

IDENTIFICATION OF MOLECULAR MARKERS ASSOCIATED WITH
RESISTANCE TO *ASPERGILLUS FLAVUS* IN MAIZE

A Thesis

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TABLE OF CONTENTS

| | |
|--|----|
| ACKNOWLEDGEMENTS..... | ii |
| LIST OF TABLES..... | iv |
| LIST OF FIGURES..... | v |
| ABSTRACT..... | vi |
| CHAPTER 1 INTRODUCTION..... | 1 |
| CHAPTER 2 MATERIALS AND METHODS..... | 5 |
| 2.1 Plant Material and DNA Extraction..... | 5 |
| 2.2 Laboratory-based Infection Resistance Screening..... | 5 |
| 2.3 <i>In vitro</i> Pollen Germination in the Presence of <i>A. flavus</i> | 5 |
| 2.4 Genotyping and Construction of Linkage Map..... | 6 |
| 2.5 Statistical and QTL Analysis..... | 7 |
| 2.6 Discriminant Analysis..... | 8 |
| CHAPTER 3 RESULTS..... | 9 |
| 3.1 Map Construction..... | 9 |
| 3.2 Phenotypic Evaluation..... | 9 |
| 3.3 QTL Analysis..... | 10 |
| 3.4 Discriminant Analysis..... | 11 |
| CHAPTER 4 DISCUSSION..... | 13 |
| CHAPTER 5 SUMMARY AND CONCLUSIONS..... | 18 |
| REFERENCES..... | 20 |
| VITA..... | 26 |

LIST OF TABLES

| | |
|--|----|
| 3. 1 Means, analysis of variance and broad sense heritability (H^2) estimate results for percent kernels uninfected (PKU) and number of pollen germinated (NPG)..... | 10 |
| 3. 2 QTL and its associated marker (interval) for percent kernels uninfected (PKU) and transformed number of pollen germinated (NPG)..... | 10 |
| 3. 3 Markers identified in discriminant analysis for the transformed percent kernels uninfected (PKU) and number of pollen germinated (NPG)..... | 11 |

LIST OF FIGURES

| | |
|--|----|
| 3.1 AFLP and target region amplification polymorphism (TRAP) marker based genetic linkage map of maize constructed using F ₂ population derived from of Mp313E x SC212m cross with the QTL positions for number of pollen germinated (NPG) and percent kernels uninfected (PKU) | 12 |
|--|----|

ABSTRACT

Aflatoxin contamination of maize (*Zea mays* L.) grain caused by *Aspergillus flavus* is a serious health hazard to animals and humans. Resistance to infection by *A. flavus* is poorly understood. The objectives of this investigation were to identify potential candidate markers associated with resistance in maize kernels and pollen grains to *A. flavus* using a mapping population derived from a cross between Mp313E (resistant) and SC212m (susceptible) inbred lines. The parents, F₁, and F₂ plants were planted in the field in 2005. Each F₂ was selfed to produce F_{2:3} seed. Fresh pollen collected from each F₂ plant was germinated on a growth medium in the presence of *A. flavus* conidia. Selfed seeds from parents, F₁, and F₂ plants were challenged with *A. flavus* conidial suspension and incubated using a medium-free method. Percent kernels uninfected (PKU) and number of pollen grains germinated (NPG) were recorded. A linkage map was constructed with JoinMap 3.0 using DNA profiles of all F₂ individuals produced from amplified fragment length polymorphism (AFLP) and target region amplification polymorphism (TRAP) markers. Interval mapping and multiple-QTL model (MQM) mapping analyses were performed using MapQTL 4.0 software. Three marker-QTL associations were observed for log-transformed PKU. Potential markers associated with this trait were also identified via discriminant analysis (DA). The markers identified via DA pointed to the same genomic regions as identified via the QTL mapping strategy. For log-transformed NPG, five marker-QTL associations were detected. One QTL was associated with a TRAP marker. The DA confirmed the existence of three QTL. The QTL detected for NPG were different from the QTL detected for PKU. Resistances of pollen and kernels to *A. flavus* appeared to be controlled by different genetic systems/mechanisms. Correlation between pollen germination and percent kernel

infection was negligible ($r = 0.067$), suggesting that the two traits can be improved independently.

CHAPTER 1 INTRODUCTION

Aflatoxins are carcinogenic products liberated by pathogenic fungi *Aspergillus flavus* Link ex Fr. and *A. parasiticus*. Preharvest aflatoxin contamination of maize (*Zea mays* L.) grain in the southeastern USA is a chronic problem, resulting in economic losses worth millions of dollars. Aflatoxin contamination of maize kernels poses a serious health hazard to both humans and animals (Kang and Moreno, 2002). Aflatoxin has been designated as a Group 1 category carcinogen by the International Agency for Research on Cancer (Hansen, 1993). Aflatoxin B₁ is reportedly the most potent carcinogenic toxin among the various aflatoxins (Ong, 1975). In spite of mandates to lower aflatoxin levels in foods and feeds, it has been difficult to reduce the levels of aflatoxin contamination in maize.

Efforts have been made during the past 20 years towards preventing aflatoxin contamination by following certain agronomic practices (Zuber et al., 1987; Widstrom, 1996; Kang and Moreno, 2002), but they have met with only limited success. Host-plant resistance studies have been conducted to identify resistant genotypes (Gorman and Kang, 1991; Guo et al., 1995a; Zhang et al., 1998; Li and Kang, 2005). Resistance to kernel infection by *A. flavus* and subsequent contamination of kernels is partly under genetic control (Gorman et al., 1992; Naidoo et al., 2002; Li and Kang, 2005). Some sources of resistance to *A. flavus* infection and aflatoxin accumulation have been identified (Scott and Zummo, 1988; Kang et al., 1990; Zhang et al., 1997; Li et al., 2002). Despite these investigations, the genetics of resistance to *A. flavus* remains poorly understood (Kang et al., 1990; White et al., 1997; Li and Kang, 2005). Resistance to aflatoxin contamination is a complex quantitative trait showing significant genotype x environment interaction effects (Zhang et al., 1997; Hamblin and White, 2000).

One strategy to combat aflatoxin accumulation of maize kernels is to screen and select resistant genotypes. Selection for reduced kernel infection rates could possibly reduce aflatoxin levels in maize kernels. The outer integuments of maize kernels have been implicated in resistance to *A. flavus* and aflatoxin accumulation (Guo et al., 1993 and 1995b). Laboratory-based approaches to screen for resistant genotypes should be easy, inexpensive, and less time-consuming. One of the early laboratory-based methods developed to quantify the incidence of percent kernel infection was Czepak agar medium plating (CAMP) protocol (King and Scott, 1982; Zummo and Scott, 1989). More recently, an effective, media-free, laboratory-based infection resistance screening (LIRS) method was developed to streamline the determination of percent kernel infection (PKI) (Li and Kang, 2005). In addition, screening genotypes at the microgametophytic (pollen) level could provide insights into resistance of maize to *A. flavus* infection and speed up the development of resistant lines and hybrids. Because kernels develop from fertilization between egg cells and pollen grains, either gamete could carry genes for resistance to kernel infection. Microgametophytic selection of a trait offers several advantages, such as ease of handling, presence of haploid state (avoids masking effect of dominant over recessive alleles) and genetic overlap (Hamilton and Mascarenhas, 1997).

Molecular markers play an important role in dissecting a genome and genetic architecture of a crop plant. In maize, RFLPs (Schneerman et al., 1998), randomly amplified polymorphic DNA (RAPD) (da Silva et al., 2000), simple sequence repeat (SSR) (Zhang et al., 2002) and AFLP (Zhang et al., 2002; Cai et al., 2003) markers have been employed to construct genetic linkage maps and to identify markers linked to important traits. These markers are randomly distributed throughout the genome and the identified marker-trait associations are prone to recombination, possibly leading to

breakage of linkage between marker(s) and genes of interest. Recently, a new marker technique, viz., target region amplification polymorphism (TRAP) was reported wherein one of the primers (fixed primer) is designed from a gene of interest and the other primer (arbitrary primer) is designed to target either exons or introns (Hu and Vick, 2003). The markers generated from the TRAP technique have a relatively higher probability to tag genes when compared with other random markers (Liu et al., 2005; Miklas et al., 2005; Alwala et al., 2006).

Molecular marker-assisted selection has been proposed as a complementary tool in crop improvement programs where selection of complex traits has been difficult (Xu, 2002). Molecular markers have been effectively used to tag disease resistance genes in several crops in QTL studies. In maize, previously, a few QTL studies have been reported for resistance to *A. flavus* infection and aflatoxin contamination (Paul et al., 2003; Brooks et al., 2005). One locus associated with disease resistance has been previously identified in a population derived from a Mp313E x Va35 cross (Davis et al., 2000). In another study involving the same resistant parent, Mp313E, the same QTL was confirmed to be associated with resistance (Brooks et al., 2005).

In most of currently available QTL analysis techniques, construction of a highly saturated genetic linkage map is necessary. Due to the difficulties involved in QTL analysis, such as gene-by-gene and/or gene-by-environment effects, plant breeders/geneticists have chosen an alternative approach - discriminant analysis (DA). This is a multivariate non-parametric approach, wherein an individual is categorized into a descriptive class (Fisher, 1936). The DA has an advantage over QTL analysis in that molecular markers can be identified from a group of diverse individuals in a germplasm collection without a mapping population and without a linkage map. Discriminant

analysis was previously used to identify microsatellite markers associated with agronomic traits in rice (Zhang et al., 2005), and AFLP markers associated with virus resistance in wheat (Capdevielle et al., 2002; Fahima et al., 2002) and southern root-knot nematode resistance in sweetpotato (Mcharo et al., 2004; 2005). Discriminant analysis is highly reliable when there are more than two pre-defined classes.

Until now, QTL studies have not been undertaken for the laboratory-based kernel infection phenotypic data and for *in vitro* pollen germination in the presence of *A. flavus*. The objectives of this study were to identify molecular markers associated with resistance to kernel infection and pollen germination in the presence of *A. flavus* conidia using traditional QTL and DA approaches. Although traditional QTL analysis is effective in identifying markers associated with traits, environment x QTL interactions can influence marker-assisted selection (Kang and Moreno, 2002). Because the current study was conducted in a single environment, discriminant analysis was employed to identify markers to compare with the QTL analysis-identified markers.

CHAPTER 2 MATERIALS AND METHODS

2.1 Plant Material and DNA Extraction

We used a cross between Mp313E (resistant to aflatoxin accumulation) and SC212m (susceptible) (Scott and Zummo, 1990; Scott et al., 1991). The F₁ seed was selfed to obtain F₂ seeds. In the summer of 2005, the parental inbred lines, F₁, and F₂ seeds were planted at Louisiana State Univ. Agric. Center farm at Ben Hur near Baton Rouge. A mapping population of 147 F₂ plants was used. All the individual F₂ plants were selfed to produce F_{2:3} seed. Leaf samples were collected and DNA was extracted from representative plants of the parental lines and F₁, and individual F₂ plants, using Plant DNeasy Mini Kit (Qiagen, Valencia, CA) and following the manufacturer's protocol.

2.2 Laboratory-based Infection Resistance Screening

The per-plot sampled F_{2:3} seed was washed for 1 min with sterile water and placed in a 48-well polystyrene tissue culture plate, with one kernel per well. Two replications (two plates) with a total of 96 kernels were used for each F₂ plant. Also, kernels from parents and F₁ were treated similarly. The kernels were inoculated with 40 µL conidial suspension (1×10^6 conidia/ml) of *A. flavus* isolate NRRL 3357, as previously described by Li and Kang (2005). The plates were covered with a lid and incubated in an NAPCO 6500 incubator (Juoan Industries, France). After 10 days of incubation (depending upon the *A. flavus* growth), the plates were removed and percentage of kernels uninfected (PKU) was recorded. The readings were log-transformed to normalize the distribution using Microsoft Excel 2002 before conducting statistical analyses.

2.3 *In vitro* Pollen Germination in the Presence of *A. flavus*

At anthesis, fresh viable pollen was collected from individual F₂ tassels.

A pollen-growth medium was prepared using 0.6% bacto-agar, 15% sucrose, 0.03% Calcium nitrate and 0.01% boric acid (Pfahler, 1967). The sterilized medium was poured into Petri dishes and covered with a lid. After the medium had solidified, a 5 μ L conidial suspension (1×10^6 conidia/ml) of *A. flavus* was poured in Petri dishes. A sample of fresh pollen was then sprinkled onto inoculated medium and Petri dishes were incubated at room temperature. One Petri dish was used per F₂ plant. Similarly, pollen from the parents and F₁ was inoculated on the media. Approximately equal number of pollen grains was sprinkled on each plate and the plate was divided into four sections and each section was treated as a replication. After 24 h, the number of pollen grains germinated was recorded from each section of the Petri plate. The data were log-transformed to normalize the distribution using Microsoft Excel 2002.

2.4 Genotyping and Construction of Linkage Map

For AFLP analysis, the genomic DNA was digested with *EcoR* I and *Mse* I restriction enzymes. Following the protocol of Vos et al. (1995), the digested DNA was ligated to *EcoR* I and *Mse* I adapters. Pre-amplifications were conducted using *EcoR* I +A and *Mse* I +C primers, followed by selective amplifications using two selective nucleotides. The *EcoR* I selective primers were IR-dye-labeled (either IR-700 or IR-800). Polymerase chain reaction (PCR) was conducted in a reaction volume of 10 μ L consisting of 1 μ L of 10X reaction buffer, 1.5 μ L of 25 mM MgCl₂, 1 μ L of 10 mM dNTPs, 1 μ L of 1 μ M of IR-Dye-labeled primers and 1 μ L of 10 μ M forward primer and 0.2 μ L of 5U Taq polymerase. The reactions were run on *i*-cycler (BioRad Labs, Hercules, CA). The PCR conditions for selective amplifications were as follows: initial denaturing step at 94 °C for 3 min, followed by initial 12 cycles at 94 °C for 30 s, 65 °C for 30 s (with 1 °C

decrement every cycle) and 72 °C for 1 min, then followed by 23 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, with a final extension step at 72 °C for 7 min.

Target region amplification polymorphism is a two-primer PCR-based marker technique (Hu and Vick, 2003). The forward (fixed) primer was designed from an available expressed sequence tag (EST) or gene sequence, whereas the reverse (arbitrary) primer was designed with AT- or GC-rich core sequences. The main idea was to target the genic regions of the genome rather than random portions of the genome. The designing of fixed forward primers has been described in Alwala et al. (2006). The fixed primer (5'-ACCCTCAGCAGTCTACGG-3') was designed using NBS-LRR-rich sequence of a rust-resistance gene (accession number: [AF107293](#)), whereas the arbitrary primers (5'-GACTGCGTACATGCAGACAAC-3' and 5'-GACTGC GTACATGCAGACACG-3') were designed as per Li and Quiros (2001). The PCR amplifications were performed as previously described (Alwala et al., 2006).

For the construction of a linkage map, JoinMap ver 3.0 (Van Ooijen and Voorrips, 2001) was used. A minimum LOD score of 4.0 and a maximum LOD score of 8.0 were employed for the linkage analysis using a recombination fraction of 0.4. Kosambi mapping function was used to overcome the effects of interference.

2.5 Statistical and QTL Analysis

Analyses of variance for PKU and for NPG were performed using SAS ver 9.1 (SAS Inc.). Broad-sense heritabilities for each trait were calculated as

$$H^2 = \sigma^2g / (\sigma^2g + \sigma^2e/r)$$

where H^2 is broad-sense heritability, σ^2g is genotypic variance and σ^2e is error variance.

QTL analysis was performed initially with interval mapping, followed by MQM mapping, using MapQTL ver 4.0 (Van Ooijen et al., 2002). 'Automatic selection of

cofactors' option was used to select markers as cofactors. The cofactors were used as nearby QTL in the multiple-QTL model (MQM) mapping; however, with the current version, gene-by-gene interactions could not be fitted. Permutation tests were performed to ascertain the validity of identified QTL (Doerge and Churchill, 1996).

2.6 Discriminant Analysis

Discriminant analysis was performed according to Mcharo et al. (2004). The population was divided into four groups (completely susceptible, partially susceptible, partially resistant, and completely resistant) based on the phenotypic records of each trait. Using the PROC STEPDISC procedure of SAS (SAS Inc., Cary, NC), a forward method parametric discriminant analysis was performed with criteria set to default (SLENTRY = 0.15) to select the most informative markers to assign individuals to appropriate groups. Using PROC DISCRIM, a non-parametric discriminant analysis was performed, employing the selected markers to construct and validate a class prediction function and to predict group membership.

CHAPTER 3 RESULTS

3.1 Map Construction

The linkage map is presented in Figure 1. A total of 165 polymorphic markers were scored from 17 primer combinations. Of the 165 markers, 151 were from 15 AFLP primer combinations, whereas the rest were from two TRAP primer combinations. A preliminary genetic linkage map was constructed, which comprised 48 linked markers distributed across 10 linkage groups. The cumulative genome length was 593 cM with a mean distance of 12 cM between any two linked markers. Most of the linked markers were generated via AFLPs, whereas two linked markers were from the TRAP analysis.

3.2 Phenotypic Evaluation

Mean levels of log-transformed PKU and NPG for each parent and $F_{2:3}$ are listed in Table 1. For Mp313E and SC212m, the PKU values were 1.84 and 1.44, respectively, whereas the NPG values were 2.54 and 0.71, respectively. In $F_{2:3}$, PKU ranged from 0 to 2.0 with a mean of 1.49. Likewise, NPG ranged from 0 to 2.69 with a mean of 1.59. The ranges in $F_{2:3}$ indicate transgressive segregation for both traits. Analyses of variance indicated that there was a clear-cut variation among $F_{2:3}$ for both PKU and NPG.

Variation due to replications for NPG was significant, mainly because each Petri dish was divided into four sections and variable numbers of pollen grains were observed in the different sections of the same dish. Broad-sense heritabilities were 0.58 for PKU and 0.81 for NPG. The analyses of variance are presented in Table 1. A negligible correlation ($r=0.067$) was found between PKU and NPG, meaning that these two traits could be selected independently of each other.

Table 3.1. Means, analysis of variance and broad sense heritability (H^2) estimate results for percent kernels uninfected (PKU) and number of pollen germinated (NPG)[†]

| Trait | Mp313E | SC212m | [‡] F _{2:3} | MS | F-value | P>F | R ² | CV % | H ² |
|-------|--------|--------|-------------------------------|------|---------|---------|----------------|-------|----------------|
| PKU | 1.84 | 1.44 | 1.49 (0-2.00) | 0.47 | 5.20 | <0.0001 | 0.87 | 19.96 | 0.58 |
| NPG | 2.54 | 0.71 | 1.59 (0-2.69) | 1.00 | 13.85 | <0.0001 | 0.83 | 18.04 | 0.81 |

[†]The PKU and NPG values were log-transformed before analyses

[‡]Values in the parenthesis indicate the range

Table 3. 2. QTL and its associated marker (interval) for percent kernels uninfected (PKU) and transformed number of pollen germinated (NPG).

| Trait | Linkage group | Marker interval | LOD | %Var [†] | Effect (a) [‡] |
|-------|------------------|--------------------|-----|-------------------|-------------------------|
| PKU | LG4 | E81CAA1-E71MCAA3 | 2.0 | 10 | 0.15 |
| | LG4 | E81MCAA2-E81MCAA4 | 2.0 | 10 | 0.15 |
| | LG5 | E71MCAG7-E81MCAG12 | 2.0 | 9 | -0.10 |
| NPG | LG2 | E71MCAG11 | 1.5 | 6 | 0.05 |
| | LG3 | E71MCAG6-E81MCAT7 | 2.3 | 10 | 0.15 |
| | LG4 | E81MCAA4-E71MCAA2 | 1.6 | 6 | 0.15 |
| | LG5 [§] | E82MCTC6 | 1.4 | 10 | 0.05 |
| | LG10 | MTN1803 | 1.4 | 6 | 0.15 |

[†]Phenotypic variance explained by the QTL

[‡]Estimates indicate additive effects

3.3 QTL Analysis

QTL analysis was performed following interval mapping and MQM mapping procedures. For PKU, both interval mapping and MQM mapping identified the same marker-QTL associations. The results for MQM mapping QTL analysis are presented in Table 2. Two loci were found on linkage group 4 (E81CAA1-E71MCAA3 and E81MCAA2-E81MCAA4), whereas one QTL was found on linkage group 5 (E71MCAG7-E81MCAG12). The variation explained by the three PKU-affecting QTL

ranged between 9% and 10%. Except for one, the other QTL had positive effects on PKU. Likewise, for NPG, five QTL were detected, of which four QTL (LG2, LG3, LG4 and LG10) were detected via both interval mapping and MQM mapping. One QTL on linkage group 5 was unique to interval mapping. The variation explained by observed QTL ranged from 6% to 10%. All three QTL showed positive additive effects for NPG.

Table 3. 3. Markers identified in discriminant analysis for the transformed percent kernels uninfected (PKU) and number of pollen germinated (NPG).

| Trait | DA Identified markers [†] | % classification based on the number of DA selected markers | | |
|-------|--|---|-------|-------|
| | | 5 | 10 | 15 |
| PKU | E82MCAC3, E81MCAG12 , E82MCAC17, E82MCAG8, E82MCTC4, E71MCAT5, E72MCAC14, E72MCAG11, E71MCAA1 , E71MCAA3 , E82MCAC19, E71MCAG14, E72MCAC9, E72MCAG1, E71MCAG7 | 61.69 | 91.28 | 99.20 |
| NPG | E81MCAG3, E82MCTC12, E82MCAC14, E71MCAG11 , E81MCAC7, E81MCAC2, E82MCAC11, E72MCAG2, E72MCAC13, E71MCAG4, E82MCAG3, MTN1803 , E71MCAG9, E82MCTC6 , MTN1705 | 62.15 | 91.28 | 98.40 |

[†]Markers in bold were also identified in QTL analysis

3.4 Discriminant Analysis

The mapping population was divided into four groups based on PKU and NPG data. Assuming no population structure, the discriminant analysis procedure was used to select a maximum of 15 markers for each trait (Table 3). The selected 15 markers gave 99.2% and 98.4% classification of genotypes for PKU and NPG traits, respectively. We found that to obtain a classification with > 90% probability, a minimum of 10 markers was required (Table 3). Of the DA-identified 15 markers, three markers (E81MCAG12, E71MCAA1 and E71MCAA3) for PKU and three markers (E71MCAG11, E82MCTC6 and MTN1803) for NPG were also detected via QTL analysis. Except for the two TRAP

markers identified for NPG, the rest were all derived from the AFLP technique. None of the DA-identified markers was identical for either trait.

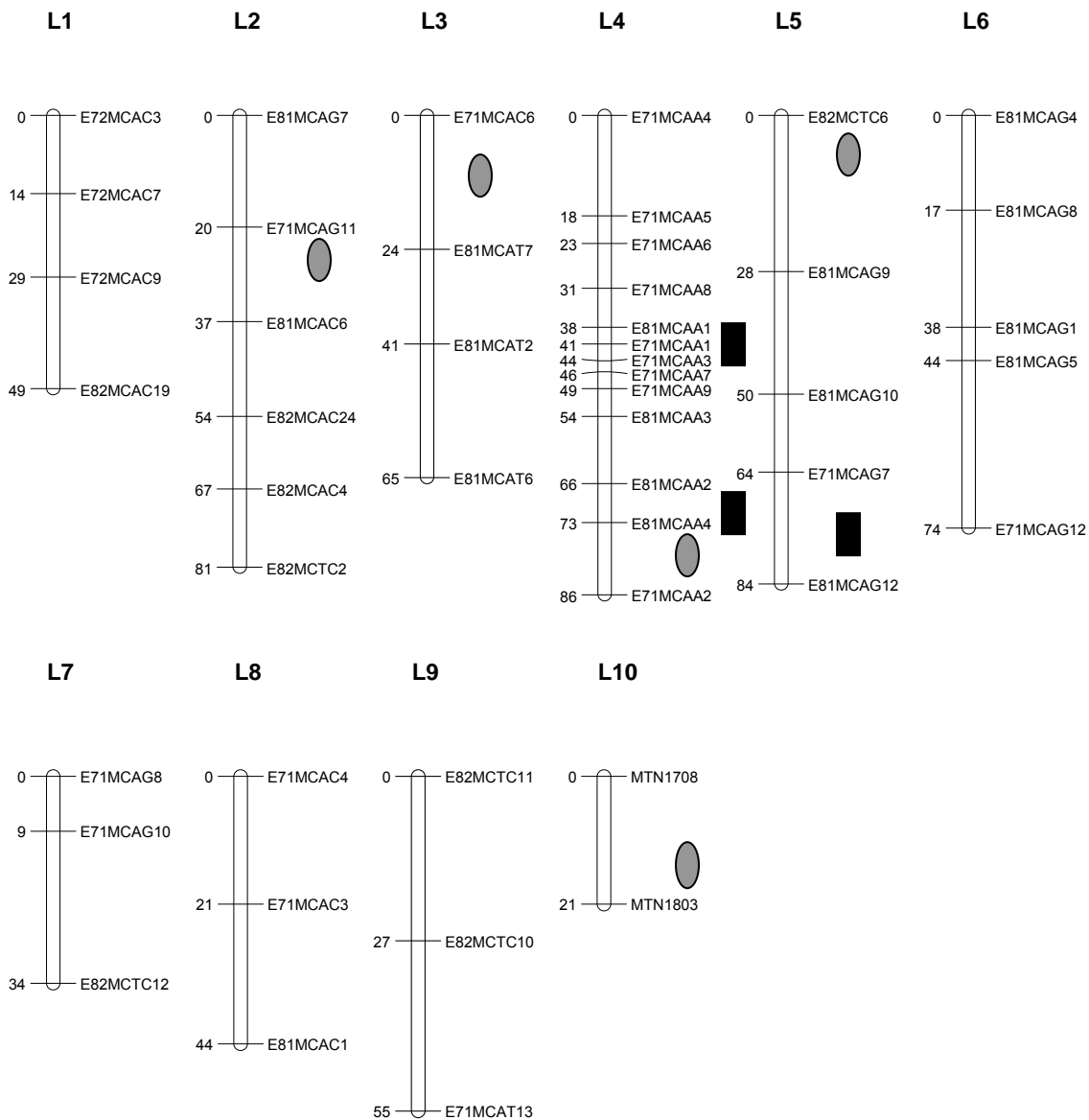


Figure 3.1. AFLP and target region amplification polymorphism (TRAP) marker based genetic linkage map of maize constructed using F_2 population derived from of Mp313E x SC212m cross with the QTL positions for number of pollen germinated (NPG) and percent kernels uninfected (PKU). Kosambi map distances and marker names are given on left and right sides, respectively, of the linkage group. Marker names starting with ‘E’ represent AFLP markers while markers starting with ‘MTN’ represent TRAP markers. Ovals represent probable QTL positions for number of pollen germinated (NPG) and rectangular boxes represent probable QTL positions for percent uninfected kernels (PKU).

CHAPTER 4 DISCUSSION

The selection intensity and the heritability estimate of a trait influence the extent of response to selection. Broad-sense heritability (H^2) estimates for resistance to aflatoxin accumulation were previously found to be 29.1% (involving Oh516 resistant parent) (Busboom and White, 2004), 32% (involving C12 parent) (Walker and White, 2001) and up to 42% (involving Mp313E parent) (Brooks et al., 2005). In this study, however, a relatively higher heritability estimate (58%) was noticed involving the same Mp313E parent. On the other hand, a much higher H^2 estimate (73%) was reported by Li (2005). The low to moderate heritability estimates signify the presence of small to medium variances in the populations. However, the presence of non-additive genetic variance cannot be discounted which might influence the total genetic variance. Moreover, as this study was taken up in a single location, the effects of genotype-by-environment interaction which were not partitioned from the σ_g^2 might also lead to an upward bias of the heritability estimate. Therefore, for quantitatively inherited traits like resistance to percent kernel infection (PKI) (Li and Kang, 2005) and NPG, selection solely on phenotype might prove inefficient. Molecular marker-assisted selection could be used as an additional selection tool to enhance the precision of the selection process.

Although pollen stage carries only half of the genome complement, studies have shown that almost all the genes (~25000 genes) in pollen are equally transcribed and translated in both gametophytic and sporophytic stages (Hamilton and Mascarenhas, 1997). A strong selection pressure can be applied at the gametophytic stage due to its haploid state and its ability to mask the dominance effects, which are more pronounced at the sporophytic stage. The gametophytic selection has contributed to improvement of traits at the sporophytic stages in many crops (Hormaza and Herrero, 1996; Clarke et al.,

2004). Several studies also documented that disease resistance levels in sunflower could be improved by gametophytic (pollen) selection (Shobarani and Ravikumar, 2007) as well as transmitted to succeeding generations (Chikkodi and Ravikumar, 2000 and 2003). In the current study, significant differences were observed between Mp313E and SC212m as well as among the segregating progeny for pollen germination (NPG) in the presence of *A. flavus* spores. Gametophytic recurrent selection schemes could be utilized in maize, wherein crosses could be made among selected resistant genotypes and susceptible genotypes are constantly removed over generations via pollen selection. Conversely, Alpe et al. (2003) observed that although source of pollen affects aflatoxin contamination, it is the genotype of ear-bearing plant that mostly imparts resistance. From this study, although resistant genotypes were observed based on pollen germination in presence of *A. flavus*, it is difficult to establish a strong causal relationship between pollen survival and resistance to *A. flavus*. Several factors such as pollen death due to reasons other than *A. flavus* toxins and/or the poor toxicity levels in the media cannot be undisputed.

Several QTL were identified, using SSR markers, for resistance to aflatoxin accumulation in the mapping populations derived from crosses involving Mp313E as the female parent (Paul et al., 2003; Brooks et al., 2005). In this study, we found QTL using AFLP and TRAP markers. The QTL for PKU had positive additive effects, except for one. It seems that there are two locations on linkage group 4 that affect PKU, which need to be resolved using additional markers. Likewise, for NPG, all the identified QTL had positive additive effects. The identified QTL were commonly detected via both interval mapping and MQM mapping, except for one QTL on linkage group 5, which was unique to interval mapping. There might be some multiple loci on linkage group 6 controlling

resistance relative to both traits. For marker-assisted selection across different locations, caution must be exercised because our experiment was conducted in only one year at a single location. Nevertheless, because all of the QTL had positive additive effects, new lines could be developed on the basis of a marker-assisted QTL pyramiding approach, to concentrate all or most of the favorable alleles in one genetic background. None of the identified QTL was common to either PKU or NPG, indicating that possibly different genetic systems/genes are involved in governing these two traits. This observation is also supported by negligible correlation between PKU and NPG. One reason could be that the pollen is haploid in state and carries half of the gene complement. On the other hand, kernels are diploid as the result of fertilization between the egg and pollen. Certain kernels may be resistant as they might have received dominant genes from the egg whereas, the pollen might have contributed the susceptible genetic complement and vice versa.

AFLP markers have been the traditional markers used for linkage mapping in many crops. Most of the AFLP polymorphisms are randomly distributed across the genome and dependent on the restriction enzymes used. A vast number of polymorphic markers for linkage analysis could be generated using methyl-insensitive *EcoR* I – *Mse* I primer combinations. Yet, the polymorphic markers might not be within actively transcribing regions of the genome as compared with hypo-methylated (*Pst* I) regions (Cedar, 1988; Mignouna et al., 2005). TRAP markers, on the other hand, might not be ideal for constructing linkage maps (Alwala et al., 2007); however, they could be used to complement AFLP markers by integrating gene/trait-based markers into already existing linkage maps (Liu et al., 2005; Miklas et al., 2006). In this study, most of the markers associated with QTL were AFLP markers, except for one marker-QTL (MTN1803),

which was a TRAP marker. Marker MTN1803 was generated using a fixed primer designed from a sequence containing NBS-LRR regions. The NBS-LRR sites have been implicated in disease resistance in many crop plants (Meyers et al., 2003; Maleki et al., 2003; Belkhadir et al., 2004). It has been documented that TRAP markers indeed target genic regions (Alwala et al., 2006) and the possibility of arbitrary primer potentially amplifying random portions of the genome is minimal due to increased T_m temperature in the PCR. Moreover, in our lab, when initially tested for potential false positives in TRAP PCR using only the arbitrary primer (as a RAPD primer), no amplification was observed. The TRAP markers were previously used to tag genes for important agronomic traits in wheat (Liu et al., 2005) and disease resistance traits in common bean (Miklas et al., 2006).

For any complex trait dissection via QTL analysis, production of large segregating populations, construction of dense linkage maps and phenotyping of quantitative traits are pre-requisites in which substantial amount of time, money and labor are invested (Zhang et al., 2005). Use of discriminant analysis is an alternative platform to QTL analysis. Genotypes can be differentiated based on the differences in variables (or markers), given the quantitative measurements (Rencher, 1992; Cruz-Castillo et al., 1994). Although dependent on several statistical assumptions, such as normality of the data and homogeneity of covariance matrices, DA proves to be robust even with minor violations of the assumptions even when marker profile categorical data are used (Zhang et al., 2005). The DA plays a prominent role especially when there are no *a priori* genetic linkage maps available. On the other hand, identification of QTL is inversely proportional to the recombination fraction between markers (Sills et al., 1995). The main difference between DA and QTL analysis is that the latter identifies markers linked to

gene(s) of interest, whereas DA identifies an array of markers that could be used to allocate an individual to a predefined (resistant) group. Recently, Mcharo et al. (2004 and 2005) identified AFLP markers associated with virus resistance and southern root-knot nematode resistance from sweet potato germplasm collections. In this investigation, DA proved to be a good supplement to QTL analysis to identify potential markers associated with resistance to kernel infection and pollen germination. It is not surprising that the markers identified via DA included those markers detected via QTL analysis. Furthermore, several markers were identified via DA, which were not detected by QTL analysis performed on the small preliminary linkage map. Previously, Aluko (2003) used a mapping population to identify markers associated with agronomic traits in rice and found common markers by using both QTL and discriminant analyses. Thus far, the results have been encouraging and clearly indicate that a combination of QTL and discriminant analyses would be beneficial in marker-assisted selection.

CHAPTER 5 SUMMARY AND CONCLUSIONS

Maize (*Zea mays* L.) is an important crop in the United States of America. In southeastern USA, preharvest aflatoxin (produced by *Aspergillus flavus* Link ex Fr .) contamination of maize kernels has been a chronic problem resulting in huge economic losses. Aflatoxin has been considered as a potent carcinogenic toxin; however, it has been difficult to reduce the aflatoxin contamination in maize. Although several sources of resistance to aflatoxin have been identified, the genetics of resistance is poorly understood. Resistance to aflatoxin contamination is considered to be a quantitatively inherited trait.

Several strategies have been proposed to combat aflatoxin contamination in maize. It has been found that the outer integuments of maize kernels have a potential role in imparting resistance to *A. flavus* and aflatoxin accumulation. In several other crops, it was noticed that the microgametophytic screening of genotypes resulted in development of resistant cultivars for certain diseases. Therefore, this study was undertaken to study the kernel resistance to aflatoxin contamination and also to screen the genotypes at microgametophytic (pollen) level using a molecular marker approach.

An F₂ mapping population was derived from a cross involving Mp313E (resistant) and SC212m (susceptible) parents was used. The pollen from each F₂ genotype were screened for germination (NPG) in presence of *A. flavus* spores and the selfed F_{2:3} seed from the segregating progeny were screened for kernel resistance (PKU) in a media-free laboratory assay. This study indicated that there was negligible correlation between the PKU and NPG indicating there might be two separate genetic systems underlying pollen germination and kernel resistance. This study also signifies PKU as a better method to screen for resistant genotypes against *A. flavus* contamination.

In this study, two types of molecular markers namely Amplified fragment length polymorphism (AFLP) and target region amplification polymorphism (TRAP) marker techniques were used to construct linkage map. Whereas, the AFLP technique was used because of its ability to produce vast number of polymorphisms, TRAP technique was used since it scans the gene rich regions to amplify polymorphisms. The results from this study further corroborates that AFLP markers are ideal for constructing linkage maps while TRAP markers could be used as an add-on to an already existing linkage maps.

From the quantitative trait loci (QTL) analysis, several markers have been identified which are associated with PKU and NPG but with low LOD scores. The low LOD scores could be attributed to the low marker density on the linkage map due to relatively small mapping population. Most of the identified markers were AFLP derived markers in addition to one TRAP derived marker. Since this study was undertaken at only one location, another methodology namely discriminant analysis (DA) was also employed to validate the QTL markers. DA is a non-parametric approach to identify marker-trait associations given the unavailability of mapping populations and/or saturated linkage maps. DA identified several markers including a few markers detected in QTL analysis and pointed to the same genomic regions as observed in QTL analysis. In addition, several additional markers were also detected by DA which were not linked on the linkage map and as such not identified by the QTL analysis. The results from this study indicate that a combination of QTL and DA might prove beneficial to an applied breeding program.

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