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Identificaton of molecular markers associated with resistance to Aspergillus flavus in maize

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

A Thesis

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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, F_1, and F_2 plants were planted in the field in 2005. Each F_2 was selfed to produce $F_{2:3}$ seed. Fresh pollen collected from each F_2 plant was germinated on a growth medium in the presence of *A. flavus* conidia. Selfed seeds from parents, F_1, and F_2 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 F_2 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 B_1 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 Czapak 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

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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 F_1 seed was selfed to obtain F_2 seeds. In the summer of 2005, the parental inbred lines, F_1, and F_2 seeds were planted at Louisiana State Univ. Agric. Center farm at Ben Hur near Baton Rouge. A mapping population of 147 F_2 plants was used. All the individual F_2 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 F_1, and individual F_2 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 F_2 plant. Also, kernels from parents and F_1 were treated similarly. The kernels were inoculated with 40 μL conidial suspension (1 x 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 F_2 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 μL conidial suspension (1 x 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 F$_2$ plant. Similarly, pollen from the parents and F$_1$ 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 μL consisting of 1 μL of 10X reaction buffer, 1.5 μL of 25 mM MgCl$_2$, 1 μL of 10 mM dNTPs, 1 μL of 1 μM of IR-Dye-labeled primers and 1 μL of 10 μM forward primer and 0.2 μL 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

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decrement every cycle) and 72 $^{\circ}$C for 1 min, then followed by 23 cycles at 94 $^{\circ}$C for 30 s, 56 $^{\circ}$C for 30 s, and 72 $^{\circ}$C for 1 min, with a final extension step at 72 $^{\circ}$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

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

where H^2 is broad-sense heritability, $\sigma^2 g$ is genotypic variance and $\sigma^2 e$ 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.
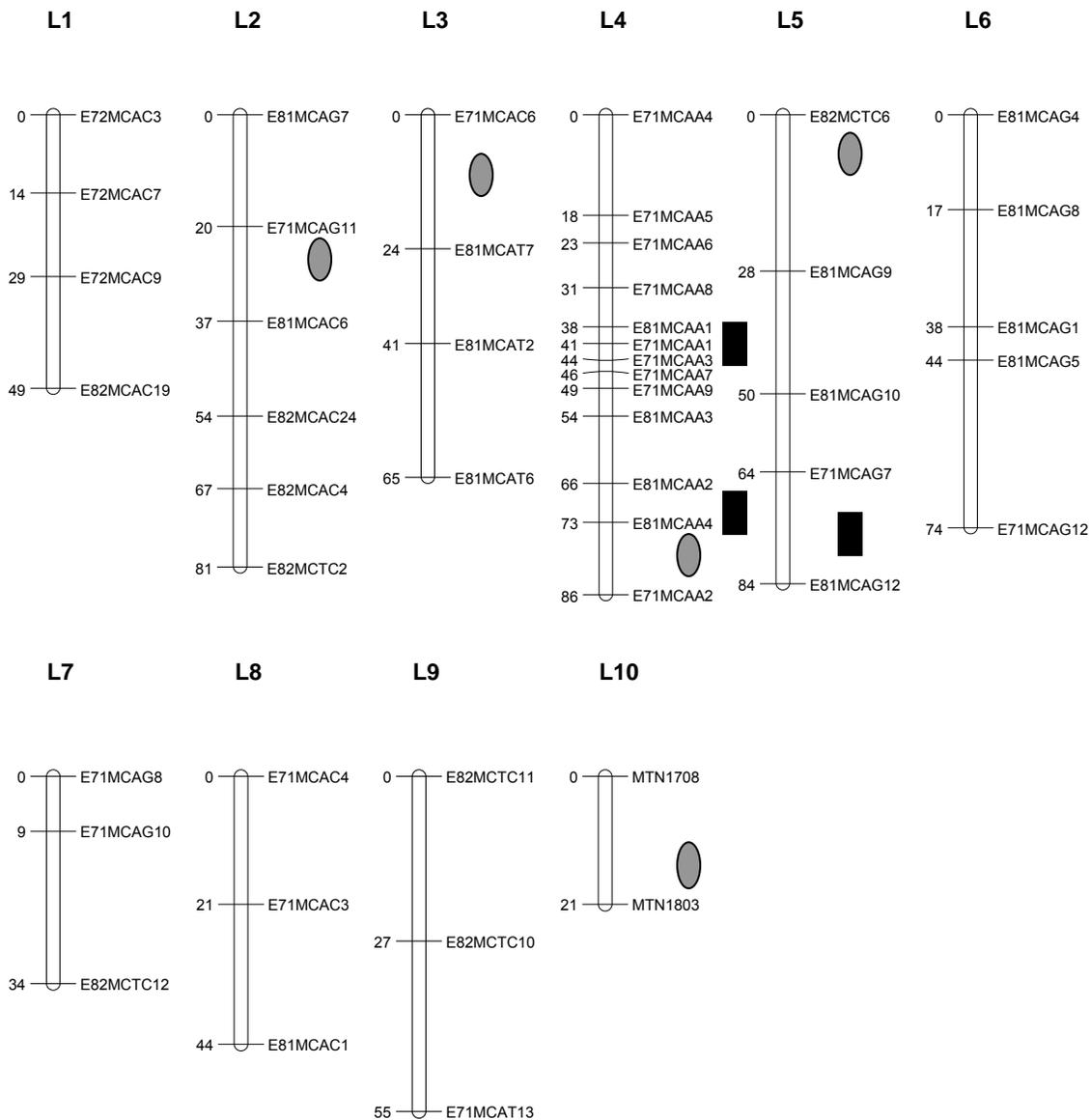
Figure 3.1. AFLP and target region amplification polymorphism (TRAP) marker based genetic linkage map of maize constructed using F_2 population derived from of Mp313E x SC212m cross with the QTL positions for number of pollen germinated (NPG) and percent kernels uninfected (PKU). Kosambi map distances 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^2_g 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

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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 F_2 mapping population was derived from a cross involving Mp313E (resistant) and SC212m (susceptible) parents was used. The pollen from each F_2 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.

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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.

REFERENCES

Alpe, M., W. P. Williams and G. L. Windham. 2003. Effect of pollen genotypes on Aspergillus flavus infection and aflatoxin accumulation in maize (abstract). Proceedings of 16th Annual Aflatoxin Elimination Workshop. p. 67.

Aluko, G. K. 2003. genetic mapping of agronomic traits from the interspecific cross of *Oryza sativa* L. and *Oryza glaberrima* Steud. Ph.D Dissertation, Louisiana State University, Baton Rouge, LA.

Alwala, S, C. A. Kimbeng, J. C. Veremis and K. A Gravois. 2007. Linkage mapping and genome analysis in a *Saccharum* interspecific cross using AFLP, SRAP and TRAP markers. (Submitted).

Alwala, S., A. Suman, J.A. Arro, J.C. Veremis, and C.A. Kimbeng. 2006. Target region amplification polymorphism (TRAP) for assessing genetic diversity in sugarcane germplasm collections. Crop Sci. 46:448–455.

Belkhadir, Y., R. Subramaniam and J. L. Dangl. 2004. Plant disease resistance protein signaling: NBS-LRR proteins and their partners. Curr. Opin. in Plant Biol 7: 391-399.

Brooks, T. D., W. P. Williams, G. L. Windham, M. C. Willcox and H. K. Abbas. 2005. Qunatitative trait loci contributing resistance to aflatoxin accumulation in the maize inbred Mp313E. Crop Sci 45: 171-174

Busboon, K. N and D. G. White. 2004. Inheritance of resistance to aflatoxin production and aspergillus ear rot of corn from the cross of inbreds B73 and Oh516. Phytopathology 94: 1107-1115

Cai, H. W., Z. S. Gao, N. Yuyama and N. Ogawa. 2003. Identification of AFLP markers closely linked to the rhm gene for resistance to southern corn leaf blight in maize by using bulked segregant analysis. Molecular Genet. Genomics 269 (3): 299-303

Capdevielle, F. M., G. K. Aluko, M. Balzarini and J. H. Oard. 2000. Application of molecular markers and discriminant analsis to identify rice lines with contrasting phenotypes for agronomic traits. *In* G. S. Khush, D. S. Brar and B. Hardy (eds.), Proceedings of the Fourth International Rice Genetics Symposium, International Rice Research Institute, Los Banos, Phillipines (abstr: p. 216).

Cedar, H. 1988. DNA methylation and gene activity. Cell 53:3-4

Chikkodi, S. B and R. L. Ravikumar. 2000. Influence of pollen selection for *Alternaria helianthi* resistance on the progeny performance against leaf blight in sunflower (*Helianthus annuus* L.). Sex. Plant Reprod 12: 222-226

Chikkodi, S.B., and R.L. Ravikumar. 2003. Effect of pollen selection for *Alternaria* blight resistance in sunflower on F_2 generation. *In* Extended summaries, National Seminar on Stress Management in Oil Seeds for Attaining Self Reliance in Vegetable Oils, Hyderabad, India. 29–31 Jan. 2003. Directorate of Oilseeds Research, Hyderabad, India

Clarke, H.J., T.N. Khan, and K.H.M. Siddique. 2004. Pollen selection for chilling tolerance at hybridization leads to improved chickpea cultivars. Euphytica 139:65–74.

Cruz-Castillo, J. G., S. Ganeshanandam, B. R. MacKay, G. S. Lawes, C. R. O. Lawoko and D. J. Woolley. 1994. Applications of canonical discriminant analysis in horticultural research. Hortscience 29: 1115-1119.

da Silva, S. C., M. V. F. Lemos and J. T. A. Osuna. 2000. RAPD marker use for improving resistance to *Helicoverpa zea* in corn. Maydica 45: 289-294

Davis, G. L., G. L. Windham and W. P. Williams. 2000. QTL analysis for aflatoxin reduction in maize. Maize Genet. Conf. Abstr 41: P139

Doerge, R. W and G. A. Churchill. 1996. Permutation tests for multiple loci affecting a quantitative character. Genetics. 142: 285-294

Fahima, T., M. S. Roder, K. Wendehake, V. M. Kirzhner and E. Nevo. 2002. Microsatellite polymorphism in natural populations of wild emmer wheat, *Triticum dicoccoides*, in Israel. Theor Appl Genet 104:17–29

Fisher, R. A. 1936. The use of multiple measurements in taxonomic problems. Ann Eugen 7:179–188

Gorman, D. P and M. S. Kang. 1991. Preharvest aflatoxin contamination in maize: Resistance and genetics. Plant Breeding 107: 1-10

Gorman, D. P., M. S. Kang, T. E. Cleveland and R. L. Hutchinson. 1992. Combining ability for resistance to field aflatoxin accumulation in maize grain. Plant Breeding 109 (4): 296-303

Guo, B. Z., J. S. Russin, T. E. Cleveland, R. L. Brown and N. W. Widstorm. 1993. The role of the pericarp of maize kernels to reduce infection and aflatoxin production by *Aspergillus flavus*. Phytopathology 83: 1417-1418

Guo, B. Z., Z.-Y. Chen, R. L. Brown, A. R. Lax, T. E. Cleveland, J. S. Russin and N. W. Widstorm. 1995a. Antifungal protein on corn kernels immunochemical localization and induction during germination. *In* J. Rubens and J. Dorner (ed) Aflatoxin elimination workshop: A decade of research progress 1988-1997 (p. 71). Food Safety and Health, USDA-ARS, Beltsville, MD

Guo, B. Z., J. S. Russin, T. E. Cleveland, R. L. Brown and N. W. Widstorm. 1995b. Wax and cutin layers in maize kernels associated with resistance to aflatoxin production by *Aspergillus flavus*. J. Food Prot. 58: 296-300

Hamblinn, A. M. and D. G. White. 2000. Inheritance of resistance to *Aspergillus* ear rot and aflatoxin production of corn from Tex6. Phytopathology 90 : 292-296

Hamilton, D.A., and J. P. Mascarenhas. 1997. Gene expression during pollen development. *In* K.R. Shivanna and V.K. Sawhney (ed.) Pollen biotechnology for crop production and improvement. Cambridge Univ. Press, New York.

Hansen, T. J. 1993. Quantitative testing for mycotoxins. Am. Asso. Cereal Chem. 38: 346-348

Hormaza, H., and M. Herrero. 1996. Male gametophytic selection as a plant breeding tool. Sci. Hortic. (Amsterdam) 65:321–333.

Hu, J.G., and B.A. Vick. 2003. Target region amplification polymorphism: A novel marker technique for plant genotyping. Plant Mol. Biol. Rep. 21:289–294.

Kang, M. S and O. J. Moreno. 2002. Maize Improvement for resistance to aflatoxins: Progress and Challenges. *In* M. S. Kang (ed). Crop Improvement: challenges in the twenty first century (p. 75). Food products press, The Haworth press Inc, NY.

Kang, M. S., E. B. Lillehoj and N. W. Widstorm. 1990. Field aflatoxin contamination of maize genotypes of broad genetic base. Euphytica 51: 19-23

King , S. B and G. E. Scott. 1982. Screening maize single crosses for resistance to preharvest infection of kernels by *Aspergillus flavus*. Phytopathology 72: 942

Li, R. 2005. A Genetic Study of Resistance to Kernel Infection by *Aspergillus Flavus* in Maize (*Zea Mays* L.). A Ph.D thesis submitted to Louisiana State University, Baton Rouge, Louisiana.

Li, R and M. S. Kang. 2005. Genetics of resistance to field kernel infection by *Aspergillus flavus* in maize. J. of Crop Improvement 15 (1): 11-31

Li, R., M. S. Kang, O. J. Moreno and L. M. Pollak. 2002. Field resistance to *Aspergillus flavus* from exotic maize (*Zea mays* L.) germplasm. Plant Genetic Resources Newsletter 130: 11-15

Liu, Z. H., J. A. Anderson, J. Hu, T. L. Friesen, J. B. Rasmussen and J. D. Faris (2005). A wheat intervarietal genetic linkage map based on microsatellite and target region amplified polymorphism markers and its utility for detecting quantitative trait loci Theor. Appl. Genet. 111: 782-794

Maleki, L., J. D. Faris, R. L. Bowden, B. S. Gill and J. P. Fellers. 2003. Physical and genetic mapping of wheat kinase analogs and NBS-LRR resistance gene analogs. Crop Sci 43: 660-670

Mcharo, M., D. R. LaBonte, C. Clark, M. Hoy and J. H. Oard. 2005. Molecular marker variability for southern root-knot nematode resistance in sweetpotato. Euphytica 144: 125-132.

Mcharo, M., D. R. LaBonte, J. H. Oard, S. J. Kays and W. L. McLaurin. 2004. Linking quantitative traits with AFLP markers in sweetpotato using discriminant analysis. Acta Hort. 637: 285-293

Meyers, B. C., A Kozik, A. Griego, H. H. Kuang and R. W. Michelmore. 2003. Genome wide analysis of NBS-LRR-encoding genes in *Arabidopsis*. Plant Cell 15: 809-834

Mignouna, H. D., Mank, R, A., Ellis, T. H. N. , van den Bosch, N.,, Asiedu, R. , Abang, M. M. , and Peleman, J 2002. A genetic linkage map of water yam (*Dioscorea alata* L.) based on AFLP markers and QTL analysis for anthracnose resistance.. / Theoretical and Applied Genetics 105 (5): 726-735Miklas, P. N., J. Hu, N. J. Grünwald and K. M. Larsen. 2006**.** Potential application of TRAP (Targeted Region Amplified Polymorphism) markers for mapping and tagging disease resistance traits in common bean Crop Sci 2006 46: 910-916.

Miklas, P. N., J. Hu, N. J. Grünwald, and K. M. Larsen. 2006. Potential application of TRAP (Targeted Region Amplified Polymorphism) markers for mapping and tagging disease resistance traits in common bean Crop Sci 2006 46: 910-916.

Naidoo, G., A. M. Forbes, A. Paul, D. G. White and T. R. Rocheford. 2002. Resistance to *Aspergillus* ear rot and aflatoxin accumulation in maize F_1 hybrids. Crop Sci. 42: 360-364

Ong, T.-M. 1975. Aflatoxin mutagenesis. Mutation Res. 32: 35-53

Paul, C., G. Naidoo, A. Forbes, V. Mikkilineni, D. G. White and T. Rocheford. 2003. Quantitative trait loci for low aflatoxin production in two related maize populations. Theor. Appl. Genetics 107 (2): 262-270

Pfahler, P. L. 1967. In vitro germination and pollen tube growth of maize (zea mays L.) pollen. Canadian J. of Botany 47: 839-845

Rencher, A. C. 1992. Interpretation of canonical discriminant functions, canonical variates and principal components. Amer. Statistician 46: 217-225.

Schneerman, M. C., W. S. Lee, G. Doyle and D. F. Weber. 1998. RFLP mapping of the centromere of chromosome 4 in maize using isochromosome for 4S. Theor. Appl. Genetics. 96: 361-366

Scott, G. E and N. Zummo. 1988. Sources of resistance in maize to kernel infection by *Aspergillus flavus* in the field. Crop Sci 28: 504-507

Scott, G. E and N. Zummo. 1990. Registration of Mp313E parental line of maize. Crop Sci 30: 1378

Scott, G. E., N. Zummo, E. B. Lillehoj, N. W. Widstrom, M. S. Kang, D. R. West, G. A. Payne, T. E. Cleveland, O. H. Calvert, and B. A. Fortnum. 1991. Aflatoxin in corn hybrids field inoculated with *Aspergillus flavus*. Agron. J. 83:595-598.

Shoba Rani, T and R. L. Ravikumar. 2007. Genetic enhancement of resistance to *Alternaria* leaf blight in sunflower through cyclic gametophytic and sporophytic selections. Crop Science 47: 529-534

Sills, G. R., Bridges, W., Al-Janabi, S. M., and B. W. S. Sobral. 1995. Genetic analysis of agronomic traits in a cross sugarcane (*Saccharum officinarum* L.) and its presumed progenitor (*S. robustum* Brandes & Jesw. Ex Grassl). Mol. Breed 1:355–363

Van Ooijen, J. W and R. E. Voorrips. 2001. JOINMAP 3.0. Software for the calculation of genetic linkage maps. Plant Research International, Wageningen, The Netherlands.

Van Ooijen, J. W., M. P. Boer, R. C. Jansen and C. Maliepaard. 2002. MapQTL 4.0: software for the calculation of QTL positions on genetic maps. Plant Research International, Wageningen, The Netherlands.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. Van de Lee, M. Hornes, A. Freijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau. 1995. AFLP: A new technique for DNA fingerprinting. Nucl. Acid Res. 23: 4407–4414.

Walker, R. D and D. G. White. 2001. Inheritance of resistance to Aspergillus ear rot and aflatoxin production of corn from C12. Plant Disease 85: 322-327.

White, D. G., T. R. Rocheford, A. M. Hamblin and a. M. Forbes. 1997. Inheritance of molecular markers associated with and breeding for resistance to *Aspergillus* ear rot and aflatoxin production in corn using Tex6. *In* J. Robens and J. Domer (eds), Aflatoxin elimination workshop: A decade of research progress 1988-1997 (p. 61). Food safety and Health, USDA-ARS, Beltsville, MD.

Widstrom, N.W. 1996. The aflatoxin problem with corn grain. Adv. Agron. 56:219-280.

Xu, Y. 2002. Global view of QTL: rice as a model. In: Kang MS (ed) Quantitative genetics, genomics and plant breeding. CAB International, pp 109–134

Zhang, N., Xu, Y., Akash, M., McCouch, S., and J. H. Oard. 2005. Identification of candidate markers associated with agronomic traits in rice using discriminant analysis. Theor Appl Genet. 110: 721-729

Zhang, Y., J. G. Simonson, G. Wang, M. S. Kang and H. F. Morris. 1998. A reliable field inoculation method for identifying aflatoxin resistant maize. Cereal Res. Commun. 26 : 245-251

Zhang, Y., M. S. Kang and R. Magari. 1997. Genetics to kernel infection by *Aspergillus flavus* in maize. Plant Breeding 116: 146-152

Zhang, Z. Y., F. Salamini and R. Thompson. 2002. Fine mapping of the defective endosperm maize mutant rgf1 using different DNA pooling strategies and three classes of molecular markers. Maydica 47: 277-286.

Zuber, M. S., E. B. Lillehoj and B. L. Renfro (eds). 1987. Aflatoxin in maize: A proceedings of the workshop. CIMMYT, Mexico, D. F.

Zummo, N and G. E. Scott. 1989. Evaluation of field inoculation techniques for screening maize genotypes against kernel infection by *Aspergillus flavus* in Mississippi. Plant Dis 73: 313-316.

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