the trait of interest (Stuber et al., 1992).

**Plant Lipoxygenases**

Plant lipoxygenases or LOX enzymes are produced by plants to catalyze the addition of molecular oxygen to polyunsaturated fatty acids (PUFAs) containing a (Z,Z)-1,4-pentadiene system to produce an unsaturated fatty acid hydroperoxides (Porta et al., 2002). Plant LOXs are monomeric proteins with a molecular weight of about 95-100kDa that consist of two domains. These are the β- barrel amino terminal domain which is about 25 - 30kDa and the α-helix carboxyl- terminal domain of about 55 - 60kDa. The exact function of the amino terminal end is as yet unknown, but it is believed that it has some involvement in membrane and substrate binding (May et al. 2000). The carboxyl terminal end harbors the catalytic site of the enzyme where the addition of molecular oxygen takes place (Schneider et al., 2007). LOX enzymes are classified based on their

positional specificity of Linoleic Acid (LA) oxygenation, which is oxygenated either at carbon atom 9 (9-LOX) or at carbon atom 13 (13-LOX) of the hydrocarbon backbone of fatty acid, leading to (9S) hydroperoxy and (13S) hydroperoxy derivatives of LA, respectively (Liavonchanka et al. 2006). The 9-LOX are subsequently used as substrates for compounds which their functions are still unknown while the 13-LOX are known as the putative producers of compounds that are known to possess anti fungal activities such as jasmonic acid (JA) and green leaf volatiles (GLV) (Nemcnenko et al., 2006). The intracellular localization of each LOX enzyme after production in the plant cell provides a hint about the physiological role and function of different LOX enzymes (Feussner and Wasternack 2002).

The identification of the genes underlying quantitative trait loci (QTLs) associated with aflatoxin accumulation resistant in resistant maize inbred lines and the development of molecular markers from within these genes can help to speed up the development of resistant germplasm. Markers developed from within the gene sequence itself (gene based markers) do not have the problem of broken linkages that can happen between generations if the marker is too far from the gene causing the trait of interest. Therefore, the identification of the specific genes underlying and causing a QTL would be of great interest. LOX genes may be some of these genes.

When plants are stressed, due to attacks from harmful invaders such as insects, bacteria or fungi, they put up some responses in order to defend themselves against such attacks by undergoing specific metabolic processes to initiate direct or indirect defense responses to counter these attacks (Maffei et al. 2006). The direct defense measures involve the secretion of defensive proteins to repel their invaders while the indirect

measures include the release of volatile organic compounds (VOC) which attracts predators in attack other plant predators. This is achieved by metabolic products derived from fatty acid biosynthesis and signaling pathways such as metabolites derived from lipoxygenase (LOX; Pare and Tumlinson 1997). Lox genes are widespread in many plant species and in some cases such as soybean and other legumes, they are abundant proteins and might also function as storage rather than defense proteins.

**Lipoxygenase Pathway**

Hydroperoxides produced by LOX reactions are the starting point of a series of other enzymatic reactions which eventually leads to the synthesis of a group of biologically active compounds collectively called oxilipins (Santino et al. 2003). In plants, the biosynthesis of oxilipins starts by the insertion of oxygen at the C9 or C13 of either linoleic (C18:2) or linolenic (C18:3) and this is the reason why plant LOX is referred to as 9-LOXs and 13-LOXs respectively. During the catalysis of insertion of molecular oxygen (oxygenation) into polyunsaturated octadecatrienoic (C18) fatty acid by LOX in plants, carbon 9 and 13 are both oxidized to form 9- hydroperoxyl 10(E),12(Z) and 13-hydroperoxyl-9(Z), 11(E)- derivatives respectively (Blee 2002). Hydroperoxide lyase, allene oxide synthase, divinyl ether synthase, reductase and peroxygenase are other enzymes which belong to different branches of the LOX pathways that further act on both 9- and 13- hydroperoxides to convert them to different compounds. Specific LOX isoforms has recently been clarified by an antisense approach and their depletion was able to influence plant development or pest /pathogen resistance (Feussner and Wasternack 2002).

The Lipoxygenase pathway starts with regio- and stereospecific dioxygenation of either linoleic acid (18:2) or linolenic acid (18:3) to yield 9-hydroperoxide and 13-hydroperoxide based on the site where the molecular dioxygenation takes place. The two products (9-hydroperoxide and 13-hydroperoxide) formed after dioxygenation are then further used as substrate for the 7 branches of LOX pathways which includes reductase, epoxy alcohol synthase, allene oxide synthase (AOS), reductase, hydroperoxide lyase (HPL), divinyl ether synthase, and LOX reactions. (Feussner and Wasternack, 2002). A common metabolic reaction which occurs by either chemical reactions or derived from enzymatic reactions in all biological processes called lipid oxidation produces a compound called oxilipins which performs various regulatory processes and also respond to biotic and abiotic stresses. This reaction mainly catalyzed by Lipoxygenase (LOX) enzymes in plants has been researched and the mode of enzymatic reactions has been revealed in recent years (Andreou et al. 2009). Metabolic pathways involved in oxilipins formation collectively known as oxilipin pathway involves the oxidation of polyunsaturated fatty acids (PUFAs) which produces metabolites via a LOX-catalyzed and also metabolites produced from the alternative oxidation reaction. The metabolism of PUFAs via the Lipoxygenase catalyzed steps and the subsequent reactions are collectively known as the Lipoxygenase pathway (Blee, 2002).

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CHAPTER III

# GENETIC CHARACTERIZATION OF THE MAIZE LIPOXYGENASE GENE FAMILY IN RELATION TO AFLATOXIN ACCUMULATION RESISTANCE


**Abstract**

Maize (*Zea mays* L.) is a staple food and one of the most important cereal grains in the world. It is prone to contamination by aflatoxin, a secondary carcinogenic metabolite produced by the fungus *Aspergillus flavus*. An efficient approach to combat the accumulation of aflatoxin is the development of a germplasm resistant to infection and spread of *A. flavus*. Lipoxygenases (LOXs) are a group of non heme iron containing dioxygenase enzymes that catalyze oxygenation of polyunsaturated fatty acids (PUFAs), and LOX derived oxilipins play critical roles in plant defense against pathogens such as *A. flavus*. The objectives of this study were to report sequence diversity and expression patterns for all LOX genes in the maize genome, and to map their effect on aflatoxin accumulation via linkage and association mapping. In total, 13 LOX genes were identified, characterized, and mapped. Genes GRMZM2G102760 in bin 5.02 and GRMZM2G104843 in bin 2.04 fell under previously published QTL in one of four mapping populations and appear to have a measurable effect on the reduction of aflatoxin in maize grains. Association mapping results find 19 of the total 215 SNPs tested from within the sequence of five genes GRMZM2G070092, GRMZM2G109130,

GRMZM2G015419, GRMZM2G104843 and GRMZM2G102760 were associated with reduced aflatoxin levels at $7.51x10^{-4} \leq p \leq 8.43 \times 10^{-5}$ according to the GLM statistics. In addition to confirming the importance of some lipoxygenases for fungal resistance, markers from within or linked to the sequence of these genes may be used for marker assisted selection and the creation of new resistant germplasm.

## Introduction

*Aspergillus flavus* is a fungus found mostly in soil, but is also found in plant products, especially in oil rich seeds such as corn, cotton and peanuts. *A. flavus* produces a secondary metabolite known as aflatoxin, which is a carcinogen, mutagen, and hepatotoxin (Geiser et al 2000). Most commercial maize hybrids are susceptible to *A. flavus* infection, which ultimately leads to high aflatoxin accumulation under environmental conditions favoring fungal growth and sporulation. Aflatoxin was first discovered and characterized in the early 1960's when more than 100,000 turkeys in England died after consuming mold contaminated peanut meal (Blount, 1961; Goldblatt, 1969). Infection by *A. flavus* and *A. parasiticus* (which can also make aflatoxin) can be recognized by yellow-green or gray-green fungal growth on the corn kernels, respectively. Why *A. flavus* produces aflatoxins is not well understood, but it has been reported that both *A. flavus* growth and the production of aflatoxins is favored by abiotic stress such as drought, high heat and nutrient deficiencies (Moreno and Kang 1999). Aflatoxins can be detected either on corn still in the field or in storage following harvest, where it continue to accumulate grain stored under humid conditions. The risk of aflatoxin contamination is higher when the grains are damaged, which creates opportunistic entry point for fungal infection.

Development of resistant germplasm is one of the most effective methods to reduce aflatoxin accumulation in maize, but the quantitative nature of the trait and the high environmental variation associated with it makes the creation of resistant germplasm difficult to achieve. Identification of maize candidate genes that contribute to aflatoxin resistance via QTL or association mapping and development of linked molecular markers for marker assisted selection (MAS) is one way to speed development of resistant maize varieties. Host plant resistance mechanisms, particularly for resistance to *A. flavus*, are slowly being uncovered (Moreno and Kang, 1999; Warburton and Williams, 2014) but many factors have yet to be determined. Nevertheless, maize breeders have been able to develop resistant germplasm using phenotypic selection procedures in the form of inbred lines including Mp313E, Mp715, Mp717 Mp420 (Campbell et al, 1997; William and Windham, 2001; Scott and Zummo, 1990.)

When plants come under insect or fungal attack, genetic and metabolic processes are initiated to help the plant respond directly or indirectly (Maffei et al, 2006). The direct measures involve the production of defensive proteins to repel or block the attack, while examples of indirect measures include the production of herbivore induced plant volatiles (HIPV) emissions, which attract insect predators (Dicke, 2009; Heil, 2006). Lipoxygenases (LOXs) are a group of non-heme iron containing dioxygenase enzymes that catalyze oxygenation of polyunsaturated fatty acids (PUFAs) such as linoleic acid, α-linolenic acid and arachidonic acid (Acosta et al, 2009), and LOX derived oxilipins play critical roles in plant defense against pathogens. Research has shown that LOX pathways are induced by a variety of biotic and abiotic stresses, and a physiological function for

LOX enzymes has been proposed for plant growth, development, and response to pathogen infection and wound stress.

The synthesis of jasmonic acid (JA) is initiated when α-linoleic acid (C18:3) is released from the membrane lipids of the chloroplast by the action of phospholipase A1 (DAD1) and converted to 12-Oxo-phytodienoic acid (OPDA) by lipoxygenase, allene oxide synthase and allene oxide cyclase (Creelman and Mullet 1997). The production of mycotoxins by fungi is partially regulated by fungal genes that also belong to the LOX family (Gao and Kolomeits, 2009), indicating a complicated interaction between hosts and pathogen using the same enxymes in both organisms. LOX activity in plants has been shown to produce metabolites essential for plant defense against pathogen infestations through fatty acid oxidation pathways (Matsui, 2006). The oxidized products of plant lipids (oxilipins) derived from well studied LOX pathways govern the interactions between host and fungal pathogen (Gao and Kolomeits 2009). The role of specific LOX isoforms are being clarified, and the deletion of LOX enzymes in maize was found to influence plant development or pest /pathogen resistance (Feussner and Wasternack, 2002). In a study of ZmLOX 3, a LOX that belongs to the 9-LOX group, the insertion of a mutator transposon into the coding sequence of this gene resulted in drastic reduction of fumonosin production on kernels infected by *Fusarium verticillioides* (Gao et al; in 2007). A mutant maize line lox3-4, in which ZmLOX 3 and 4 were knocked out, was more susceptible to *A. flavus* and aflatoxin production than the wild type (WT) maize control (Gao et al, 2008). LOX enzymes are widespread in many plant species.

Peroxidation of PUFAs results in fatty acid hydroperoxide and lipoxygenase reactions, which may initiate the synthesis of a signaling molecule or be otherwise

involved in inducing structural or metabolic changes in the cell (Brash 1999). Hydroperoxides produced by LOX reactions are the starting point of a series of other enzymatic reactions which eventually leads to the synthesis of biologically active oxilipins (Santino et al 2003). Different branches of the LOX pathways lead to the production of JA and aldehyde green leaf volatiles (GLV, Kolomeits et al 2013), which are known to help plants defend against abiotic and biotic stresses, including fungi (Feussner and Westernack, 2002). JA is a known plant hormone involved in growth and development (Creelman and Mullet, 1997) and regulates several defense genes expressed in plants in response to attack by pests and pathogens (Pena-Cortes et al, 2004; Acosta et al, 2009).

Linkage and association mapping are two complementary ways of testing the magnitude of the effect a gene on the overall phenotypic expression of a trait. Linkage or quantitative trait loci (QTL) mapping accurately measures the effect of a larger genomic region on the trait of interest because the mapping population has a balanced proportion of alleles at all polymorphic loci, giving stronger statistical power when compared to the association mapping, but establishing much larger linkage blocks, due to relatively few generations of meiosis and thus recombination. Association mapping utilizes all the diversity of many lines to identify multiple sequence polymorphisms and measure the phenotypic effect of the favorable alleles associated with the phenotype; in addition, due to a very large number of historical recombination events in an association panel, resolution can be within hundreds to a few thousand base pairs (Warburton et al; 2013). Because of the importance of the LOX gene family in fungal defense, the objectives of this study are to characterize all genes that belong to the lipoxygenase gene family in

maize through expression pattern and sequence polymorphisms and to map the phenotypic effect of these genes in up to four known QTL mapping populations and one association mapping panel.

## Materials and Methods

### Database search for maize LOX genes and information

A search was carried out on five databases (Gramene, MaizeGDB,Unigene, Maizecyc and Uniprot) to find any previously published Lipoxygenase genes in maize and also to seek any gene or protein with Lipoxygenase activity (GO: 0016165) that is responsible for the end product of any of the seven Lipoxygenase pathways as reported by Feussner and Wasternack; (2002). A literature search was also conducted to find any maize lipoxygenase genes that were not included in the online resources. A total of thirteen genes were found on chromosomes 1 (ZmLox 3, 4, 9 and 13), 2 (ZmLox 6 and 8), 3 (ZmLox 1, 2 and 12), 4 (ZmLox 10), 5 (ZmLox 5 and 11), and 10 (ZmLox 7) (Table 3.1). This information was used to identify the coordinates of these genes in the maize B73 reference genome and extract the reference DNA sequence of each gene, for BLAST alignment and polymorphism identification.

A sequence alignment was carried out for all genes on the same chromosome with coordinates that are physically close to each other to ensure they are not the same gene given different names by different authors and databases. LOX genes with high sequence homology included ZmLOX 4 and ZmLOX 5 on chromosome 1 and 5, respectively, and ZmLOX 1 and ZmLOX 2, a pair of closely linked genes on chromosome 3 (<40kb apart)., Aligning the sequences of these genes against each other was also done to explore the possibility that one arose from the other in a recent duplication event. In addition,

ZmLOX 1 and 2 are either the same gene with a very large intron, or two genes very close together on chromosome 3. These genes have different gene identifiers but only one identifier in the maize B73 reference sequence (GRMZM2G156861). Due to a huge intron (>100kb) that is present in between exon 1 and exon 2 of the gene GRMZM2G156861 (ZmLOX 1 or 2) and also a (>16kb) intron present within exon 2 and exon 3 of gene I.D GRMZM2G109056 (ZmLOX 4), a blast search was carried out on a maize database (Gramene) and also the NCBI database to determine if the introns are due to the presence of a transposable element in the maize genome.

In order to gain more insight into relationship between genes and possible gene functions, two more databases were used in the characterization of the lipoxygenase gene family. The genome wide atlas of lipoxygenase transcription during maize development adapted from Sekhon et al. [36] and Qteller [35] was searched for expression pattern of each LOX gene identified in maize. Finally, the PIECE (Plant Intron Exon Comparison and Evolution) Database http://wheat.pw.usda.gov/piece was used to construct a phylogenetic tree (Figure 2) to determine structural relationships between the LOX genes and to provide clues about the evolutionary history of the genes. The PIECE database uses the pfam database (V26.0) to classify all the plant genes and use the FastTree program to build the phylogenies.

**Extracting SNPs for candidate genes using GBS data**

An in-house maize hapmap database was created to store Genotype By Sequencing (GBS) data for 273 maize inbred lines that form the aflatoxin association mapping panel described in Warburton et al (2014). The database describes where the variants occur in the genome of each inbred line and how alleles are distributed between

the different lines. This hapmap database was used to identify Single Nucleotide Polymorphisms (SNPs) or Insertion/Deletion (InDel) polymorphisms within the coordinates of each of the candidate genes (and extending up to 500 kilobases up-and downstream). An average of 20 SNPs were found for each candidate gene, and the allelic variant for each SNP in each of the 273 maize inbred lines was extracted. Only extracted SNPs with a minor allele frequency of greater than 5% were used to carry out the genetic mapping. If a sufficient number of SNPs were found within the coding sequence of the gene, SNPs further up- and downstream were not sought.

**Aflatoxin association mapping**

TASSEL software (Trait analysis by association, evolution and linkage, Bradbury et al, (2007) was used for aflatoxin association mapping for each of the candidate genes. It employs two models (the general linear model (GLM) and the mixed linear model (MLM)) to determine association. We ran MLM to determine associations between the SNP and InDel sequence polymorphisms and aflatoxin levels within the association mapping panel according to Elshire et al (2011). The panel of 273 diverse inbred lines had been testcrossed to a common tester and phenotyped for aflatoxin levels in inoculated, replicated field trials and reported in Warburton et al. (2013).

**Genetic linkage mapping**

The phenotypic and previously published genotypic data obtained from the four $F_{2:3}$ QTL mapping populations were combined with the new genetic data for each ZmLOX sequence. Single Nucleotide Polymorphisms found to be associated with aflatoxin levels in the candidate gene association analysis were converted to individual

SNP assays using the KASP system from LGC Genomics (Hurts, UK). These were used to screen the parents of four QTL mapping populations and where polymorphisms were validated, were then scored in all $F_{2:3}$ QTL mapping families. In addition, insertion/deletion (InDel) markers from genes ZmLOX 1, ZmLOX 2, ZmLOX 3 and ZmLOX 4, and Short Sequence Repeat (SSR) markers within 1,000kb upstream and downstream of the coordinates for each of the candidate gene were sought in MaizeGDB and used for linkage mapping analysis for other ZmLOX genes where no polymorphic SNPs or InDels were found. Due to the high duplication of the ZmLOX genes and also because some of the genes are within less than 1000kb from each other (ZmLOX 1 and 2, and ZmLOX 3 and 4) some ZmLOX pairs were treated as a single QTL, as it would not be possible to tell which of the genes is responsible for the phenotypic effect on the trait (if either) using linkage mapping.

The four QTL mapping populations included Mp313E (resistant) x B73 (susceptible) Brooks et al. (2005), Mp313E (resistant) x Va35 (susceptible) Wilcox et al. (2013), Mp715 (resistant) x T173 (susceptible) Warburton et al. (2011) and Mp717 (resistant) x NC300 (susceptible) Warburton et al. (2011). All markers were amplified via PCR according to the manufacturers' suggestions . The PCR products of the SSR and InDel markers were electrophoresed and visualized on a 4% agarose gel with ethidium bromide. SNP markers were visualized using the OMEGA plate reader by BMG LABTECH GMBH, (Orthenberg, Germany). The allele information obtained for every individual in the mapping populations in which the markers segregated was used to map and test the phenotypic effects on aflatoxin resistance for each candidate gene. Markers used to test each ZmLOX gene, including type, location, and which mapping population

they were run in are found in Table 3.2. Quantitative trait analysis for each of the ZmLOXs was carried out using the QTL cartographer which carries out the compisite interval mapping to estimate the 0.05 significant threshold for each QTL (Figure 3.2)

## Results

### Identification of maize lipoxygenase genes

A total of 13 lipoxygenase genes were identified through a search carried out on numerous online databases and a brief description of all maize lipoxygenase genes (ZmLOX) with the gene identification, gene bank accession numbers, Uniprot identification and chromosomal locations can be found in Table 3.1. ZmLOX 1, 2 and 12 (GRMZM2G156861, GRMZM2G106748) are all found on chromosome 3; according to NCBI BLAST (Geer et al. (2010), ZmLOX 1 and 2 are 89% identical, and share the same gene I.D. (GRMZM2G156861). They are physically ~40kb apart, and the B73 V3 reference sequence treats them as one gene with a very large intron. Genetically, we treat them as one locus in the QTL mapping analysis.

ZmLOX 3, 4, 9 and 13 (GRMZM2G109130, GRMZM2G109056, GRMZM2G017616, GRMZM5G822593 respectively) are all located on chromosome 1. ZmLOX 3 and 4 are 79% identical and are < 50kb apart. They do have different gene identifiers in the B73 V3 reference, but at such close proximity, QTL mapping will not distinguish the genetic effects of the two (although association mapping may). Although ZmLOX 6 and 8 (GRMZM2G040095 and GRMZM2G104843) are both located on chromosome 2 and although they are located on the same chromosome, there was no significant similarities found within the sequence of both genes. ZmLOX 5 and 11 (GRMZM2G102760 and GRMZM2G009479) are both located on chromosome 5, these

pairs of genes are sufficiently distant to map independently and there was also no significant similarities found between the sequences of the two genes ZmLOX 7 and ZmLOX10 are located on chromosome 4 and chromosome 10, respectively.

The gene structure for each ZmLOX was identified including the number of introns and exons present within each gene sequence, and this information was used to create a phylogenetic relationship tree (Figure 1). Two other genes GRMZM2G018275 (Chr2: 43,746,150 - 43,747,663) and GRMZM2G087245 (Chr4: 180,815,028 - 180,816,217) were identified by the PIECE phylogenetic analysis; however, these are not LOX genes and no gene has been associated with these gene I.Ds in maize. They are probably artifacts created by the Pfam database, which approximates the maximum likelihood of the relatedness of proteins, not genes. This also causes multiple transcripts of each gene to be entered separately into the phylogenetic tree (Figure 3.1). Clustering also occurred on LOX function, as genes from the 9-LOX functional group clustered together, and genes from the 13-LOX group together in a distinct cluster.

**Linkage and Association Mapping**

The linkage was used to determine the phenotypic effect of each marker linked to the lipoxygenase genes and to confirm the QTL position in the maize genome. Mapping results in one or more mapping populations of the InDel and SSR markers identified within each gene sequence or closely linked SSR markers are presented in Table 3.3. An SSR that is linked to GRMZM2G104843 [ZmLOX 8, also known as the mutant tassel seed 1 (ts1)] in bin 2.04 mapped right under one of the perviously identified QTLs present on chromosome 2 of the the MpB population (Figure 3.4) with a LOD score of 5.6 and explaining about ($R^2$) 5% of the phenotypic variation observed in this population

in one environment. The QTL was associated with an additive gene action and the allele causing the reduction of aflatoxin came from the resistant parent (Mp313E). ZmLOX8 is part of the pathway that provides substrates for the synthesis of JA Christensen et al in 2013, and the ts1 mutation results in the lack of sufficient JA to properly form male floral structures instead of female. JA is also known to be involved in direct and indirect mechanisms for plant resistance to fungal and insect attack (Browse, 2009; Koo and Howe, 2009), and annotation of ZmLOX8 includes fungal resitance as a biological process in the Gramene maize genome database.

Another previously published QTL was highlighted in this linkage mapping exercise after gene GRMZM2G015419 (ZmLOX 10) bin 4.09 mapped directly under a QTL of LOD 2.6 that explains approximately 5% of the phenotypic variation observed in the MpT population in one environment. The gene also mapped correctly in two other populations (MpT and MpVa) but no QTL was identified at this location in these mapping populations. ZmLOX 10 has also been reported to play an important role in the biosynthesis of green leaf volatiles (GLVs), a group of compounds that possess both anti-insect and anti-fungal properties (Prost et al, 2005; Matsui et al, 2006). These GLVs have also been reported to induce the expression of other defensive genes (Bate and Rothstein, 1998). There is evidence that ZmLOX 8 and ZmLOX 10 work synergistically, although the enzymes they express are located in different cellular compartments, and the genes are found on different chromosomes in the maize genome. A reduced expression of JA by ZmLOX 8 leads to diminished levels of (GLVs) by ZmLOX 10 Chrustensen et al (2013). Such an epistatic interaction could not be detected in the QTL mapping populations used in this study, as they only had ~ 200 $F_{2:3}$ families each.

Two markers, (one InDel and one SSR) linked to gene GRMZM2G156861 (ZmLOX 1 and 2 in bin 3.06) according to the IBM2 2008 Neighbors map in MaizeGDB, mapped within the confidence interval but very close to the edge of another QTL on chromosome 3 of the another MpT mapping population. ZmLOX 1 or 2 (or both) could therefore possibly be responsible for the phenotypic variation associated with this QTL. All other ZmLOXs in the maize genome mapped outside of previously identified QTLs (Table 3.2) however, six of the markers mapped in this study did help narrow previously reported QTL intervals; although they were not presumed to be the causal gene for the QTL, they did help to fine-map them and reduce the interval for future marker introgression of the QTL. These included markers linked to GRMZM2G017616 (ZmLox 9), GRMZM2G106748 (ZmLOX 12), GRMZM2G102760 (ZmLOX 5), GRMZM2G070092 (ZmLOX 7), GRMZM2G109130 (ZmLOX 3) and GRMZM2G109056 ( ZmLOX 4) (Table 3.2).

For the association mapping, a total of 215 SNPs were identified within the genetic sequence of all the ZmLOX genes using the in-house hapmap database (Supplementary Table 1). Of all the 215 SNPs, 19 were identified as associated to aflatoxin accumulation resistance according to the general linear model (GLM) of TASSEL, with p-values that ranged between $7.51 \times 10^{-4} \leq p \leq 8.43 \times 10^{-5}$ (Table 3.3). Ideally, the same SNPs with a relatively low p-value would be polymorphic in one or more of the QTL mapping populations in order to confirm the effect of the locus via QTL mapping as well, but none of the associated SNPs could be converted into a polymorphic KASP assay in this study. This may have been because of the high sequence duplication within the ZmLOX gene family, or they may have simply been monomorphic in all four

populations. Only one SNP was found from within or linked to the sequence of ZmLOX 4 and 11 (GRMZM2G109056 and GRMZM2G009479), which is too small a number to be confident that negative results are truly representative of the effects of these genes.

**Sequence Evolution**

Of all 13 LOX genes found in maize, GRMZM2G109056 (ZmLOX 4) located in bin 1.09 and GRMZM2G102760 (ZmLOX 5) located in bin 5.02 are the most identical, with a sequence similarity of 94% according to a BLAST search carried out using the NCBI alignment tool. Both genes consist of 9 exons and 8 introns, but the second intron spans ~ 11kb in ZmLOX 4 and only ~ 500bps in ZmLOX 5. A blast search of this intron was conducted on the NCBI (a general database for DNA sequences) and GRAMENE databases (a maize database) and both databases matches the intron to multiple genes as a huge intron and therefore can be assumed that this intron is a retroelement, and since nearly 85% of the maize genome is composed of hundreds of transposable element families that are randomly dispersed across the whole genome (Schnable et al 2012), this strengthens the assumptions that the intron might be a retrotransposon such as Ji, huck and opie which are the most common retrotransposons present within the maize genome (Phillip et al; 2005) . ZmLOX 4 and 5 are only 40-67% identical to other ZmLOXs (Fuente et al, 2012).

The linked pair of genes ZmLOX 1 and ZmLOX 2 share the same gene I.D. (GRMZM2G156861) in all maize databases and taken separately, are the next most similar paralogs in the Lox gene family. This is common with tandemly duplicated genes in the maize genome, which may result in duplication following transposable element insertion (with or without subsequent excision). Genes GRMZM2G070092 (ZmLOX 7)

and GRMZM2G104843 (ZmLOX 8) are a set of segmentally duplicated genes with near identical sequences (Fuente et al 2013) both having 7 exons and 6 introns. The first 2 exons of both genes share 83% and 93% identity and the intron between them is 83% identical. Another set of segmentally duplicated genes are GRMZM2G015419 ZmLOX 10, with 3 exons and 2 introns and GRMZM2G009479 ZmLOX 11, with 5 exons and 4 introns, which share 94% sequence identity (Fuente et al 2013) and both belongs to the 13-LOX group as the ZmLOX 7 and 8. Genes GRMZM2G109130 (ZmLOX 3), GRMZM2G109056 (ZmLOX 4), GRMZM2G017616 (ZmLOX 9), GRMZM5G822593 (ZmLOX 13) are all on chromosome 1, and ZmLOX 3 and 4 are only 4kb apart from each other and share a sequence identity of 80% (and both are 9-LOX genes). ZmLOX 9 and 13 are much further away, and not similar to ZmLOX3 and 4, nor each other (and both are 13-LOX genes).

**Discussion**

All ZmLOXs were found on six of the ten chromosomes present in the maize genome, and all mapped to these locations as expected. Maize LOX genes are divided into two major functional groups: 9-hydroperoxides (9-LOXs) and 13- hydroperoxides (13-LOXs), depending on the carbon where their molecular dioxygenation takes place. ZmLOX 1, 2, 3, 4 and 5 all belong to the 9-LOXs group and their functions are still not well known, while ZmLOX 7, 8, 9, 10, 11, and 13 all belongs to the 13-LOXs group and are known or putative producers of JA and GLVs (Nemchenko et al, 2006; Gao et al, 2008; Part et al, 2010). Compounds produced by the various lipoxygenase pathways belonging to the 13-LOX group includes hydroperoxide lyase (HPL) and allene oxide synthase (AOS) branches, whose final product, GLV and JA, play a very important role

in plant immunity against predatory insects and fungi (Engelberth et al 2004; 2011). The composite interval mapping results of this study (Figure 3.4) can be compared with published information to suggest a biological role of some of these genes in aflatoxin accumulation resistance.

Genes GRMZM2G104843 (ZmLOX 8) and GRMZM2G015419 (ZmLOX 10) are reported to direct and indirect roles in plant defense against herbivory and fungal resistance by producing the substrates used in the biosynthesis of JA and GLVs respectively Christensen et al (2013); however, due to the physical separation of both genes in the maize genome, it has been suggested that the only interaction between both genes will be as a result of signaling crosstalk of their products; this has not yet been demonstrated in laboratory assays (Christensen et al, 2013). ZmLOX 8 mapped directly under a QTL of LOD value 5.6 (Table3.2). ZmLOX 10 generates 13S-HPOTE, which is required for synthesis of GLVs, but this only happens in the presence of five of the other 13-LOXs (and especially gene GRMZM2G009479, ZmLOX 11) (Nemchenko et al 2006). ZmLOX10 was found beneath a QTL for aflatoxin accumulation resistance with a LOD value of 2.6.

ZmLOX 5 (and its near identical homolog ZmLox4) belongs to the 9-LOX family; ZmLOX 5 is expressed in silks (Park et al; 2010) and mapped directly under another QTL found in bin 5.02 with a LOD value of 2.4. The near identical homolog ZmLOX4 was neither associated nor linked to a QTL for aflatoxin accumulation resistance, and it has a very different expression pattern than ZmLOX 5, as it is expressed primarily in the roots (Park et al, 2010). This may explain the lack of association with

aflatoxin levels in maize grain, and we may speculate that ZmLOX4 may have more to do with resistance to pests that attack corn roots.

Although most of the ZmLOXs identified in this study with an effect on aflatoxin accumulation resistance explainied less than 5% of the phenotypic variation observed in the populations measured, it will still be informative to verify the effect of the resistant alleles through the creation of transgenic lines, near isogenic lines (NILs), or knock-out mutants to verify the effect of these genes in a different background other than the background present in the mapping populations of this study. Genes GRMZM2G104843 (ZmLOX 8) and GRMZM2G015419 (LmLOX 10) both explain approximately 5% of the phenotypic variation, which may be a large enough effect to justify further studies. The expression pattern of ZmLOXs varies from tissue to tissue and are expressed at different times in the life of the plant (Table 3.4a). For example GRMZM2G102760 (ZmLOX 5) is expressed more in silks and immature seeds while almost at the same time, gene GRMZM2G070092 (ZmLOX 7) is expressed more in the tassel. Gene GRMZM2G040095 is moderately expressed in every tissue at any given time in the life of the plant Sehkon et al (2011) (Supplemental Figure 1)

Table 3.1 Maize lipoxygenase gene family.

| Name | Gene I.D. | Gen Bank accession | UniProt | Description | Bin | Position (bp, V3 B73 reference sequence) | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Chr | From | To |
| ZmLox1 | GRMZM2G156861 | DQ335760 | Q9LKL4 | Lipoxygenase 1 | 3.06 | 3 | 168,738,873 | 168,742,524 |
| ZmLox2 | - | DQ335761 | A1XCH8 | Lipoxygenase 2 | 3.06 | 3 | 168 695, 543 | 168,699, 133 |
| ZmLox3 | GRMZM2G109130 | AF329371 | - | Lipoxygenase 3 | 1.09 | 1 | 264,266,381 | 264, 271, 190 |
| ZmLox4 | GRMZM2G109056 | DQ335762 | M1HFG0 | Lipoxygenase 4 | 1.09 | 1 | 264,275,083 | 264, 291, 510 |
| ZmLox 5 | GRMZM2G102760 | DQ335763 | A1XCI0 | Lipoxygenase 5 | 5.02 | 5 | 12,285, 656 | 12, 290,564 |
| ZmLox 6 | GRMZM2G040095 | DQ335764 | A1XCI1 | Lipoxygenase 6 | 2.02 | 2 | 4,192, 152 | 4, 196, 263 |
| ZmLox 7 | GRMZM2G070092 | DQ335765 | A1XCI2 | Lipoxygenase 7 | 10.04 | 10 | 120,237, 308 | 120,241, 527 |
| ZmLox 8 | GRMZM2G104843 | DQ335766 | A1XCI3 | Lipoxygenase 8 | 2.04 | 2 | 45, 820,737 | 45, 825, 105 |
| ZmLox 9 | GRMZM2G017616 | DQ335767 | A1XCI4 | Lipoxygenase 9 | 1.02 | 1 | 16, 573, 827 | 16, 580, 722 |
| ZmLox 10 | GRMZM2G015419 | DQ335768 | A1XCI5 | Lipoxygenase 10 | 4.09 | | 233,626,682 | 233,629,283 |
| ZmLox 11 | GRMZM2G009479 | DQ335769 | Q06XS2 | Lipoxygenase 11 | 5.04 | 5 | 123,239,668 | 123,243,697 |
| ZmLox12 | GRMZM2G106748 | DQ335770 | A1XCI7 | Lipoxygenase 12 | 3.04 | 3 | 93,841,905 | 93,845,764 |
| ZmLox 13 | GRMZM5G822593 | - | - | Lipoxygenase 13 | | 1 | 188,148,388 | 88, 153, 483 |

NOTE:Maize lipoxygenase gene family. Gene I.D. (identification) and UniProt protein identification numbers are used as unique identifiers of each lipoxygense in the study. Bin location indicatesgenetic mapping location according to MaizeGDB, and Position indicates the physical interval in relation to the B73 maize reference genome.

Table 3.2 List of InDELs and SSRs used to map the phenotypic effect of each ZmLOXs to the QTL mapping populations, the estimated marker starting positions and also the populations in which they segregate.

| # | Gene Name | Gene I.D | Marker | Marker type | Tested Bin # | Segregating population | Est Marker position | LOD Score[a] |
|---|---|---|---|---|---|---|---|---|
| 1 | ZmLOX 1 & 2 | GRMZM2G156861 | FxLOX_2738 | InDEL | 3.06 | MpB, MpT | 168,862,738 | 2.6 (MpB), NS (MpT) |
| 2 | ZmLOX 3 | GRMZM2G109130 | Phi 037 | SSR | 1.08 | MpT, MpVa | 226,891,043 | NS in both populations |
| 3 | ZmLOX 4 | GRMZM2G109056 | Phi 037 | SSR | 1.08 | MpT, MpVa | 226,891,043 | NS in both populations |
| 4 | ZmLOX 5 | GRMZM2G102760 | Umc 2303 | SSR | 5.03 | MpT, MpB | 179,766,869 | 2.4 (MpT), NS in MpB |
| 5 | ZmLOX 6 | GRMZM2G040095 | - | - | - | - | - | |
| 6 | ZmLOX 7 | GRMZM2G070092 | Umc 1453 | SSR | 10.04 | MpT, MpNc | 115,571,589 | NS in both populations |
| 7 | ZmLOX 8 | GRMZM2G104843 | Bnlg 1018<br>Bnlg 1909 | SSR<br>SSR | 2.04<br>2.05 | MpB, MpNc<br>MpT, MpVa, | 40,890,003<br>47,170,490 | 5.6 (MpB), NS in MpNc<br>NS in both population |
| 8 | ZmLOX 9 | GRMZM2G017616 | Umc 1976 | SSR | 1.03 | MpT, MpNc | 21,419,759 | NS in both populations. |
| 9 | ZmLOX 10 | GRMZM2G015419 | Umc 2287 | SSR | 4.09 | MpB, MpT, MpVa | 213,902,712 | 2.6 (MpT), NS in other populations |
| 10 | ZmLOX 11 | GRMZM2G009479 | - | - | - | - | - | - |
| 11 | ZmLOX 12 | GRMZM2G106748 | Umc 1968 | SSR | 3.04 | MpB, MpNc | 95,266,767 | NS in both populations. |
| 13 | ZmLOX 13 | GRMZM5G822593 | | - | - | - | - | - |

NOTE: [a]LOD score was set at a default threshold (2.5) of the composite interval mapping (CIM).

Table 3.3 A summary of ZmLOXs association mapping result.

| Trait | Marker | Chr | Marker $P^2$ | Marker_$R^2$ |
|---|---|---|---|---|
| CSta09LSM | S10_120219787 | 10 | 1.26E-04 | 0.05347 |
| CSta10LSM | S10_120220589 | 10 | 4.39E-04 | 0.04231 |
| Lubb10LSM | S1_264183077 | 1 | 4.85E-04 | 0.04107 |
| AveLSM | S10_120219787 | 10 | 7.51E-04 | 0.02745 |
| Star10LSM | S4_233627058 | 4 | 7.92E-04 | 0.02767 |
| StRa10LSM | S2_4188974 | 2 | 0.00257 | 0.06793 |
| Star09LSM | S3_168838069 | 3 | 0.00286 | 0.02136 |
| Lubb10LSM | S1_264172554 | 1 | 0.00417 | 0.02835 |
| CSta10LSM | S10_120220419 | 10 | 0.00424 | 0.02836 |
| CSta09LSM | S10_120219854 | 10 | 0.00484 | 0.02306 |
| Lubb10LSM | S1_264172489 | 1 | 0.00487 | 0.02631 |
| CSta09LSM | S10_120220348 | 10 | 0.00574 | 0.02119 |
| CSta09LSM | S10_120219582 | 10 | 0.00584 | 0.02205 |
| CSta09LSM | S5_12277329 | 5 | 0.00611 | 0.0265 |
| Star09LSM | S5_12277329 | 5 | 0.00751 | 0.01943 |
| Star10LSM | S4_233626196 | 4 | 0.00876 | 0.01778 |
| Star10LSM | S4_233626197 | 4 | 0.00876 | 0.01778 |
| Star10LSM | S4_233626821 | 4 | 0.00895 | 0.01703 |
| AveLSM | S10_120219582 | 10 | 0.00895 | 0.01323 |

NOTE: The number of SNPs found within each of the gene sequences with p values that range between $7.51 \times 10^{-4} \leq p \leq 8.95 \times 10^{-3}$as calculated by the GLM statistics and also the maximum $R^2$ value for each of the associated candidate genes

Table 3.4 A table showing the expression data of all ZmLOXs in different tissues at different stages in the life of the plant

| Gene_Name | gene_I.D | chr | Anthers | Developing_ear | Endosperm_25DAP | Ovule2 | Silk | Tassel | Pollen |
|---|---|---|---|---|---|---|---|---|---|
| ZmLox 1 & 2 | GRMZM2G156861 | 3 | 2.50 | 51.65 | 13.57 | 242.10 | 39.52 | 15.22 | 0.006 |
| ZmLox 3 | GRMZM2G109130 | 1 | 14.17 | 14.88 | 9.93 | 37.20 | 5.77 | 57.38 | 0.03 |
| ZmLox 4 | GRMZM2G109056 | 1 | 10.27 | 32.37 | 2.11 | 54.87 | 19.91 | 21.26 | 0.39 |
| ZmLox 5 | GRMZM2G102760 | 5 | 25.52 | 194.21 | 5.33 | 549.3 | 557.53 | 180.07 | 0.06 |
| ZmLox 6 | GRMZM2G040095 | 2 | 0.45 | 85.50 | 13.29 | 197.31 | 26.03 | 113.55 | 0 |
| ZmLox 7 | GRMZM2G070092 | 10 | 6.87 | 0.41 | 0.88 | 0.70 | 0.44 | 1.58 | 0 |
| ZmLox 8 | GRMZM2G104843 | 2 | 4.27 | 2.96 | 2.24 | 1.96 | 3.12 | 13.57 | 0 |
| ZmLox 9 | GRMZM2G017616 | 1 | 9.45 | 16.40 | 3.07 | 6.21 | 18.88 | 3.21 | 0 |
| ZmLox 11 | GRMZM2G009479 | 5 | 0.27 | 33.05 | 6.43 | 132.10 | 669.06 | 53.38 | 0 |
| ZmLox 12 | GRMZM2G106748 | 3 | 1.54 | 0.32 | 22.42 | 8.09 | 0.23 | 2.34 | 0.51 |
| ZmLox 13 | GRMZM5G822593 | 1 | 0.71 | 0.061 | 0.07 | 0.17 | 0 | 1.83 | 0.04 |

NOTE: Not all data shown. Created using the maize Qteller software. All values are absolute.

Table 3.5 Visualize Expression Link

| |
|---|
| http://qteller.com/qteller3/bar_chart.php?name=GRMZM2G156861 |
| http://qteller.com/qteller3/bar_chart.php?name=GRMZM2G109130 |
| http://qteller.com/qteller3/bar_chart.php?name=GRMZM2G109056 |
| http://qteller.com/qteller3/bar_chart.php?name=GRMZM2G102760 |
| http://qteller.com/qteller3/bar_chart.php?name=GRMZM2G040095 |
| http://qteller.com/qteller3/bar_chart.php?name=GRMZM2G070092 |
| http://qteller.com/qteller3/bar_chart.php?name=GRMZM2G104843 |
| http://qteller.com/qteller3/bar_chart.php?name=GRMZM2G017616 |
| http://qteller.com/qteller3/bar_chart.php?name=GRMZM2G009479 |
| http://qteller.com/qteller3/bar_chart.php?name=GRMZM2G106748 |
| http://qteller.com/qteller3/bar_chart.php?name=GRMZM5G822593 |

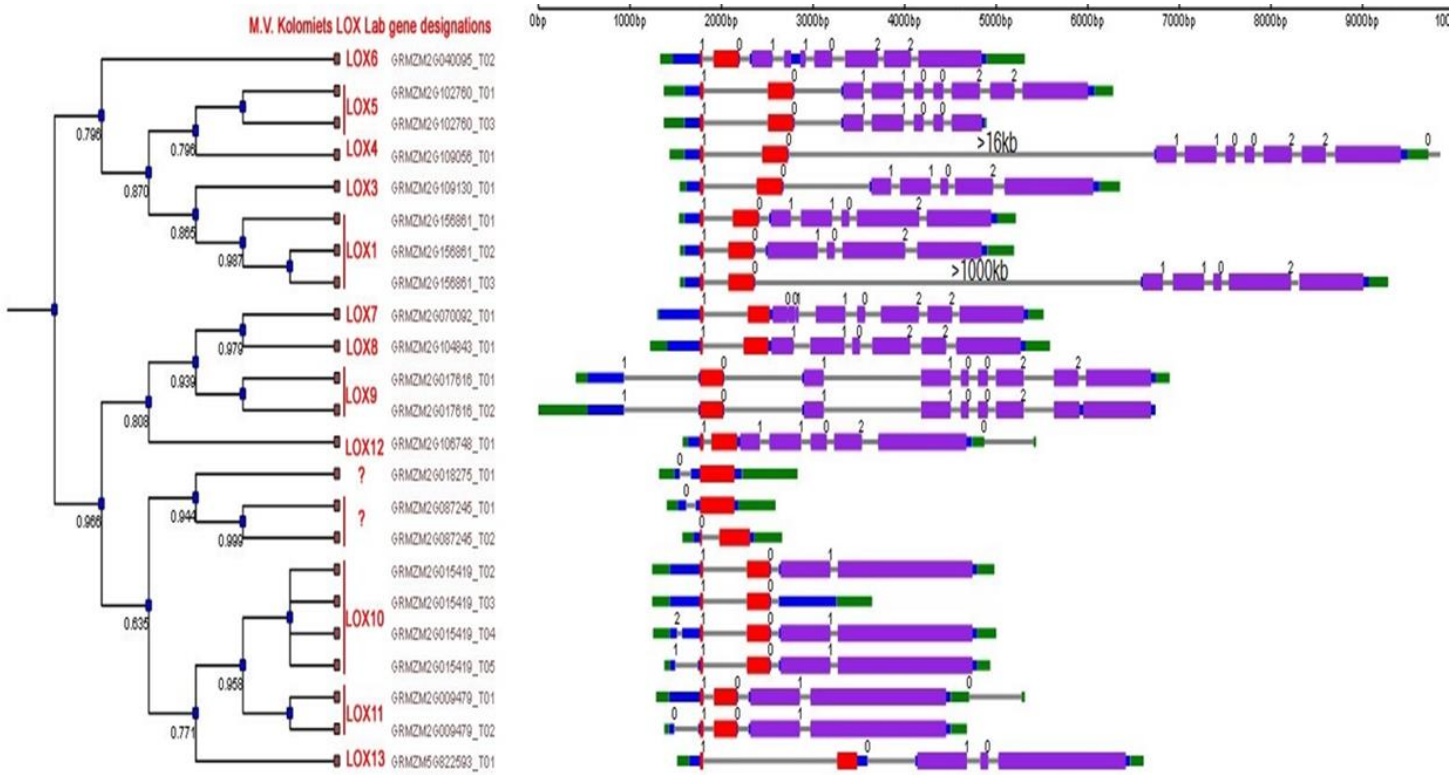


Figure 3.1 Phylogenetic tree analysis of all 13 maize LOX genes, including Gene and transcript I.D. for each of the gene.

NOTE: The tree was constructed using the PIECE (Plant Intron and Exon Comparison Evolution) database. Green/blue color are 5' and 3' untranslated regions (UTRs), gray color corresponds to gene introns, and purple color to gene exons.

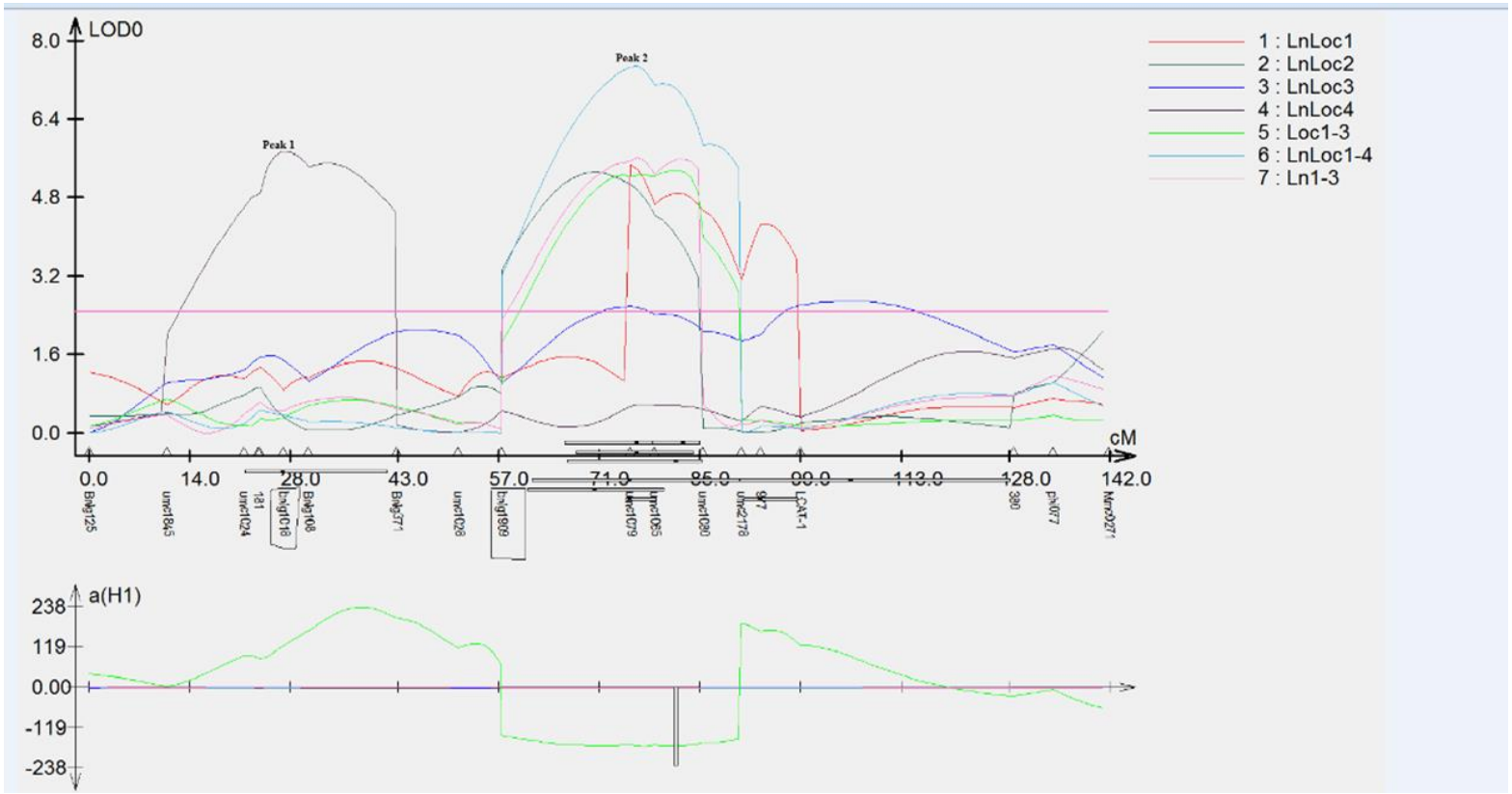


Figure 3.2 Composite interval mapping results of the MpB population (chromosome 2) across a total of six environments and the average.

NOTE: GRMZM2G104843 in bin 2.04 mapped right under a QTL. The X axis represents the genetic length of the chromosome and the Y axis represents the LOD significance set at 2.5. Each peak above the threshold represents distinct QTLs in the chromosome.

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CHAPTER IV

# CONSTRUCTING MAIZE NEAR ISOGENIC LINES (NILS) TO TEST GENOMIC REGIONS FOR RESISTANCE TO AFLATOXIN ACCUMULATION IN MAIZE


**Abstract**

*Aspergillus flavus* is an opportunistic saprophytic and/or pathogenic fungus of maize that may infect the living plant when it is under stress. When it is able to overcome infection barriers and sporulate, it initiates the production of a secondary metabolite known as aflatoxin, a carcinogenic substance that negatively affects the health of consumers of contaminated maize, and thus causes farmers in hot or humid areas of the world great economic losses. There have been many efforts to combat either the invasion of maize by this fungus or its production of aflatoxin. One of the many programs that have been practiced to combat this issue is the generation of maize that is resistant to the fungus or the accumulation of its toxins. The mapping of molecular markers has helped in the identification of Quantitative Trait Loci (QTLs) that are correlated to aflatoxin accumulation resistance. For validation and detailed study of QTLs, the creation of near isogenic lines (NILs) is a valuable tool, because lines that are nearly isogenic to susceptible maize for specific regions, carrying resistance alleles only in this one region but otherwise identical to the susceptible line, can help validate the location and physical effects of different regions on resistance.

## Introduction

*Aspergillus flavus* is a fungi that is mostly found in soil and plant tissue samples, and it is an opportunistic pathogen of many crops. Under the right conditions for growth, *A. flavus* will infect and produce a carcinogenic secondary substance known as aflatoxin (Orum et al 1997). Carcinogenic aflatoxin B1 (AFB1), produced by the fungus, is one of the major food safety concerns of maize. Aflatoxins were first discovered in the early 1960's in England when a very large group of turkeys died after consuming a grain with a high level of aflatoxin accumulation (Richard and Payne; 2003). This secondary metabolite is of great concern to both human and animal health because of its damaging effect on development and immune systems and extreme carcinogenic properties. Thus, most countries have strict regulations to limit accumulation of aflatoxin in maize and all other agricultural products that are susceptible to aflatoxin (Wang and Tang 2005).

Maize (*Zea mays* L.) is a staple food for much of the world population especially in many developing countries, and in tropical environments it is often contaminated by aflatoxin B1 (Castells et al 2007). The U.S Food and Drug Administration (FDA) prohibits interstate commerce of maize grains with an aflatoxin concentration equal to or greater than 20ng/g (Brown et al 1993). Multiple studies have been published on finding Quantitative Trait Loci (QTLs) that are associated with aflatoxin resistance, and dozens of the QTLs have been identified in maize lines that are resistant to aflatoxins (Windstrom et al, 2003, Mideros et al; 2009). Researchers can use different population structures such as backcross (BC), $F_2$, double haploids, testcrossed progenies, half sib and full sib families, F2 derived families, recombinant inbred lines (RIL) and diverse inbred population structures for detecting and mapping QTLs and for subsequent confirmation

of the detected QTLs in different genetic backgrounds. Another way to confirm and validate QTLs is the use of near isogenic lines (NILs) which are lines that differ only in one region, such as a QTL of interest. The creation of NILs is used for detailed study of QTLs that have been previously detected in other populations and are thus known to contribute to the trait of interest. Near isogenic lines can be used for the verification, mapping and incorporation of desired QTL into an elite cultivar that has all other desirable phenotypic characteristics except for the one that the QTL of interest controls (Eshed and Zamir 1995; Kaeppler, 1997). The fine-mapping of the NILs using molecular markers can in some instances be an effective approach in detecting new QTLs (Osborn et al 1987). NILs are useful for the accurate estimation of the effects of a QTL on a particular trait, and NILs carrying more than one QTL at a time are suitable for determining epistatic interactions, genetic linkage and genomic architecture of a trait (Pea et al 2013). The use of NILs to verify and fine map QTLs has been successful in maize (Graham et al, 1997), rice (Yu et al, 1991), soybeans (Muehlbaure et al, 1988) and tomato (Brouwer and St Clair 2004) among other species.

The QTL regions are identified in a mapping population with the use of molecular markers and estimates of the level at which the QTL contribute to the trait of interest are calculated (Kaeppler, 1997). NILs for aflatoxin accumulation resistance are derived by the initial crossing of a resistant line with known QTL that contribute to aflatoxin resistance to a susceptible line, and subsequently backcrossing the progeny derived from the initial $F_1$ to the susceptible parent for five to six generations (Szalma et al; 2007) to create a line that differs from the susceptible parent in just the QTL of interest initially present in the resistant parent. In this project, the construction of the NIL will be based on

one SNP of interest per NIL so that the effect of the each SNP on the trait can be tested separately. Conventional backcrossing is a process whereby a desired trait from a donor parent is transferred into an elite recurrent parent that has all other desirable phenotypic characteristics (Soto-Cerda; 2013). Identification of molecular markers linked to various genes and QTLs allows the marker assisted selection of these genes or QTLs during the introgression process via a series of backcrossing to the recurrent (susceptible) parent to create the NILs, and prevents the QTL from being lost in the process. A final selfing step fixes the QTL in the NIL in homozygous form.

Molecular markers are used to identify which alleles have been inherited by progeny after each generation. In the final step, both the susceptible parent and the NIL will be grown and phenotyped together to determine the effect of the genomic regions identified by a QTL or SNP haplotype on aflatoxin accumulation resistance (Kaeppler; 1997). Despite the large amount of QTLs that has been identified in various experiments and published in the literature, the transfer of these QTL into elite germplasm for validation and for the improvement of elite cultivars is very rare (Robertson et al; 2005). However, the use of NILs to validate QTL can help instill confidence in these QTL for breeders who wish to use them in the improvement of quantitative traits via marker assisted selection (Stuber et al, 1992). Results presented in this project are preliminary and will not complete the backcrossing to the required level, because I can only go as far as the BC2 generation in this project for my master's thesis. I intend to continue the project for my PhD in the immediate future.

## Materials and Methods

### Genetic stock and plant material

A total of 11 unrelated maize inbred lines (7 aflatoxin resistant lines and four aflatoxin susceptible lines, Table 4.1) were used to start the creation of near isogenic lines (NILs). Individual pairs of resistant and susceptible lines were crossed to generate $F_1$ progenies. The $F_1$ progeny obtained from each cross was backcrossed to the original susceptible parent to create the $BC_1$ generation. Twenty-five Single Nucleotide Polymorphisms (SNPs) from the in-house maize genome wide association studies (GWAS) hapmap database with the highest influence on aflatoxin accumulation (according to p value and $R^2$ from Warburton et al., 2015) was chosen as described in the following section. These SNP makers were used to obtain allelic information from all the inbred lines used for the creation of the NILs to know which lines to cross (as parents had to be polymorphic for the SNPs of interest). The aim of the crossing was to produce two to three NILs for each SNP region, each in a different susceptible genetic background. After each generation of backcrossing to the recurrent parent (the susceptible parent in this case), the progeny will consist of 50% more of the recurrent parent than the previous generation (Figure 4.1). When the $BC_1$ was again backcrossed to the susceptible parents for the creation of a segregating $BC_2$ population, marker assisted selection was carried out using the set of 25 SNPs (Table 4.3) to keep plants with the alleles from the resistant parent. The plants that were heterozygous for the resistant line's allele at one or more of the target loci were selected to be carried into the next generation of backcrossing. Plants homozygous for the susceptible recurrent allele were discarded to eliminate plants without the desired allele at the loci to be validated for resistance to aflatoxin and also to

reduce labor, as fewer plants are carried on to the next generation. Backcrossing with selection will continue until the $BC_3$ generation, which will be selfed twice to create the $BC_3S_2$ generation (Figure 4.2); plants selected with the SNP markers will be fixed for the regions to be tested and thus represent the end of the NIL derivation.

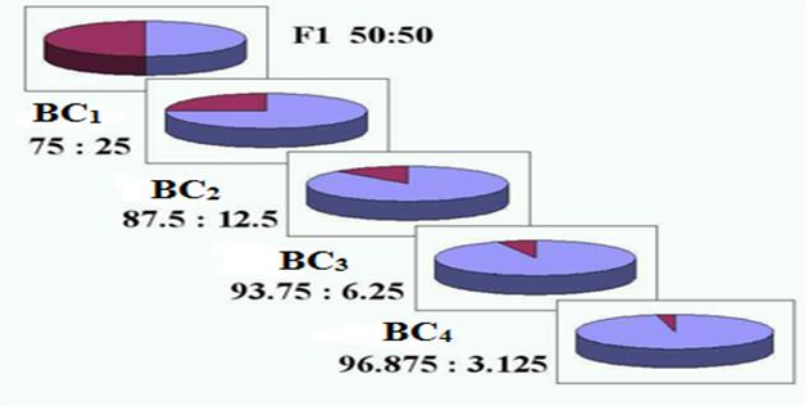


Figure 4.1 The genome of the donor parent is reduced by 50% after each generation of backcrossing. Byrne and Richardson; 2005.

**Identification of SNP markers and development of KASP assays**

Warburton et al, (2013; 2015) described the Genotype by Sequencing (GBS) data used in this study, which was generated according to Elshire et al (2011) for a panel of 273 diverse maize inbred lines containing aflatoxin accumulation resistant and susceptible genotypes and stored in an in-house hapmap database. The database presents the variation in the genomic sequence and allelic distribution of each line for all GBS data. The SNPs of interest from the GWAS study of Warburton et al. (2015), those associated with aflatoxin accumulation with the lowest p-values ($2.87 \times 10^{-10} < p < 9.78 \times$

$10^{-5}$) were found in the hapmap database for every line in the panel (Table 4.3). Those SNPs that were polymorphic between as many potential parents as possible and that displayed a minor allele frequency (MAF) greater than 5% were used for the creation of the NILs.

KASP assays were designed for the SNPs of interest by finding 100bp of DNA sequence from both upstream and downstream of the SNP of interest in the B73 reference genome Lawrence et al; (2008) and the assay was ordered from LGC genomics (Hurts UK) and tested for amplification and polymorphism on the 11 inbred lines used for the creation of the NILs. The KASP assays were used to select the individual progeny that carried the SNP allele of interest (from the resistant parent) from one generation to the next.

Table 4.1 Hapmap data showing SNPs within each extracting region and the SNP calls within each inbred lines used for constructing the NILs.

| S/N | rs# | alleles | Chr. location | CML5[a] | Ki3[a] | CML69[a] | CML348[a] | Mp313E[a] | Mp715[a] | NC388[a] | Mo17[b] | Va35[b] | NC258[b] | B73[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | S1_272220142 | A/G | 1 | G | A | A | G | A | G | A | A | G | A | A |
| | S1_272220150 | A/C | 1 | A | A | A | A | C | A | A | A | A | A | C |
| 1 | S1_272220177 | A/G | 1 | G | A | G | G | A | G | A | A | G | A | A |
| | S1_272220762 | T/C | 1 | N | T | T | N | N | N | T | T | N | T | T |
| | S1_272220785 | G/A | 1 | G | G | G | G | N | G | G | G | G | G | G |
| | S1_272220818 | A/C | 1 | A | A | C | A | N | A | C | C | A | C | C |
| | S1_272220826 | G/C | 1 | G | C | G | G | N | G | G | G | G | G | G |
| 2 | S1_272220852 | A/G | 1 | G | A | A | G | N | G | A | A | G | A | A |
| | S1_272272944 | T/G | 1 | G | T | T | N | N | G | T | T | G | T | T |
| | S1_272272950 | T/C | 1 | T | T | T | N | N | T | T | T | T | T | T |
| | S1_272272952 | G/A | 1 | A | G | G | N | N | A | G | G | A | G | G |
| | S1_272272953 | G/A | 1 | G | G | G | N | N | G | G | G | G | G | G |
| 3 | S1_272272977 | A/G | 1 | A | G | G | N | N | A | A | A | A | A | G |
| | S1_280635905 | A/T | 1 | A | T | A | A | A | T | T | A | N | A | A |
| | S1_280635931 | T/C | 1 | T | C | T | T | T | C | C | T | N | T | T |
| | S1_280635934 | T/A | 1 | T | A | T | T | T | A | A | T | N | T | T |
| | S1_280635950 | C/G | 1 | C | G | C | C | C | G | G | C | N | C | C |
| | S1_280635958 | C/T | 1 | C | T | C | C | C | T | T | C | N | C | C |
| 4 | S1_280635967 | A/G | 1 | A | G | A | A | A | G | G | A | N | A | A |
| | S2_22947761 | C/G | 2 | C | C | G | C | G | C | G | C | C | G | C |
| | S2_22947846 | C/T | 2 | C | C | C | C | C | N | T | C | C | C | C |
| | S2_22947850 | A/C | 2 | A | A | A | A | A | N | N | A | A | A | A |
| | S2_22947856 | C/A | 2 | C | C | C | A | C | C | A | C | C | C | C |
| 5 | S2_22947859 | A/C | 2 | A | A | A | A | N | N | N | A | A | C | C |
| 6 | S2_153128978 | G/A | 2 | A | G | N | A | N | A | N | G | G | G | G |
| 7 | S2_183190432 | C/T | 2 | N | T | N | N | N | T | N | N | C | C | C |

Table 4.1 (Continued)

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | S2_188872757 | T/C | 2 | T | T | T | C | T | T | T | T | N | C | T |
| | S2_188872760 | T/G | 2 | T | T | T | T | G | T | T | T | N | T | T |
| | S2_188872774 | C/T | 2 | C | C | C | C | T | T | C | C | N | C | C |
| 8 | S2_188872782 | C/T | 2 | C | C | C | C | T | T | C | C | N | C | C |
| | S2_188872896 | G/T | 2 | G | G | G | G | G | G | G | G | G | G | G |
| | S2_188872906 | C/T | 2 | C | C | C | T | C | C | C | C | C | T | C |
| | S2_188872911 | C/T | 2 | C | N | C | C | T | T | C | C | T | C | C |
| | S2_188872925 | G/A | 2 | G | G | G | G | G | G | A | G | G | G | A |
| 9 | S2_188872945 | C/T | 2 | C | C | C | C | C | C | C | C | C | C | C |
| | S2_205035169 | G/T | 2 | G | G | G | N | N | G | G | T | G | G | G |
| | S2_205035174 | G/A | 2 | G | G | G | N | N | G | G | G | A | G | A |
| | S2_205035222 | A/C | 2 | C | C | C | N | A | C | A | A | A | A | A |
| | S2_205035226 | T/C | 2 | T | C | C | N | T | T | T | T | T | T | T |
| 10 | S2_205035273 | G/A | 2 | G | G | G | N | G | G | G | G | G | G | G |
| 11 | S3_127577411 | A/G | 3 | G | A | A | A | A | G | N | A | A | A | A |
| | S3_217358320 | A/T | 3 | A | A | A | A | A | A | A | A | A | A | A |
| | S3_217358327 | G/A | 3 | G | G | G | G | G | G | G | G | G | G | G |
| | S3_217358330 | G/T | 3 | G | G | G | G | G | G | G | G | G | G | G |
| | S3_217358336 | G/C | 3 | G | G | G | G | G | G | G | G | G | G | G |
| 12 | S3_217358368 | A/T | 3 | T | A | A | A | T | T | A | A | A | A | A |
| | S3_217808740 | A/T | 3 | N | N | A | N | A | T | T | A | A | A | A |
| | S3_217808747 | T/C | 3 | N | T | T | N | T | C | C | T | T | T | T |
| | S3_217808798 | C/A | 3 | N | A | C | N | C | A | A | C | A | C | C |
| 13 | S3_217808846 | C/T | 3 | N | N | C | C | C | T | T | C | T | C | C |
| | S3_217820554 | A/G | 3 | A | A | A | A | A | A | A | A | G | A | A |
| | S3_217820587 | G/A | 3 | G | G | G | G | A | A | A | G | G | G | G |
| | S3_217820604 | C/T | 3 | C | C | C | C | T | T | T | C | C | C | C |
| 14 | S3_217820634 | G/A | 3 | N | N | G | N | A | A | A | G | A | N | G |

Table 4.1 (Continued)

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | S3_217820636 | G/A | 3 | N | N | G | N | A | A | A | G | A | N | G |
| | S3_217820637 | C/A | 3 | N | N | C | N | A | A | A | C | N | N | C |
| | S3_217820646 | C/G | 3 | C | C | G | C | C | C | C | C | C | G | G |
| | S3_217820647 | G/A | 3 | G | G | A | G | G | G | G | G | G | A | A |
| | S3_217820650 | G/C | 3 | G | G | C | G | G | G | G | G | G | C | C |
| | S4_26653745 | C/T | 4 | C | C | C | C | C | C | T | T | C | C | C |
| | S4_26653796 | C/G | 4 | G | G | C | G | N | G | N | C | C | C | C |
| | S4_26653812 | C/T | 4 | C | C | T | C | C | C | N | C | C | C | C |
| 15 | S4_26653817 | A/C | 4 | A | A | C | A | A | A | N | A | A | A | A |
| | S5_20311258 | A/T | 5 | T | A | N | T | N | A | A | T | N | N | N |
| 16 | S5_20311281 | A/T | 5 | A | A | N | A | N | T | T | A | N | N | A |
| | S5_206795038 | T/C | 5 | T | T | T | T | T | T | T | T | T | T | T |
| | S5_206795116 | G/T | 5 | T | N | T | G | G | N | N | G | G | G | G |
| | S5_206795119 | C/G | 5 | G | N | G | C | C | N | N | C | C | C | C |
| 17 | S5_206795160 | T/A | 5 | A | N | A | T | T | N | N | T | T | T | T |
| | S6_74967436 | G/A | 6 | G | G | G | G | G | G | G | G | G | G | G |
| | S6_74967447 | G/C | 6 | C | C | C | G | G | C | C | G | G | G | G |
| | S6_74967491 | G/A | 6 | A | N | A | G | G | A | N | G | G | G | G |
| | S6_74967512 | C/T | 6 | T | C | C | C | C | C | C | C | C | C | C |
| | S6_74967515 | C/T | 6 | C | N | T | C | C | T | N | C | C | C | C |
| | S6_74967516 | C/G | 6 | C | N | G | C | C | G | N | C | C | C | C |
| | S6_74967517 | C/G | 6 | C | N | G | C | C | G | N | C | C | C | C |
| 18 | S6_74967532 | C/T | 6 | C | N | T | C | C | T | N | C | C | C | C |
| | S6_121311660 | C/G | 6 | C | C | C | C | C | C | C | C | C | G | C |
| | S6_121311683 | G/A | 6 | G | G | G | G | G | G | G | G | G | A | G |
| | S6_121311700 | C/T | 6 | C | C | C | C | C | T | C | T | T | C | C |
| | S6_121311711 | C/T | 6 | N | N | T | T | C | C | T | C | C | C | C |
| 19 | S6_121311723 | A/G | 6 | A | A | A | A | A | A | A | A | A | G | A |

Table 4.1 (Continued)

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | S7_155754021 | G/A | 7 | N | A | A | A | N | N | A | N | N | N | G |
| 20 | S7_155754047 | C/G | 7 | N | C | C | C | N | N | C | N | N | N | C |
| | S7_155752542 | G/A | 7 | N | G | N | G | G | G | G | G | G | N | G |
| | S7_155752569 | A/C | 7 | N | A | N | A | A | A | A | C | N | N | A |
| | S7_155752575 | C/T | 7 | N | T | N | T | C | C | C | C | C | N | C |
| 21 | S7_155752599 | G/A | 7 | N | A | N | A | G | G | G | G | G | N | G |
| | S8_94752214 | A/T | 8 | N | A | N | A | N | N | A | A | A | T | A |
| | S8_94752242 | T/C | 8 | C | T | C | T | C | C | T | T | T | T | T |
| | S8_94752243 | G/T | 8 | T | G | T | G | T | T | G | G | G | G | G |
| | S8_94752244 | A/T | 8 | N | A | N | A | N | N | A | A | A | A | A |
| | S8_94752246 | T/C | 8 | C | T | C | T | C | C | T | T | T | T | T |
| | S8_94752247 | A/C | 8 | C | A | C | A | C | C | A | A | A | A | A |
| | S8_94752248 | C/A | 8 | N | C | N | C | N | N | C | C | C | C | C |
| | S8_94752249 | T/A | 8 | A | T | A | T | A | A | T | T | T | T | T |
| | S8_94752251 | C/T | 8 | T | C | T | C | T | T | C | C | C | C | C |
| | S8_94752253 | T/C | 8 | C | T | C | T | C | C | T | T | T | T | T |
| | S8_94752254 | G/T | 8 | T | G | T | G | T | T | G | G | G | G | G |
| | S8_94752255 | G/T | 8 | T | G | T | G | T | T | G | G | G | G | G |
| | S8_94752257 | C/G | 8 | G | C | G | C | G | G | C | C | C | C | C |
| | S8_94752258 | G/A | 8 | A | G | A | G | A | A | G | G | G | G | G |
| 22 | S8_94752280 | T/C | 8 | C | T | C | T | C | T | T | T | T | T | T |
| 23 | S9_107333254 | A/G | 9 | A | G | G | A | N | G | G | A | A | A | A |
| | S9_117048726 | G/A | 9 | A | A | A | A | N | A | A | G | G | G | G |
| | S9_117048730 | T/C | 9 | T | T | T | T | N | T | T | T | T | T | T |
| 24 | S9_117048731 | C/T | 9 | T | T | T | T | N | T | T | C | C | C | C |
| | S10_95855746 | G/A | 10 | G | N | G | G | G | G | G | G | G | G | G |
| | S10_95855756 | T/G | 10 | T | N | T | T | T | T | T | T | T | T | T |
| 25 | S10_95855766 | C/T | 10 | T | N | C | C | T | T | T | C | C | C | C |

Table 4.1 (Continued)

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | S10_95855774 | G/A | 10 | G | N | G | G | G | G | G | G | G | G |
| | S10_95855780 | C/T | 10 | C | N | C | C | C | C | C | C | C | C |
| | S10_95855783 | G/A | 10 | G | N | G | G | G | G | G | G | G | G |
| | S10_95855839 | A/T | 10 | A | T | A | A | A | N | A | T | A | T | T |
| | S10_95855840 | T/C | 10 | T | N | T | T | T | N | T | C | T | C | C |

[a]Resistant lines, bSusceptible lines. There are between 1 to 15 SNPs linked to each of the SNPs within the region according to the GWAS study. SNP assays were designed for the SNPs in green.

**SNP Genotyping and genomic distribution.**

Leaf samples were collected from all 11 inbred lines that serve as the parents of the NILs. DNA was extracted from these parents to verify that each KASP assay genotyped the parents in the same way as did the original GBS data. Plants from the $BC_1$ generation were genotyped as the first generation segregating population for marker assisted selection. From all plants to be genotyped, leaf tissue samples were collected from individual plants, frozen to -80$^{o}$C, lyophilized and ground to a fine powder. DNA was extracted as described by Saghai - Maroof (1984) using the CTAB (cetyltrimethylammonium bromide) method. The DNA samples were genotyped with 25 SNPs via KASP as described below and allele calling was carried out using the klustal caller software for the OMEGA plate reader by BMG LABTECH GMBH, Orthenberg, Germany.

Before genotyping, the 25 SNPs were individually tested to ensure they mapped to the correct location in the maize genome using one of four previously constructed mapping populations. KASP assays work using a 94KDa recombinant thermostable DNA polymerase (KlearTaq). The amplification of the DNA at targeted loci using the KASP assay involves the use of two specially constructed mixtures, the SNP specific KASP assay mix and the KASP master mix. KASP assays enable bi-allelic scoring of SNPs at specified loci through competitive allele-specific PCR. The KASP assay mix contains 3 primers, two allele specific forward primers which each harbors a unique tail sequence connected to a universal FRET (fluorescence resonant energy transfer) sequence, and one common reverse primer. The KASP Master mix also contain two universal FRET cassettes labeled with FAM or HEX dye (one for each allele), which

fluoresce at different wavelengths thereby making the difference in the genotype call for different alleles when read by a fluorescent-based plate reader. The PCR conditions for the KASP assays designed in this study are presented in table 4.2 below.

Table 4.2 Thermal cycling conditions using KlearTaq

| Step | Temperature | Time | Number of cycles |
|---|---|---|---|
| 1 | 95ºC | 15 mins | 1 cycle |
| 2 | 95 ºC | 30 sec | 34 cycles |
| | 61 ºC | 30 sec | |
| | 72 ºC | 1 min/kb | |
| 3 | 72 ºC | 5 mins | 1 cycle |

The amplification pattern of two specific alleles (including the two homozygous classes and the heterozygous class) in the KASP assay for 96 individuals in a 96-well microtiter plate is shown in the klustal plot software in Figure 4.3 of the end-point fluorescent read after the amplification process. One of the two fluorescent signals is generated if the genotype of a given SNP is homozygous while both signal are generated if the genotype is heterozygous for the given SNP.

Table 4.3 List of SNPs associated with aflatoxin resistance levels (p < 10-4) in 7 environments and potentially causing the association.

| Region | Environment | SNPs | BIN | F | P | R2 | Proximity* | Gene |
|---|---|---|---|---|---|---|---|---|
| 1 | Star10LSM | S1_272220177 | | 23.12 | 2.78E-06 | 0.0994 | gene contains marker | GRMZM2G049349 |
| 2 | Star10LSM | S1_280635905 | 1.10 | 25.10 | 1.14E-06 | 0.1007 | gene contains linked SNP 55 KB away, LD > 0.8 | GRMZM2G009958 |
| 3 | AveLSM | S2_22947761 | 2.03 | 22.05 | 4.24E-06 | 0.0761 | gene contains marker | GRMZM2G003784 |
| 4 | Star10LSM | S2_153128978 | 2.06 | 20.98 | 8.53E-06 | 0.1105 | gene contains marker | GRMZM2G155437 |
| 5 | Star10LSM | S2_183190432 | 2.06 | 23.27 | 3.80E-06 | 0.1382 | gene is 5 KB from marker and lies in LD window = 0.5 | GRMZM2G037574 |
| 6 | Star09LSM | S2_188872774 | 2.07 | 21.35 | 6.08E-06 | 0.0754 | gene contains marker | GRMZM2G026065 |
| 7 | Star10LSM | S2_205035222 | 2.08 | 23.26 | 2.38E-06 | 0.0812 | gene contains marker | GRMZM2G166337 |
| 8 | Star10LSM | S3_127577411 | 3.05 | 21.89 | 4.85E-06 | 0.0915 | gene is 18 KB from marker but not in LD | GRMZM2G333619 |
| 9 | Star10LSM | S3_217358368 | 3.09 | 23.73 | 1.85E-06 | 0.0797 | gene contains marker | GRMZM2G089525 |
| 10 | Lubb09LSM | S3_217808747 | 3.09 | 27.33 | 3.83E-07 | 0.1121 | gene contains marker | GRMZM2G052991 |
| | AveLSM | S3_217808747 | | 16.71 | 6.00E-05 | 0.0689 | | |
| 11 | CSta09LSM | S3_217820604 | 3.09 | 15.67 | 9.66E-05 | 0.0541 | gene contains marker | GRMZM2G053047 |
| 12 | Star10LSM | S3_217832603 | 3.09 | 21.56 | 5.65E-06 | 0.0808 | gene contains marker | GRMZM2G053140 |
| 13 | AveLSM | S4_26406913 | 4.04 | 16.74 | 5.63E-05 | 0.0557 | gene contains marker | GRMZM2G003814 |
| 14 | StRa10LSM | S4_26653796 | 4.04 | 24.31 | 1.52E-06 | 0.0902 | gene contains marker | GRMZM2G111261 |
| 15 | Star10LSM | S5_20311281 | 5.03 | 23.04 | 3.04E-06 | 0.1082 | gene contains marker | GRMZM2G545615 |
| 16 | AveLSM | S5_185650835 | 5.05 | 23.31 | 2.63E-06 | 0.1049 | gene is 5 KB from marker and in LD = 0.3 | GRMZM2G028736 |
| 17 | Star10LSM | S5_206795119 | 5.07 | 22.94 | 3.58E-06 | 0.1313 | gene contains marker | GRMZM2G105874 |
| 18 | Star10LSM | S6_74967491 | 6.01 | 23.12 | 2.73E-06 | 0.0947 | gene contains marker | GRMZM2G005499 |

Table 4.3 (Continued)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 19 | Star10LSM | S6_121311711 | 6.05 | 20.68 | 8.26E-06 | 0.0747 | gene contains marker | GRMZM5G841142 |
| 20 | Star10LSM | S7_155754021 | 7.03 | 21.89 | 7.09E-06 | 0.1548 | gene is 8.6 KB from marker and lies in LD window > 0.5 | GRMZM2G444075 |
| 21 | Star10LSM | S8_94752247 | 8.02 | 25.30 | 8.98E-07 | 0.0890 | gene contains marker | GRMZM2G147221 |
| 22 | AveLSM | S9_107333254 | 9.04 | 22.52 | 3.47E-06 | 0.0845 | gene is 1KB from marker but no nearby LD info | GRMZM2G331766 |
| 23 | AveLSM | S9_117048726 | 9.04 | 43.12 | 2.87E-10 | 0.1620 | gene contains marker | GRMZM2G108619 |
| 24 | Star09LSM | S10_95855766 | 10.04 | 23.80 | 2.04E-06 | 0.0950 | gene contains linked SNP 38 KB away, LD > 0.3 | GRMZM2G407650 |
| 25 | Star09LSM | S10_109718061 | 10.04 | 20.60 | 8.86E-06 | 0.0794 | gene contains marker | GRMZM2G159675 |
| 26 | AveLSM | S10_125923329 | 10.04 | 24.44 | 1.34E-06 | 0.0839 | gene contains marker | GRMZM2G058573 |
| 27 | Star10LSM | S10_139081513 | 10.06 | 24.04 | 1.71E-06 | 0.0870 | gene contains marker | GRMZM2G148467 |

NOTE: Linkage Disequilibrium LD was measured by the $R^2$ value. Bin location of all SNPs and association statistics such as the p value and the $R^2$ value is shown. Gene name corresponds to the MaizeGDB database and the SNP locations are given in reference to V2 of the maize reference sequence.

**Proposed Theory**

Testing phenotypic effect of the alleles of each SNPs (effect on the trait under study) and the inheritance of each SNP will be done by observing the differences in aflatoxin accumulation resistance averaged over individuals that inherited the desired SNPs from the parents and those that did not due to Mendelian inheritance. The implementation of this proposed theory will start at the $BC_3S_2$ stage of this project when the NILs would have been created and the testing of the phenotypic effect of each SNP will be underway. The resistant allele of each SNPs will have been completely backcrossed and fixed in the heterozygous state in a susceptible background after the series of marker assisted backcrossing to the $BC_3$ stage followed by selfing to get the $BC_3S_1$ and $BC_3S_2$ generation. The phenotypic effect of each SNP for the fixed NIL carrying it will be calculated as explained by Kaeppler (1997), using the formula for the calculation of the linear model as follows:

$$y_{jk} = \mu + \gamma_j + e_{jk}$$

where $y_{jk}$ represents the phenotypic value of the *k*th replication of the *j*th line, $\mu$ represents the mean of the two lines, $\gamma_j$ represents the effect of the *j*th line and $e_{jk}$ is the residual error where k =1,2,...n = number of replications; (Kaeppler, 1997). To test the null hypothesis of equality of the means of the created NIL pairs, analysis of variance (ANOVA) will be employed and the difference between the averages of all pairs of lines that are tested in the study will be used to determine if a QTL is present in the individuals tested or not.

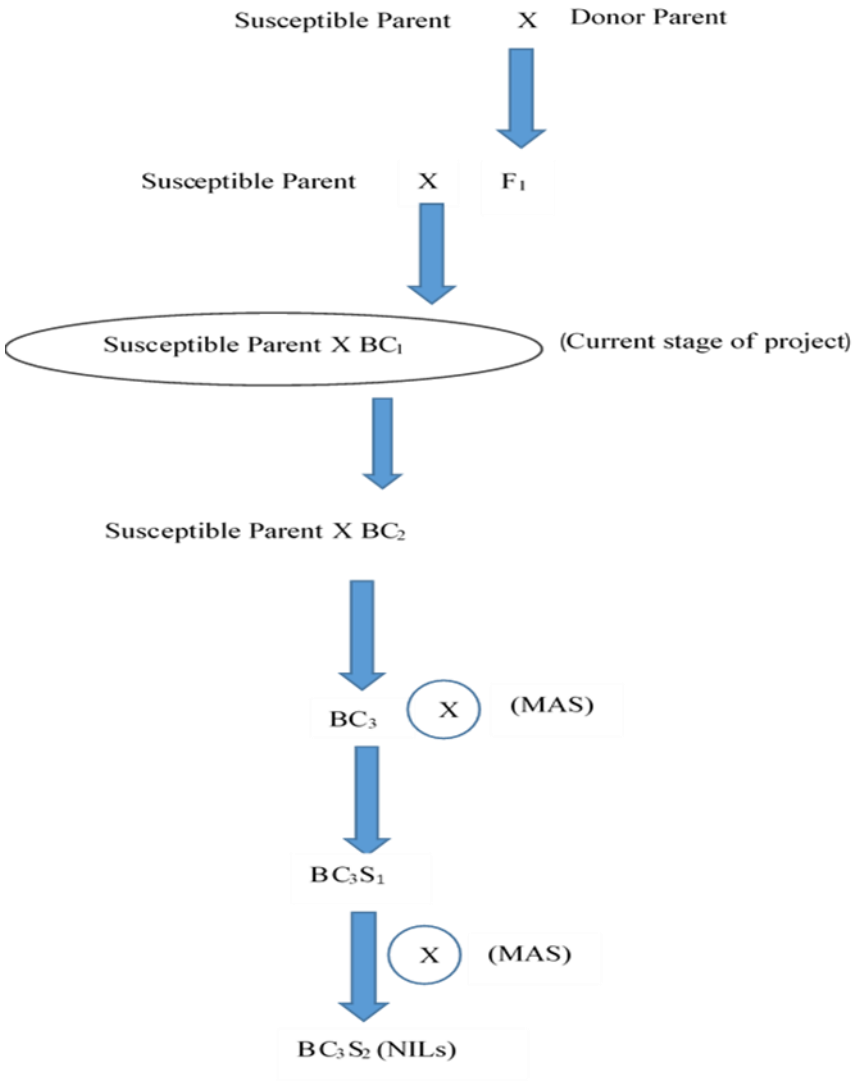


Figure 4.2 A scheme showing how plant materials was created.

**Results and discussion to date**

The creation of NILs requires at least five to six generations of maize backcrosses and subsequent selfing, but it is very important to determine which of the segregating individuals in each family carry the desired SNP or QTL allele from all the progeny derived each year of backcrossing. To date, 15 KASP assays have been designed and

tested, but only 6 of the 25 desired SNPs have been successfully converted to working assays and are polymorphic in the parents used for the creation of the NILs. Testing one of the SNP assays on all the individual progeny in the BC1 generation of all crosses shows that approximately 40% (8 out of 20 individuals per family on average) inherited the allele of interest from their parents. This is a bit lower than the 50% expected, but within the range of probability. More assays are being designed and will be ready for the next step in the project, being carried out now.

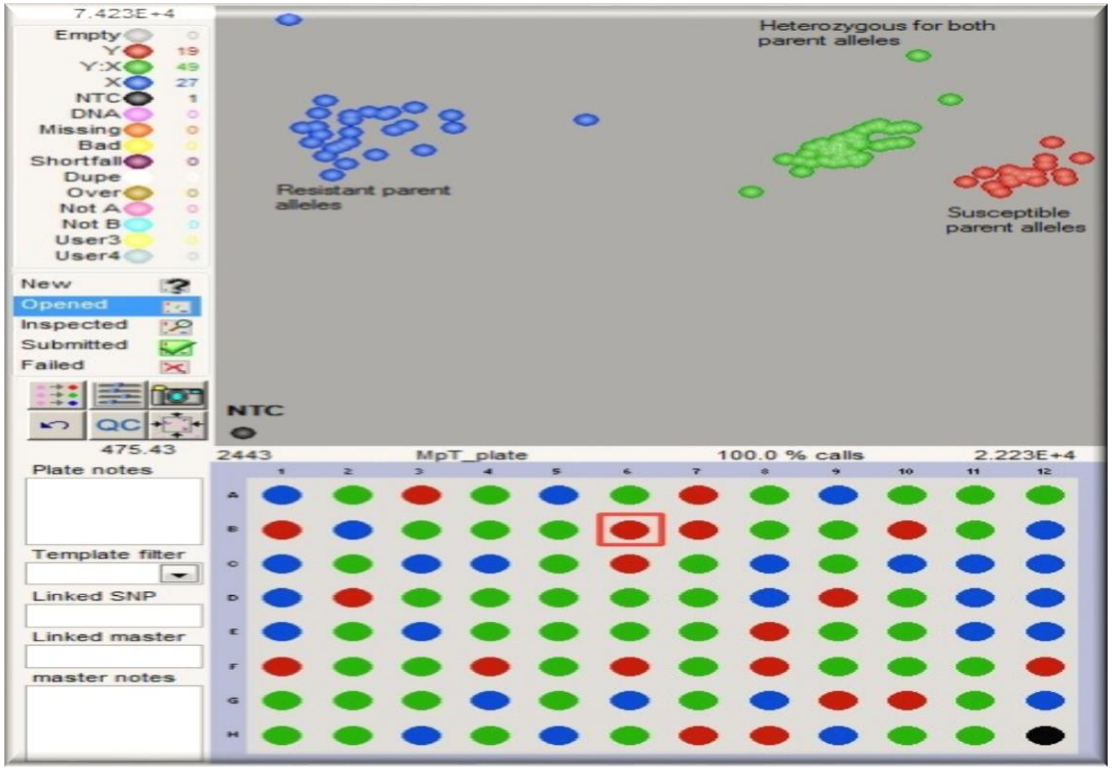


Figure 4.3 A typical genotyping clustal plot showing the genotypes of 95 individuals (including the parents) and one negative template controls (NTC).

NOTE: Genotypes homozygous for the resistant allele reported by the FAM dye in blue, genotypes homozygous for the susceptible allele reported by the HEX dye in red, and heterozygous genotypes contains both resistant and susceptible alleles in green.

The 6 SNPs that have been successfully converted to working assays were tested on the QTL mapping population which they expected to be polymorphic according to the hapmap data. Since the resistant parents used in the creation of the QTL mapping population (Mp313E and Mp715) are present as parents of the NILs contained but in a different susceptible background, it will give an idea of the heritability of the SNPs. The results shows that out 184 individuals in the mapping population, 50 of the individuals were homozygous for the resistant's parent alleles, 35 individuals are homozygous for the susceptible parents allele while the remaining 99 individuals were heterozygous for both alleles from both parents (Figure 4.3). Only one plate with 92 individuals is shown.

**Considerations for the use of NILs**

At the $BC_3$ generation it is expected that the genome of all the individual NILs will carry ~97% of the recurrent parents and ~3% of the donor parent, including the desired alleles for the favorable SNPs (Figure 4.1). Each NIL is designed to examine a specific SNP or chromosomal region and the effect it has on aflatoxin accumulation resistance. NILs are also very important as they can be used in physically observing the effect of the introgressed SNP or region, although not all traits can be physically differentiated visually when grown side by side with the susceptible (recurrent) parent. If aflatoxin levels are not different between the NIL pairs, tests to determine the fungal biomass via qPCR may show a difference in this case NILs are also a very useful tool in studying the interactions of two or more SNPs/chromosome regions in the same background and this will provide insight on the epistatic interactions between the SNP/loci of interest (Kaeppler; 1997). Initial results by Williams et al (unpublished) provide an indication of these interactions, where the phenotypic effect of 2 or 3 QTLs

providing resistance from Mp313E, a resistant maize inbred line, was measured in a susceptible background (Va35) and the effect of two QTL together was, in some cases, to make the plant more resistant to aflatoxin accumulation than expected based on the phenotypic effect of single QTL (Table 4.4). Furthermore, the created NILs can be very useful for studying high resolution mapping if smaller chromosomal regions are introgressed than were mapped in the original QTL mapping populations (since multiple generations of meiosis can provide more recombination and thus a smaller chromosomal region being tested).

Table 4.4 Validation of QTLs in NILs

| QTLs | Phen. Effect | ppb aflatoxin 2012 | ppb aflatoxin 2014 |
|---|---|---|---|
| 2.05 | 15% | 289 | 231 |
| 3.05 | 5% | 538 | 401 |
| 4.06 | 10% | 303 | 278 |
| 4.09 | 14% | 157 | 368 |
| 3.05, 4.06 | 5%+10% | - | 242 |
| 2.05, 4.09 | 15%+14% | - | 258 |
| 2.05, 3.05 | 15%+5% | - | 36 |
| 3.05, 4.09 | 5%+14% | - | 28 |
| 4.06, 4.09 | 10%+14% | - | 10 |
| 2.05, 3.05, 4.09 | 15% + 5% + 14% | - | 82 |
| 2.05, 3.05, 4.06 | 15% + 5% + 10% | - | 12 |
| None | - | 690 | - |
| Va35 | - | 748 | 411 |
| Mp313E | - | 26 | 1 |

Table adapted from Williams et al; (unpublished).

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# CHAPTER V

# CONCLUSION

Aflatoxin produced by *A. flavus* poses a serious threat to public health and causes high economic losses for farmers of cotton, maize, and some other crops. This study is one of the ongoing effort to make maize more resistant to *A. flavus* and aflatoxin accumulation. Various government and non-government organization and also the USDA-ARS Corn Host Plant Resistance Research Unit (CHPRRU) are also working on how to make maize more resistant to *A. flavus* and aflatoxin accumulation. The overall objectives of this study are

1. To identify all the ZmLOXs and report their sequence diversity and expression patterns.
2. To map their effects on aflatoxin accumulation resistance via linkage (QTL mapping) and association mapping.
3. To create Near Isogenic Lines via Marker Assisted Selection, with the ultimate goal of validating SNPs identified in a previous GWAS study for association accumulation resistance.

Lipoxygenase enzymes are known to catalyze the addition of molecular oxygen to poly-unsaturated fatty acid which are subsequently used in a series of pathway producing the jasmonic acid and methyl jasmonate, two compounds that are known to contribute to plant resistance to pest and pathogens. Lipoxygenase gene family in maize were

identified and their influence on *A. flavus* and aflatoxin accumulation was determined in this study using the aflatoxin association mapping and the QTL mapping methods. Both methods show that three of the ZmLOXs have measurable effect on aflatoxin accumulation.

Creation of Near isogenic lines (NILs) is very important for detailed studying the effect of a gene or a QTL on aflatoxin accumulation resistance. It also helps to separate the effect of the QTL from other genetic effect. NILs are different in just the loci of interest and thus helping to differentiate the effect of that loci from other loci. This project is still an ongoing project that I will continue to work on for my PhD in the nearest future.