Characterization Of Two Genes For Resistance To Aflatoxin Accumulation In Maize (Zea Mays L.)

J Erik Mylroie

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CHARACTERIZATION OF TWO GENES FOR RESISTANCE TO AFLATOXIN ACCUMULATION IN MAIZE (Zea mays L.)

By

John Erik Mylroie

A Dissertation
Submitted to the Faculty of Mississippi State University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Molecular Biology in the Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology

Mississippi State, Mississippi

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CHARACTERIZATION OF TWO GENES FOR RESISTANCE TO AFLATOXIN ACCUMULATION IN MAIZE (Zea mays L.)

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Maize (Zea mays L.) is one of the world’s largest food crops and thus any pathogens of maize are of great importance. Aspergillus flavus is one of these pathogens and it produces a carcinogenic metabolite called aflatoxin. Efforts to reduce infection by A. flavus and subsequent aflatoxin accumulation include the development of maize lines resistant to aflatoxin accumulation. However, resistant lines that have been developed contain agronomically unfavorable traits. Gene-based markers would allow for easier transfer of resistance from resistant inbred lines into maize lines with good agronomic traits.

The focus of this research was the development of gene-based markers for resistance to aflatoxin accumulation. To this end, two genes were characterized for their association with reduced aflatoxin accumulation in maize. A gene coding for a photosystem II3 protein shown to be differentially regulated between maize lines Mp313E (resistant) and Va35 (susceptible) was used to develop the marker MpM1. This marker was shown to be associated with resistance to aflatoxin accumulation in three F$_{2:3}$
mapping populations derived from Mp313E x B73, Mp313E x Va35, and Mp715 x T173 and identified a new quantitative trait locus (QTL) on chromosome 4.

The second gene chosen was the chitinase A gene \((chiA)\), which has been shown to inhibit fungal growth and is differentially regulated between resistant and susceptible lines of maize. \(ChiA\) also had an association with reduced aflatoxin accumulation in the three \(F_{2:3}\) mapping populations and identified a new QTL in the Mp313E x Va35 population. Together, MpM1 and \(chiA\) were associated with 27\% of the phenotypic variation in one environment of the Mp313E x B73 population. These markers represent the first two gene-based markers developed for resistance to aflatoxin accumulation, and the methodology developed in this study can be used to screen other candidate genes for potential use as gene-based makers.

Key words: maize, aflatoxin, molecular markers, \textit{Aspergillus flavus}
DEDICATION

I would like to dedicate this dissertation to my wife Robika Mylroie, my parents John and Joan Mylroie, my in-laws Ravi and Melalie Modak, and my brothers Leif and Lars Mylroie. Thank you for all of your support and always believing in me.
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my major professor and mentor Dr. Paul Williams. Without his support this dissertation would not have been possible. He has been a constant source of encouragement, guidance, and knowledge throughout my entire Ph.D. process. I would also, like to thank the rest of my committee, Dr. Marilyn Warburton, Dr. Din-Pow Ma, Dr. Ashli Brown, and Dr. Brain Baldwin for their help and guidance throughout the entire Ph.D. process. If it takes a town to raise a child, then it definitely takes a committee to graduate a student. I would like to especially thank Dr. Warburton for her assistance helping me understand the concepts and applications of mapping and QTL analysis. Also, I would like to thank Dr. Scott Willard for always answering my questions and being an excellent department head.

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TABLE OF CONTENTS

DEDICATION .................................................................................................................... ii

ACKNOWLEDGEMENTS ............................................................................................... iii

LIST OF TABLES ............................................................................................................ vii

LIST OF FIGURES ......................................................................................................... viii

CHAPTER

I. INTRODUCTION ...................................................................................................1
  Literature Cited ........................................................................................................6

II. LITERATURE REVIEW ......................................................................................10

  Aspergillus flavus and Aflatoxin ...........................................................................10
  Aspergillus and Aspergillus flavus ....................................................................10
  Aflatoxin ..............................................................................................................12
  Aflatoxin and human and animal health ..........................................................15
  Maize ....................................................................................................................18
  Aspergillus flavus, aflatoxin, and maize ..........................................................20
  Post-harvest and Pre-harvest prevention of A. flavus and aflatoxin
    contamination in maize ....................................................................................23
  Harvest and Post-harvest methods ..................................................................23
  Pre-harvest control methods other than host plant resistance .....................25
  Host plant resistance .........................................................................................27
  Marker Assisted Selection (MAS) ....................................................................29
  Quantitative Trait Loci (QTL) .............................................................................30
  Gene-based markers ..........................................................................................36
    Candidate gene identification .........................................................................36
    Identification of polymorphisms in candidate genes ..................................41
  Literature Cited .....................................................................................................44

III. DEVELOPMENT OF A GENE - BASED MARKER CORRELATED TO
    REDUCED AFLATOXIN ACCUMULATION IN MAIZE ..............................63
IV. USE AND VALIDATION OF CHITINASE A MAKER ASSITED SELECTION OF RESISTANCE TO AFLATOXIN ACUMULATION IN MAIZE

Abstract..................................................................................................................88
Introduction............................................................................................................89
Methods ..................................................................................................................92
   DNA Isolation ......................................................................................................92
   Primer Design and Verification .........................................................................93
   PCR Purification and Sequencing ......................................................................94
   Design of Genotyping Primers .........................................................................95
   Population Screening and Genotyping .............................................................99
   Mapping and QTL Analysis .............................................................................100
Results....................................................................................................................101
   Primer Verification, Sequencing, and Alignment Results ................................101
   Genotyping of F2:3 Mapping Populations .........................................................106
   QTL Mapping and Analysis ...........................................................................106
Discussion..............................................................................................................111
Literature Cited ....................................................................................................116

V. SUMMARY ........................................................................................................120

Literature Cited ....................................................................................................128

APPENDIX

A MULTIPLE ALIGNMENTS FOR CANDIDATE GENES NOT USED FOR GENE-BASED MARKER DEVELOPMENT ........130
# LIST OF TABLES

2.1. Secondary Metabolites Produced by Species in *Aspergillus* section *Flavi* ...........................................................................................................................................13

2.2 U.S. Food and Drug Administration action levels for total aflatoxins in food and feed.............................................................................................................................................19

3.1 Primers used for PCR and sequencing.........................................................................................69

3.2 Sequences of probe and primers used for genotyping F$_{2:3}$ populations........70

3.3 Least significant difference (LSD) analysis of aflatoxin accumulation of each class of lines grouped by genotype in Mp313E x B73 F$_{2:3}$ population from Mississippi State, MS field location in 2000...........................................................................................................................................78

3.4 QTL analysis of phenotypic effects of MpM1................................................................................80

4.1 Primers for sequencing and genotyping of *chiA* ........................................................................96

4.2 QTL analysis of phenotypic effects of *chiA* in the Mp313E x B73 and Mp313E x Va35 mapping populations and in multiple environments..............................................................................................................................................109

5.1 Least significant difference (LSD) analysis of aflatoxin accumulation of each class of lines grouped by genotype in Mp313E x B73 F$_{2:3}$ population for the MpM1 and *chiA* markers ..................................................126
LIST OF FIGURES

2.1. The structures of the major aflatoxins .............................................................14
2.2 Clustered genes of aflatoxin biosynthetic pathway ........................................16
2.3 Ranges and medians of total aflatoxin limits in food for different regions between 1995 and 2003..............................................................18
2.4 *Aspergillus flavus* life cycle ..........................................................22
3.1 Gene structure of chloroplast precursor (AW424439) and location of primers and probes used for genotyping..............................................70
3.2 Multiple alignment of AW424439 gene ................................................75
3.3 Melting peaks obtained from the parents and F1 of Mp313E x B73 population of F2:3 families................................................................76
3.4 Parents and F1 from the Mp715 x T173 population of F2:3 families as visualized by polyacrylamide gel electrophoresis and ethidium bromide using the AW424489SHRT primer set ..............................77
3.5 Chromosomal location of MpM1 and QTL in Mp313E x B73 population ..............................................................79
4.1 Alignment of Mp313E and B73 *chiA* gene segment amplified by ChitinaseA primer pair.................................................................97
4.2 Alignment of Mp313E and Va35 *chiA* gene segment amplified by ChitinaseA primer pair.................................................................98
4.3 PCR products of the genomic DNA from Mp313E, Mp715, B73, and Va35 amplified with ChitinaseA primer pair.................................102
4.4 Multiple alignment of the *chiA* gene segment ........................................103
4.5 Alignment of *chiA* gene segment with B73 reference genome..............104
4.6 PCR products using the ChiAMP3VA35 primer pair.................................105
4.7 PAGE showing the PCR products of the Mp715 x T173 mapping population screened using the ChiAMP3Va35 primer pair ..............107
4.8 QTL Cartographer output showing the chiA QTL on chromosome 2 .......108
4.9 Chromosomal locations of chiA QTL in Mp313E x B73 and Mp313E x Va35 populations .............................................................................110
4.10 Multiple alignment of chiA segments from maize lines LH82, Mp313E, Mp715, and B73 .............................................................................115
A.1 Multiple alignment of AW216267 .........................................................131
A.2 Multiple alignment of AW244196 .........................................................132
A.3 Multiple alignment of AW400128 .........................................................133
A.4 Multiple alignment of AW438153 .........................................................134
A.5 Multiple alignment of BE128894 ................................................................135
A.6 Multiple alignment of AW179553 .........................................................136
A.7 Multiple alignment of AW360365 .........................................................136
CHAPTER I
INTRODUCTION

*Aspergillus flavus* is a fungal pathogen of maize (*Zea mays* L.) and other crops such as peanut (*Arachis hypogaea* L.), cotton (*Gossypium hirsutum*), pistachio (*Pistacia vera* L.), and almond (*Prunus dulcis*). *A. flavus* and other members of the *Aspergillus* genus produce a secondary metabolite called aflatoxin which is carcinogenic, teratogenic, and mutagenic (Castegnaro and McGregor 1998; Scheidegger and Payne 2005). The health risks associated with aflatoxin contamination of corn grain has led to the establishment of action limits for acceptable aflatoxin contamination levels. In the United States, the action limit established by the United States Food and Drug Administration for total aflatoxins in food stuffs destined for human consumption is 20 parts per billion (ppb) (FDA 2011). This restriction results in economic losses for maize producers totaling hundreds of millions of dollars each year (Robens and Cardwell 2005; AMCOE 2010).

The economic and health concerns related to aflatoxin accumulation have led to research in methods to mitigate aflatoxin contamination in maize and other grains. These methods include pre-harvest measures like biocontrol or cultural practices and post-harvest measures such as proper grain storage or decontamination of maize grain (King and Prudente 2005; Abbas et al. 2009). Of the proposed methods to reduce aflatoxin
accumulation in maize, host plant resistance is one of the most promising long term solutions (Gorman and Kang 1991; Campbell and White 1995a; Williams et al. 2008). Through traditional breeding and screening methods several maize lines resistant to aflatoxin accumulation have been developed including Mp313E, Mp715, Mp717, Tex6, and the GT-MAS:gk population (Scott and Zummo 1990; McMillian et al. 1993; Campbell and White 1995b; Williams and Windham 2001, 2006; Clements and White 2005). However, these lines lack the agronomic performance of commercial cultivars, and the highly quantitative nature of resistance has hampered efforts to transfer resistance into commercial lines (Campbell and White 1995a; Hamblin and White 2000; Bertrán et al. 2002; Warburton et al. 2011).

The sequence of genes directly involved in resistance to aflatoxin accumulation can be used to develop gene-based markers that would be an important tool in moving resistance from developed lines into elite breeding lines. Candidate genes can be identified from Quantitative Trait Loci (QTL) mapping studies, genomics studies, proteomics studies, or from information about plant defense factors. The most progress in identifying regions of the maize chromosome associated with resistance to aflatoxin accumulation has been made through QTL studies focusing on resistance to aflatoxin accumulation and/or *A. flavus* infection (Davis et al. 1999; Willcox et al. 2000; Paul et al. 2003; Widstrom et al. 2003; Brooks et al. 2005; Robertson-Hoyt et al. 2007; Alwala 2008; Warburton et al. 2009, 2010). However, no mapped loci account for more than 20% of the phenotypic variation in one environment has been identified, and the physical size of most QTL hinders moving the entire QTL into susceptible lines effectively (Willcox et al. 2000; Paul et al. 2003; Brooks et al. 2005; Robertson-Hoyt et al. 2007;
Warburton2009, 2010). Despite drawbacks, QTL have been very important in identifying areas of the maize genome associated with resistance to aflatoxin accumulation and give researchers a narrower region to screen for genes associated with resistance to aflatoxin accumulation.

Genomics and proteomics studies give researchers a list of genes or proteins that are differentially regulated between different maize lines or different maize lines under different treatments. Resistant and susceptible maize lines infected with *A. flavus* make ideal candidates for these studies, and the gene/proteins shown to be differentially regulated make excellent candidate genes for gene-based markers. Genomics studies such as microarray studies have been used to investigate differences in gene expression between resistant and susceptible maize lines (Lou 2008; Kelley et al. 2009; Lou 2010). However, only Kelley et al. (2009) looked specifically at differential maize response when challenged with *A. flavus*. This microarray study focused on genes that were differentially expressed between Mp313E (resistant) and Va35 (susceptible) four days after infection with *A. flavus*. A total of 234 genes were identified as differentially regulated between the two lines, and give a good starting point for a list of candidate genes for gene-based marker development.

Proteomics studies have investigated proteins differentially regulated between resistant and susceptible lines and differences when lines were challenged with *A. flavus* or aflatoxin (Brown et al. 2003; Peethamberan et al. 2009; Brown et al. 2010; Pechanova 2011). Other studies have identified specific proteins associated with resistance to *A. flavus* infection and/or aflatoxin accumulation including β-1,3-glucanases, the chitinase family of proteins, trypsin inhibitors, and catalase proteins (Brown et al. 1993,1995; Wu
et al. 1994; Huang et al. 1997; Russin et al. 1997; Chen et al. 1998, 2006; Gembeh et al. 2001; Wilson et al. 2001; Moore et al. 2004; Magbanua 2007). The chitinase family of proteins is shown to be important in many of these studies and is an example of a protein (or protein family) that would be useful as a gene-based marker and warrants further investigation (Wu et al. 1994; Moore et al. 2004; Peethambaran et al. 2009; Pechanova 2011).

Despite QTL studies, genomics, and proteomics work, there has been little progress in developing gene-based markers for resistance to aflatoxin accumulation. Development of gene-based markers associated with resistance to aflatoxin accumulation would greatly benefit efforts to develop maize lines with resistance to aflatoxin accumulation and desired agronomic qualities. Even markers in genes that are tested but turn out to be not associated with resistance to aflatoxin accumulation will be useful for increasing marker densities in maize mapping populations especially since such genes are frequently chose from inside important QTL regions; thus they may still help to fine map already identified QTL.

To help identify and test gene-based markers for \textit{A. flavus} and aflatoxin accumulation resistance, information from QTL studies, “omics” studies, and other investigations into resistance factors were combined with modern molecular and genetics techniques to develop and test gene-based markers for resistance to aflatoxin accumulation. The main objectives of this research study were:

I. Develop a methodology for screening candidate genes for their usefulness as gene-based markers.
II. Use candidate genes from a microarray study of Mp313E (resistant) vs. Va35 (susceptible) infected with *A. flavus* (Kelley et al. 2009) and previous studies involving the chitinase A gene (Moore et al. 2004; Peethambaran et al. 2009) to develop gene-based markers from the DNA sequence of these genes.

III. Test the association of some of these markers with aflatoxin accumulation resistance in a pilot study to demonstrate successful use of the screening methodology developed in Objective I.
Literature Cited


Russin JS, Guo BZ, Tubajika KM, Brown RL, Cleveland TE, Widstrom NW (1997) Comparison of kernel wax from corn genotypes resistant or susceptible to Aspergillus flavus. Phytopathology 87: 529-533


CHAPTER II
LITERATURE REVIEW

Aspergillus flavus and aflatoxin

Aspergillus and Aspergillus flavus

The genus Aspergillus is composed of approximately 250 species of fungi classified under the order Ascomycota (Scheidegger and Payne 2005; Geiser et al. 2007). The genus Aspergillus has been classified into 8 subgenera and 22 sections with around one third of the members of the genus producing telomorphs (Scheidegger and Payne 2005; Peterson et al. 2008; Geiser 2009). Aspergillus was traditionally classified using morphological, cultural, and biochemical characteristics (Scheidegger and Payne 2005; Peterson et al. 2008). However, recent advances have led to the use of molecular biology techniques such as restriction length polymorphisms, DNA sequencing, and DNA melting curve analysis for the further classification of Aspergillus subgenera, sections, and subspecies (Scheidegger and Payne 2005; Geiser et al. 2007; Peterson et al. 2008; Wu 2009a).

Aspergillus flavus has been recently included in subgenus Circumdati while traditionally it was classified in the subgenus Aspergillus; however, in both cases A. flavus is sorted into section Flavi (Scheidegger and Payne 2005; Peterson et al. 2008). A.
flavus, like many of the other Aspergilli, is a saprophytic fungus that spends most of its life-cycle in the soil, on detritus, or as a parasite on organisms such as maize (Zea mays L.) (Scheidegger and Payne 2005; Hedayati et al. 2007; Bennett 2010). The fungus is most often found in regions located between 16 to 35 degrees north or south latitude and optimally grows at a temperature of around 37°C, although the fungus has been shown to grow at temperatures as low as 12°C and as high as 48°C (Klich 2002; Scheidegger and Payne 2005; Hedayati et al. 2007; Payne et al. 2008). A. flavus reproduces asexually through conidia which are produced from the conidiaphore. Additionally, the A. flavus mycelia can produce specialized structures called sclerotia which can survive long periods of time and harsh conditions and subsequently produce conidia and hyphae for further colonization (Wicklow and Donahue 1984; Wicklow et al. 1993; Hedayati et al. 2007). A. flavus was once thought to produce only by asexual means, but recent research has shown that A. flavus and its relatives may actually have sexual states that allow for the exchange of genetic information and account for the genetic diversity among A. flavus (Geiser 1996, 1998; Horn et al. 2009a, 2009b).

There is great diversity among Aspergillus genera, and there is great diversity within A. flavus itself (Scheidegger and Payne 2005). The sclerotia produced by A. flavus are used as a mode of classification for the fungal species. Strains that produce large sclerotia (> 400 µm in diameter) are classified as L strains, and those producing small sclerotia (< 400 µm in diameter) are classified as S strains (Cotty 1989; Scheidegger and Payne 2005). Geiser et al. (1998) placed A. flavus strains into reproductively isolated groups named I and II. Vegetative compatibility groups (VCG) have also been used to describe the genetic diversity among A. flavus strains. Fungi that share vegetative
compatibility can form heterkaryon (Bayman and Cotty 1993). Other methods such as restriction fragment length polymorphism (RFLP) analysis, simple sequence repeats (SSRs), and melting curve analysis have helped to identify and characterize the diversity found in *A. flavus* (Wu 2009a).

**Aflatoxin**

Aflatoxin is a secondary metabolite produced by *A. flavus* and other *Aspergillus* species including: *A. parasiticus*, *A. nomius*, *A. bombycis*, and *A. pseudotamariii*. Besides aflatoxin these and other *Aspergillus* section *Flavis* species produce other secondary metabolites (Table 2.1) (Kurtzman et al. 1987; Payne and Brown 1998; Ito et al. 2001; Peterson et al. 2001; Richard and Payne 2002; Scheidegger and Payne 2005).

There are four major forms of aflatoxin produced by fungi, B1, B2, G1, and G2, with names based on their fluorescence under ultraviolet light (B = blue and G = green) (Figure 2.1) (Richard and Payne 2002; Klich 2007). Aflatoxin M1 and M2 are not produced by fungi but have been found in milk from dairy animals that consumed aflatoxin contaminated grain (Stoloff 1980; Richard and Payne 2002; Scheidegger and Payne 2005). *A. flavus* produces predominantly the B1 and B2 forms of aflatoxin while *A. parasiticus* produces all four forms of aflatoxin (Moreno and Kang 1999; Scheidegger and Payne 2005; Chang 2010). Aflatoxin production by *A. flavus* occurs optimally between 28 to 30 °C, with other environmental and nutritional conditions such as pH, humidity, oxidative stress, carbon source, and nitrogen source being important factors (Schroeder and Hein 1967; Bhatnagar et al. 2002, 2006; Georgianna and Payne 2009).
Table 2.1 Secondary Metabolites Produced by Species in *Aspergillus* section *Flavi*

<table>
<thead>
<tr>
<th>Species</th>
<th>Aflatoxins</th>
<th>Other Secondary Metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus avenaceus</em></td>
<td>B, G</td>
<td>Avenaciolide</td>
</tr>
<tr>
<td><em>Aspergillus bombycis</em></td>
<td></td>
<td>Kojic acid</td>
</tr>
<tr>
<td><em>Aspergillus caelatus</em></td>
<td></td>
<td>Kojic acid, Aspergillic acid, cyclopiazonic acid,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>leporine, paspaline, pseurotin</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>B, G</td>
<td>Kojic acid, nominine, paspaline, paspalinine</td>
</tr>
<tr>
<td><em>Aspergillus lanosus</em></td>
<td></td>
<td>Griseofluvin, kojic acid, met I</td>
</tr>
<tr>
<td><em>Aspergillus leporis</em></td>
<td></td>
<td>Antibiotic Y, kojic acid, leporine, pseurotin</td>
</tr>
<tr>
<td><em>Aspergillus nominus</em></td>
<td>B, G</td>
<td>Aspergillic acid, kojic acid, nominine, pseurotin, tenuazonic acid</td>
</tr>
<tr>
<td><em>Aspergillus oryzae</em></td>
<td></td>
<td>Cyclopiazonic acid, kojic acid</td>
</tr>
<tr>
<td><em>Aspergillus parasiticus</em></td>
<td>B, G</td>
<td>Aspergillic acid, kojic acid, parasiticol, parasiticolide A</td>
</tr>
<tr>
<td><em>Aspergillus pseudotamarii</em></td>
<td>B</td>
<td>Cyclopiazonic acid, kojic acid</td>
</tr>
<tr>
<td><em>Aspergillus sojae</em></td>
<td></td>
<td>Kojic acid</td>
</tr>
<tr>
<td><em>Aspergillus tamarii</em></td>
<td></td>
<td>Cyclopiazonic acid, fumigaclavine A, kojic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Asperlicine, kojic acid, kotanins, met I, nominine, ochratoxin A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and B, paspaline</td>
</tr>
</tbody>
</table>

Reproduced from Scheidegger and Payne 2005.
Figure 2.1 The structures of the major aflatoxins.

Major aflatoxins are: AFB1, AFB2, AFM1, AFM2, AFG1, and AFG2. Adopted from http://www.food-info.net/uk/tox/afla.htm

The aflatoxin biosynthesis pathway is a polyketide pathway with acetate and malonyl used as precursors (Payne and Brown 1998; Yu et al. 2002; Yu et al. 2004; Bhatnagar et al. 2006). The pathway is composed of a 70 kb, 25 gene cluster located on A. flavus chromosome 3 (Yu et al. 2004; Bhatnagar et al. 2006; Georgianna and Payne 2009) (Figure 2.2). Transcriptional regulation of these genes is controlled by two key genes, aflR and aflS (Payne and Brown 1998; Yu et al. 2002; Bhatnagar et al. 2006; Price 2006; Georgianna and Payne 2009). Other proposed regulatory mechanisms include: antisense RNAs, G-protein signaling, Ras signaling, cAMP signaling, and chromosomal remodeling (Payne and Brown 1998; Bhatnagar 2006; Smith 2008; Georgianna and Payne 2009).
Aflatoxin and human and animal health

Aflatoxin is extremely toxic and carcinogenic with aflatoxin B1 being the most common and toxic of the four aflatoxins (McCann et al. 1975; Wild and Gong 2010). As a human and animal health concern, aflatoxin truly came into the public eye in the 1960’s with the outbreak of Turkey X disease in England and the identification of *A. flavus* in contaminated grain and aflatoxin as the causative agent (Blount 1961; Seargent et al. 1961; Wogan 1966). Subsequent cases of aflatoxin exposure have been documented in dogs (*Canis familiaris*), cattle (*Bos taurus*), and swine (*Sus scrofa*) (Richard and Payne 2002). In animals such as pigs, horses (*Equus caballus*), cows, poultry (Galliformes), and dogs, chronic exposure to aflatoxin can cause weight loss, hemorrhages, nasal discharge, discolored bodily waste, and death (Miller and Wilson 1994). Of further concern is the presence of the M1 form of aflatoxin in the milk of dairy animals fed contaminated food stocks (Stoloff 1979).

Of greater concern for the human population is the possibility of acute and chronic exposures to aflatoxin. There have been numerous documented cases of aflatoxin related illness and death in Asia and Africa, and these areas are still the regions with the greatest danger of outbreaks of exposure (Krishnamachari et al. 1975a, 1975b; Ngindu et al. 1982; Lewis et al. 2005; Shephard 2005). The epidemiology of aflatoxin shows that the main target organ is the liver. In the most severe cases of acute aflatoxicosis, the result is liver failure and death, whereas chronic low does exposure can lead to a myriad of other health problems including cancer (Williams et al. 2004; Wild and Gong 2010).
Figure 2.2 Clustered genes of aflatoxin biosynthetic pathway.

Adopted from Yu et al. 2004.
Research has shown a correlation between exposure to aflatoxin and incidences of liver cancer, specifically hepatocellular carcinoma (HCC) (Wogan 1992; Cullen and Newberne 1994; Chuang et al. 2009; Wild and Gong 2010). In fact, aflatoxin exposure combined with hepatitis B virus has been shown to increase the risk of HCC additively (Wu et al. 2009; Wild and Gong 2010). This may be due to the mutagenic nature of aflatoxin which can intercalate into DNA and form a AFB1-N7-guanidine adduct once the aflatoxin has been metabolized by the body into the AFB1-8,9-exo-epoxide (Essigmann et al. 1977; Loechler 1994; Wild and Gong 2010). The formed DNA adduct is most commonly responsible for a G to T mutation, which happens to be the same mutation commonly found in codon 249 of the p53 tumor suppressor gene from patients with HCC (Bailey et al. 1996; Smela et al. 2001; Chuang et al. 2009; Wild and Gong 2010). Besides HCC, aflatoxin can result in weight loss, stunted growth, immunosuppression, and hemolysis (Verma and Raval 1991; Roebuck and Maxuitenko 1994; Yu et al. 2002; Williams et al. 2004; Wild and Gong 2010).

Due to the human and animal health concerns related to aflatoxin exposure, the United States and other countries have imposed strict action levels for the amount of aflatoxin allowed in food stuffs with some countries even regulating for Aflatoxin B1 specifically (van Egmond and Jonker 2005) (Figure 2.3). In the United States, the Food and Drug Administration (FDA) has imposed an action level of 20 parts per billion (ppb) for all food products destined for human consumption (Table 2.2), but the European Union has an even more strict action level of 4 ppb (van Egmond and Jonker 2005; Klich 2007). These action levels are put in place to help prevent exposure to aflatoxin, but the
regulations also result in economic losses for farmers and producers with conservative estimates for direct and indirect costs totaling at least $500 million dollars annually (Robens and Cardwell 2005).

![Figure 2.3](image)

**Figure 2.3** Ranges and medians of total aflatoxin limits in food for different regions between 1995 and 2003.

Adopted from van Egmond and Jonker 2005

**Maize**

*Zea mays* L. (maize/corn) is a diclinous monoecious monocot from the family *Poaceae* with a genetic make up of $2n = 2x = 20$. Maize is an annual plant that grows rapidly and reaches an average height of 2.5 meters at maturity (USDA 2011). It is an open pollinated crop with wind being the main mode of pollination. Maize can grow in a wide range of environments and can be found and grown today from regions at 40°S latitude to 50°N latitude (Tenaillon and Charcosset 2011).
Table 2.2  U.S. Food and Drug Administration action levels for total aflatoxins in food and feed

<table>
<thead>
<tr>
<th>Commodity</th>
<th>Concentration (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All products, except milk, designated for humans</td>
<td>20</td>
</tr>
<tr>
<td>Brazil nuts</td>
<td>20</td>
</tr>
<tr>
<td>Peanuts and peanut products</td>
<td>20</td>
</tr>
<tr>
<td>Pistachios nuts</td>
<td>20</td>
</tr>
<tr>
<td>Corn for immature animals and dairy cattle</td>
<td>20</td>
</tr>
<tr>
<td>Corn and peanut products for breeding beef cattle, swine and mature poultry</td>
<td>100</td>
</tr>
<tr>
<td>Corn and peanut products for finishing swine of 100 pounds or greater</td>
<td>200</td>
</tr>
<tr>
<td>Corn and peanut products for finishing beef cattle</td>
<td>300</td>
</tr>
<tr>
<td>Cottonseed meal (as a feed ingredient)</td>
<td>300</td>
</tr>
<tr>
<td>All other feedstuffs</td>
<td>20</td>
</tr>
<tr>
<td>Milk</td>
<td>0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>U.S. Food and Drug Administration (FDA) Guidance for Industry: Action Levels for Poisonous or Deleterious Substances in Human Food and Animal Feed (http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/ChemicalContaminantsandPesticides/ucm077969.htm#afla).

<sup>b</sup>Aflatoxin M1

Table partially reproduced from Richard and Payne 2002

Maize domestication began in the Balsas region of Mexico approximately 9000 years ago (Piperno et al. 2009; Ranere et al. 2009; Tenaillon and Charcosset 2011). The recent research shows that maize as it is known today originated in this region from its ancestor *Zea mays* ssp. *parviglumis* which is a subspecies of teosinté (Matsuoka et al. 2002; Van Heerwaarden et al. 2011). Domesticated maize moved south into Guatemala and South America, and moved north through Mexico into regions which are now part of the southwestern United States, and to the rest of the continental United States (Matsuoka et al. 2002; Tenaillon and Charcosset 2011). It then moved to Europe and the rest of the world from the Americas after the arrival of Europeans to the new world (Tenaillon and Charcosset 2011).
Today, maize, rice (*Oryza sativa*) and wheat (*Triticum aestivum*) are the world’s main staple crops, and maize is the 2\(^{nd}\) largest worldwide in area harvested (ha) behind wheat and ahead of rice but 1\(^{st}\) in yield (Mg/ha) among grain crops (FAOSTAT 2009). In the United States, maize is the number one crop in terms of acres harvested with 81.4 million acres harvested in 2010, and it is also the number one crop in terms of select crop value at a total of $65.97 billion in 2010 (NCGA 2011). The United State is the world’s largest producer of maize followed by: China, the European Union, Brazil, and Mexico (NCGA 2011). Worldwide maize exports are also led by the United States followed by Argentina and Brazil (NCGA 2011). The main uses of maize in the United States are feed and residual (38.7\%), fuel/ethanol (36.5\%), export (14.5\%), and high fructose corn syrup (3.8\%) (NCGA 2011).

*Aspergillus flavus*, aflatoxin, and maize

In the United States and the world, maize is an important crop both nutritionally and economically. Therefore, any pathogen that adversely affects maize production, consumption, or sales is important from both human health and economic perspectives. One of these pathogens is *A. flavus* which can also infect: peanuts, cotton, and tree nuts, such as pistachios. *A. flavus* is an opportunistic pathogen that infects maize through the silks or through plant damage caused by insects (Smart 1990; Payne 1992, 1998; Miller 1995). Furthermore, plants under biotic stresses such as high temperature stresses and inadequate nutrition have been shown to be more susceptible to infection by the fungus (Miller 1995). *A. flavus* contaminates the maize ear when conidial spores land on the silks or damaged kernels via wind distribution or are carried by insects (the likely cause
of kernel damage) (Miller 1995; Payne 1998) (Figure 2.4). The main source of the *A. flavus* spores that infect maize plants comes from: fungal spores, sclerotia, or mycelia that have over wintered in the soil on debris such as old cobs, kernels, or other plant tissues (Wicklow and Donahue 1984; Zummo and Scott 1990; Wicklow et al. 1993; Payne 1998; Hedayati et al. 2007). *A. flavus* spores that land on the silks will grow down the silk channels, gain entry to the ear, and begin infection on the kernel surface (Marsh and Payne 1984). Once at the ear the fungus may gain entry to the kernels via the rachillae by growing through the aerenchyma to the floral axis and into the pericarp (Smart 1990). Scheidegger and Payne (2005) suggest that the fungus takes the path of least resistance to gain entry into the ear and kernels and thus insect damage would make a simple mode of entry for the fungus although it is not necessary for infection. From this primary infection the fungus can continue to grow through different kernels and the cob and eventually produce aflatoxin.

Aflatoxin contamination of maize is influenced by many environmental factors, including abiotic and biotic stresses (Gorman and Kang 1991; Payne 1998; Moreno and Kang 1999). Abiotic stresses such as: high heat, high humidity and drought have all been shown to correlate with higher levels of aflatoxin accumulation in maize (Gorman and Kang 1991; Payne 1998; Moreno and Kang 1999; Chen 2004; Gou 2008). Furthermore, biotic stresses such as insect damage also lead to increased aflatoxin accumulation in maize (Lillehoj 1980a; Williams et al. 2002, 2003, 2005). These stresses are common in the southeastern United States, an area that ranges from Georgia to Texas, making aflatoxin contamination in maize an almost annual problem in the region and regions with similar climatic conditions in other parts of the world such as Africa and Asia.
Contamination is more of a sporadic problem in the midwestern United States “Corn Belt” but outbreaks do occur (Robens and Cardwell 2005).

Figure 2.4  Aspergillus flavus life cycle.
Image from: http://www.aspergillusflavus.org/aflavus/

The economic costs of aflatoxin contamination in maize can be extremely high. In 1998 losses in maize from aflatoxin contamination across the Southeast were estimated at $85 to $100 million dollars, and the yearly estimated losses due to aflatoxin accumulation in the United States are around $200 million dollars (Windham and Williams 2002; AMCOE 2010). These numbers only take into account crop losses due to aflatoxin contamination and do not account for testing costs and also lost crop potential.
where farmers may avoid planting valuable maize crops for fear of aflatoxin contamination (Robens and Cardwell 2005). It is estimated that the total cost of aflatoxin contamination across all crops due to crop loss and research and monitoring costs is between $500 million and $1.5 billion annually, making aflatoxin contamination a very expensive problem (Robens and Cardwell 2005).

**Post-harvest and Pre-harvest prevention of *A. flavus* and aflatoxin contamination in maize**

**Harvest and Post-harvest methods**

Aflatoxin contamination is unavoidable at this time in the United States and other parts of the world. Therefore, there has been much time and effort put into understanding how to limit initial aflatoxin contamination or decrease aflatoxin contamination during harvest and post-harvest. There are many practices during harvest that can be used to help limit aflatoxin contamination in maize. Timely harvesting of maize is important in reducing aflatoxin contamination, preventing further fungal growth, kernel breakage from over-drying and damage from insects and animals (Bruns 2003; Kayaa et al. 2005; Hell et al. 2008). Delayed harvesting can result in a large increase in fungal growth and aflatoxin titer, and it is important that there be a minimal delay between harvesting and the next important step of seed drying (Sétamou et al. 1997; Kayaa et al. 2005; Hell et al. 2008). Drying of corn during the harvest process can eliminate initial or further aflatoxin accumulation in maize grain. It is recommended that maize is dried to a moisture content of < 14% to prevent further fungal growth and thus subsequent aflatoxin production.
Processes during harvesting such as properly setting and cleaning combines as well as monitoring air flow to remove foreign matter are all important in reducing the risk of increasing or spreading aflatoxin contamination during harvesting (Bruns 2003; Munkvold 2003).

Post-harvest steps besides proper drying that can impact aflatoxin include: sorting of damaged and contaminated grain, low moisture and/or well ventilated storage, removal of old grain from storage containers, and pest monitoring (Munkvold 2003; Bruns 2003; Hell et al. 2008; Choudhary and Kumari 2010). Although not a big problem in the United States, proper storage of maize grain is an important factor in preventing aflatoxin contamination in Africa and Asia (Kayaa 2005; Shephard 2005; Shier et al. 2005; Hell et al. 2008).

Decontamination of grain is another post-harvest method of mediating aflatoxin contamination in maize. There are numerous methods of chemical detoxification, natural detoxification and toxin binding to try and rid maize or other contaminated food stuffs of aflatoxin (Moreno and Kang 1999; King and Prudente 2005; Hell et al. 2008). Some of the chemical detoxification methods include ammoniation, hypochlorination, acidification, alkalation, and bisulfite treatments (Moreno and Kang 1999; King and Predente 2005). The most widely used and studied of these chemical detoxification methods is ammoniation of contaminated grain. This method has been shown to reduce AFB1 levels more than 95% in maize grain and has been investigated for use in removing contamination during the fermentation process (Weng et al. 1994; Burgos-Hernández et al. 2002; King and Prudente 2005; Hell et al. 2008). However, this method has only been approved for use in animal feed and still has not been approved in all states or for use on
human food (Moreno and Kang 1999; Hell et al. 2008). Other methods of chemical
decontamination have been shown to have some efficacy, but further research is
necessary to determine cost effectiveness, safety, and how the process impacts the target
grain (King and Prudente 2005; Hell et al. 2008). Natural methods of decontamination
include: use of microorganisms to help to help degrade aflatoxin in food, use of *A. flavus*
itself to degrade the toxin, or use of natural binders such as bentonite clay (Bata and
Lásztity 1999; Moreno and Kang 1999; King and Prudente 2005; Hell et al. 2008; Wu et
al. 2009c). Heat treatments, microwaves, and ultra violet radiation have also been tested
for efficacy in removing aflatoxin contamination from grain (Moreno and Kang 1999).

**Pre-harvest control methods other than host plant resistance**

Although harvest and post-harvest methods of preventing or reducing aflatoxin
contamination are important and can be effective, the most effective method of reducing
aflatoxin contamination is prevention of aflatoxin accumulation or even *A. flavus*
infection in maize through pre-harvest methods. These methods include a wide-range of
options from cultural practices to host plant resistance.

As discussed earlier, abiotic factors such as: heat, drought stress, and nutrient
deficiency play a large role in *A. flavus* infection and aflatoxin accumulation in maize
(Moreno and Kang 1999; Guo et al. 2005; Abbas et al. 2009). Although many of these
factors cannot be prevented, they can be mitigated through cultural practices that aim to
limit their impact on the growing maize plant. High temperatures have been shown to
favor both *A. flavus* infection and aflatoxin contamination in maize (Jones et al. 1980;
earlier planting date is one way to avoid higher temperatures in some regions, but they may be unavoidable in certain regions of the United States including the Southeast. Drought is another key abiotic stress and irrigation of fields can be useful in lowering drought stress and reducing aflatoxin accumulation in maize (Jones et al. 1981b; Payne et al. 1986; Bruns 2003). However, this is not economically feasible in many current areas of maize production. A lack of nitrogen or other nutrients can also increase aflatoxin accumulation in maize, and thus proper fertilization is recommended to reduce risk for aflatoxin contamination (Jones and Duncan 1981; Payne et al. 1989; Bruns 2003). Other cultural practices used in management of aflatoxin accumulation include: fungicides, tillage, weed control, and plating density (Payne et al. 1986; Rodriguez-del-Bosque 1996; Bruns 2003; Guo et al. 2005). Cultural practices should be tailored to the specific region where maize is being grown and modified for each individual growing season to account for year-to-year variation, but cultural practices alone are still not enough to prevent or even greatly affect aflatoxin accumulation (Bruns 2003; Gou et al. 2005).

Insect damage to the maize ear is another factor allowing *A. flavus* to gain a foothold and subsequent production of aflatoxin by the fungus (Wilson et al. 1979; Lillehoj 1980; Windham et al. 1999; Williams et al. 2002, 2003, 2005a). Therefore, management of insects is another pre-harvest method of controlling aflatoxin accumulation in maize. Insecticides are one management possibility, but add a high cost to production; and thus, the use of insect resistant corn such as *Bt* or other resistant lines is useful and often more cost effective in reducing insect damage (Williams et al. 2002, 2005a; Wu et al. 2005; Abbas et al. 2009).
Another pre-harvest prevention method is the application of biological control agents to maize. These agents, usually non-toxigenic fungi, operate under the theory that these safe (or at least less harmful) organisms will out-compete the dangerous toxin producing fungal strains thus reducing or eliminating the toxin and the risk posed by the toxigenic strains. Biological control has been shown to reduce aflatoxin levels in maize and in other crops both in *in vitro* studies and in field tests (Cotty 1989; Ehrlich 1987; Wicklow et al. 1988; Brown et al. 1991; Dorner et al. 1999; Dorner 2005; Abbas et al. 2006; Atehnkeng et al. 2008; Cotty et al. 2008). Progress has been impressive enough in preventing *A. flavus* infections and aflatoxin accumulation in maize that a commercial product Afla-Guard® (Aflasafe™ in Africa) has even been developed and marketed. Biological control requires that the non-toxigenic strain of fungus be applied to the field/soil in high concentrations and before the toxigenic/wild strains can proliferate enough to gain a foothold or infect the maize plant (Dorner 2005; Cotty et al. 2008). Therefore, the biocontrol must be added to the field before it is known whether *A. flavus* infection and aflatoxin accumulation will be a problem that year, and it is an extra added cost to maize production.

**Host plant resistance**

The most promising avenue of preventing *A. flavus* infection / aflatoxin accumulation in maize is through the development of resistant lines of maize (Gorman and Kang 1991; Widstrom 1996; Kang and Moreno 2002). Breeding programs were started in the late 1970’s and early 1980’s to identify, develop, and enhance sources of resistance to *A. flavus* / aflatoxin accumulation. These programs began by screening:
open-pollinated varieties, hybrids, and inbred lines of field corn, popcorn, and sweet corn, to find maize with resistance to *A. flavus* / aflatoxin accumulation (Lillehoj et al. 1975, 1980b; McMillan et al. 1982; Zuber et al. 1983; Widstrom et al. 1984, 1987; Scott and Zummo 1988, 1990a; Kang et al. 1990; Gorman and Kang 1991; Campbell and White 1995a; Williams et al. 2005b). Initial screenings involved only natural infections, and resistance was based solely on percent kernel infection by *A. flavus* (Scott and Zummo 1987; Windham et al. 2005). Given the sporadic and heavily environmentally influenced nature of *A. flavus* infection and subsequent aflatoxin contamination, a more uniform and consistent method of inoculation techniques was needed and developed (Scott and Zummo 1987; Widstrom et al. 1987; Brown et al. 1999; Windham et al. 2005; Windham and Williams 2007; Hawkins 2008; Williams et al. 2008a). Screening of maize lines was made more efficient by the development of techniques such as the pin-bar and side needle injections methods. The finding of cheaper, quicker methods for aflatoxin quantification such as enzyme-linked immunoabsorbent assay (ELISA) and the Vicam AflaTest® has allowed for the screening of resistant genotypes by percent infection and aflatoxin accumulation (Scott and Zummo 1987; Campbell and White 1995; Clements and White 2005; Windham et al. 2005).

Through improved selection procedures, breeding programs were able to develop resistant germplasm in the form of inbred lines such as: Mp313E, Mp715, Mp717, Mp420, Tex 6, Mo18W, LB31, CI2, and MI82 as well as the GT-MAS:gk population (Scott and Zummo 1990b, 1992; McMillian et al. 1993; Campbell and White 1995a,b; Campbell et al. 1997; Williams and Windham 2001, 2006; Maupin et al. 2003; Clements and White 2005). However, most resistant sources and lines contain undesirable
agronomic traits such as, late maturity and tight husk coverage, when compared with commercial cultivars, and are unsuitable for immediate use in the formation of commercial hybrids (Betrán et al. 2002; Clements and White 2005; Williams et al. 2008b). Further complicating matters is the fact that resistance to aflatoxin accumulation is a quantitative trait with low to moderate heritability, encoded by many genes, and is highly influence by environmental factors (Stoloff and Lillehoj 1981; Widstrom et al. 1984; Campbell and White 1995a; Hamblin and White 2000; Williams et al. 2008b). Therefore despite breeding progress, there are no elite maize breeding lines or commercial hybrids with any appreciable level of resistance to aflatoxin accumulation (Windham and Williams 1998; Abbas 2002).

**Marker Assisted Selection (MAS)**

Breeding programs have been successful in creating inbred lines with resistance to *A. flavus* / aflatoxin accumulation; however, there has been difficulty in transferring resistance from inbred lines to more agronomically favorable commercial lines. A difficulty in moving the resistance is that resistance to *A. flavus* infection or aflatoxin accumulation is a quantitative trait which is highly influenced by the environment in which the maize is grown (Stoloff and Lillehoj 1981; Widstrom et al. 1984; Campbell and White 1995a; Hamblin and White 2000; Williams et al. 2008b). There are many genes that appear to influence resistance both directly and indirectly through other traits. Therefore, traits such as: maturity, husk coverage, insect resistance, kernel wax type, and other factors can be selected and may increase *A. flavus* infection or aflatoxin accumulation resistance but they may not be acceptable in elite breeding lines, or they
may bring other undesirable traits along with them. Thus, researchers have started the task isolating the genetic components of resistance to aflatoxin accumulation. Isolation of certain chromosomal regions, genes, or proteins that are causative or linked to resistance to *A. flavus* / aflatoxin accumulation will allow for easier transfer of resistance from inbred lines to commercial breeding lines and hybrids through marker assisted selection (MAS).

Marker Assisted Selection has been successfully employed in many crops including: rice, barley (*Hordeum vulgare* L.) and wheat (Koebner 2003; Hospital 2009; Varshney 2009a). In maize, MAS has been used in breeding programs in both the public and private sectors for improving yield, resistance to stresses, and increasing nutritional components including the Quality Protein Maize (QPM) varieties (Willcox et al. 2002; Danson et al. 2006; Eathington et al. 2007; Ribaut and Ragot 2007; Prasanna et al. 2010). Other investigators have already begun the process of validating markers for use in MAS programs (Chen et al. 2010a). However, despite the abundance of QTL studies and identification of possible markers, Xu and Crouch (2008) explain that MAS is still in its infancy and has not reached its expected utility, especially in the public sector (Ragot and Lee 2007). This is expected to change due to the decreasing expense involved in identifying markers and higher throughput methods for genotyping populations for one or more markers.

**Quantitative Trait Loci (QTL)**

Quantitative trait loci (QTL) are regions of the genome associated with a particular quantitative trait. Identification of QTLs requires both the phenotypic
information for a given trait such as: disease resistance for a population of segregating individuals, genotypic information on the same individuals, and a linkage map created based on the same genetic information. Creating a linkage map requires three main steps starting with the creation of a segregating mapping population (Young 2000; Collard et al. 2005; Robertson et al. 2005). In breeding for resistance, the parents in the mapping population should differ for the trait(s) of interest. In the case of resistance to *A. flavus* / aflatoxin accumulation, a resistant inbred parent should be crossed with a susceptible parent, and the segregating population should be developed through self-pollination of the F₁ offspring of the cross ( Tanksley 1993; Collard et al. 2005; Robertson et al. 2005). The size of the mapping population should be at least 50 individuals, but greater linkage map resolution is achieved with larger mapping populations (Young 2000; Collard et al. 2005). Populations smaller than 100 individuals can suffer from the Beavis effect, which means the effect of identified QTL may be significantly overestimated (Beavis 1998; Xu 2003).

The second step is the discovery and characterization of polymorphisms to be used as markers (Young 2000; Collard et al. 2005). To be useful in creating a linkage map the marker must be polymorphic between the two parents with the F₁ distinguishable from the two parents as well. These markers can be: SSR, RFLP, amplified fragment length polymorphisms (AFLP), single nucleotide polymorphisms (SNP), or others (Tanksley 1993; Mohan et al. 1997; Gupta et al. 2001; Rafalski 2002; Collard et al. 2005; Robertson et al. 2005). In QTL analysis performed for resistance to *A. flavus* / aflatoxin accumulation, AFLP and RFLP markers as well as SSR markers made publicly available by the Maize Genetics and Genomics Database (MaizeGDB) have been used.
These polymorphisms and new polymorphisms available following large scale sequencing efforts, such as Insertions/Deletions (indels), are often visualized on agarose or polyacrylamide gels, but recent advancements in technology have allowed for the visualization of SNP markers using probes. Once visualized, the individuals in the mapping population are scored on whether they are like parent A (usually denoting the parent containing the trait of interest), B (the other parent), or the F₁ for the given marker. This step is repeated for every maker that will be used on the mapping population.

The final step in creating a linkage map is the genetic mapping itself (Young 2000; Collard et al. 2005). Linkage maps are created based on the principle that markers closer together will experience recombination less frequently than two markers which are far apart from one another (Collard et al. 2005). These recombination frequencies among markers can be used to determine the relative distances between markers and combine groups of markers into linkage groups which represent chromosomes (Collard et al. 2005). The genetic distances between markers can be calculated using recombination fractions and these distances are presented in centiMorgans (cM) (Collard et al. 2005). Due to the number of markers necessary to gain the resolution needed in creating a linkage map for QTL analysis, it is generally not possible to perform the genetic mapping by hand. Therefore, researchers use mapping software like JoinMap (Kyazma B.V.; Wageningen, Netherlands) that is capable of handling the large amounts of data that are produced by genotyping a mapping population (Young 2000; Collard et al. 2005). Once finished, the linkage map gives the researcher a map that shows on which chromosome
markers are located and the relative genetic distance between markers (Collard et al. 2005).

Once the linkage map has been created, the marker information can be combined with the phenotypic information for the trait(s) of interest to perform QTL analysis. In resistance to *A. flavus* / aflatoxin accumulation, the phenotypic information is usually fungal proliferation measured by ear rot ratings, aflatoxin accumulation, or a combination of both. The markers can then be analyzed to determine whether there are significant differences among the genotypic classes with respect to the phenotype being measured. In other words, is the phenotype associated with individuals with marker class A significantly different than the phenotype associated with marker class B? If there are significant differences among the classes, then significant phenotypic variation can be attributed to the marker or a region between two markers (Tanksley 1993; Young 1996; Collard et al. 2005). As in linkage mapping, QTL analysis is made possible due to recombination frequencies between a marker and the QTL; thus, the closer a marker is to the QTL the more likely that the marker and the QTL will be inherited together (Collard et al. 2005). This means that the marker can be used to track the QTL. Common methods in QTL analysis include: single marker analysis, simple interval mapping and composite interval mapping (Collard et al. 2005). Much like the construction of a linkage map, QTL analysis requires software such as QTL Cartographer (NCSU; Raleigh, NC) (Tanksley 1993; Young 1996; Mohan 1997; Collard et al. 2005).

Previous studies have identified QTL associated with resistance to *A. flavus* / aflatoxin accumulation; however, none have been able to identify QTL that account for more than 20% of the phenotypic variation for resistance to aflatoxin accumulation in the
study (Davis et al. 1999; Willcox et al. 2000; Paul et al. 2003; Widstrom et al. 2003; Brooks et al. 2005; Robertson-Hoyt et al. 2007; Warburton 2009, 2010). Moreover, there is little overlap among QTL from different environments and even less among QTL resulting from different populations, although chromosomes 2, 4, and 5 do have QTL that show up in at least one environment in multiple studies (Willcox et al. 2000; Paul et al. 2003; Busboom and White 2004; Brooks et al. 2005; Robertson-Hoyt et al. 2007; Warburton 2009, 2010). These variations in QTL significance and locations can be attributed to (and cause) the high QTL x environment interactions, the environmental influence on aflatoxin production, different methods of *A. flavus* inoculation, and the use of different resistant and susceptible lines in many of these studies (Paul et al. 2003; Busboom and White 2004; Brooks et al. 2005; Robertson et al. 2005; Robertson-Hoyt et al. 2007; Warburton 2009, 2010).

QTL for resistance to aflatoxin accumulation can be used in MAS to help introgress resistance found in inbred lines into elite commercial cultivars. By selecting for individuals containing markers tightly linked to the genetic regions associated with resistance, the trait can be transferred from resistant inbreds into select commercial lines (Mohan 1997; Collard et al. 2005; Robertson et al. 2005). This can be done early in the planting season, thus allowing researchers to make only those pollinations among plants carrying the desirable markers and discarding individuals not carrying the desired markers. In breeding for resistance to aflatoxin accumulation, this is particularly helpful because phenotypic evaluation for resistance to aflatoxin accumulation can only be conducted after pollinations have been made. By identifying individuals with the desired markers before pollinations, selection for resistance and development of new resistant
lines can be quicker and with less dependency on environmentally influenced phenotypic evaluations (Mohan 1997; Collard et al. 2005; Robertson et al. 2005). However, MAS using QTL does have some drawbacks. QTL mapping usually identifies large regions of the chromosome that can be difficult to move into elite lines, as recombinations may break up a large QTL. Mapping may also identify multiple QTL, each conferring only minor amounts of resistance. When moving large or multiple QTL, undesirable traits may piggyback along with resistance traits in a situation known as linkage drag (Zeven et al. 1983; Young 1996; Robertson 2005). Linkage drag is due to the fact that a large QTL may contain many other genes besides the gene(s) affecting the trait of interest; therefore, when markers linked to the QTL of interest are selected other gene(s) that are agronomically unfavorable may be selected for along with the gene(s) of interest. Linkage drag can especially be a problem when transferring traits from wild sources into commercial lines (Collard et al. 2005). Moreover, the larger the QTL region the more likely recombination between the marker(s) and the actual gene(s) conferring resistance will occur, thus limiting the markers usefulness in MAS.

To alleviate issues of linkage drag, high-resolution or fine mapping may be used. This process involves a larger number of markers and increased population sizes to more finely link markers to gene(s) controlling the trait of interest, thus narrowing the size of the QTL (Asins 2002; Collard et al. 2005). A smaller QTL means that the markers used to track the QTL are like closer to the actual gene(s) controlling the trait, and this means that it is less likely deleterious gene(s) will be transferred along with the gene(s) of interest. Many SSR markers are available in maize but not all of them are polymorphic in each population, and they may not be evenly distributed throughout the genome.
Therefore, it is important that investigators always look for new markers that help to narrow the distance between markers and the gene(s) controlling the trait of interest. These may be new SSR markers or markers based on SNPs or insertions / deletions (indels) in genes found to be linked to or causative in the trait of interest. Moreover, if the polymorphic gene is closely linked or causative to the trait, then the gene-based marker can be used for MAS (Gupta et al. 2001; Ayeh 2008). However, even though polymorphisms are abundant in the maize genome, identification of polymorphisms useful for marker design can be difficult (Jones et al. 2009).

Development of gene-based markers requires two steps: 1) identification of candidate genes and 2) identification of polymorphisms in candidate genes to be used in development of gene-based markers.

**Gene-based markers**

**Candidate gene identification**

Ways to identify candidate genes to be used as new markers for QTL elucidation or gene-based marker MAS include: proteomics studies, genomics studies, physiological studies, and bio-informatics studies. Work to identify proteins and other factors in maize related to resistance to *A. flavus* infection and aflatoxin accumulation have been successful in identifying proteins and kernel properties that may be important in resistance to *A. flavus* / aflatoxin contamination (Kang and Moreno 2002; Chen et al. 2006; Brown et al. 2010). Many of these proteins and factors have been studied exclusively in maize kernels (Brown et al. 1993, 1995; Wu et al. 1994; Huang et al. 1997;
Russin et al. 1997; Chen et al. 1998; Gembeh et al. 2001; Wilson et al. 2001; Magbanua 2007). Non-protein factors such as: diterpanoid phytoalexins, anthocyanins, aldehydes, and phenolic compounds may also play a role in resistance to *A. flavus* and aflatoxin (Gembeh et al. 2001; Kang and Moreno 2002; Schmelz et al. 2011). Some of the key proteins that have been identified are: β-1,3-glucanase, the chitinase family of proteins, trypsin inhibitors, and catalase proteins (Wu et al. 1994; Brown et al. 1995; Chen et al. 1998; Kang and Moreno 2002; Moore et al. 2004; Magbanua 2007; Peethambaran 2009). Other proteins may even play a role in inhibiting the production of aflatoxin by *A. flavus* (Brown et al. 1993; Huang et al. 1997; Chen et al. 2006). Experiments in comparative proteomics have helped to expand the list of proteins that may be involved in resistance to *A. flavus* / aflatoxin accumulation by focusing on all proteins expressed and also examining tissues other than the maize kernel (Brown et al. 2003; Peethambaran 2009; Brown et al. 2010; Pechanova et al. 2011). Although these experiments have helped to give a better insight into how resistant and susceptible genotypes may respond to *A. flavus* infection / aflatoxin accumulation at the proteomic level, the unknowns about the host / pathogen interaction outweigh what is known (Brown et al. 2010; Pechanova et al. 2011).

Identifying proteins involved in resistance to *A. flavus* / aflatoxin accumulation is important to understanding how resistance may work and is expressed; however, identifying the genes that code for these proteins and control other resistance factors is essential for transferring resistance into elite production lines of maize. Efforts to identify the genes controlling or linked to resistance to *A. flavus* / aflatoxin accumulation involve many techniques such as: analyzing genes responsible for expression of
resistance associated proteins (RAPs) or factors, analysis of differential expression patterns between susceptible and resistant maize lines, and bioinformatics research comparing the results of multiple expression studies to look for commonalities among experiments.

The genes that code for proteins associated with resistance to *A. flavus* / aflatoxin are ideal candidates for gene-based markers. Resistance associated proteins can be translated into gene sequences and identified in the maize reference genome sequence. These genes may be mapped to existing linkage maps from QTL analysis to see if the coding gene falls into or near known QTL, and further examined for association with *A. flavus* / aflatoxin resistance. Many studies have taken the genes coding RAPs and tracked their expression in susceptible and resistant lines under different stress treatments (Wilson et al. 2001; Fountain et al. 2010; Brown et al. 2010; Huffaker et al. 2011). If the gene for a RAP is differentially expressed in one maize line versus another, or under one condition versus another then, it makes a stronger case for that gene as a good candidate for gene-based marker development. Validation of the role of RAPs in resistance to aflatoxin accumulation has also been conducted through RNA interference (RNAi) gene silencing (Chen et al. 2009; 2010b; Brown et al. 2010). These studies showed that silencing of the genes encoding the RAPs resulted in increased susceptibility to *A. flavus* / aflatoxin accumulation.

Another method for identifying candidate genes for resistance to *A. flavus* / aflatoxin accumulation is through the use of functional genomics techniques such as microarray analysis and new sequencing like high throughput RNA sequencing technologies (RNAseq) (Bhatnagar et al. 2008). Microarray analysis allows for
examination of gene expression differences between different types of tissues or maize genotypes, or the same tissues and / or genotypes under different environmental conditions.

Two studies conducted using the resistant line Tex6 examined the expression of defense and stress related genes in Tex6 at multiple time points, and identified numerous genes that were differentially expressed in over time and in response to drought (Lou 2008; Lou 2010). While neither experiment focused directly on response to A. flavus / aflatoxin accumulation, Luo et al. (2010) compared the expression of 30 genes in Tex6 versus B73 via quantitative RT-PCR and found differences in gene expression patterns between the two lines. It has been suggested in other studies that there is a link between drought stress and aflatoxin accumulation, and thus genes important for drought resistance may make good candidate genes for aflatoxin accumulation as well (Jones et al. 1981b; Payne et al. 1986; Bruns 2003; Chen et al. 2004; Wang et al. 2008; Luo et al. 2010). A different microarray study of tissue from developing ears from resistant line Mp313E and susceptible line Va35 collected two days after inoculation with A. flavus strain NRRL 3357 (toxigenic) identified 234 genes that were differentially expressed between the lines (Kelly et al. 2009). At the time, 28 of these genes had been mapped near known QTL for resistance to aflatoxin accumulation (Brooks et al. 2005; Kelley et al. 2009).

Next generation sequencing technologies allow researches to gather sequence information for the entire genome of the target organism (Edwards and Batley 2010; Varshney et al. 2009b; Wang et al. 2009). Furthermore, RNAseq technologies give an understanding of both gene expression between two experimental conditions and the raw
sequence data for those expressed genes simultaneously (Varshney et al. 2009b; Wang et al. 2009). This sequence data is highly informative for SNP discovery especially given the recent release of the maize genome (Schnable et al. 2009). High throughput sequencing studies in maize by Emrich et al. (2007) and Barbazuk et al. (2007) demonstrated the ability of this technology to identify not only SNPs, but also unique transcripts as well. Barbazuk et al. (2007) used this technology to identify almost 5000 SNPs in more than 2400 genes between B73 and Mo17. Furthermore, this technology can be used to examine tissue specific gene expression and look at tissue specific gene sequences (Emrich et al. 2007). The applications this new technology in maize breeding are evident, and next generation DNA sequencing technology will allow for the sequencing of specific maize lines such as those with resistance to *A. flavus* / aflatoxin accumulation. Sequencing of susceptible and resistant maize lines will allow for the identification of polymorphisms more quickly and in greater numbers than through other methods, and RNAseq technologies allow for greater insight into which genes are expressed in response to fungal infection and aflatoxin accumulation.

With the increase in sequence data, genomics data, and proteomics data, an understanding of bioinformatics and how it may aid in the identification of candidate genes is important. Mammadov et al. (2011) used SNP data from multiple public databases to develop a list of 162 SNPs from almost 130,000 public markers. Kelley et al. (2010) developed a database at Mississippi State University by integrating various “omics” data along with sequence data, and QTL mapping data to help identify candidate genes for resistance to *A. flavus* / aflatoxin contamination and the development of gene-based markers. This database, called the Corn Fungal Resistance Associated
Sequence Database (CFRAS-DB), allows researchers to examine information from many different types of experiments simultaneously and design queries to extract the most information from the various datasets. Furthermore, it has the ability to be continuously updated with new information from other experiments to further expand the data available to researchers.

*Identification of polymorphisms in candidate genes*

Identification of polymorphisms (SNPs and indels) in the sequence of genes involved in or linked to resistance is the second step in the development of gene-based markers and can be accomplished by various methods (Ganal et al. 2009). The most direct method of discovering polymorphisms in target genes is direct sequencing of the gene in multiple maize lines. The sequence from these lines can be compared using one of the many available alignment software packages and/or algorithms to determine if any polymorphisms exist between the lines. This is a very reliable way to discover polymorphisms with a false discovery rate at or below 5% (Gupta et al. 2001; Rafalski 2002; Ganal et al. 2009). Another method of polymorphism discovery is the use of existing sequence data. Expressed sequence tags (ESTs) are available for numerous maize genes, and ESTs from different maize lines may be compared with each other or with newly sequenced genes (Gupta et al. 2001; Rafalski 2002; Ganal et al. 2009; van Oeveren and Janssen 2009).

High throughput sequencing technologies allow for the marriage of the two previously described methodologies for finding polymorphisms. These technologies offered by companies such as Roche 454 Life Sciences and Illumina allow for the
generation of nearly full genome sequences of individuals from species including maize and published reports have already demonstrated their usefulness in identifying polymorphisms between different maize lines (Barbuzak et al. 2007; Edwards and Batley 2009; Ganal et al. 2009; van Oeveren and Jansen 2009; Varshney et al. 2009b). Reliability of this method of polymorphism discovery is greatly enhanced by the availability of a good reference sequence; and thus, due to the recent publication of the B73 maize genome, this method of SNP discovery has become a more reliable option for maize researchers (Ganal et al. 2009). A study by Mammadov et al. (2010) identified 120 high-quality SNPs from two diverse inbred maize lines using the Complexity Reduction of Polymorphic Sequences technology. Other methods for polymorphism discovery include: array based technologies, the sequencing of overlapping bacterial artificial chromosomes (BACs), and high resolution melting curve chemistry (Gupta et al. 2001; Ganal 2009).

Genotyping of discovered polymorphisms in a larger panel of maize lines can be accomplished by the sequencing methods above, but this is expensive. A simple alternative is to genotype indels via PCR amplification and separation by electrophoresis on agarose (for larger indels) or polyacrylamide gel electrophoresis (PAGE), which has a higher resolution, for smaller indels. Gel based methods may sometimes be used for genotyping SNPs. If the SNP alters a restriction site in the gene-based marker, then RFLP analysis may be used to genotype the individuals for the target polymorphism (Gupta et al. 2001). However, this is a time consuming polymorphism visualization method.
Newer methods of genotyping SNPs allow for higher throughput and the possibility of automation. Labeled probes that fluoresce in the presence of one polymorphisms and not the other are one option for SNP or indel identification. Equipment like the Roche Light Cycler 480 can then measure the intensity of the fluorescence to differentiate between the two homozygotes and the heterozygote (Gupta et al. 2001; Ding and Jin 2009). Labeled primers may also be used to genotype SNPs in a PCR reaction that distinguishes the two alleles of the polymorphism via a fluorescent plate reader (Warburton et al. 2011). High resolution melting is another technology that can be used to genotype SNPs and is based on the principal that the polymorphisms between the two parents will result in differences in melting temperatures that can be detected by analyzing machinery (Grievnik and Stowell 2008; Kristensen and Dobrovic 2008). There are various other methods for detecting SNPs including: primer extension, DNA and microarray chips, and invasive cleavage (Gupta et al. 2001; Semagn et al. 2006; Ayeh 2008). The method used must be based on numerous factors including: type and number of polymorphisms, equipment, and cost.


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CHAPTER III

DEVELOPMENT OF A GENE - BASED MARKER CORRELATED TO REDUCED AFLATOXIN ACCUMULATION IN MAIZE

Abstract

Aflatoxins are carcinogenic and toxic metabolites produced by the fungus Aspergillus flavus during infection of maize (Zea mays L.) and seed oil crops. Climatic conditions in the southeastern United States favor A. flavus infection and aflatoxin contamination in maize, making it an issue for farmers in the region. One of the most promising avenues to combat aflatoxin contamination is the development of resistant maize lines. However, this has proven difficult due to a lack of gene-based markers for resistance. Previous studies have identified candidate genes that were differentially expressed in response to A. flavus infection. One gene, encoding a chloroplast precursor, was found to contain multiple polymorphisms that were used to design a marker designated Mississippi Marker 1 (MpM1). The marker differentiates between the “resistant” and “susceptible” forms of the gene. This marker was used to screen three populations of F_{2:3} mapping families, where it was found to map to chromosome 4 and was associated with a significant effect for resistance to aflatoxin accumulation in all three populations. Furthermore, the marker MpM1 identified a previously unknown QTL for resistance to aflatoxin accumulation on maize chromosome 4. MpM1 is the first
gene-based marker developed specifically for resistance to aflatoxin accumulation in maize and can now be integrated into existing marker assisted selection programs aimed at incorporating resistance into elite maize breeding lines.

Introduction

Aspergillus flavus is a pathogenic mold fungus of many crops including: maize (Zea mays L.), cotton, peanuts, and tree nuts. Although A. flavus is not a strong pathogen, it produces the toxic secondary metabolite, aflatoxin, which is a health concern to consumers and thus an economic threat to growers. It is not known exactly why A. flavus produces aflatoxin, but it is known that both A. flavus colonization of maize and aflatoxin production are favored by abiotic stresses (drought, high heat, and nutrient deficiencies) (Moreno and Kang 1999). This toxin, in small amounts, can cause hepatocellular carcinoma in humans and can be deadly if ingested in high amounts by humans, small animals, and livestock (Castegnaro and McGregor 1998). Due to the human and animal health concerns associated with aflatoxin, many countries have imposed limits on the amount of aflatoxin acceptable for foodstuffs destined for human and animal consumption, including an FDA action limit of 20 ng/g for grain destined for consumer products in the United States (Park and Liang 1993). These restrictions can result in significant economic losses for farmers and food producers whose products exceed set aflatoxin limits (Vardon et al. 2003; Windham and Williams 2002). The health and economic problems resulting from A. flavus infection and aflatoxin contamination have resulted in many programs aimed at reducing or preventing A. flavus infection and aflatoxin contamination in maize. One promising avenue is the
development of maize lines that are genetically resistant to *A. flavus* infection or aflatoxin accumulation (Williams et al. 2003).

Several sources of natural resistance have been identified (Campbell and White 1995; Scott and Zummo 1988, 1990, 1992; Williams and Windham 2001, 2006). However, many of these lines exhibit undesirable agronomic traits that make their introduction into breeding programs with elite commercial lines difficult. Molecular markers for resistance to *A. flavus* / aflatoxin accumulation would allow for easier introgression of this resistance into commercial lines while maintaining the agronomic integrity of the commercial lines. Previous Quantitative Trait Loci (QTL) studies have identified chromosomal regions associated with this natural resistance to aflatoxin accumulation (Davis et al. 1999; Willcox et al. 2000; Paul et al. 2003; Brooks et al. 2005; Warburton et al. 2009, 2010). However, QTL mapping often identifies large regions of the chromosome associated with the desired trait that may also contain genes encoding undesirable traits from the donor line (a phenomenon known as linkage drag). Moving a large genomic region is also difficult because recombination may occur between markers linked to, but not in, the gene of interest and the gene itself, limiting the utility of these linked markers in Marker Assisted Selection (MAS) to lines that have been specifically mapped.

Gene-based markers overcome these limitations because they are located within a gene known to encode the trait of interest, and thus can be used to transfer only that gene into desired lines through MAS without the associated problems of recombination or linkage drag. Single nucleotide polymorphisms (SNPs) or insertions / deletions (indels) found within a target gene can be used to develop gene-based specific markers for
tracking a specific trait (Gupta et al. 2001; Ayeh 2008; Mammadov et al. 2011). SNPs are stable and very abundant in the maize genome and usually biallelic (Gupta et al. 2001; Ching et al. 2002; Rafalski 2002). SNPs and indels used for development of a gene-based marker may be found in either the coding or non-coding region of the gene.

Previous microarray studies of resistant (Mp313E) and susceptible (Va35) maize lines collected 2 days after infection with *A. flavus* (NRRL 3357) identified 234 genes that showed differential expression between lines (Kelley et al. 2009). This information was combined with previous QTL experiments to identify genes occurring at known genetic locations that were used as a starting point for the development of gene-based makers for aflatoxin accumulation resistance. From this group, 11 gene sequences were chosen based on genetic location relative to known QTL or based on putative gene functions or roles.

The goals of this project were: (i) to test our methodology in identifying genes with SNPs or indels between resistant and susceptible lines; (ii) to screen F2:3 QTL mapping families of resistant x susceptible lines with markers developed based on the identified polymorphisms; and (iii) to create, map, and characterize one gene-based marker to determine its contribution to aflatoxin accumulation resistance, as a proof of concept that this methodology will be useful to create tools to speed aflatoxin resistance breeding in maize.
Methods

DNA Sequencing for SNP Discovery

The sequence for the selected 10 genes from the microarray study of Kelley et al. (2009) was retrieved using the NCBI sequence database (http://www.ncbi.nlm.nih.gov/). The EST accession numbers of the selected genes were AW216267, AW244196, AW400128, AW438153, BE128894, AW179553, AW225099, AW331008, AW360565, and AW424439 (Table 3.1). Using the EST sequence of the differentially expressed genes, a primer pair was designed for each of the 10 genes using Primer 3 software v. 0.40 (http://frodo.wi.mit.edu/primer3/) (Table 3.1) and ordered from Sigma – Genosys (The Woodlands, TX) (Rozen and Skaletsky 2000). The primers were then verified using PCR and tested for amplification using Zea mays lines Mp313E (resistant) and B73 or Va35 (susceptible). The thermocycling steps were: initial denaturation at 95°C for 4 minutes followed by 35 cycles of 94°C for 1 minute, 55°C for 2 minutes (anneal temperature was optimized based on individual primer pairs), 72°C for 1.5 minutes, followed by a final extension step of 72°C for 5 minutes. PCR products were electrophoresed on a 1.5% agarose gel and visualized with ethidium bromide. PCR products that gave a good, single band were purified using Qiagen QIAquick™ PCR Purification Kit (Qiagen Inc.; Valencia, CA). After purification, sequencing reactions were prepared using Big Dye® chemistry from Applied Biosystems Inc. (Foster City, CA). The sequencing reactions were then analyzed using an Applied Biosystems 3730xl DNA Analyzer. Sequencing was performed on at least one of two resistant (Mp313E, Mp715) and at least one of two susceptible (B73, Va35) maize genotypes, and alignment
of sequences was performed using DNAMAN software v. 5.2.9 (Lynnon Corporation; Pointe-Claire, Quebec, Canada).

**Probe Design**

A probe was designed to exploit the SNP(s) or indel polymorphisms identified in the AW424439 gene, such that the two homozygotes and the heterozygote could all be differentiated. This Hybprobe was designed by TIB MolBiol (Adelphia, NJ) for use with the Roche LightCycler® 480 and the Genotyping Master Kit (Roche Applied Science; Indianapolis, IN) (Table 3.1). Additional primers were also designed for specific use with the probe and Genotyping Master Kit to reduce the fragment size generated by the original primer. The second amplified fragment was of optimal size for use with the probe and kit (Table 3.2; Figure 3.1).

An additional primer pair that amplified a smaller fragment was later designed using the Primer 3 software version 0.40 (http://frodo.wi.mit.edu/primer3/) to take advantage of the same polymorphisms in the target gene that was used to design the probe (Rozen and Skaletsky 2000). This polymorphism was an indel causing a size difference large enough to be seen using polyacrylamide gel electrophoresis (PAGE). This alternate primer pair was used to screen the other two F2:3 families via PAGE (Table 3.2; Figure 3.1).
Table 3.1 Primers used for PCR and sequencing.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AW216267F</td>
<td>GGGAACTTGGAGAAATCTGG</td>
<td>62.3</td>
</tr>
<tr>
<td>AW216267R</td>
<td>TGAAATGTGTTTCTGTACG</td>
<td>63.5</td>
</tr>
<tr>
<td>AW244196F</td>
<td>GATGCAAACCGTGATCTGC</td>
<td>63.1</td>
</tr>
<tr>
<td>AW244196R</td>
<td>GTCTGCTGTACCTGGAAACC</td>
<td>63.5</td>
</tr>
<tr>
<td>AW400128F</td>
<td>ACATTCGACGAGGAGACCC</td>
<td>63.2</td>
</tr>
<tr>
<td>AW400128R</td>
<td>TGGACCAACGAGTCACG</td>
<td>63.6</td>
</tr>
<tr>
<td>AW438153F</td>
<td>GGATTTCAAGCGAGGTTCC</td>
<td>62.2</td>
</tr>
<tr>
<td>AW438153R</td>
<td>TTTTTTACAGACAGACCAGTACACC</td>
<td>62.0</td>
</tr>
<tr>
<td>BE128894F</td>
<td>ATGAAGGGAATTGCAAAGC</td>
<td>63.9</td>
</tr>
<tr>
<td>BE128894R</td>
<td>AGATCAGCTCACGGCATAGC</td>
<td>64.4</td>
</tr>
<tr>
<td>AW179553F</td>
<td>GGACACTCCGTACTCATCG</td>
<td>62.1</td>
</tr>
<tr>
<td>AW179553R</td>
<td>CGTGCAAGTTCTTACCGG</td>
<td>62.5</td>
</tr>
<tr>
<td>AW225099F</td>
<td>ATTCATTTCCCTCAATACG</td>
<td>62.4</td>
</tr>
<tr>
<td>AW225099R</td>
<td>GGTGTTGTGTCTCTCTCTCTTG</td>
<td>62.9</td>
</tr>
<tr>
<td>AW331008F</td>
<td>CACTTGATCAGCCTGAAAGC</td>
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<tr>
<td>AW331008R</td>
<td>TGCTGTGGCTATCGTACCC</td>
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<tr>
<td>AW360565F</td>
<td>CAGGGTGGAGAACAAGAAGC</td>
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<tr>
<td>AW360565R</td>
<td>CGACTGGCTTGGATGTGTC</td>
<td>63.6</td>
</tr>
<tr>
<td>AW424439F</td>
<td>CTGCAGCCAACCACATCTACC</td>
<td>63.3</td>
</tr>
<tr>
<td>AW424439R</td>
<td>CGTACTTGTCGACGAACCTGG</td>
<td>63.3</td>
</tr>
</tbody>
</table>

Primers used for PCR amplification and sequencing of the 10 candidate genes chosen for screening as potential gene – based markers
Table 3.2  Sequences of probe and primers used for genotyping F_{2:3} populations.

<table>
<thead>
<tr>
<th>Primer / Probe Name</th>
<th>Sequence</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Probe for Genotyping</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensor 2</td>
<td>GCTTTTCGTCGGCCGCCGTT-FL&lt;sup&gt;a&lt;/sup&gt;</td>
<td>70.1</td>
</tr>
<tr>
<td>Anchor 2</td>
<td>LC640&lt;sup&gt;b&lt;/sup&gt;-GCCGGGTGTGGTTCTGACTTCTGAGCTGAGT-PH&lt;sup&gt;c&lt;/sup&gt;</td>
<td>74.2</td>
</tr>
<tr>
<td><strong>Primers for Genotyping</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B73 F2</td>
<td>TCAAGACCGACAAAGCCCTAC</td>
<td>56.7</td>
</tr>
<tr>
<td>B73 R2</td>
<td>ACCACGAATTTCAGCTCCAT</td>
<td>55.3</td>
</tr>
<tr>
<td><strong>Primers for Polyacrylamide Analysis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AW424439SHRTF</td>
<td>AAGAAGATCAAGACCGACAAAGC</td>
<td>63.5</td>
</tr>
<tr>
<td>AW424439SHRTR</td>
<td>ATCACGACGACGACTCAGC</td>
<td>64.3</td>
</tr>
</tbody>
</table>

Probe and primers for the AW424439 gene used with Roche LightCycler® 480 and the Genotyping Master Kit for genotyping Mp313E x B73 F_{2:3} population, and Primers used for genotyping the Mp313E x Va35 and Mp715 x T173 F_{2:3} populations using polyacrylamide gel electrophoresis

<sup>a</sup> Fluorescein
<sup>b</sup> Light Cycler Red Dye (640)
<sup>c</sup> Phosphate
<sup>d</sup> Further information regarding TIB MolBiol probes can be found at: http://www.tib-molbiol.com/lightcycler/products/hybpr_amounts.html

Figure 3.1  Gene structure of chloroplast precursor (AW424439) and location of primers and probes used for genotyping.

The figure shows the location of the primers and probes that were used to genotype the three populations of F_{2:3} families (Table 1). The probe sensor lies directly over the GG indel and the G/C SNP. Note that the figure shows the location of probes and primers on the B73 allele of the gene.
Population Screening and Genotyping

A previously studied mapping population of F\textsubscript{2:3} families derived from the cross of B73 x Mp313E (Brooks et al. 2005), was screened using the probe designed for the Roche LightCycler® 480, and the Genotyping Master Kit. Only 158 F\textsubscript{2} individuals from the F\textsubscript{2:3} population were screened due to a lack of DNA for some of the individuals. Runs were performed in a 96-well Roche plate at a volume of 10 μL (8 μL of master mix and 2 μL of template DNA). The LightCycler® run was performed as follows: a pre-incubation run of 1 cycle at 95°C for 10 minutes with a ramp rate of 4.4°C/s; amplification run of 45 cycles at 95°C for 10 seconds with a ramp rate of 4.4°C/s, 50°C for 10 seconds (with a single acquisition) with a ramp rate of 2.2°C/s, and 72°C for 12 seconds with a ramp rate of 4.4°C/s; a melting curve for 1 cycle which went from 95°C held for 1 minute at a ramp rate of 4.4°C/s then down to 40°C held for 1 minute at a ramp rate of 2.2°C/s and with 2 acquisitions taken per 1°C; and ending with a cooling step run for 1 cycle at 40°C for 30 seconds at a ramp rate of 2.2°C/s.

Two other previously studied mapping populations of F\textsubscript{2:3} families from the crosses between Mp313E (resistant) x Va35 (susceptible) and Mp715 (resistant) x T173 (susceptible) were screened using the shorter, redesigned primer for PCR amplification followed by high resolution non-denaturing 7% PAGE visualized with ethidium bromide. A total of 162 F\textsubscript{2} individuals were screened in the Mp313E x Va35 population and 224 F\textsubscript{2} individuals were screened in the Mp715 x T173 population.

All F\textsubscript{2:3} mapping populations had been previously phenotyped for aflatoxin accumulation in the grain in replicated field trials in multiple fields and years (Willcox 2000, Brooks et al 2005, Warburton et al. 2010); each field/year combination was treated
as a separate environment. The Mp313E x Va35 population was grown and tested at
three different locations Mississippi State, MS (1997 – 2000), Stoneville, MS (2000), and
Westlaco, TX (2000). The Mp313E x B73 population was grown and tested in two
locations Mississippi State, MS (2000-2002) and Stoneville, MS (2000), and the Mp715
x T173 population was grown and tested at Mississippi State, MS (2003-2006).

Statistical Analysis

An analysis of variance (ANOVA) was conducted to determine whether there was
a significant difference in aflatoxin accumulation among genotypes with 0, 1, or 2 copies
of the resistance allele. The means of the three genotype classes were compared using
Fisher’s Protected least significance difference (LSD). The genotypic data for the MpM1
marker from each family in the F2:3 mapping population was combined with the aflatoxin
accumulation data obtained for the same F2:3 population from the Mississippi State, MS
field location in 2000; aflatoxin data has been previously reported for the Mp313E x B73
population in Brooks et al.(2005). The LSD analysis and ANOVA (performed using
Proc GLM) were performed using the SAS® (SAS Institute Inc.; Cary, NC) software
using a significance level of \( \alpha = 0.05 \). The replications for each entry were treated as
individuals, thus resulting in a N of 442.

Mapping and QTL Analysis

The mapping data from the three F2:3 populations were used to map the MpM1
marker to the maize genome. The combined genotypic and phenotypic data were used
for QTL and phenotypic effect analysis in each population. Genetic mapping was
performed using JoinMap and the Maximum Likelihood mapping function (Kyazma B.V.; Wageningen, Netherlands). QTL Cartographer (North Carolina State University; Raleigh, NC) was used for the QTL analysis, using the Composite Interval Mapping function. A LOD score of 2.4, which is the default LOD score used by QTL cartographer, was used to identify the most significant QTL, but all QTL identified with LOD ≥ 2.0 were measured for phenotypic effect of the marker across all environments.

Results

Primer Verification and Sequencing

All of the primer pairs amplified successfully and showed only a single band on agarose gel. Sequencing and alignment revealed that only four of the sequenced genes had polymorphisms among the different lines. These genes were AW424439, AW438153, BE128894, and AW216267. AW424439 had a G/C SNP and a GG indel that were polymorphic between the resistant lines versus the susceptible lines sequenced (Figure 3.2). This was the only gene that had polymorphisms that were conserved between the resistant and susceptible lines sequenced. Seven of the nine other genes gave reliable sequence; the sequences of these seven genes can be found in the Appendix (A.1-A.7). Based on the conservation of the SNP/Indel between the resistant and susceptible lines in AW424439, a probe was developed to screen the gene as a potential marker (Figure 3.2). The sequence for the B73 allele AW424439 gene was aligned to B73 reference sequence using the MaizeGBD BLAST tool (http://blast.maizegdb.org). The alignment showed a 100% homology between the AW424439 sequence and the B73
reference sequence and the AW424439 sequence aligned with nucleotides 27,096,340 to 27,096,835 on chromosome 4.

**Familial Screening**

The probe was used to screen a population of F\textsubscript{2:3} families from the QTL mapping population developed from Mp313E x B73. DNA from a total of 158 F\textsubscript{2} individuals (which had been selfed to create the F\textsubscript{2:3} families that were phenotyped for aflatoxin accumulation) was genotyped based on melting peaks (Figure 3.3). Following the melting curve analysis, 42 individuals were genotyped as having 2 copies of the “resistant” allele (homozygous resistant), 82 individuals were genotyped as heterozygous for the allele, and 34 individuals were genotyped as having 2 copies of the “susceptible” allele (homozygous susceptible).

The redesigned primer pair was used to genotype two more F\textsubscript{2:3} mapping populations to verify marker results in independent genetic backgrounds. Screening of 162 F\textsubscript{2} individuals from Mp313E x Va35 showed that 43 individuals were homozygous for the “resistant” allele, 96 individuals were heterozygous, and 23 individuals were genotyped as homozygous for the “susceptible” allele.

A total of 224 F\textsubscript{2} individuals from a population of F\textsubscript{2:3} families of Mp715 x T173 were also screened. This screen identified 66 individuals as homozygous for the “resistant” allele, 110 individuals as heterozygous, and 48 individuals as homozygous for the “susceptible” allele (Figure 3.4).
Figure 3.2  Multiple alignment of AW424439 gene.

Multiple alignment of Mp313E (resistant), Mp715 (resistant), B73 (susceptible), and Va35 (susceptible) from the sequencing of AW424439 using the primer pair AW424439F and AW424439R (Table 1). Alignment showed multiple indels and SNPs and was performed using DNAMAN. Gray color indicates a 100% match among the four sequences; pink color indicates a match among three of the sequences; blue color indicates a match between two of the sequences.
Figure 3.3  Melting peaks obtained from the parents and \(F_1\) of Mp313E x B73 population of \(F_{2:3}\) families.

Differences in fluorescence due to a higher affinity of the probe for B73 than Mp313E allowed for the genotyping of 158 \(F_2\) individuals from the population of \(F_{2:3}\) families based on the polymorphisms of the AW424439 gene between Mp313E and B73. Analysis was done using the Roche LightCycler® 480, genotyping probe, genotyping primer (Table 1; Figure 2) and Roche Genotyping Master
Figure 3.4   Parents and F₁ from the Mp715 x T173 population of F₂:₃ families as visualized by polyacrylamide gel electrophoresis and ethidium bromide using the AW424489SHRT primer set.

Mp715, T173, and the F₁ can be seen in lanes 2-4, respectively. The sizes of the main bands are all around 150 base pairs (bp). 7% polyacrylamide gel used and size standards in lanes one and five are a Bio-Rad EZ Load 100 base pair molecular ruler

QTL Location and Effect

ANOVA and LSD analysis was run on the individuals from the Mp313E x B73 population to determine if statistically significant differences in aflatoxin levels could be attributed to the polymorphisms. ANOVA analysis using the GLM procedure showed a significant difference between at least two of the genotypes (p-value = 0.0031). Further analysis using LSD breakdown showed that individuals with at least one copy of the “resistant” allele accumulated significantly less aflatoxin than individuals that had no copies of the “resistant” allele (Table 3.3). It was decided that these polymorphisms in
the gene were suitable to use as a marker, which was subsequently name Mississippi Marker 1 (MpM1).

Table 3.3 Least significant difference (LSD) analysis of aflatoxin accumulation of each class of lines grouped by genotype in Mp313E x B73 F2:3 population from Mississippi State, MS field location in 2000

<table>
<thead>
<tr>
<th>In Transformed Mean$^a$</th>
<th>Geometric Mean (ng/g)$^b$</th>
<th>N</th>
<th>Number of Resistance Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.652 a</td>
<td>775.43</td>
<td>103</td>
<td>0</td>
</tr>
<tr>
<td>6.3539 b</td>
<td>573.76</td>
<td>212</td>
<td>1</td>
</tr>
<tr>
<td>6.2833 b</td>
<td>536.59</td>
<td>127</td>
<td>2</td>
</tr>
</tbody>
</table>

LSD = 0.2072
Means followed by the same letter do not significantly differ at $\alpha = 0.05$
$^a$ mean of natural log transformed values;
$^b$ values converted back to original scale expressed as nanograms of toxin per gram of ground maize material

Marker MpM1 mapped to maize chromosome 4, bin 4.03 between previously mapped SSR markers phi079 and umc2082 (Figure 3.5), in all three F2:3 populations. QTL analysis of the Mp313E x B73 highlighted a peak with a significant LOD score (greater than 2.4) in one environment, and the percentage of phenotypic variation in aflatoxin accumulation that could be attributed to MpM1 in that environment was 10.9%. Other environments had identifiable LOD peaks; however, they were not significant at a LOD of 2.4. The MpM1 marker identified a previously unknown QTL region, 19-cM in length, located between the marker MpM1 and the SSR marker umc2082. Resistance was contributed by Mp313E, and the mode of action for resistance was identified as largely additive (Table 3.4).
Figure 3.5  Chromosomal location of MpM1 and QTL in Mp313E x B73 population.

Chromosome map showing location of MpM1 maker, previously unidentified QTL located between MpM1 and umc2082, and other markers and QTL identified in the Mp313E x B73 population.
Table 3.4  QTL analysis of phenotypic effects of MpM1.

<table>
<thead>
<tr>
<th>Population</th>
<th>Environment^d</th>
<th>Chromosome</th>
<th>Bin</th>
<th>Add^e</th>
<th>Dom^f</th>
<th>% Var^g</th>
<th>LOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>B73 x Mp313E^a</td>
<td>MSU 2002</td>
<td>4</td>
<td>4.03</td>
<td>-0.448</td>
<td>0.115</td>
<td>10.9</td>
<td>2.4</td>
</tr>
<tr>
<td>Va35 x Mp313E^b</td>
<td>MSU 1997</td>
<td>4</td>
<td>4.03</td>
<td>-0.191</td>
<td>0.078</td>
<td>6.1</td>
<td>2.0</td>
</tr>
<tr>
<td>T173 x Mp715^c</td>
<td>MSU 2004</td>
<td>4</td>
<td>4.03</td>
<td>0.075</td>
<td>-0.383</td>
<td>&lt; 1</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Analysis conducted in the three mapping populations and in multiple environments; only significant effects (at LOD ≥ 2.0) are presented.

^a Brooks et al. 2005  
^b Willcox et al. 2000  
^c Warburton et al. 2010  
^d R.R. Foil Plant Science Research Center, Mississippi State University, MS  
^e Additive Effect  
^f Dominance Effect  
^g Proportion of phenotypic variance explained by MpM1

In the Mp313E x Va35 population, the MpM1 marker was also significant in one environment, and in other environments had identifiable, but not significant, LOD peaks. MpM1 was responsible for 6.1% of the phenotypic variation in the significant environment. Resistance to aflatoxin accumulation was contributed by Mp313E, and the mode of gene action for MpM1 in this genetic background was also additive (Table 4).

The Mp715 x T173 mapping population was created with a resistant donor inbred, Mp715, that was unrelated to Mp313E (and thus may have different QTLs for resistance). Indeed, MpM1 had a smaller effect in this population, but was still significant in one environment at the LOD = 2.0 level, and was responsible for less than 1.0% of the phenotypic variation. In this population, Mp715 contributed the allele for resistance, which had a mainly dominant gene action (Table 3.4).
Discussion

Previous work examining DNA polymorphisms in maize has shown high numbers of SNPs. In an investigation of 18 maize genes using 36 inbred lines, an average of one SNP per 31 bp in non-coding regions and one polymorphism per 124 bp in coding regions was found (Ching et al., 2002). In this study, results from sequencing the 10 candidate genes showed multiple SNPs and indels between B73 (susceptible) and Mp313E (resistant), as well as among other resistant and susceptible lines. These results are in agreement with previous SNP discovery research in maize.

However, only one of the genes screened, AW424439, showed polymorphisms that were consistently conserved between the resistant and susceptible lines sequenced in this study. This gene, EST accession AW424439, encodes a photosystem II3 protein (accession NP_001148037). This gene was shown to be up-regulated in maize line Va35 and down-regulated in maize line Mp313E (Kelley et al. 2009). It has been reported that plants down regulate multiple genes associated with metabolism and shift resources towards defense in response to pathogen infection (Somssich and Hombrick 1998; Mysore et al. 2003). With the expression pattern of the chloroplast precursor gene showing this response in the resistant Mp313E line and the opposite in the susceptible Va35 line, there is reason to believe that this gene may be linked to or associated with a the active systemic response to fungal infection.

The identified differences between the resistant and susceptible lines allowed the development of a marker that can successfully distinguish among the possible allelic genotypes found in the three screened F_{2;3} populations, using either a probe or a simple
PAGE assay. QTL analyses of different mapping populations using Mp313E as the resistant parent have identified various QTLs associated with significant resistance to aflatoxin accumulation in maize. Two large QTL have been found on chromosome 4 in bins 4.06 and 4.08 in multiple mapping populations (Paul et al. 2003; Brooks et al. 2005; Willcox et al. 2000). Although the gene in which MpM1 is found maps to chromosome 4, this marker uncovered a new QTL located in bin 4.03, quite distant to the previously recorded QTLs on chromosome 4. This QTL was undetected in previous studies most likely due to a lack of marker density in that region. Analysis via QTL Cartographer showed that in all mapping populations, MpM1 showed significant phenotypic effects for resistance to aflatoxin accumulation in at least one environment. The most significant contribution by the MpM1 marker explained 10.9% of the variation associated with resistance to aflatoxin accumulation in the Mp313E x B73 population when the mapping population was grown in 2002 at the Mississippi State University, R. R. Foil Plant Science Research Center. Other populations had lower significant phenotypic effect associated with the marker, but still showed measurable phenotypic effects in at least one environment.

It must be noted that the QTL uncovered by MpM1 was not significant in all environments, and the LOD had to be lowered to 2.0 to measure the phenotypic effect in some of the environments in the Mp313E x Va35 and the Mp715 x T173 populations. Aflatoxin accumulation is a trait of low heritability and very high genotype by environment interactions, and inbred lines displaying good resistance in one location may show considerably less resistance in a different location, or even a different year at the same location (Windham and Williams 2002). Likewise, QTLs associated with
resistance in one environment may not be significant in others (Brooks et al 2005, Warburton et al. 2009). This is due to growth of the fungus and production of the toxin being dependent on the temperature, humidity, and soil moisture conditions, among other factors, in which the maize (and fungus) are growing (Moreno and Kang 1999). Therefore, it is not surprising that QTL identified by this marker may not be identified in every environment; indeed, this is the norm in QTL identification of this and other polygenic traits. In addition, the finding of significance of MpM1 in the Mp715 x T173 population is of considerable importance because Mp715 is a resistant parent unrelated to Mp313E. This shows that even though the effect is not as strong as in the Mp313E derived populations, MpM1 is still associated with a measurable phenotypic effect in a population derived from a different resistant parent (Mp715). The chloroplast precursor gene from which MpM1 was derived may be important in reducing aflatoxin accumulation in many different lines; however, further studies would be needed to confirm its role in resistance.

The methodology to identify a candidate gene and then to find SNPs and indels found in the target sequence allowed for the development of a gene specific probe that was used to screen F$_{2:3}$ family-derived QTL mapping populations. From our examination of genotypic and phenotypic differences between these F$_{2:3}$ families, it is clear that genotyping with MpM1 can successfully aid in distinguishing the resistant individuals from the more susceptible individuals in a fast and economical manner, making MpM1 the first gene-based maker developed for tracking resistance to aflatoxin accumulation in maize. Polymorphism differences among a diverse range of maize genotypes may require mapping of the effect before use in unrelated germplasm. However, it is not
expected to suffer from recombination or linkage drag as are QTL linked but non-gene
based markers, and thus MpM1 can now be incorporated into MAS methods aimed at
introducing resistance to aflatoxin accumulation into elite commercial lines. In addition
to the one gene-based marker presented here, this demonstrates a viable alternative to
genome-wide QTL mapping for rapidly pinpointing genes and gene-based markers
associated with resistance to aflatoxin accumulation in maize. Work is ongoing in the
analysis of other candidate genes identified from other expression and proteomics studies.
Literature Cited


Mp313E mapping population in multiple environments. Aflatoxin / Fumonisin Workshop

Abstract: 120


CHAPTER IV
USE AND VALIDATION OF CHITINASE A MAKER ASSITED SELECTION OF RESISTANCE TO AFLATOXIN ACUMULATION IN MAIZE

Abstract

Maize (Zea mays L.) is a major agricultural commodity and food staple in the United States and globally. Aspergillus flavus, which produces the carcinogenic secondary metabolite aflatoxin, is one of the pathogens that can negatively affect maize and is thus important from a human health and economic perspectives. Many programs to combat A. flavus infection and aflatoxin accumulation have focused on the development of maize lines resistant to A. flavus and aflatoxin accumulation. Molecular markers would speed up the selection for resistance to aflatoxin accumulation, but there are currently not enough to make rapid selection gains for this trait. The chitinase family of genes has been shown to have antifungal activities, and the gene chitinase A (chiA) has specifically been studied for its antifungal activities against A. flavus and other fungi. Therefore, chiA was sequenced to find polymorphisms between resistant and susceptible maize lines which were verified to be associated with resistance in three of F2:3 populations. ChiA was found to be associated with resistance to aflatoxin accumulation in all three populations, and a new quantitative trait loci (QTL) was identified in the Mp313E x Va35 population. An easily scored marker developed from one of the
polymorphisms can now be used to select for resistance to aflatoxin accumulation in marker assisted breeding programs. The entire chitinase family of genes warrants further investigation as a source of gene-based markers for resistance to aflatoxin accumulation.

**Introduction**

*Aspergillus flavus* is a fungal pathogen of maize that produces the toxic secondary metabolite aflatoxin. Aflatoxin is carcinogenic, teratogenic, immunosuppressive, and can be acutely toxic to humans and other animals (Castenargo and McGregor 1998; Hedayati et al. 2007). Of the four main forms of aflatoxin (B1, B2, G1, and G2) produced by fungus, B1 is considered the most toxic and is the primary form produced by *A. flavus*, followed by B2 (Bhatnagar et al. 2002; Scheidegger and Payne 2005). After infection of maize by *A. flavus*, biotic and abiotic stresses such as high heat, high humidity, and insect herbivory can influence *A. flavus* colonization and the amount of aflatoxin produced by the fungus (Moreno and Kang 1999).

Due to the health risks associated with exposure to aflatoxin, 76 countries have imposed limits on the allowable total aflatoxin contamination in food (van Egmond and Janker 2004). In the United States, this aflatoxin action limit is 20 ng/g; in the EU, it is 4 ng/g (van Egmond and Janker 2004). Because grain contaminated at higher levels that this limit is usually destroyed or of limited commercial value, the economic impact of these action limits is estimated to be approximately $200 million annually in direct losses in maize alone (AMCOE 2010). In the southern United States where climatic conditions are favorable for fungal growth and aflatoxin contamination, losses are an annual concern for maize producers (Windham and Williams 1998).
The health concerns and economic burdens related to aflatoxin contamination have led to many areas of research focused on reducing both *A. flavus* infection and aflatoxin accumulation in maize. These areas include post-harvest methods such as decontamination of grain and pre-harvest methods such as biological control and cultural practices including irrigation, insecticides, and fungicides. (Cleveland et al. 2003; Munkvold 2003). Because decontamination and bio-control are an added expense to the farmer/producer, and the efficacy of cultural practices is variable, the most promising branch of research for reducing accumulation of aflatoxin is through breeding programs aimed at increasing host plant resistance. Currently, no commercial hybrids contain any appreciable level of resistance, so breeding programs currently work to identify, develop, and enhance natural sources of resistance with an ultimate goal of creating resistant elite inbred maize lines (Windham and Williams 1998; Abbas 2002; Williams et al. 2005). The results of these breeding programs have been development several breeding lines with resistance to aflatoxin accumulation, including: Mp313E, Mp715, and Mp717 developed by the USDA-ARS Corn Host Plant Resistance Research Unit (Scott and Zummo 1990; Williams and Windham 2001, 2006). However, resistance to aflatoxin accumulation has been found to be difficult to transfer into elite lines or hybrids due to the fact that it is a quantitative trait with low heritability and a high sensitivity to environmental factors (Campbell and White 1995; Hamblin and White 2000). Furthermore, the resistant lines display many undesirable agronomic traits making them unsuitable for immediate use in commercial breeding programs (Betrán et al. 2002).

Marker assisted selection (MAS) would allow for faster development of elite maize lines with resistance to aflatoxin accumulation while excluding undesirable
Quantitative trait loci (QTL) associated with resistance to aflatoxin accumulation have been identified in multiple studies (Davis et al. 1999; Willcox et al. 2000; Paul et al. 2003; Widstrom et al. 2003; Brooks et al. 2005; Robertson-Hoyt et al. 2007; Alwala 2008; Warburton et al. 2009, 2010). QTL contain one or more genes encoding resistance, and multiple linked markers are easily identified within each QTL. Using an entire QTL for marker-assisted selection (MAS) is not ideal because it usually contains a large region of the chromosome, which is difficult to move and may include genes encoding undesirable traits along with the gene(s) of interest. If the gene underlying the QTL could be identified, markers from within the sequence of the gene would be ideal for MAS because they do not suffer from the drawbacks listed above. Candidate genes underlying a QTL, or even those as-yet unidentified via QTL analysis, can be identified through numerous methods including: genomic studies, proteomic studies, identification of defense proteins, and physiological studies.

Chitinase proteins are a group of defense proteins that can limit fungal growth by enzymatically degrading chitin, a component of fungal cell walls (Selitrennikoff 2001; Tiffin 2004). Chitinase proteins have been isolated from maize leaf, silk, and kernel tissues, and they appear to be expressed in response to fungal infection and also may be constitutively expressed in some maize genotypes (Huynh et al. 1992; Cordero et al. 1994; Wu et al. 1994; Moore et al. 2004; Peethambaran et al. 2009). Their anti-fungal properties have been investigated against A. flavus and shown to reduce fungal growth (Huynh 1992; Moore et al. 2004). Proteomics studies by Peethambaran et al. (2009) and Pechanova et al. (2010) have shown differential expression of chitinase proteins in resistant maize lines versus susceptible maize lines. Thus, the chitinase
family of proteins identifies logical candidate genes for association with resistance to aflatoxin accumulation. Of the chitinase genes, chitinase A \((\text{chi}A)\) was identified by Peethambaran et al. (2009) as being significantly more highly expressed in resistant maize line Mp313E than other resistant or susceptible maize lines. Moreover, a study by Huynh et al. (1992) identified \text{chi}A as having higher anti-fungal activity than chitinase B \((\text{chi}B)\). The objectives of this study were: (i) to sequence \text{chi}A in multiple resistant and susceptible maize genotypes and discover any polymorphisms that may be used in further characterization of this gene; (ii) to genotype three F\(_{2:3}\) QTL mapping populations with the discovered polymorphisms; (iii) to map and characterize \text{chi}A with the genotypic and phenotypic data from these populations, and to determine the contribution of \text{chi}A resistance to aflatoxin accumulation and its potential usefulness as a gene-based marker.

Methods

DNA Isolation

DNA used for primer verification and sequencing extracted from five week old maize plants grown in a greenhouse. All the leaves from each plant were harvested, stored on ice, and frozen in liquid nitrogen. Tissue was lyophilized for four days and stored at – 80°C. The lyophilized tissue was then ground, and DNA was extracted using the CTAB extraction method (Saghai-Maroof et al. 1984). DNA for genetic mapping in the three F\(_{2:3}\) mapping populations was obtained in a similar manner, as reported previously (Willcox et al. 2000; Brooks et al. 2005; Warburton et al. 2010). DNA quality
and quantity was verified using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE).

Primer Design and Verification

The nucleotide sequence for *chiA* was retrieved from the European Nucleotide Archive (ENA) which is maintained by the European Molecular Biology Laboratory's European Bioinformatics Institute (EMBL-EBI) (http://www.ebi.ac.uk/ena/). The nucleotide sequence was referenced through UniProt (http://www.ebi.ac.uk/uniprot/) from the protein id Q6JBK8 for *chiA* from Peethambaran et al. (2009). The nucleotide accession number for *chiA* is AY532775 and was sequenced from *Zea mays* ssp. *parviglumis* cultivar PI331783 (Tiffin 2004). From the nucleotide sequence, two primers (ChitinaseA-F 5’- TGCCAGCCTAACTTCTGC -3’ and ChitinaseA-R 5’-GGTGCACGTTGTTCATCC -3’) were designed to amplify a region spanning ≈ 600 base pairs (bp) (Table 4.1). The primer pair was designed using Primer3 on-line software version 0.4.0 (http://frodo.wi.mit.edu/primer3/) and ordered from Sigma-Genosys (The Woodlands, TX) (Rozen and Skaletsky 2000).

The ChitinaseA primer pair was verified via polymerase chain reaction (PCR) on four maize genotypes, Mp313E, Mp715, B73, and Va35. PCR was performed on an Eppendorf Mastercycler® personal (Eppendorf AG, Hamburg, Germany) using *Taq* DNA polymerase and buffer from Sigma Life Sciences (Sigma-Aldrich, Inc., St. Louis, MO). The PCR mixture contained 1µL of genomic DNA from each genotype, 1µL of ChitinaseA-F and ChitinaseA-R primers (10 µM), 5 µL of 10x PCR buffer containing MgCl₂, 1 µL dNTP mixture (10 mM) from Invitrogen™ (Invitrogen Corporation,
Carlsbad, CA), and 40 µL of autoclaved, distilled water for a final total volume of 50 µL. The PCR steps were as follows: Initial denaturation at 95°C for 4 minutes followed by 35 cycles of 94°C for 1 minute, 56°C for 2 minutes, 72°C for 1.5 minutes, followed by a final elongation step of 72°C for 5 minutes. Amplified products from PCR were analyzed via agarose gel electrophoresis on a 1.5 % (w/v) agarose gel using a 1x TAE buffer and visualized by ethidium bromide staining, de-staining in water, and UV illumination.

**PCR Purification and Sequencing**

For sequencing, PCR was repeated using the ChitinaseA primer pair but with Invitrogen Platinum® Taq DNA polymerase, buffer, and MgCl₂ mixture (Invitrogen Corporation, Carlsbad, CA). The components of Platinum® Taq PCR mixture were 0.2 µL of Platinum® Taq, 5 µL of 10x buffer, 1.5 µL of 50 mM MgCl₂, 1 µL of genomic DNA (Mp313E, Mp715, B73, Va35), 1 µL of the ChitinaseA primer pair, 1 µL dNTP (10 mM) mixture from Invitrogen™, and 39.3 µL of autoclaved, distilled water for a final volume of 50 µL. The cycle parameters for PCR remained the same as for primer verification. After PCR amplification was complete, PCR products were visualized using gel electrophoresis of a 1.5 % agarose gel (w/v) and ethidium bromide staining to assure successful amplification and presence of only single bands. The PCR products were purified using the Qiagen QIAquick™ PCR Purification Kit (Qiagen Inc., Valencia, CA) and following the manufactures protocols with the final elution being conducted using 30 µL of autoclaved, distilled water.

Sequencing was conducted by the Iowa State University DNA Facility (Ames, IA, http://www.dna.iastate.edu/index.html) using Big Dye® chemistry from Applied
Biosytsems Inc. (Foster City, CA) for the sequencing reaction, which was analyzed using an Applied Biosystems 3730xl DNA Analyzer (Life Technologies Corporation, Carlsbad, CA). As per facility guidelines, the purified PCR products were diluted to 16.25 ng/µL, and the primers (ChitinaseA-F and ChitinaseA-R) were diluted to 5 µM. Sequences were aligned using DNAMAN software v. 5.2.9 (Lynnon Corporation; Pointe-Claire, Quebec, Canada), and chromatographs were viewed using Chromas software version 2.33 (Technelysium Pty Ltd, Brisbane, Australia, http://www.technelysium.com.au/chromas.html).

Design of Genotyping Primers

Based on polymorphisms in \textit{chiA} among the maize genotypes sequenced, two sets of primers were designed for use in genotyping via polyacrylamide gel electrophoresis (PAGE) (Table 4.1; Figure 4.1 and Figure 4.2). The primers were designed using Primer3 on-line software version 0.4.0 (http://frodo.wi.mit.edu/primer3/) and ordered from Sigma-Genosys (The Woodlands, TX) (Rozen and Skaletsky 2000). Primer pair ChiAMp3B73 was designed around an insertion/deletion (indel) found between Mp313E and B73 (Figure 4.1), and primer pair ChiAMp3Va35 was designed around an indel found between Mp313E and Va35 (Figure 4.2). Short sequence repeat (SSR) primer pair umc1783 was designed by the Maize Mapping Project (MMP) around a TTA repeat and was designed from the EST AW574496 (http://www.maizemap.org/; http://www.maizegdb.org/cgi-bin/displayssrrecord.cgi?id=292369)
Table 4.1 Primers for sequencing and genotyping of chiA.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’ to 3’)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primer for Sequencing</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ChitinaseA-F</td>
<td>TGCCAGCCTAACTTCTGC</td>
<td>61.7</td>
</tr>
<tr>
<td>ChitinaseA-R</td>
<td>GGTGCACGTGGTTCATCC</td>
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<td><strong>Primers for Genotyping</strong></td>
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<tr>
<td>ChiAMp3B73F</td>
<td>CTGCAGCAAGTTCGGCTACT</td>
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<td>ChiAMp3B73R</td>
<td>CGCTCCGGGTGTAGAAGTT</td>
<td>64.1</td>
</tr>
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<td>ChiAMp3Va35F</td>
<td>CTTCAACGGCATAAGAACC</td>
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<td>umc1783-F</td>
<td>ATTCATTTCAGTCGAAACAAAGTG</td>
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</tr>
<tr>
<td>umc1783-R</td>
<td>GCTAGCTCGTCCAAATAATGCT</td>
<td>66.2</td>
</tr>
</tbody>
</table>

Genotyping primers were designed from the sequences of Mp313E, Mp715, B73, and Va35, and primers were used to genotype the Mp313E x Va35 and Mp715 x T173 F2:3 populations using PAGE. Sequence of umc1783 primer was obtained from MaizeGDB (http://www.maizegdb.org/)
Figure 4.1  **Alignment of Mp313E and B73 chiA gene segment amplified by ChitinaseA primer pair.**

Figure shows the location of the ChiAMp3B73F primer and the ChiAMp3B73R primer as well as the indel which the primer pair was designed around for genotyping. Gray color indicates a 100% match between the sequences; a blue color indicates a mismatch between the two sequences.
Figure 4.2  Alignment of Mp313E and Va35 \textit{chiA} gene segment amplified by ChitinaseA primer pair.

Figure shows location of the ChiAMP3Va35F primer and ChiAMP3Va35R primer. Also shown are the indels which the primers were designed around for genotyping. Grey color indicates a 100\% match between the two sequences; blue indicates a mismatch between the two sequences.
Population Screening and Genotyping

The Mp313E (resistant) x B73 (susceptible) population of 184 F_{2:3} families were screened by Brooks et al. (2005) with 225 SSR primers from the Maize Genetics and Genomics Database (MaizeGDB; http://www.maizegdb.org/). SSR markers were screened using either 4% agarose gel or PAGE, and one of the markers used in the screening was marker umc1783. The PCR parameters for used were: 95^\circ C for 1 minute, decrease in temperature from 65^\circ C to 55^\circ C by decreasing 1 degree each cycle and repeated 30 times once 55^\circ C is reached, this cycle was 1 minute, 72^\circ C for 1.5 minutes (Brooks et al. 2005).

The Mp313E x Va35 population of 93 F_{2:3} families was screened using a primer pair of ChiAMp3Va35F and ChiMp3Va35R. The cycle parameters for the PCR were as follows: Initial denaturation at 95^\circ C for 2 minutes followed by 35 cycles of 94^\circ C for 1 minute, 62^\circ C for 1 minute, 72^\circ C for 2 minutes, followed by a final elongation step of 72^\circ C for 5 minutes. The PCR products were visualized and scored using a 7% PAGE stained with ethidium bromide.

For the Mp715 x T173 population of 180 F_{2:3} families, a primer pair of ChiAMp3Va35F and ChiMp3Va35R was used, and a PCR cycle of: Initial denaturation at 95^\circ C for 2 minutes followed by 35 cycles of 94^\circ C for 1 minute, 62^\circ C for 1 minute, 72^\circ C for 2 minutes, followed by a final elongation step of 72^\circ C for 5 minutes. The PCR products for the population was visualized and screened in the same manner as the Mp313E x Va35 population of F_{2:3} families.
All mapping populations had been previously phenotyped for aflatoxin accumulation in the grain in replicated field trials in multiple fields and years (Willcox 2000, Brooks et al 2005, Warburton et al. 2010). Each field/year combination was treated as a separate environment. The Mp313E x Va35 population was grown and tested at three locations Mississippi State, MS (1997 – 2000), Stoneville, MS (2000), and Westlaco, TX (2000). The Mp313E x B73 population was grown and tested in two locations Mississippi State, MS (2000-2002) and Stoneville, MS (2000), and the Mp715 x T173 population was grown and tested at Mississippi State, MS (2003-2006).

Mapping and QTL Analysis

The mapping data from the three F2:3 populations were used to map the $\text{chiA}$ marker to the maize genome. The combined genotypic and phenotypic data were used to analyze the QTL position and phenotypic effect of each marker in each population. Genetic mapping was performed using JoinMap (Kyazma B.V.; Wageningen, Netherlands) and the Maximum Likelihood mapping function. QTL Cartographer (North Carolina State University; Raleigh, NC) was used for the QTL analysis, using the Composite Interval Mapping function. A LOD score of 2.4, which is the default LOD score used by QTL cartographer, was used to identify the most significant QTL. Single marker analysis was used to test for an association between $\text{chiA}$ and phenotypic variation in the Mp715 x T173 mapping population. The phenotypic assessment used was accumulation of aflatoxin measured in ng/g and transformed via natural log transformation to normalize distributions. A chi-square test was performed on all three
mapping populations to determine whether there were significant deviations from the expected 1:2:1 marker segregation ratio.

**Results**

**Primer Verification, Sequencing, and Alignment Results**

The primer pair ChitinaseA amplified in Mp313E, Mp715, B73, and Va35 (Figure 4.3). In relation to the ladder, the amplified band was approximately 660 bp in size. This was larger than the expected fragments size of 600 bp based on the sequence used to design the primers.

A multiple alignment of all four sequenced maize inbred lines revealed multiple polymorphisms among the four lines in the form of single nucleotide polymorphisms (SNP) and indels (Figure 4.4). The four lines were also aligned with the mRNA sequence for *chiA* from B73 as published by Hyunh et al. (1992). This alignment showed an almost 100% homology with the gene segment sequenced from B73 using the ChitinaseA primer pair (Figure 4.4). When the B73 *chiA* segment sequence was aligned to the B73 reference genome at MaizeGDB (B73 RefGen_v2) using BLAST, the segment had a 100% homology to the reference genome and the first referenced hit was to the sequence for *chiA* (Figure 4.5). The *chiA* segment of B73 aligned to nucleotides 33,534,296 to 33,534,967 on maize chromosome 2 which falls in bin 2.04 in the maize genome.

The genotyping primer pair ChitAMp3Va35 was tested on the parents and F<sub>1</sub> from the Mp313 x Va35 and Mp715 x T173 mapping populations. It was shown to be
able to distinguish among the two parents and the F1 in each population (Figure 4.6A and 4.6B).

![Figure 4.3](image)

Figure 4.3  PCR products of the genomic DNA from Mp313E, Mp715, B73, and Va35 amplified with ChitinaseA primer pair.

Products were visualized on a 1.5 % agarose gel stained with ethidium bromide. The lanes contain the following from left to right: Invitrogen 1 kb plus ladder (Invitrogen Corporation, Carlsbad, CA), Mp313E, Mp715, B73, B73, and Va35. The amplified bands are approximately 660 bp in size.
Figure 4.4 Multiple alignment of the \( \text{chi}A \) gene segment.

Multiple alignment of the amplified gene segment using the ChitinaseA primer pair with Mp313E, Mp715, B73, and Va35, as well as the mRNA sequence from \( \text{chi}A \) as published by Hyunh et al. (1992). The multiple alignment was done using DNAMAN software v. 5.2.9 (Lynnon Corporation; Pointe-Claire, Quebec, Canada). Gray color indicates a 100% match among all five sequences; a pink color indicates a match among four of the sequences; a blue color indicates a match among three of the sequences; white indicates a match between two or fewer of the sequence.
Figure 4.5  
Alignment of \textit{chiA} gene segment with B73 reference genome.

Alignment results from a BLAST to the B73 maize reference genome (B73 RefGen_2) from MaizeGDB (http://www.maizegdb.org/) with \textit{chiA} segment amplified from B73 genomic DNA. Query is the amplified \textit{chiA} B73 gene segment and Sbjct is the B73 reference genome. The amplified segment of B73 had a 100\% homology the B73 reference genome.
Figure 4.6  PCR products using the ChiAMp3VA35 primer pair.

(A) PCR products of the genomic DNA from Mp313E, Va35, and F₁ hybrid (MpVF₁) amplified with the ChiAMp3Va35 primer pair. Products were visualized on a 2.5 % agarose gel stained with ethidium bromide. The lanes contain the following from left to right: Invitrogen 1 kb plus ladder (Invitrogen Corporation, Carlsbad, CA), Mp313E, Va35, and MpVF₁. (B) PCR products of the genomic DNA from Mp715, T173, and F₁ hybrid (MpTF₁) amplified with the ChiAMp3Va35 primer pair. Products were visualized on a 2.5 % agarose gel stained with ethidium bromide. The lanes contain the following from left to right: Invitrogen 1 kb plus ladder (Invitrogen Corporation, Carlsbad, CA), Mp715, T173, and MpVF₁.
Genotyping of F$_2$:3 Mapping Populations

The Mp313E x B73 population of F$_2$:3 families was originally screened by Brooks et al. (2005) with the umc1783 SSR primer pair (which amplifies in the chiA gene). However, the results from the umc1783 marker were not reported in that article. A total of 184 families were screened and 30 were homozygous for the Mp313E allele, 37 were homozygous for the B73 allele and 88 were heterozygous. Four were genotyped as being either homozygous B73 or heterozygous, three were genotyped as being either homozygous Mp313E or heterozygous, and 22 could not be genotyped at all.

The Mp313E x Va35 population was screened here using the ChiAMp3Va35 primer pair. A total of 93 families were screened, of which 19 were homozygous for the Mp313E allele, 22 were homozygous for the Va35 allele, and 43 were heterozygous. One family was genotyped as either homozygous Va35 or heterozygous and eight could not be scored.

The Mp715 x Va35 population was screened using the ChiAMp3Va35 primer pair (Figure 4.7). Of 180 families screened using the primer pair, 53 were homozygous for the Mp715 allele, 33 were homozygous for the T173 allele, and 94 were heterozygous. Chi-square tests were performed on all three populations, and none of the mapping populations significantly deviated from an expected 1:2:1 ratio at $\alpha = 0.05$.

QTL Mapping and Analysis

Analysis with JoinMap placed chiA in bin 2.04 in all three populations. In the Mp313E x B73 population the chiA marker umc1783 defined one side of a large QTL
that was present in all but one of the environments tested and this QTL was defined on
the other side by marker mmc0271 (Figure 4.8, 4.9A). This QTL was previously
reported in Brooks et al. (2005); however, the significance of umc1783 was not reported.
The maximum LOD score was 9.0, and the marker was shown to account for a maximum
of 22.5% of the phenotypic variation (accumulation of aflatoxin) (Table 4.2).

![PAGE showing the PCR products of the Mp715 x T173 mapping population screened using the ChiAMp3Va35 primer pair.](image)

PCR products scored were ~ 200 base pair in size. Individuals were genotyped as being A (two copies of Mp715 chiA allele), B (two copies of T173 chiA allele) or H (heterozygous). The heterozygous individuals display a heteroduplex set of bands between 400 and 500 base pairs. 7% polyacrylamide gel used and ladder is a Bio-Rad EZ Load 100 base pair molecular ruler
Figure 4.8 QTL Cartographer output showing the \textit{chi}A QTL on chromosome 2.

QTL output of QTL analysis from the Mp313E x B73 population. The horizontal pink line signifies the LOD cut-off significance score of 2.4. The different colored lines show different environments.

In the Mp313E x Va35 population, \textit{chi}A was genotyped using the ChiMp3Va35 primer pair and defined one side of a new QTL located between markers ChiMp3Va35 and umc5a (4.9B). This QTL was not present in all environments. The maximum LOD score of this QTL was 3.4 and accounted for a maximum of 16.3\% of the phenotypic variation (Table 4.2).
ChiA was genotyped in the Mp715 x T173 population using the ChiAMp3Va35 primer pair. The marker was not significant at a LOD of 2.4, but a peak was detectable with a LOD of 2.1. Due to the fact that the marker was not significant at the default cut off point in the population, single marker analysis was used to determine whether or not there was a significant association between chiA and aflatoxin accumulation. The results showed that at $\alpha = 0.05$ there was a significant relationship between chiA and the phenotypic variation for aflatoxin accumulation.

Table 4.2 QTL analysis of phenotypic effects of chiA in the Mp313E x B73 and Mp313E x Va35 mapping populations and in multiple environments.

<table>
<thead>
<tr>
<th>Population</th>
<th>Environment</th>
<th>Chromosome</th>
<th>Bin</th>
<th>Add $^c$</th>
<th>Dom $^f$</th>
<th>% Var $^g$</th>
<th>LOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mp313E x B73</td>
<td>MSU 2000</td>
<td>2</td>
<td>2.04</td>
<td>-0.2849</td>
<td>-0.1648</td>
<td>10.3</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>MSU 2001</td>
<td>2</td>
<td>2.04</td>
<td>-0.5383</td>
<td>-0.1153</td>
<td>22.5</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>Stoneville 2000</td>
<td>2</td>
<td>2.04</td>
<td>-0.3585</td>
<td>-0.1572</td>
<td>15.4</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>Overall</td>
<td>2</td>
<td>2.04</td>
<td>-0.2372</td>
<td>-0.1825</td>
<td>9.8</td>
<td>6.8</td>
</tr>
<tr>
<td>Mp313E x Va35</td>
<td>MSU 1997</td>
<td>2</td>
<td>2.04</td>
<td>-0.2944</td>
<td>0.1022</td>
<td>16.3</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>Overall</td>
<td>2</td>
<td>2.04</td>
<td>-0.3389</td>
<td>0.0026</td>
<td>14.2</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Only significant effects (at LOD $\geq 2.4$) are presented.

$^a$ Brooks et al. 2005  
$^b$ Willcox et al. 2000  
$^d$ R.R. Foil Plant Science Research Center, Mississippi State University, MS  
$^e$ Additive Effect  
$^f$ Dominance Effect  
$^g$ Proportion of phenotypic variance explained by chiA
Figure 4.9 Chromosomal locations of chiA QTL in Mp313E x B73 and Mp313E x Va35 populations.

(A) Location of the QTL on chromosome 2 in the Mp313E x B73 population. The QTL is defined by chiA (umc1783) and mmc0271. (B) Location of the QTL on chromosome 2 in the Mp313E x Va35 population. The QTL in this population is defined by chiA (ChiAMp3Va35) and umc5a. Gene name is in italics and actual marker name is in ( ).
Discussion

This research was focused on the potential use of one of the many maize genes from the chitinate family as a marker for resistance to aflatoxin accumulation. Chitinases are a class of proteins used by plants in defense against fungal pathogens (Selitrennikoff 2001; Theis and Stahl 2004; Tiffin 2004). Due to their antifungal properties, chitinases have been studied for their role in resistance to fungal pathogens in maize (Huynh et al. 1992; Cordero et al. 1994; Wu et al. 1994; Moore et al. 2004; Peethambaran et al. 2009; Naumann and Wicklow 2010; Pechanova et al. 2010). With specific evidence pointing to chiA as a protein associated with response of maize to \textit{A. flavus}, \textit{chiA} was chosen as a candidate for potential use as a gene-based marker for resistance to aflatoxin accumulation (Wu et al. 1994; Moore et al. 2004; Peethambaran et al. 2009; Pechanova et al. 2010).

Gel electrophoresis and sequencing results showed that the amplified fragment was larger than expected. However, this was not surprising since the primers were designed from an EST sequence, and thus the larger size of the amplified fragment can be attributed to an intron that was not present in the EST sequence used to design the primers. Indels found among the lines allowed for the development of two primer pairs that could be used in genotyping of the Mp313E x B73, Mp313E x Va35, and Mp715 x T173 populations of F$_{2;3}$ families. There are multiple chitinases in the maize genome and the possibility of gene duplication in the maize genome. The 100% homology between our B73 sequence and the reference genome was good validation that the gene segment amplified via PCR was in fact \textit{chiA}. Furthermore, the BLAST search allowed the discovery of a SSR marker, umc1783, in \textit{chiA} that had already been mapped in the
Mp313E x B73 mapping population, but the effects of this particular marker were not reported (Brooks et al. 2005). In our re-analysis, umc1783 defined one end of a large QTL on chromosome 2 (that had already been reported by Brooks et al. 2005), and this QTL had a maximum LOD of 9.0 in one environment and was significant in all but one environment (MSU 2001). The newly designed chiA primers (ChiAMp3Va35 pair) allowed for the genotyping of both the Mp313E x Va35 and the Mp715 x T173 mapping populations because in these populations the umc1783 marker was not polymorphic. In the Mp313E x Va35 population, the chiA marker helped to uncover a new, previously unreported QTL. However, the LOD was lower in this population and the QTL was not significant in as many environments as it was in the Mp313E x B73 population.

In the Mp715 x T173 population, chiA did not uncover a new QTL and a maximum LOD of 2.1 was observed, which is lower than the accepted limit of 2.4. However, single maker analysis indicated that at $\alpha = 0.05$, there was a significant association between chiA and resistance to aflatoxin accumulation. Therefore, though the relationship between chiA and aflatoxin accumulation is not strong enough to show a LOD great than 2.4, there is still an association between chiA and reduced aflatoxin accumulation in the Mp715 x T173 population. Thus, chiA has been shown to have a significant association with resistance to aflatoxin accumulation in three populations and chiA was associated with strong effects in the Mp313E x B73 and Mp313 x Va35 populations. However, this association does not prove that chiA is the causative gene in the QTL. The association between chiA and resistance may be because chiA is linked to the actual gene(s) conferring resistance to aflatoxin accumulation in the QTL. More
research on the chiA and its effects will be necessary before any definitive conclusion on
chiA’s role in resistance to aflatoxin accumulation can be drawn.

Nevertheless, the association between \textit{chiA} and resistance to aflatoxin
accumulation is not surprising given the results of previous research on \textit{chiA} and the
antifungal properties of its gene product. Proteomics research found that chitinase
proteins were significantly differentially regulated between resistant and susceptible lines
of maize in different tissues when challenged with \textit{A. flavus} under control conditions
(Peethambaran et al. 2009; Pechanova et al. 2010). The ChiA protein, one of the
chitinases studied by Peethambaran et al. (2009), showed significantly higher activity in
resistant maize lines Mp313E and Mp420 than in susceptible lines SC212m and Mp339.

Huynh et al. (1992) reported antifungal activities of chiA from maize line B73,
and that this activity was higher than chiB activity. Building on this research, Moore et
al. (2004) investigated the properties of a chitinase purified from resistant maize line
Tex6. This chitinase was found to be most similar to chiA and chiB, but it differed
somewhat in its amino acid sequence. Interestingly, the chitinase isolated in that study
from Tex6 was more effective at inhibiting fungal growth \textit{in vitro} than chiA or chiB from
B73 (Moore et al. 2004). Finally, a study in 2010 by Naumann and Wicklow found that
chiA protein from maize line LH82 was more resistant to modification from a protein
secreted by the fungus \textit{Stenocarpella mayadis} than was chiA from B73. \textit{S. mayadis} is a
fungus that causes ear rot in maize, and LH82 is resistant to this rot while B73 is
susceptible. Nucleic acid and amino acid alignments showed that LH82 differed
significantly from B73 chiA in sequence and that some of the genomic sequence changes
resulted in amino acid changes in signal, binding, and activity domains of the chitinase
protein. Interestingly, the Mp313E, Mp715, and LH82 alleles of \textit{chiA} share two common polymorphisms that differentiate them from B73 (Figure 4.10). Therefore, it is possible that Mp313E and Mp715 may share some modifications that make them more resistant to fungal proteins that inhibit chiA’s activity. However, more research into the protein properties of Mp313E and Mp715 chiA would be necessary before a complete conclusion could be drawn, and these protein properties would need to be compared to those of the Tex6 chitinase.

The results from this study and previous research suggest that \textit{chiA} may play an important role in maize resistance to \textit{A. flavus} and subsequent aflatoxin accumulation (Huynh et al. 1992; Moore et al. 2004; Peethambaran et al. 2009; Naumann and Wicklow 2010; Pechanova et al. 2010). Therefore, the \textit{chiA} marker should be used to create Near Isogenic Lines of maize in order to validate the effect of \textit{chiA} on resistance and determine whether \textit{chiA} is causative or only linked to the causative gene(s). Furthermore, it would be prudent to investigate \textit{chiA}’s effect on resistance to aflatoxin accumulation in other mapping populations and resistant lines other than Mp313E and Mp715.

Investigation of the properties of the chiA in lines such as Mp313E and Mp715 could help to determine any functional similarities between chiA from Mp313E and Mp715 and previously studied chiAs from LH82 and Tex6. Finally, these results and other research suggest that further investigation of other maize chitinases for their potential use as gene-based markers for resistance to aflatoxin accumulation is warranted.
Figure 4.10  Multiple alignment of chiA segments from maize lines LH82, Mp313E, Mp715, and B73.

Multiple polymorphisms can be observed among the lines as well as some which are conserved between LH82, Mp313E, and Mp715 and B73. Gray color indicates a 100% match among all four sequences; pink color indicates a match among three of the sequences; blue color indicates a match between two sequences
Literature Cited


CHAPTER V
SUMMARY

*Aspergillus flavus* and its secondary metabolite aflatoxin present a public health concern and is an economic burden that impacts farmers of maize, cotton, peanuts, and other crops. This project is part of an ongoing effort to incorporate resistance to aflatoxin accumulation into elite maize lines. The research presented in this study builds on research conducted by investigators at the USDA-ARS Corn Host Plant Resistance Research Unit, Mississippi State University, and other government and non-government labs focused on understanding *Aspergillus flavus*, aflatoxin, and the host/pathogen interaction. The overall goal of this project was the development of gene-based markers for resistance to aflatoxin accumulation, specifically the goals of this project were:

I. Develop a methodology for screening candidate genes for their usefulness as gene-based markers.

II. Use candidate genes from a microarray study of Mp313E (resistant) vs. Va35 (susceptible) infected with *A. flavus* (Kelley et al. 2009) and previous studies involving the chitinase A gene (Moore et al. 2004; Peethambaran et al. 2009) to develop gene-based markers from the DNA sequence of these genes.
III. Test the association of some of these markers with aflatoxin accumulation resistance in a pilot study to demonstrate successful use of the screening methodology developed in Objective I.

The methodology used in this study to test and develop gene-based markers was as follows: (i) identify candidate genes and obtain sequences for candidate genes from NCBI or maizeGBD, (ii) design primers for candidate genes and sequence the gene or part of gene in multiple resistant and susceptible lines of maize to find polymorphisms between resistant and susceptible lines, (iii) design a screening method for chosen polymorphisms (this can be a probe, primers for PAGE, or other methods), (iv) screen the marker in populations of $F_{2:3}$ families to genotype the families, (v) map the marker in each of the mapping populations screened and perform QTL analysis to determine phenotypic effect of the marker and its potential use as a gene-based marker for resistance to aflatoxin accumulation.

This methodology was successful in characterizing two genes associated with resistance to aflatoxin accumulation and developing two gene-based markers (MpM1 and $chiA$); the methodology could be used in the future to develop more markers associated with resistance. Furthermore, this general methodology was adopted and modified by the USDA-ARS Corn Host Plant Resistance Research Unit for development of a candidate gene verification platform (Warburton et al. 2011). This method, which can be expensive and time consuming, currently involves sequencing of each candidate gene in multiple maize lines to determine whether or not useful polymorphisms exist between resistant and susceptible maize lines. In progress is a project to sequence the complete genome from 300 resistant and susceptible maize lines that will help accelerate the process of
identifying polymorphisms and would eventually be of lower expense due to the proliferation of high-throughput sequencing technologies.

The first gene-based marker developed using this methodology was the MpM1 marker developed from a gene encoding a photosystem II3 protein (accession AW424439). This gene was chosen from a list of genes shown to be differentially regulated in a microarray study between Mp313E and Va35 two days after infection with *A. flavus* (Kelley et al. 2009). Nine other genes were also examined from the list of differentially regulated genes, but only AW424439 had polymorphisms that were conserved between the resistant and susceptible lines. The Mp313E x B73 mapping population was screened using a probe based on a GG indel and a G/C SNP. While this method worked well, the probe was expensive and thus a new primer pair was developed around the indel to screen the Mp313E x Va35 and Mp715 x T173 populations using PAGE. Mapping and QTL analysis showed that MpM1 mapped to the same location in each population and showed significant effects for resistance to aflatoxin accumulation in all three mapping populations. In the Mp313E x B73 population the MpM1 maker accounted for 10.9% of the phenotypic variation in one environment. However, in the Mp313E x Va35 and Mp715 x T173 populations the maker accounted for less of the phenotypic variation, and the LOD had to be lowered to 2.0 to measure the effect.

Whether the photosystem II3 gene from which MpM1 was derived plays a direct role in resistance to aflatoxin accumulation cannot be confirmed from this research, and thus more research would be needed into the specific gene and protein action. However, it is linked to a resistance effect, and the fact that this gene is upregulated in the susceptible line and down regulated in the resistant line may indicate that it is part of an
overall pathogen response pathway that is more efficient in resistant lines than in the susceptible lines. This would agree with research that shows plants shift resources from plant metabolism to defense pathways in response to pathogen infection (Somssich and Hombrick 1998; Mysore et al. 2003).

The second gene-based marker developed was based on polymorphisms found in the chitinase A gene \((chiA)\). The chitinase A gene product has been shown to have antifungal properties and is upregulated in resistant maize lines (Hyunh et al. 1992; Wu et al. 1994; Moore et al. 2004; Peethambaran 2009; Pechanova 2010). Furthermore, other chitinase genes have been shown to be important in maize’s response to fungal pathogens, making \(chiA\) a logical choice as a candidate gene for development of a gene-based marker.

All mapping and QTL analyses were performed using primers designed around indels found between the resistant and susceptible maize lines or using the previously designed SSR marker umc1783. Mapping showed that the \(chiA\) marker mapped to the same location in all populations and that the marker showed significant effects for resistance to aflatoxin accumulation in the Mp313E x B73 and Mp313E x Va35 populations in multiple environments. The maximum effect in the Mp313E x B73 population was 22.5%, and the maximum effect in the Mp313E x Va35 population was 16.3%. There was a noticeable peak in the Mp715 x T173 mapping population, but its LOD was just below 2.4 and thus its effect was not measured. However, single maker analysis showed that at \(\alpha = 0.05\) there was a significant association between \(chiA\) and resistance to aflatoxin accumulation in the Mp715 x T173. This suggests that in the Mp715 x T173 population there is a weak association between \(chiA\) and resistance to
aflatoxin accumulation, but that either the association or the gene effect is not strong enough to be significant at a LOD of 2.4 using CIM.

Both the MpM1 and chiA showed a significant association with resistance to aflatoxin accumulation in multiple populations and in at least one environment. Therefore, it was decided to test the effect of having both markers and determine if there were any epistatic interactions between the genes. This process involves performing CIM for the entire mapping data set for each population and then performing Multiple Interval Mapping (MIM) using the QTL that were identified by CIM. This process can be time consuming, so an ANOVA was first conducted to determine whether there was a significant difference in aflatoxin accumulation among the genotypic combinations that could occur for individuals containing MpM1 and chiA. Individuals were scored based on their genotype for each gene, and any individuals with missing data were discarded. This gave classes of AA, AH, AB, BA, BB, BH, HA, HB, and HH where A means homozygous for the “resistant” form of the gene and B means homozygous for the “susceptible” form of the gene, and H means heterozygous; the first marker corresponded to chiA and the second to MpM1. Therefore, an individual that was AA was homozygous for the “resistant” forms of chiA and MpM1 genes. The means of the nine genotype classes were compared using Analysis of Variance (ANOVA) and Fisher’s Protected least significance difference (LSD). Statistical analyses were conducted using SAS® (SAS Institute Inc.; Cary, NC) at $\alpha = 0.05$. This test was only performed for the Mp313E x B73 populations because it was the only population where both MpM1 and chiA were significant in the same environment (Mississippi State, MS 2000). The statistical analysis showed that individuals that were homozygous for the two
“susceptible” alleles accumulated significantly more aflatoxin than individuals that were homozygous for the “resistant” alleles (Table 5.1).

Based on the results from the statistical analysis it was concluded that MIM should be performed to determine if there was any interaction between the markers. The MIM analysis showed that there was no epistasis between the MpM1 and the $\chi_A$ markers. However, the MIM showed that the effects of the two markers were additive and that together the two markers were associated with 27% of the phenotypic variation in that environment. Therefore, it would be advantageous to move both markers in concert when breeding for resistance to aflatoxin accumulation.

The results from this study are very promising, but also show the difficulties when working with a complex, quantitative trait such as resistance to aflatoxin accumulation. Though both markers were found to have significant effects in multiple populations; however, no marker was significant in every environment, and only the Mp313E x B73 population had significant effects at both MpM1 and $\chi_A$ in the same environment. This, however, is the norm for aflatoxin accumulation which has high genotype by environment interactions and for which even the largest QTL vary between environments and populations (Windham and Williams 2002; Brooks et al. 2005; Warburton et al. 2009).
Table 5.1  Least significant difference (LSD) analysis of aflatoxin accumulation of each class of lines grouped by genotype in Mp313E x B73 F2:3 population for the MpM1 and \( chiA \) markers.

<table>
<thead>
<tr>
<th>In Transformed Mean(^a)</th>
<th>Geometric Mean (ng/g)(^b)</th>
<th>N</th>
<th>Genotype Classes(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4672 ( a )</td>
<td>1749.70</td>
<td>8</td>
<td>BB</td>
</tr>
<tr>
<td>7.0556 ( ab )</td>
<td>1159.33</td>
<td>33</td>
<td>BH</td>
</tr>
<tr>
<td>6.9780 ( bc )</td>
<td>1072.77</td>
<td>17</td>
<td>HB</td>
</tr>
<tr>
<td>6.7913 ( bcd )</td>
<td>890.07</td>
<td>4</td>
<td>AB</td>
</tr>
<tr>
<td>6.6994 ( bcd )</td>
<td>811.92</td>
<td>11</td>
<td>AH</td>
</tr>
<tr>
<td>6.6686 ( bcd )</td>
<td>787.29</td>
<td>5</td>
<td>BA</td>
</tr>
<tr>
<td>6.6627 ( bcd )</td>
<td>782.66</td>
<td>40</td>
<td>HH</td>
</tr>
<tr>
<td>6.5421 ( cd )</td>
<td>693.74</td>
<td>10</td>
<td>AA</td>
</tr>
<tr>
<td>6.4752 ( d )</td>
<td>648.85</td>
<td>18</td>
<td>HA</td>
</tr>
</tbody>
</table>

Aflatoxin accumulation means are from the mean of Mississippi State, MS field locations in 2000-2002 and Stoneville, MS 2000.

LSD = 0.4521

Means followed by the same letter do not significantly differ at \( \alpha = 0.05 \)

\(^a\) mean of natural log transformed values

\(^b\) values converted back to original scale expressed as nanograms of toxin per gram of ground maize material

\(^c\) the first letter represents the genotype of the \( chiA \) allele and the second letter represents the genotype of the MpM1 allele.

Overall, this research is a considerable step forward in efforts to transfer resistance from inbred lines such as Mp313E into elite commercial breeding lines.

MpM1 and \( chiA \) represent the first gene-based markers developed specifically for resistance to aflatoxin accumulation. Furthermore, the methodology developed in this study has already been incorporated into other research models and may be further
modified as more genetic information becomes available. Although the genes which these markers identify must first be validated in controlled field conditions, these markers have already helped to identify previously unknown QTL, better defined existing QTL, and are additive when present in the same individual. Also, the results from the \textit{chiA} marker suggest that the other chitinase genes may be useful in developing more gene-based markers and reinforce research suggesting chitinases’s role in maize resistance to \textit{A. flavus} and aflatoxin accumulation. Both of these markers can soon be integrated into MAS programs focused on integrating resistance into elite maize breeding lines.
Literature Cited


APPENDIX A

MULTIPLE ALIGNMENTS FOR CANDIDATE GENES NOT USED FOR GENE-BASED MARKER DEVELOPMENT
Figure A.1  Multiple alignment of AW216267.

Multiple alignment of Mp313E (resistant), Mp715 (resistant), B73 (susceptible), and Va35 (susceptible) from the sequencing of AW216267 using the primer pair AW216267F and AW216267R (Table 3.1). Gray color indicates a 100% match among the four sequences; pink color indicates a match among three of the sequences; blue color indicates a match between two of the sequences.
Multiple alignment of Mp313E (resistant) and Va35 (susceptible) from the sequencing of AW244196 using the primer pair AW244196F and AW244196R (Table 3.1). Gray color indicates a 100% match between the Va35 and Mp313E sequences.
Figure A.3  Multiple alignment of AW400128.

Multiple alignment of Mp313E (resistant) and Va35 (susceptible) from the sequencing of AW400128 using the primer pair AW400128F and AW400128R (Table 3.1). Gray color indicates a 100% match between the Mp313E and Va35 sequences.
Figure A.4  Multiple alignment of AW438153.

Multiple alignment of Mp313E (resistant), Mp715 (resistant), B73 (susceptible), and Va35 (susceptible) from the sequencing of AW438153 using the primer pair AW438153F and AW438153R (Table 3.1). Gray color indicates a 100% match among the four sequences; pink color indicates a match among three of the sequences.
Figure A.5  Multiple alignment of BE128894.

Multiple alignment of Mp313E (resistant), Mp715 (resistant), B73 (susceptible), and Va35 (susceptible) from the sequencing of BE128894 using the primer pair BE128894F and BE128894R (Table 3.1). Gray color indicates a 100% match among the four sequences; blue color indicates a match between two of the sequences.
Figure A.6  Multiple alignment of AW179553.

Multiple alignment of Mp313E (resistant) and Va35 (susceptible) from the sequencing of AW179553 using the primer pair AW179553F and AW179553R (Table 3.1). Gray color indicates a 100% match between the Mp313E and Va35 sequences.

Figure A.7  Multiple alignment of AW360365.

Multiple alignment of Mp313E (resistant) and Va35 (susceptible) from the sequencing of AW360365 using the primer pair AW360365F and AW360365R (Table 3.1). Gray color indicates a 100% match between the sequences; blue color indicates a mismatch between the Mp313E and Va35 sequences.