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B.Z. Guo · R.G. Li · N.W. Widstrom · R.E. Lynch
T.E. Cleveland

Genetic variation within maize population GT-MAS:gk and the relationship with resistance to *Aspergillus flavus* and aflatoxin production

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Abstract *Aspergillus flavus* (Link:Fr.) infection and aflatoxin contamination of maize (*Zea mays* L.) grain are an extremely serious problem. Maize genotypes resistant to *A. flavus* attack are needed. Maize breeders and plant pathologists must identify resistance sources and incorporate resistance into adapted breeding material. Maize population GT-MAS:gk has been released for use as a resistance source. In this study, we surveyed the genetic variation in this population and made the breeders/plant pathologists aware of the heterogeneous nature in this maize population by using RAPD analysis and correlated the RAPD marker association with the resistance to *A. flavus* and aflatoxin production. Of 40 RAPD primers, only 15 gave sufficient numbers of reproducible and readily scored polymorphic bands suggesting that this population was highly homogeneous. However, genetic distances, ranging from 0.08 to 0.28 and averaging 0.17, suggest that there is variation within the population. Cluster analysis distinguished three major polymorphic groups. Laboratory bioassay revealed that group I contained the most resistant individuals, i.e., those with less aflatoxin production. Group II had the least resistance, and group III was intermediate. This study showed that the maize population GT-MAS:gk is heterogeneous and individuals are different in resistance to *A. flavus* and aflatoxin production. Resistance should be confirmed through progeny testing before further development. The RAPD marker OPX-04, which may be associated with the resistance trait, has been cloned and further characterization will be pursued.

Keywords Aflatoxin · Heterogeneous · Population · Resistance · RAPD markers · *Zea mays*

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B.Z. Guo (✉) · N.W. Widstrom · R.E. Lynch
USDA-ARS,
Insect Biology and Population Management Research,
Laboratory, Tifton, GA 31793-0748, USA
e-mail: bguo@tifton.cpes.peachnet.edu
Fax: (912)-387-2321

R.G. Li
Department of Entomology, University of Georgia,
Coastal Plain Experiment Station, Tifton, GA 31793, USA

T.E. Cleveland
USDA-ARS, Southern Regional Research Center,
New Orleans, LA 70179, USA

Introduction

Contamination of food and feed grains by aflatoxins is considered one of the most serious safety problems both in the United States and throughout the world. The fungi *Aspergillus flavus* (Link:Fr.) and *Aspergillus parasiticus* (Speare) produce aflatoxins on a number of crops, but aflatoxin contamination is most serious on maize (*Zea mays* L.), peanut (*Arachis hypogaea* L.), cotton (*Gossypium hirsutum* L.) seed and tree nuts. *A. flavus* appears to be the primary aflatoxin-producing fungus on these commodities although *A. parasiticus* also occurs frequently on peanut. Both fungi produce a family of related aflatoxins; the aflatoxins most commonly produced by *A. flavus* are B1 and B2, whereas *A. parasiticus* produces two additional aflatoxins, G1 and G2. B1 is the most carcinogenic and prevalent of the aflatoxins and thus receives most attention in mammalian toxicology (Goldblatt 1969; Squire 1981; Cleveland and Bhatnagar 1992). Foodstuffs contaminated with aflatoxin have been associated with increased incidence of liver cancer in humans (Hsieh 1989; Henry et al. 1999).

Plant breeding and host-plant resistance traditionally have been used to manage plant diseases and most likely will play a key role in reducing losses to pre-harvest aflatoxin contamination. In 1977, Zuber (1977) proposed the pursuit of genetic differences to improve resistance to aflatoxin contamination, followed with genetic investigations into the mechanisms of resistance (Zuber et al. 1978, 1983). Research studies have shown that resistance to infection and aflatoxin biosynthesis are genetically controlled (Thompson et al. 1984; Widstrom et al. 1984; Darrah et al. 1987; Gardner et al. 1987; Campbell

and White 1995; Guo et al. 1995, 1996; Campbell et al. 1997; Zhang et al. 1997). Efforts have been made to reduce grain contamination by aflatoxin through host-plant resistance, but the relative lack of techniques for the identification of resistant germplasm, if it exists, has slowed progress in plant improvement programs. There are several reasons for this, including non-uniform infestation procedures and lack of reproducibility across environments (Zuber 1977; Widstrom and Zuber 1983; Widstrom et al. 1984). Perhaps the greatest hindrance has been the absence of precise physical or biochemical markers responsible for, or associated with, resistant traits. In practical terms, there are no correlated traits currently known that breeders can use to effectively select for resistance. Efficient measurement of traits is an important aspect of breeding and selection. The ability to easily differentiate between plants that are resistant and susceptible to *A. flavus* and aflatoxin biosynthesis is critical to the selection and development of resistant lines and the successful transfer of resistance to elite lines.

Widstrom et al. (1987) selected maize population GT-MAS:gk from an infected ear which showed consistent resistance to aflatoxin accumulation when compared to susceptible lines (Widstrom et al. 1987; Guo et al. 1995, 1997, 1998; Russin et al. 1997). This population was released as resistant germplasm (McMillian et al. 1993). A sister population, GT-MAS:pw,nf, was less resistant to *A. flavus* and was maintained for comparison within a common genetic background (Widstrom et al. 1987; Brown et al. 1993).

Since GT-MAS:gk is a population and not an inbred line, segregation for many agronomic traits and resistance to *A. flavus* have been observed (N.W. Widstom, personal communication). In additions, aflatoxin levels tested in this population were inconsistent (Campbell and White 1995; Guo et al. 1995). In order to demonstrate if the variability of resistance exists in this population, and to demonstrate the heterozygous nature of this maize population, we examined the genetic variation using randomly amplified polymorphic DNA (RAPD) markers and tested the S_1 families resistance/susceptibility to *A. flavus* and aflatoxin production in a laboratory bioassay.

Materials and methods

Plant material and initial self-pollination

The population GT-MAS:gk was identified and released as germplasm resistant to *A. flavus* and has been maintained at the USDA-ARS Insect Biology and Population Management Laboratory, Tifton, GA. We planted 100 kernels of GT-MAS:gk in the greenhouse and labeled them individually for DNA analysis. Plants were self-pollinated and produced enough seeds (S_1 families) for a resistance assay in the laboratory. As a comparison for genetic analysis, GT-MAS:pw,nf (the sister population of GT-MAS:gk) was also planted.

Genomic DNA extraction

Leaves were collected from individual plants of GT-MAS:gk and stored immediately at –80°C. Bulked leaf samples from GT-MAS:pw,nf were also collected. Freeze-dried leaf samples were ground with a Cyclotec 1093 sample mill (Fisher Scientific, Atlanta, Ga.) and stored at –20°C until DNA was extracted. Genomic DNA was extracted according to the protocol of Hillis et al. (1990) with modification (Guo et al. 2001). Ground tissue (0.1 g) from each individual plant of GT-MAS:gk and the bulked sample from GT-MAS:pw,nf were added to a 1.5-ml microfuge tube along with 1.0 ml of CTAB extraction buffer (4% mixed alkyltrimethylammonium bromide, 1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0) containing 0.5% (w/v) sodium bisulfite to substitute for β-mercaptoethanol (Hämäläinen et al. 1997). Each tube was mixed thoroughly using a sterilized toothpick, to make sure that the leaf tissue was uniformly distributed in solution, and vortexed briefly before incubating at 65°C for 45 min.

Phenol/chloroform/isoamyl alcohol (25:24:1, 500 µl) was added to each tube. Tubes were inverted briefly by hand and rocked gently for 10 min. After centrifugation at 13,000 rpm for 5 min, the upper aqueous phase was transferred to a new 1.5-ml microfuge tube. An equal volume of chloroform/isoamyl alcohol (24:1) was added, and extracted by gentle inversion. The samples were centrifuged at 13000 rpm for 5 min and the upper aqueous layer was removed to a new 1.5-ml tube to which a 2× vol of 100% ethanol was added. The tubes were inverted gently several times and the DNA was allowed to precipitate for 20 min at –20°C. The samples were centrifuged for 20 min at 13000 rpm and the supernatant was discarded. The DNA pellet was rinsed with 70% ethanol and then air-dried for 15 min at room temperature. DNA was dispersed into 100 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0). The DNA content of each sample was measured using fluorescence spectrophotometry (DyNAQuant 200, Hoefer Pharmacia Biotech, San Francisco, Calif.).

RAPD polymerase chain reaction and scoring

Following an initial screening of 40 decamer oligonucleotides (Operon Kits X and Y, Operon Technologies, Alameda, Calif.) for polymorphism, 15 were used to amplify DNA samples from GT-MAS:gk and a bulked sample from GT-MAS:pw,nf. The nucleotide sequence of each primer was randomly generated with the only specification that it possess a 60–70% GC content and has no palindromes of greater than four base pairs (Williams et al. 1990). The DNA amplification protocol reported by Kresovich et al. (1994) was followed. Reactions were carried out in a DNA thermal cycler programmed for 45 cycles of 1 min at 94°C, 5 min at 38°C, a 3-min ramp up to 72°C, and 2 min at 72°C. Amplification products were analyzed by gel electrophoresis in 2% agarose. Molecular sizes of all amplification products were estimated by using a 1-kb ladder (BRL, Bethesda, Md.). Each amplification run was performed three times for each DNA sample for consistency. Amplification bands were scored as present or absent.

Resistance evaluation

After initial screening of 100 S_1 families, 11 were selected and tested for resistance to *A. flavus* and aflatoxin accumulation using a laboratory screening method as described (Brown et al. 1993; Guo et al. 1995). There were 15 replications for each family. Immediately following incubation, kernels were rated for fungal growth (0–5) as described by Guo et al. (1995). Kernels then were dried in a forced-air oven at 60°C for 2 days to stop further toxin synthesis.

Aflatoxin B1 in each replication was determined using the official methods of the American Oil Chemists Society (Anonymous 1989) with modification (Guo et al. 1995). The final volume of each sample was 2 ml of benzene:acetonitrile (98:2, v:v), and 30 µl from each sample was spotted on silica gel thin-layer chro-

matography (TLC) plates (Aldrich Chemical Company, Milwaukee, Wis.). Plates were developed in anhydride ether:methanol:water (96:3:1). Aflatoxins were identified and quantified directly on TLC plates and compared with a commercial aflatoxin B and G mixture (Sigma, St. Louis, Mo.) using a Shimadzu dual wavelength flying spot scanning densitometer with a fluorometry attachment (Model CS-9301PC, Shimadzu Corporation, Kyoto, Japan).

Data analysis

The genetic similarities (GS) between pairs of individual plants were calculated from RAPD data for all possible pairs by the formula: S(x, y)=2 N(x, y)/[N(x)+N(y)], where N(x, y) is the total number of bands common to lines x and y, and N(x) and N(y) are the total number of bands present in x and y, respectively. The genetic distance (GD) is converted from GS (GD=1–GS). Using the algorithm of Dice (Dice 1945; Nei and Li 1979) in the "RAPDistance" computer program (Armstrong et al. 1994), values of GD may range from 0 (identical profiles for all markers in the two samples) to 1 (no bands in common). Relationships among individual samples were summarized in an unrooted neighbor-joining tree (Saitou and Nei 1987) which was constructed with "NJTREE" software (version 2.0) and produced by the "TDRAW" option in the "RAPDistance package" (Armstrong et al. 1994).

Results

To select primers with both high levels of polymorphism and reproducible RAPD profiles, we did a preliminary analysis using a total of 40 primers and six representative DNA samples. Using this approach, we selected 15 primers producing intensively stained and reproducible polymorphic bands after two PCR reactions. These 15 primers were used to detect polymorphisms among the 32 samples from the GT-MAS:gk population and one bulked sample from GT-MAS:pw,nf, the susceptible sister population, yielding a total of 137 bands. Among the DNA samples, 102 bands were polymorphic (Table 1).

Table 1 List of decamer oligonucleotides utilized for random priming, their sequences, and fragments amplified among the maize population GT-MAS:gk

Primer identification	Sequence (5′ to 3′)	Number of fragments	
		Total	Variable
OPX-01	CTGGGCACGA	7	5
OPX-03	TGGCGCAGTG	9	5
OPX-04	CCGCTACCGA	10	6
OPX-06	ACGCCAGAGG	6	2
OPX-09	GGTCTGGTTG	10	8
OPX-20	CCCAGCTAGA	10	9
OPY-04	GGCTGCAATG	6	4
OPY-05	GGCTGCGACA	9	6
OPY-06	AAGGCTCACC	6	5
OPY-07	AGAGCCGTCA	11	7
OPY-10	CAAACGTGGG	10	9
OPY-11	AGACGATGGG	18	15
OPY-14	GGTCGATCTG	6	4
OPY-15	AGTCGCCCTT	6	5
OPY-20	AGCCGTGGAA	13	12
Total		137	102

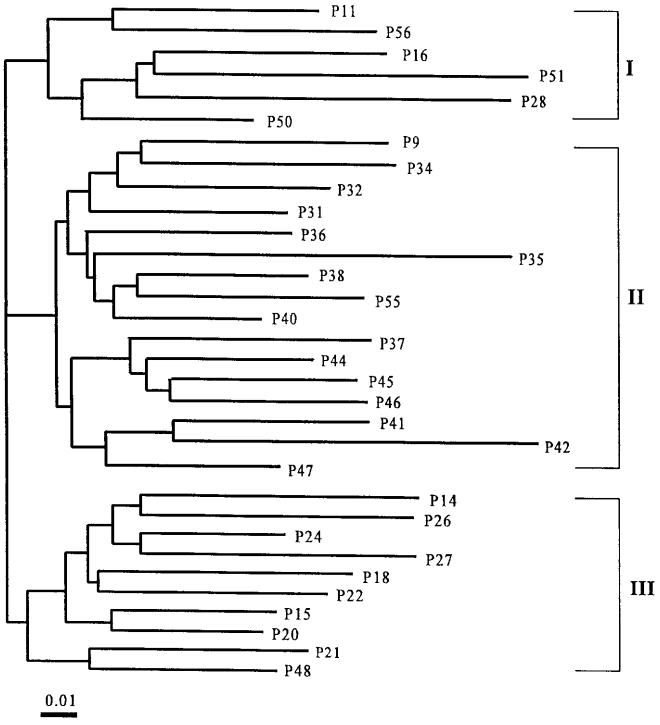

Fig. 1 Dendrogram of neighbor-joining tree of individual samples from maize population GT-MAS:gk, resistant to *A. flavus* and aflatoxin production. It was generated by the "NJTREE" procedure of "RAPDistance" (Armstrong et al. 1994) for Dice genetic distance indices (Dice 1945)

The 102 polymorphic bands were used to generate the genetic distance matrix (Table 2). Genetic distances among the 32 individuals within the population GT-MAS:gk ranged from 0.08 for the most-similar individuals (P15 and P20) to 0.28 for the more-variable ones (P28 and P35), with an average of 0.17. A comparison of the bulked DNA sample of the population GT-MAS:pw,nf with the individual samples of GT-MAS:gk showed a range of genetic distances from 0.27 to 0.33, with an average of 0.29. Results showed that these two sister populations, GT-MAS:gk and GT-MAS:pw,nf, were more distant genetically than the individuals within the GT-MAS:gk population (Table 2).

The cluster-analysis dendrogram using the neighbor-joining tree procedure showed three major groups within the GT-MAS:gk population (Fig. 1) containing 6, 16 and 10 individuals, respectively. The primer OPX-04 produced polymorphic bands among the samples (Fig. 1) and one polymorphic band corresponded well with this dendrogram. All six samples in group I had this polymorphic band, while only two samples in group III had this band. As an example, 14 samples were amplified using primer OPX-04 (Fig. 2). This polymorphic band located above the size marker of 506 bp was only present in group I and absent in groups II, III and GT-MAS:pw,nf (Fig. 2). This polymorphic DNA band has been cloned and sequenced with a 642-bp length (data not shown). Another polymorphic band, about 298 bp

Table 2 Genetic distance matrix of individuals within maize population GT-MAS:gk and a bulked sample of the GT-MAS:pw,nf population (pw,nf)

Plant #	9	11	14	15	16	18	20	21	22	24	26	27	28	31	32
9	0.00														
11	0.18	0.00													
14	0.17	0.13	0.00												
15	0.15	0.14	0.13	0.00											
16	0.20	0.18	0.18	0.13	0.00										
18	0.18	0.17	0.19	0.13	0.15	0.00									
20	0.11	0.13	0.15	0.08	0.14	0.10	0.00								
21	0.15	0.14	0.14	0.14	0.18	0.14	0.11	0.00							
22	0.17	0.17	0.17	0.14	0.17	0.13	0.15	0.15	0.00						
24	0.16	0.13	0.13	0.10	0.15	0.11	0.10	0.14	0.13	0.00					
26	0.20	0.13	0.15	0.16	0.17	0.16	0.14	0.17	0.13	0.11	0.00				
27	0.18	0.17	0.17	0.15	0.18	0.15	0.15	0.17	0.14	0.11	0.15	0.00			
28	0.21	0.21	0.19	0.15	0.17	0.18	0.16	0.18	0.21	0.18	0.22	0.24	0.00		
31	0.12	0.13	0.16	0.15	0.15	0.17	0.15	0.15	0.14	0.12	0.16	0.15	0.23	0.00	
32	0.13	0.16	0.20	0.14	0.17	0.15	0.14	0.14	0.15	0.13	0.18	0.19	0.22	0.12	0.00
34	0.13	0.15	0.18	0.18	0.21	0.18	0.15	0.17	0.14	0.15	0.17	0.19	0.27	0.13	0.13
35	0.21	0.22	0.27	0.20	0.19	0.25	0.21	0.24	0.20	0.23	0.26	0.25	0.28	0.16	0.19
36	0.14	0.13	0.19	0.12	0.18	0.17	0.15	0.14	0.13	0.15	0.19	0.19	0.21	0.11	0.11
37	0.19	0.16	0.21	0.14	0.14	0.13	0.15	0.17	0.17	0.15	0.19	0.21	0.22	0.14	0.16
38	0.16	0.14	0.17	0.13	0.17	0.16	0.12	0.14	0.13	0.14	0.17	0.16	0.18	0.13	0.14
40	0.14	0.12	0.17	0.10	0.15	0.16	0.11	0.15	0.14	0.13	0.13	0.15	0.22	0.10	0.11
41	0.16	0.15	0.20	0.15	0.20	0.20	0.15	0.16	0.20	0.15	0.19	0.22	0.20	0.14	0.16
42	0.20	0.24	0.24	0.22	0.24	0.26	0.21	0.20	0.23	0.21	0.25	0.24	0.25	0.19	0.19
44	0.15	0.14	0.18	0.13	0.16	0.17	0.13	0.14	0.18	0.14	0.19	0.18	0.17	0.13	0.14
45	0.18	0.16	0.21	0.14	0.15	0.15	0.16	0.17	0.19	0.16	0.21	0.19	0.18	0.15	0.17
46	0.16	0.20	0.23	0.15	0.17	0.16	0.16	0.18	0.18	0.18	0.22	0.18	0.18	0.15	0.16
47	0.14	0.14	0.19	0.14	0.18	0.15	0.13	0.14	0.17	0.14	0.19	0.21	0.21	0.11	0.11
48	0.13	0.16	0.18	0.17	0.20	0.16	0.13	0.11	0.15	0.18	0.20	0.19	0.18	0.14	0.14
50	0.14	0.15	0.20	0.15	0.14	0.14	0.14	0.16	0.16	0.17	0.19	0.18	0.16	0.11	0.14
51	0.25	0.19	0.24	0.22	0.16	0.21	0.21	0.23	0.21	0.18	0.21	0.22	0.21	0.21	0.22
55	0.16	0.16	0.17	0.13	0.18	0.17	0.14	0.16	0.12	0.15	0.17	0.17	0.22	0.11	0.14
56	0.19	0.12	0.18	0.18	0.16	0.18	0.17	0.16	0.10	0.19	0.22	0.21	0.21	0.16	0.19
pw,nf	0.27	0.30	0.30	0.28	0.31	0.30	0.25	0.31	0.33	0.30	0.32	0.33	0.29	0.28	0.29

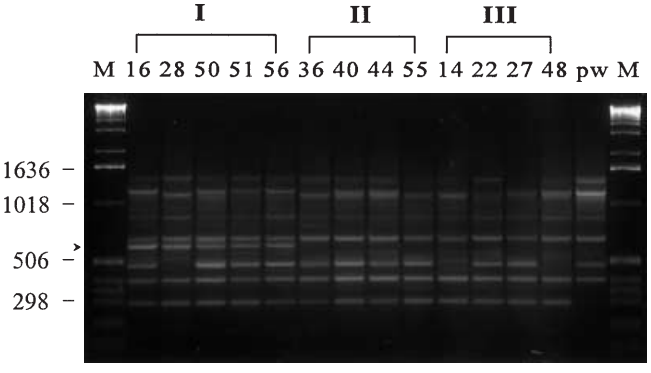

Fig. 2 Randomly amplified polymorphic DNA (RAPD) profiles of samples within maize population GT-MAS:gk and a bulked sample from population GT-MAS:pw,nf (*pw*) analysed with primer OPX-04. The *arrow* (>) shows that a polymorphic band is present in group I only, which may be associated with the resistance to *A. flavus* and aflatoxin production. DNA size markers (*M*) in base pairs was used. *I*, *II*, and *III* are cluster groups in the dendrogram (Fig. 1)

in size, was present in all GT-MAS:gk samples, but absent in GT-MAS:pw,nf (Fig. 2).

Resistance evaluation of the S_1 families from the population GT-MAS:gk showed *A. flavus* growth ratings and aflatoxin concentrations ranging from 0.5 to 4.5 and 53

Table 3 *A. flavus* fungal growth and aflatoxin concentration on kernels from S_1 families of maize population GT-MAS:gk and kernels from population GT-MAS:pw,nf (pw,nf)

S1#	Fungal growth rate[a]	Aflatoxin B1 (ng/g)[b]	Resistance to *A. flavus*[c]	Clustered group
16	1.0	120±52 c	R	I
28	0.5	53±16 c	R	I
50	1.5	159±68 c	R	I
51	1.0	83±30 c	R	I
56	1.5	107±57 c	R	I
34	4.0	982±107 a	S	II
40	4.5	1106±250 a	S	II
55	4.0	1408±178 a	S	II
14	3.5	583±96 b	S	III
27	4.5	790±241 b	S	III
48	3.0	381±183 b	I	III
pw,nf	4.0	985±351 a	S	N/A

[a] Fungal growth rating was rated as follows: 0, mycelium visible on kernel surface but no sporulation; 1, 1 to 20%; 2, 21 to 40%; 3, 41 to 60%; 4, 61 to 80%; 5, 81 to 100% of the kernel surface covered by conidiophores bearing conidia

[b] Mean±SE. Each mean was averaged from 15 replication. Means followed by the same letter did not differ significantly ($P>0.05$)

[c] R=resistant; S=susceptible; I=intermediate

Table 2 (contineud)

34	35	36	37	38	40	41	42	44	45	46	47	48	50	51	55	56	pw,nf
0.00																	
0.18	0.00																
0.14	0.16	0.00															
0.18	0.20	0.13	0.00														
0.17	0.18	0.11	0.14	0.00													
0.13	0.15	0.10	0.12	0.09	0.00												
0.15	0.22	0.15	0.16	0.15	0.13	0.00											
0.18	0.23	0.20	0.24	0.20	0.20	0.15	0.00										
0.17	0.22	0.11	0.12	0.12	0.11	0.13	0.16	0.00									
0.18	0.20	0.15	0.11	0.15	0.15	0.15	0.15	0.11	0.00								
0.18	0.17	0.13	0.12	0.12	0.12	0.15	0.19	0.09	0.09	0.00							
0.15	0.17	0.11	0.14	0.12	0.12	0.13	0.15	0.10	0.09	0.14	0.00						
0.14	0.20	0.13	0.17	0.11	0.12	0.17	0.23	0.14	0.18	0.14	0.15	0.00					
0.17	0.17	0.13	0.13	0.11	0.11	0.15	0.20	0.13	0.12	0.11	0.14	0.12	0.00				
0.25	0.20	0.24	0.21	0.21	0.21	0.23	0.26	0.18	0.17	0.21	0.19	0.22	0.14	0.00			
0.17	0.18	0.13	0.17	0.10	0.10	0.19	0.22	0.16	0.19	0.16	0.16	0.13	0.15	0.24	0.00		
0.19	0.18	0.17	0.17	0.15	0.16	0.16	0.21	0.19	0.15	0.16	0.17	0.18	0.12	0.18	0.16	0.00	
0.27	0.28	0.32	0.32	0.29	0.29	0.28	0.33	0.29	0.30	0.29	0.28	0.28	0.27	0.32	0.30	0.28	0.00

to 1408 ng/g, respectively (Table 3). In group I, the average fungal growth rating was 1.1, and aflatoxin concentration averaged 104 ng/g. In groups II and III, the fungal growth rating averaged 4.2 and 3.7, and aflatoxin concentration averaged 1165 and 585 ng/g, respectively. The sister population GT-MAS:pw,nf had a fungal growth rating of 4.0 and aflatoxin concentration of 985 ng/g (Table 3). Aflatoxin concentrations were significantly lower in Groups I and III than in Group II, but aflatoxin levels were not different between Group II and the sister population GT-MAS:pw,nf.

Discussion

In this research, we surveyed the variability within the maize population GT-MAS:gk using RAPD markers and assessed the relationship between RAPD polymorphism and the resistance of S_1 families to *A. flavus* and aflatoxin production. Our results are in agreement with those of Widstrom et al. (1987) and McMillian et al. (1993), in which heterozygosity was expected in this population and the resistance trait was not fixed. McMillian et al. (1993) released maize germplasm GT-MAS:gk as a source of resistance to aflatoxin accumulation. It was derived from a visibly segregating hybrid ear that was infected by *A. flavus* (Widstrom et al. 1987). A less-resistant sister population GT-MAS:pw,nf was also selected from this segregating hybrid ear (Widstrom et al. 1987).

We demonstrated that considerable variation among the individual plants within GT-MAS:gk was detectable using RAPD markers. However, only 15 out of 40 primers (i.e., 37.5%) resulted in polymorphic bands, which suggested that this population is highly homogeneous. Comparison of these two sister populations revealed that the genetic distance among individual plants within GT-MAS:gk was smaller than between it and its susceptible sister population GT-MAS:pw,nf. This result is in close agreement with the fact that GT-MAS:gk was selected and maintained as a resistant population and that GT-MAS:pw,nf was selected and maintained as a separate susceptible population.

In the dendrogram obtained from the cluster analysis in this study, individual plants within this population were separated into three main groups on the basis of 102 polymorphic RAPD markers. Group I had six individuals, Group II had 16 individuals, and Group III had ten individuals. On the other hand, the resistance evaluation of the S_1 families showed a relationship with the dendrogram clustering analysis. Group I contained the

most-resistant individuals with less accumulation of aflatoxin, whereas groups II and III contained individuals with higher aflatoxin accumulation. Compared with the susceptible sister population GT-MAS:pw,nf, groups I and III had significantly lower aflatoxin concentrations. Numerous studies support a positive correlation between the genetic diversity of parental lines and hybrid performance in a number of species (Lee et al. 1989; Smith et al. 1990; Zhang et al. 1995; Ajmone Marsan et al. 1998; Garcia et al. 1998). Analysis by the RAPD method may also provide a better knowledge of the genetic relationship among the individuals within this population. The genetic differences between individuals, revealed by the RAPD markers, may be linked with the resistance traits. Further study needs to be carried out to answer the quantitative nature of these traits with a larger population (Guo et al. 2000). We have made a selection for S_5 generations, and field and laboratory evaluations (unpublished data) are in agree with the S_1 families in this test. Genetic mapping of the resistance traits in this population has been conducted using RFLP (Guo et al. 2000).

A. flavus infection and subsequent aflatoxin contamination of maize grain is an extremely serious problem in the southeastern United States and in other parts of the world. Limited progress has been made in developing and identifying sources for resistance and incorporating improved resistance into breeding material. This study shows a potential for the better utilization of resistance traits from GT-MAS:gk, e.g. pericarp wax (Guo et al. 1995, 1996; Russin et al. 1997) and antifungal proteins (Guo et al. 1997, 1998; Chen et al. 1998). The results in this study clearly show the genetic divergence within this population. Resistance in the S_1 families has been tested and one RAPD marker with OPX-04 has been identified for potential association with the resistance trait. This polymorphic band has been cloned and sequenced (unpublished data) and further characterization of this marker will be done in order to use the trait in a breeding program for developing resistant inbred lines.

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References

Ajmone Marsan P, Castiglioni P, Fusai F, Kuiper M, Motto M (1998) Genetic diversity and its relationship to hybrid performance in maize as revealed by RFLP and AFLP markers. Theor Appl Genet 96:219–227

Anonymous (1989) Aflatoxin in corn, official method Aj3–87. In: Official methods and recommended practices. American Oil Chemists' Society, Champsign, Illinois

Armstrong JS, Gibbs AJ, Peakall R, Weiller G (1994) The RAPDistance package. http://life.anu.edu.au/molecular/software/rapd.html

Brown RL, Cotty PJ, Cleveland TE, Widstrom NW (1993) Living maize embryo influences accumulation of aflatoxin in maize kernels. J Food Prot 56:967–971

Campbell KM, White DG (1995) Evaluation of corn genotypes for resistance to *Aspergillus* ear rot, kernel infection, and aflatoxin production. Plant Dis 79:1039–1045

Campbell KM, Hamblin AM, White DG (1997) Inheritance of resistance to aflatoxin production in the cross between corn inbreds B73 and LB31. Phytopathology 87:1144–1147

Chen Z-Y, Brown RL, Lax AR, Guo BZ, Cleveland TE, Russin JS (1998) Resistance to *Aspergillus flavus* in corn kernels is associated with a 14-kDa protein. Phytopathology 88:276–281

Cleveland TE, Bhatnagar D (1992) Molecular strategies for reducing aflatoxin levels in crops before harvest. In: Bhatnagar D, Cleveland TE (eds) Molecular approaches to improve food quality and safety. Van Nostrand Reinhold, New York, pp 205–228

Darrah LL, Lillehoj EB, Zuber MS, Scott GE, Thompson D, West DR, Widstrom NW, Fortnum BA (1987) Inheritance of aflatoxin B1 levels in maize kernels under modified natural inoculation with *Aspergillus flavus*. Crop Sci 27:869–872

Dice LR (1945) Measures of the amount of ecologic association between species. Ecology 26:297–302

Garcia E, Jamilena M, Alvarez JI, Arnedo T, Oliver JL, Lozano R (1998) Genetic relationships among melon breeding lines revealed by RAPD markers and agronomic traits. Theor Appl Genet 80:833–840

Gardner CAC, Darrah LL, Zuber MS, Wallin JR (1987) Genetic control of aflatoxin production in maize. Plant Dis 71:426–429

Goldblatt LA (1969) Aflatoxin: scientific background, control, and implications. Academic Press, New York

Guo BZ, Russin JS, Cleveland TE, Brown RL, Widstrom NW (1995) Wax and cutin layers in maize kernels associated with resistance to aflatoxin production by *Aspergillus flavus*. J Food Prot 58:296–300

Guo BZ, Russin JS, Cleveland TE, Brown RL, Damann KE (1996) Evidence for cutinase production by *Aspergillus flavus* and its possible role in infection of corn kernels. Phytopathology 86:824–829

Guo BZ, Chen ZY, Brown RL, Lax AR, Cleveland TE, Russin JS, Mehta AD, Selitrennikoff CP, Widstrom NW (1997) Germination induces accumulation of specific proteins and antifungal activities in corn kernels. Phytopathology 87:1174–1178

Guo BZ, Brown RL, Lax AR, Cleveland TE, Russin JS, Widstrom NW (1998) Protein profiles and antifungal activities of kernel extracts from corn genotypes resistant and susceptible to *Aspergillus flavus*. J Food Prot 61:98–102

Guo BZ, Widstrom NW, Butron A, Wilson DM, Snook ME, Cleveland TE, Lynch RE (2000) Control of preharvest aflatoxin contamination in maize through pyramiding resistance genes to ear-feeding insects and invasion by *Aspergillus* spp. Phytopathology 90:S32

Guo BZ, Zhang ZJ, Li RG, Widstrom NW, Snook ME, Lynch RE, Plaisted D (2001) Restriction fragment polymorphism markers associated with silk maysin, antibiosis to corn earworm (Lepidoptera: Noctuidae), in a dent and sweet corn cross. J Econ Entomol 94:564–571

Hämäläinen JH, Watanabe KN, Valkonen JPT, Arihara A, Plaisted RL, Pehu E, Miller L, Slack SA (1997) Mapping and marker-assisted selection for a gene for extreme resistance to potato virus Y. Theor Appl Genet 94:192–197

Henry SH, Bosch FX, Troxell TC, Bolger PM (1999) Reducing liver cancer – global control of aflatoxin. Science 286:2453–2454

Hillis DM, Larson A, Davis SK, Zimmer EA (1990) Nucleic acids III: sequencing. In: Hillis DM, Moritz C (eds) Molecular systematics. Sinauer, Sunderland, Massachusetts, pp 318–370

Hsieh DPH (1989) Potential human health hazards of mycotoxins. In: Natori S, Hashimoto K, UenoY (eds) Mycotoxins and phycotoxins. Elsevier, Biomedical Press, Amsterdam, pp 69–80

Kresovich S, Lamboy WF, Li R, Ren J, Szewc-McFadden AK, Bliek SM (1994) Application of molecular methods and statistical analyses for discrimination of accessions and clones of vetiver grass. Crop Sci 34:805–809

Lee M, Godshalk EB, Lamkey KR, Woodman WL (1989) Association of restriction length polymorphism among maize inbreds with agronomic performance of their crosses. Crop Sci 29: 1067–1071

McMillian WW, Widstrom NW, Wilson DW (1993) Registration of GT-MAS:gk maize germplasm. Crop Sci 33:882

Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76:5269–5273

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

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Smith OS, Smith JSC, Brown SL, Tenborg RA, Wall SJ (1990) Similarities among a group of elite maize inbreds measured by pedigree. Theor Appl Genet 80:833–840

Squire (1981) Ranking animal carcinogens: A proposed regulatory approach. Science 214:877

Thompson DL, Rawlings JO, Zuber MS, Payne GA, Lillehoj EB (1984) Aflatoxin accumulation in developing kernels of eight maize crosses after inoculation with *Aspergillus flavus*. Plant Dis 68:465–467

Widstrom NW, Zuber MS (1983) Prevention and control of aflatoxin in corn: sources and mechanisms of genetic control in the plant. In: Diener UL, Asquith RL, Dickens JW (eds) Aflatoxin and *Aspergillus flavus* in corn. South Coop Ser Bull 279, Alabama Agr Exp Station, Auburn, pp 72–76

Widstrom NW, Wilson DM, McMillian WW (1984) Ear resistance of maize inbreds to field aflatoxin contamination. Crop Sci 24:1155–1157

Widstrom NW, McMillian WW, Wilson DM (1987) Segregation for resistance to aflatoxin contamination among seeds on an ear of hybrid maize. Crop Sci 27:961–963

Williams JGK, Kubelik AR, Livak WJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535

Zhang Q, Gao YJ, Saghai Maroof MA, Yang SH, Li JX (1995) Molecular divergence and hybrid performance in rice. Mol Breed 1:133–142

Zhang Y, Kang MS, Magari R (1997) Genetics of resistance to kernel infection by *Aspergillus flavus* in maize. Plant Breed 116:146–152

Zuber MS (1977) Influence of plant genetics on toxin production in corn. In: Rodricks JV, Hesseltine CW, Mehlman MA (eds) Mycotoxins in human and animal health. Pathotox Publishers, Park Forest South, Illinois, pp 173–179

Zuber MS, Calvert OH, Kwolek WF, Lillehoj EB, Kang MS (1978) Aflatoxin B1 production in an eight-line diallel of *Zea mays* infected with *Aspergillus flavus*. Phytopathology 68: 1346–1349

Zuber MS, Darrah LL, Lillehoj EB, Josephson LM, Manwiller A, Scott GE, Gudauskas RT, Horner ES, Widstrom NW, Thompson DL, Bockholt AJ, Brewbaker JL (1983). Comparison of open-pollinated maize varieties and hybrids for preharvest aflatoxin contamination in the southern United States. Plant Dis 67:185–187