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Control of preharvest aflatoxin contamination in maize by pyramiding QTL involved in resistance to ear-feeding insects and invasion by *Aspergillus* spp.

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Abstract

Several resistance sources and resistance mechanisms to aflatoxin formation and corn earworm (*Helicoverpa zea* Boddie) damage to maize (*Zea mays* L.) have been identified. Based on this knowledge, experiments were initiated toward achievement of the following objectives: (1) to confirm earlier determinations on resistance traits of germplasm sources and to identify quantitative trait loci (QTL) associated with each of the traits, and (2) upon estimation of the degree of QTL effects on each trait, to generate a maize population, with chemical and physical resistance to *Aspergillus* spp. and ear-feeding insects, for inbred development. A 2-year field experiment to evaluate selected genotypes inoculated with *A. flavus* and infested with corn earworm revealed that significant variation exists among the genotypes for aflatoxin contamination and corn earworm damage. The protection of maize ears against aflatoxin contamination was primarily dependent on resistance to fungal infection and ear-feeding insects, and excellent husk coverage and tightness. A major QTL (*p1*) identified on chromosome 1S had effects of 54.0, 42.1, and 28.3% on the phenotypic variability for concentrations of silk maysin, 3′-methoxymaysin+apimaysin, and chlorogenic acid, respectively. Markers/QTLs for husk phenotypic traits and total aflatoxin concentrations have been determined, but more detailed mapping of these chromosomic regions will be necessary to locate precise markers/QTLs for husk traits and aflatoxin production. Realizing the complexity of the *Aspergillus*–aflatoxin-maize system and the factors affecting aflatoxin contamination, we are directing our program toward marker-assisted breeding to enhance or improve general genetic resistance to ear-feeding insects and invasion by *Aspergillus* spp.
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1. Introduction

Contamination by aflatoxin, a metabolite of *Aspergillus* spp. fungi, in maize grain was documented as a preharvest problem by Anderson et al. (1975). The seriousness of the problem was not fully recognized until 1977 when widespread contamination occurred, especially in the southeastern US. High levels of contamination were reported in 1977 by Alabama (Gray et al., 1982), by Georgia (Wilson et al., 1979) and by North Carolina (Hesseltine et al., 1981). A clear pattern of heavy contamination in the South was already emerging prior to 1977, and was reported by Lillehoj et al. (1975). Other studies by many of the same authors confirmed much higher contamination levels in Florida, Georgia and South Carolina than in adjacent states to the north or in the Corn Belt (Lillehoj et al., 1980).

The field contamination process is known to be influenced by numerous factors, several of which cannot be controlled by the producer, such as temperature, humidity, evapotranspiration, water availability to the crop and fungus, and many other factors that produce stress on the plant (McMillian et al., 1985; Widstrom, 1992). A recurring theme in southern grown maize, however, is the influence of insects on ultimate contamination levels (Widstrom et al., 1975). The association of aflatoxin contamination with insect damage has been a common thread connecting several reports (Barry et al., 1992). Insects have been implicated as effective vectors of the fungus to developing ears of field corn (Barry et al., 1985). The effect of husk tightness and coverage, subsequently has an important influence on aflatoxin contamination, especially as it relates to prevention of entry by insects into the ears of southern grown hybrids (McMillian et al., 1987).

Tight husks that cover the ear completely are necessary to insure silk feeding while the corn earworm enters the ear. Silk feeding is necessary to allow maysin, an antibiotic compound produced by silks of some maize lines, opportunity to prevent normal growth of corn earworm larvae (Widstrom and Snook, 1997). Some maize lines have sufficient maysin in their silks to prevent larval development beyond the first instar (Snook et al., 1997). Sufficient genetic variation is available to allow development of dent and sweetcorn lines with very high silk maysin concentrations (Guo et al., 1999b). Several quantitative trait loci (QTL) influencing maysin concentration are known, as are a number of flavonoid pathway genes controlling maysin concentration in silks (McMullen et al., 1998; Lee et al., 1998). Therefore, these loci are available for use in the development of high maysin lines.

Several germplasm sources are available that have at least moderate plant resistance to invasion by *Aspergillus* fungi and contamination by the fungal metabolite aflatoxin (Scott et al., 1991; McMillian et al., 1993). Genetic variation for resistance to aflatoxin contamination is difficult to measure, and in addition, none of the sources is considered as having a high level of resistance (Zuber et al., 1983; Darrah et al., 1987). Breeding strategies to control preharvest contamination have been proposed (Widstrom and Zuber, 1983). Most of them have emphasized measurement of infection or the concentration of aflatoxin as the selected trait, but some recent studies have suggested that endosperm characteristics (Widstrom et al., 1984), kernel proteins (Guo et al., 1999a), kernel wax and cutin layers on the surface of kernels (Guo et al., 1995; Russin et al., 1997), and other unknown compounds in the embryo (Guo et al., 1996) are important resistance mechanisms. In view of what is presently known about the importance of resistance to insects, maysin concentration of the silks, accompanying husk cover, and plant resistance to infection and aflatoxin production by *Aspergillus* spp., we propose combining these important traits into a single genotype. Our objectives are; (1) to confirm previous determinations on resistance traits of resistance sources, and to identify QTL associated with each of these traits: resistance to ear-feeding insects, silk maysin concentration in the silks, husk tightness and coverage, and aflatoxin production, and (2) upon estimation of the degree of QTL effects for each trait, to generate a maize population with chemical and physical resistance to *Aspergillus* spp. and ear-feeding insects for inbred development using marker-assisted selection (MAS).

2. Materials and methods

2.1. Field testing

Field evaluations were conducted to demonstrate the potential for combining various types of resistance to control preharvest aflatoxin contamination. Six inbred, or near-inbred lines were chosen as the source germplasm for pyramiding the traits of interest. Each source had been identified from numerous previous studies as having the unique traits we were seeking to combine. The sources and their general ratings for several traits are given in Table 1. These sources were chosen so that at least two or more would rate favorably for each desirable trait to be pyramided.

The germplasms chosen were tested in a field experiment conducted during the years 1998 and 1999. $F_{2:3}$ families were evaluated in 1999 and 2000. One-half of the plots were silk-inoculated with an aflatoxin producing isolate of *A. flavus* and half were also infested with 30 corn earworm eggs/plant applied to the silks in an agar medium, giving four infestation–inoculation treatment combinations. Inoculations were made when silks began to turn brown and infestations were made at the full-silk stage. The experiments were planted on May 5, 1998, May 12, 1999, and April 14, 2000 in a randomized complete block arrangement with four replications. Inoculation and infestation treatment combinations were randomized within germplasm sources. Plots were single rows, 6-m long with 90-cm between rows and 20-cm plant spacing within rows.

Ten plants were rated for corn earworm damage at 21 days after infestation and ears from 10 plants were harvested at 60 days after silking, dried at 60 °C, shelled and fine-ground for aflatoxin analysis. Data for aflatoxin contamination, corn earworm damage and days to silking were analyzed by partitioning years and germplasm sources, with individual degrees of freedom broken out for infestation–inoculation treatment combinations. Years and replications were assumed random while germplasm sources and treatment combinations were considered as being fixed.

2.2. RLFP mapping population

Our approach was to develop F_2 populations and $F_{2:3}$ families from single crosses chosen to address the contribution of specific traits of silk maysin levels, husk coverage, and aflatoxin contamination. The F_2 mapping population for this study was developed from the cross GT-MAS:gk (A1) × GT119 and $F_{2:3}$ families were produced by self-pollination of individual F_2 ears. The inbred

Table 1
Germplasm sources evaluated for corn earworm damage, aflatoxin contamination and maturity in 1998 and 1999 at Tifton, GA, USA, and general evaluation of related traits

Trait	Year	GT-MAS:gk(A1)	GT-MAS:gk(A2)	SC102	GE37	GT119	ZC2451(P)C3	Mean
Corn earworm rating[a]	1998	7.7	7.5	6.8	5.7	5.3	2.4	6.3*
	1999	8.7	5.2	6.0	5.2	5.6	1.5	5.7
Aflatoxin concentration[b]	1998	5.52	4.85	3.98	4.78	3.24	3.24	4.67**
	1999	4.45	2.52	2.39	1.52	2.40	2.01	3.13
Days to silking	1998	60	61	62	70	60	52	60**
	1999	60	63	66	74	62	63	63
Husk coverage and tightness		Poor	Poor	Good	Medium	Good	Excellent	–
Maysin concentration		Medium	Medium	High	High	Low	Medium	–
Agronomic type		Good	Good	Poor	Good	Medium	Poor	–

*, ** 1998 values are significantly different from those of 1999 at $P \leqslant 0.05$ and $P \leqslant 0.01$, respectively.

[a] 0 = no damage, 1 = silk damage, 2 = damage to 1 cm beyond ear tip, and 3..... $n = (n-1)$ cm damage by corn earworm penetration.

[b] Concentration transformed to $\ln(\text{ng g}^{-1}+1)$.

GT-MAS:gk (A1) subsequently referred to as GT-A1, was developed from the population GT-MAS:gk (McMillian et al., 1993), which was released as a breeding population resistant to *A. flavus*. GT-A1 has a silk maysin concentration of about 0.6%, is resistant to *A. flavus* producing relative low aflatoxin levels (Li et al., 1998; Guo et al., 2001), but has loose husks. Inbred GT119 was developed by selfing within the breeding population DDSynB (C3) (Widstrom et al., 1988). GT119 has a negligible amount of silk maysin, is susceptible to aflatoxin contamination and possesses good husk coverage and tightness.

F_2 plants and $F_{2:3}$ family rows were grown in the field. Whorl tissue was collected from F_2 plants, DNA was extracted, and genotypes of individuals in the F_2 population were determined by restriction fragment length polymorphism (RFLP) analysis on 250 F_2 plants, using the University of Missouri-Columbia core RFLP markers (Gardiner et al., 1993; Davis et al., 1999) and probes to cover specific regions of maize chromosomes. The 250 $F_{2:3}$ families were grown in a randomized complete block design with three replications during the summer of 1999 and 2000. Silks were bulked 3 days after silk emergence from plants within $F_{2:3}$ family rows, weighed, and stored in cold methanol. Concentrations of silk maysin, apimaysin, 3′-methoxymaysin, and chlorogenic acid were determined by reversed phase high performance liquid chromatography (HPLC) (Snook et al., 1989). Husk tightness and coverage were evaluated 12 days after silking on $F_{2:3}$ plants using a subjective scale from 0 (loose husks and ear exposed) to 5 (extremely tight with complete ear coverage). Plants within $F_{2:3}$ rows were bulk-sib pollinated, and 20 days after midsilk, plants were inoculated by inserting a pinboard, dipped into an *A. flavus* spore suspension, through the husk into the midportion of the abaxial side of the ear. At 40 days after inoculation, ears from each row were harvested and dried at 60 °C. Dried ear samples were shelled and all grains were ground for analysis. A 10-g well-mixed sub-sample from each row-sample was used to determine B1, B2, G1, and G2 aflatoxin concentrations (ng g^{-1} dry matter) by HPLC (Thean et al., 1980).

Analyses of the phenotypic data for silk chemicals, husk traits, and aflatoxin levels were carried out using analysis of variance, general linear model, and correlation procedures of SAS, statistical analysis software (SAS, 1990). Markers and interactions that were significant at the 0.01 probability level for husk tightness and total aflatoxin concentration in the single-factor and two-way analyses of variance, respectively, were introduced in the multiple-factor analyses in order to find the best multiple-locus model. A linkage map for the population was constructed with the program MAPMAKER/EXP using maximum likelihood procedures, and chromosomal position, significance, and the percentage contribution to traits were determined through interval mapping with MAPMAKER/QTL. Genotypic class means were calculated using the least squares means.

3. Results

Even though the study received irrigation during the growing season, the warmer than usual temperatures and reduced rainfall of 1998 resulted in an unfavorable growing environment. These conditions significantly reduced the time from planting to silking by 3 days (Table 1). Corn earworm ratings and aflatoxin concentrations were thus significantly higher in 1998 than in 1999. The differences were probably due to the favorable conditions for insect activity and aflatoxin development during the warmer and drier season of 1998.

The analysis of variance indicated significant differences between years and among germplasm sources for earworm damage, aflatoxin contamination and days to silking. The interaction between years and germplasm sources was significant for the aflatoxin contamination and days to silking data, therefore, the analyses were not combined over years. However, the means from year to year were not unexpectedly different, giving credence to their general ratings shown in Table 1. The analyses provided no evidence of significant differences between infested and non-infested plots or between plots inoculated and non-inoculated with *A. flavus*. The plantings were

intentionally made in mid-April–mid-May, rather late for this area, so that we could take advantage of naturally occurring insects and inoculation to guarantee exposure to the injury we were measuring, or at least minimize escapes.

Chemical concentrations of silk antibiotic compounds were expressed as percentages of fresh silk weight. Concentrations of maysin, 3′-methoxymaysin, apimaysin and chlorogenic acid were determined, but 3′-methoxymaysin and apimaysin were pooled to simplify the analytical process (Guo et al., 1999b). A major QTL (*p1*) on chromosome 1S was identified (Table 2). The *p1* locus explained 54.0, 42.1, and 28.3% of the phenotypic variability for silk maysin, 3′-methoxymaysin + apimaysin, and chlorogenic acid concentrations, respectively (Table 2). Marker *csu1066*, on the centromeric region of chromosome 2, was another common marker for maysin, 3′-methoxymaysin plus apimaysin (3′M + A) and chlorogenic acid (Table 2). However, this marker explained less phenotypic variability for maysin and 3′M + A (6.8 and 9.2%, respectively) compared to the 20.4% of phenotypic variability for chlorogenic acid. The *p1* locus explained about twice the phenotypic variability for maysin concentration than for chlorogenic acid. The *p1* locus and *csu1066* were included in the multiple-locus model for each antibiotic compound (Table 3), which accounted for 59.3% of the maysin concentration variability. The loci *p1*, *csu1066*, and *php20608* were included in the multiple-factor model accounting for 54.2% of the 3′M + A concentration variability (Table 3). For chloro-

Table 3
Multiple-factor[a] analyses of variance for silk maysin, 3′M + A, and chlorogenic acid concentrations in the $F_{2:3}$ population derived from the single cross GT-A1 × GT119

Locus	Bin[b]	Maysin	3′M + A	Chlorogenic acid
p1	1.03	0.0001	0.0001	0.0001
csu1066	2.05	0.0010	0.0001	0.0001
php20608	4.10	–	0.0001	–
p1 × *csu32*		–	–	0.0030
R^2 (%)[c]		59.3	54.2	53.3

[a] Level of probability for the *F*-test in a multiple-factor analysis of variance.
[b] QTL positions refer to the UMC 1999 Maize Map Standard (Davis et al., 1999). Chromosome was dissected into Bin regions.
[c] Percentage of total phenotypic variance explained by the multiple-locus model for each compound.

Table 2
Single-factor analyses of variance for silk maysin, 3′M + A, and chlorogenic acid concentrations in the $F_{2:3}$ families derived from the single cross GT-A1 × GT119

Locus	Bin[a]	Maysin		3′M + A		Chlorogenic acid	
		*P**	R^2 (%)[b]	*P**	R^2 (%)[b]	*P**	R^2 (%)[b]
p1	1.03	0.0001	54.0	0.0001	42.1	0.0001	28.3
asg45	1.04	0.0001	47.8	0.0001	41.3	0.0001	25.2
bn19.11b	1.04	0.0001	30.4	0.0001	22.2	0.0001	23.2
csu3	1.05	0.0001	20.4	0.0001	17.0	0.0001	15.0
umc67	1.06	0.0001	9.3	0.0002	8.9	0.0007	7.6
asg62	1.07	0.0068	4.9	–	–	–	–
umc34	2.04	–	–	0.0017	6.3	0.0018	6.2
csu1066	2.05	0.0011	6.8	0.0001	9.2	0.0001	20.4
umc176	2.06	0.0036	7.5	0.0021	8.1	0.0001	11.3
csu32	3.02	–	–	0.0064	5.0	–	–
bn18.45b	4.08	–	–	0.0003	8.0	–	–
php20608	4.10	0.0029	5.7	< 0.0001	9.5	–	–

[a] Locus positions refer to the UMC 1999 Maize Map Standard (Davis et al., 1999). Chromosome was dissected into Bin regions.
[b] Percentage of phenotypic variance explained by each locus.
* Level of probability for the *F*-test in a single-factor analysis of variance. Markers significant at $P < 0.01$ level of probability are presented.

genic acid, *csu32* was not significant in the single-factor model (Table 2), but it was significant when it was combined with the *p1* in a multiple-factor model along with loci *p1* and *csu1066* contributing to a total of 53.3% of the variability (Table 3).

We measured the husk phenotypic trait in 1999 and 2000. Single factor analysis detected four significant markers ($P < 0.05$) on chromosomes 3L, 4L and 7S in 1999 (Table 4). Each marker accounted for less than 10% of the phenotypic variation. We identified a significant ($P < 0.001$) epistatic interaction affecting overall husk coverage which was retained in the multiple-locus model. The multiple-locus model, including chromosomes 4L, 7L, and the epistatic interaction 4S × 8S, explained a total of 27.9% of the phenotypic variance.

Using 2000 field data for husk tightness, we identified four significant ($P < 0.001$) markers on chromosomes 1S, IL, 3L, and 7L (Table 4). One marker on chromosome 3L accounted for 12.7% of the variation and other individual markers' effects on phenotypic variance were less than 10%. Three markers were retained in the multiple-factor model with a total contribution of 28.8% of the variation. Markers on chromosomes 3L and 7L were significant in both years.

Simple correlation coefficients among different aflatoxin compounds (B1, B2, G1, and G2) were highly significant. Therefore, analyses were made for the concentration of total aflatoxin compounds. Among genotypes, means for B1, B2, G1, and G2 concentrations were 50.1, 4.2, 0.3, 0.2 ng g^{-1} (dry matter), respectively. B1 and B2 were the predominant aflatoxin compounds, as expected, since inoculations were made with *A. flavus*, a B1 and B2 producer (Moreno and Kang, 1999).

Single-factor analyses of variance for total aflatoxins detected only one marker that was significant at 0.01 level of probability and explained 5.3% of the phenotypic variability. Multiple-factor analysis of variance indicated that markers *umc176* on chromosome 2L, *csu3* on chromosome 1S, and the interaction *umc176* × *umc3* explained 24.7% of the phenotypic variability for aflatoxin concentration (Table 5). Homozygous genotypes with markers *umc176* for alleles from GT-A1 and *csu3* for alleles from GT119 had aflatoxin levels significantly higher than any other

Table 4
Significance of loci in the analyses of variance for husk tightness in the $F_{2:3}$ population derived from the single cross GT-A1 × GT119 in year 1999 and 2000

Locus	Bin[a]	Single-factor[b]		Multiple-factor[b]		R^2 (%)[c]	
		1999	2000	1999	2000	1999	2000
bnl9.11b	1.04	–	0.0078	–	–	–	5.0
csu3	1.05	–	0.0008	–	–	–	7.3
asg62	1.07	–	0.0011	–	< 0.0001	–	6.8
bn15.37	3.06	–	0.0004	–	–	–	8.5
bn16.16	3.07	–	0.0004	–	–	–	7.9
umc17	3.08	0.0365	< 0.0001	–	< 0.0001	3.3	12.7
php10080	3.08	–	0.0005	–	–	–	7.6
bn18.45b	4.08	< 0.0001	–	0.0005	–	9.6	–
asg49	7.03	0.0017	–	–	–	6.5	–
umc254	7.04	0.0004	0.0005	0.0024	< 0.0001	7.6	7.6
umc168	7.06	–		0.0034	–	–	5.7
npi386 × *npi220a*	4.04 × 8.01	–	–	0.0011	–	–	–
Total R^2 (%)[d]	–	–	–	–	–	27.9	28.8

[a] Map coordinates from the Maize Map Standard (Davis et al., 1999).
[b] Probability of significance.
[c] Percentage of phenotypic variance explained for each locus.
[d] Percentage of phenotypic variance explained by the multiple-locus model.

Table 5
Multiple-factor analysis for total aflatoxin concentration in the (GT-A1 × GT119) $F_{2:3}$ population

Marker	Bin[a]	Significance	Favorable allele donor
csu3	1.05	0.0001	GT-A1
umc176	2.06	0.0001	GT119
csu3 × *umc176*		0.0001	
$R^{2b} = 24.7\%$			

[a] Map coordinates from the Maize Map Standard (Davis et al., 1999).
[b] Percentage of the phenotypic variability explained.

genotypic class (Table 6). However, homozygous genotypes with marker *umc176* associated with alleles from GT119 and *csu3* associated with alleles from GT-A1 had aflatoxin levels not significantly different from zero (Table 6).

4. Discussion

There is certainly strong evidence for sufficient resistance to insects although ratings of resistance to aflatoxin were less consistent and convincing. At present, we have no resistance sources to *A. flavus* infection or aflatoxin contamination that can be classified as excellent or outstanding. In fact, the good ratings given in the aflatoxin column of Table 1 are optimistic, and valid only under moderate to favorable environmental conditions that did not exist in either 1998 or 1999. Resistance for this trait is still in need of much improvement. Crosses among our germplasm sources, and incorporation of others that may become available, will be used to formulate a breeding population in which MAS can be practiced.

Table 6
Means and standard errors for the interaction between markers *umc176* and *csu3* in the (GT-A1 × GT119) $F_{2:3}$ population

umc176[a]	*csu3*[a]	Aflatoxin concentration (ng g^{-1})
A	A	50.72±26.84 b[b]
A	H	63.17±21.91 b
A	B	294.01±37.95 a
H	A	64.39±16.56 b
H	H	48.64±10.74 b
H	B	52.81±20.29 b
B	A	10.17±33.95 b
B	H	50.63±18.41 b
B	B	70.74±30.99 b

[a] A is the homozygous genotype for the allele from parent GT-A1, B is the homozygous genotype for the allele from parent GT119, and H is the heterozygous genotype.
[b] Means followed by the same letter did not differ significantly at the 0.05 level of probability according to *t*-test.

The low amounts of aflatoxin contamination for ZC2451(P)C3 and GT119 are most probably a result of the husk cover and tightness of these entries. However, the higher aflatoxin concentrations of GT-A1 and GT-A2 are likely due to poor husk coverage and insect damage (Table 1). The combination of husk cover and maysin concentration are known to be responsible for the low corn earworm rating for ZC2451(P)C3. Ear-feeding damage by insects has been consistently associated with increased contamination by aflatoxin and, as such, is considered a major contributor to the breakdown of the natural resistance in corn kernels to *A. flavus* (Widstrom et al., 1975; Barry et al., 1985, 1992).

QTL analyses showed the essential role of the *p1* locus in controlling maysin and maysin related compounds. The regulatory role of *p1* in maysin synthesis has been demonstrated by other authors (Byrne et al., 1996). The main role of *p1* in the flavonoid pathway leading to maysin related compounds has been indirectly determined, since *p1* determines the silk-browning genotype which may be related to concentrations of maysin and its analogues (Levings and Stuber, 1971; Guo et al., 1999b).

It is interesting to note that marker *csu1066* has a higher association (20.4%) with chlorogenic acid than maysin (6.8%) and 3′M+A (9.2%). Marker *csu1066* is flanking to locus *umn1* (acc) which encodes acetyl-CoA carboxylase. Acetyl-CoA catalyzes the first committed step in fatty acid biosynthesis and provides malonyl-CoA, a precursor of phenylpropanoid/flavonoid pathway, for the synthesis of a variety of important secondary metabolites and for malonylation (Lee et al., 1998; Davis et al., 1999). The high correlation between marker *csu1066* and concentration of chlorogenic acid in this population may indicate that the phenylpropanoid pathway is regulated, at least in part, via feedback regulation of early pathway

steps by the accumulation of malonyl-CoA as demonstrated by C4H (cinnamic acid 4-hydroxylase's down regulation of PAL (L-phenylalanine ammonia-lyase) (Blount et al., 2000). This feedback regulation may stimulate the pathway leading to chlorogenic acid production.

In order to obtain the best responses for antibiosis in a MAS program, individuals selected in advanced generations from the cross GT-A1 × GT119 should carry favorable alleles for the *p1* locus along with favorable alleles for markers on chromosomal regions 2C–2L, 3S, and 4L (Tables 2 and 3). These markers should be determined in a more detailed mapping study since, in the present study, separation between some consecutive markers on linkage groups is not short enough to guarantee a strong linkage between the marker and the QTL. However, the genotype mean of homozygous *p1* was 0.462% total polyphenols, which is twice more than the 0.2% threshold necessary for effective resistance to corn earworm (Wiseman et al., 1992; Snook et al., 1993) and it may be possible to use *p1* alone as the marker to select progenies with higher silk maysin or total antibiotic compounds in silks.

Two factors could be responsible for the failure to detect major QTLs for traits of husk coverage and aflatoxin formation, the lack of enough markers in some chromosomal regions in order to obtain a good coverage of the whole genome, achieving a higher correlation with the quantitative traits, and the complex pattern that apparently controls kernel resistance to aflatoxin contamination. A preponderance of dominance and/or epistatic effects for aflatoxin accumulation was reported by Gardner et al. (1987). Gorman et al. (1992) observed that aflatoxin production in crosses among seven maize synthetics was largely influenced by the environment and that genetic variation was not significant. Preliminary data present here suggests that epistatic effects (*umc176* × *csu3*) could be very important in the inheritance of host resistance to aflatoxin accumulation. Campbell and White (1995) observed significant deviations from the additive-dominance model when studying the inheritance of resistance to *Aspergillus* ear rot using generation mean analysis. A more detailed mapping of the chromosomic regions 1S and 2L will be necessary to locate precise QTLs for aflatoxin production in the segregating population used for this study.

5. Conclusion

In this paper, we report our progress from 2 years field experimentation to demonstrate strategies for prevention of aflatoxin contamination in the southern US, based on breeding procedures for pyramiding various traits. Also, we report preliminary results from QTL analyses to identify loci associated with the traits: silk chemical concentration, husk coverage, and aflatoxin concentrations. We have identified two types of resistance, resistance to *A. flavus* infection and aflatoxin production, and resistance to corn earworm, the most destructive ear-feeding insect, enhanced by maysin in silks, and have determined that multiple traits influence both types of resistance. The research has demonstrated a potential for combining both types of resistance to control preharvest aflatoxin contamination.

Since the data generated on QTL analyses for husk coverage and aflatoxin production are being used to set the stage for progress toward our long-term goal, and QTL for aflatoxin production are based on a 1 year study in 1999, verification and re-examination of these results will follow on this and other populations. However, our field studies demonstrated that prevention of ear-feeding insects and husk coverage are essential for reduction and/or elimination of aflatoxin contamination in southern US produced corn, requirements that are different from those for corn production in the US corn belt. The protection of maize ears against aflatoxin contamination is primarily dependent on resistance to fungal infection, resistance to ear-feeding insects, and excellent husk coverage and tightness. The germplasm sources in Table 1 are being used to generate a maize population with chemical and physical resistance to *Aspergillus* spp. and ear-feeding insects for inbred development using MAS.

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References

Anderson, H.W., Nehring, E.W., Wichser, W.R., 1975. Aflatoxin contamination of corn in the field. Agric. Food Chem. 23, 775–782.

Barry, D., Widstrom, N.W., Darrah, L.L., McMillian, W.W., Riley, T.J., Scott, G.E., Lillehoj, E.B., 1992. Maize ear damage by insect in relation to genotype and aflatoxin contamination in preharvest maize grain. J. Econ. Entomol. 85, 2492–2495.

Barry, D., Zuber, M.S., Lillehoj, E.B., McMillian, W.W., Adams, N.J., Kwolek, W.F., Widstrom, N.W., 1985. Evaluation of two anthropod vectors and inoculators of developing maize ears with *Aspergillus flavus*. Environ. Entomol. 14, 634–636.

Blount, J.W., Korth, K.L., Masoud, S.A., Rasmussen, S., Lamb, C., Dixon, R.A., 2000. Altering expression of cinnamic acid 4-hydroxylase in transgenic plants provides evidence for a feedback loop at the entry point into the phenylpropanoid pathway. Plant Physiol. 122, 107–116.

Byrne, P.F., McMullen, M.D., Wiseman, B.R., Snook, M.E., Musket, T.A., Theuri, J.M., Widstrom, N.W., Coe, E.H., 1996. Quantitative trait loci and metabolic pathways: genetic control of the concentration of maysin, a corn earworm resistance factor, in maize silks. Proc. Natl. Acad. Sci. USA 93, 8820–8825.

Campbell, K.W., White, D.G., 1995. Inheritance of resistance to *Aspergillus* ear rot and aflatoxin in corn genotypes. Phytopathology 85, 886–896.

Darrah, L.L., Lillehoj, E.B., Zuber, M.S., Scott, G.E., Thompson, D., West, D.R., Widstrom, N.W., Fortnum, B.A., 1987. Inheritance of aflatoxin B1 levels in maize kernels under modified natural inoculation with *Aspergillus flavus*. Crop Sci. 27, 869–872.

Davis, G.L., McMullen, M.D., Baysdorfer, C., Musket, T., Grant, D., Staebell, M., Xu, G., Polacco, M., Koster, L., Melia-Hancock, S., Houchins, K., Chao, S., Coe, E.H., Jr., 1999. A maize map standard with sequenced core markers, grass genome reference points and 932 expressed sequence tagged sites (ESTs) in a 1736-locus map. Genetics 152, 1137–1172.

Gardiner, J.M., Coe, E.H., Melia-Hancock, S., Hoisington, D.A., Chao, S., 1993. Development of a core RFLP map in maize using an immortalized F2 population. Genetics 134, 917–930.

Gardner, C.A.C., Darrah, L.L., Zuber, M.S., Wallin, J.R., 1987. Genetic control of aflatoxin production in maize. Plant Dis. 71, 426–429.

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

Gray, F.A., Faw, W.F., Boutwell, J.L., 1982. The 1977 corn-aflatoxin epiphytotic in Alabama. Plant Dis. 66, 221–222.

Guo, B.Z., Cleveland, T.E., Brown, R.L., Widstrom, N.W., Lynch, R.E., Russin, J.S., 1999a. Distributijon of antifungal proteins in maize kernel tissues using immunochemistry. J. Food Prod. 62, 295–299.

Guo, B.Z., Li, R.G., Widstrom, N.W., Lynch, R.E., Cleveland, T.E., 2001. Genetic variation within maize population GT-MAS:gk and the relationship with resistance to *Aspergillus flavus* and aflatoxin production. Theor. Appl. Genet. 103, 533–539.

Guo, B.Z., Russin, J.S., Brown, R.L., Cleveland, T.E., Widstrom, N.W., 1996. Resistance to aflatoxin contamination in corn as influenced by relative humidity and kernel germination. J. Food Prod. 59, 276–281.

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

Guo, B.Z., Widstrom, N.W., Wiseman, B.R., Snook, M.E., Lynch, R.E., Plaisted, D., 1999b. Comparison of silk maysin, antibiosis to corn earworm larvae (Lepidoptera: Noctuidae), and silk browning in crosses of dent × sweet corn. J. Econ. Entomol. 92, 746–753.

Hesseltine, C.W., Rogers, R.F., Shotwell, O.L., 1981. Aflatoxin and mold flora in North Carolina in 1977 corn crop. Mycologia 73, 216–218.

Lee, E.A., Byrne, P.F., McMullen, M.D., Snook, M.E., Wiseman, B.R., Widstrom, N.W., Coe, E.H., 1998. Genetic mechanisms underlying apimaysin and maysin synthesis and corn earworm antibiosis in maize (*Zea mays* L.). Genetics 149, 1997–2006.

Levings, C.S., Stuber, C.W., 1971. A maize gene controlling silk browning in response to wounding. Genetics 69, 491–498.

Li, R., Guo, B.Z., Widstrom, N.W., Lynch, R.E., Cleveland, T.E., 1998. Genetic diversity in GT-MAS:gk population and possible PCR and RFLP markers linked to resistance to *Aspergillus flavus*. Proceedings of the USDA-ARS Aflatoxin Elimination Workshop, St. Louis, MO.

Lillehoj, E.B., Kwolek, W.F., Vandegraft, E.E., Zuber, M.S., Calvert, O.H., Widstrom, N.W., Futrell, M.C., Bockholt, A.J., 1975. Aflatoxin production in *Aspergillus flavus* inoculated ears of corn grown at diverse locations. Crop Sci. 15, 267–270.

Lillehoj, E.B., Kwolek, W.F., Zuber, M.S., Horner, E.S., Widstrom, N.W., Guthrie, W.D., Turner, M., Sauer, D.B., Findley, W.R., Manwiller, A., Joesephson, L.M., 1980. Aflatoxin contamination caused by natural fungal infection of preharvest corn. Plant and soil 54, 469–475.

McMillian, W.W., Widstrom, N.W., Wilson, D.M., 1987. Impact of husk type and species of infesting insects on

aflatoxin contamination in preharvest corn at Tifton, Georgia. J. Entomol. Sci. 22, 307–310.

McMillian, W.W., Widstrom, N.W., Wilson, D.M., 1993. Registration of GT-MAS:gk maize germplasm. Crop Sci. 33, 882.

McMillian, W.W., Wilson, D.M., Widstrom, N.W., 1985. Aflatoxin contamination of preharvest corn in Georgia: a six-year study of insect damage and visible *Aspergillus flavus*. J. Environ. Qual. 14, 200–202.

McMullen, M.D., Byrne, P.F., Snook, M.E., Wiseman, B.R., Lee, E.A., Widstrom, N.W., 1998. Quantitative trait loci and metobolic pathways. Proc. Natl. Acad. Sci. USA 95, 1996–2000.

Moreno, O.J., Kang, M.S., 1999. Aflatoxins in maize: the problem and genetic solutions. Plant Breeding 118, 1–16.

Russin, J.S., Guo, B.Z., Tubajika, K.M., Brown, R.L., Cleveland, T.E., Widstrom, N.W., 1997. Comparison of kernel wax from corn genotypes resistant or susceptible to *Aspergillus flavus*. Phytopathology 87, 529–533.

SAS Institute, Inc., 1990. SAS/STAT User's Guide, SAS Institute, Inc., Cary, North Carolina, USA.

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

Snook, M.E., Gueldner, R.C., Widstrom, N.W., Wiseman, B.R., Himmelsbach, D.S., Harwood, J.S., Costello, C.E., 1993. Levels of maysin and maysin analogues in silks of maize germplasm. J. Agric. Food Chem. 41, 1481–1485.

Snook, M.E., Widstrom, N.W., Gueldner, R.C., 1989. Reversed-phase high-performance liquid chromatographic procedure for the determination of maysin in corn silks. J. Chromatogr. 477, 439–447.

Snook, M.E., Wiseman, B.R., Widstrom, N.W., Wilson, R.L., 1997. Chemicals associated with maize resistance to corn earworm and fall armyworm. In: Insect Resistant Maize: Recent Advances and Utilization. Mihm J.A. (Ed.), Proceedings of the International Symposium held at CIMMYT. 27 Nov.–3 Dec., Mexico, D.F, 1994, pp. 37–45.

Thean, J.E., Lorenz, D.R., Wilson, D.M., Rodgers, K., Gueldner, R.C., 1980. Extraction, cleanup, and quantitative determinations of aflatoxins in corn. J. Assoc. Off. Anal. Chem. 63, 631–633.

Widstrom, N.W., 1992. Aflatoxin in developing maize: interactions among involved biota and pertinent econiche factors. In: Bhatnagar, D., Lillehoj, E.B., Arora, D.K. (Eds.), Handbook of Applied Mycology, vol. 5. Marcel-Dekker, New York, pp. 23–58.

Widstrom, N.W., McMillian, W.W., Wilson, D.M., Garwood, D.L., Glover, D.V., 1984. Growth characteristics of *Aspergillus flavus* on agar infused with maize kernel homogenates and aflatoxin contamination of whole kernel samples. Phytopathology 74, 887–890.

Widstrom, N.W., Snook, M.E., 1997. The maysin chronicle: background, identification, quantification, and inheritance in maize silks. Recent Res. Devel.in Agric. & Food Chem. 1, 301–316.

Widstrom, N.W., Sparks, A.N., Lillehoj, E.B., Kwolek, W.F., 1975. Aflatoxin production and lepidopteran insect injury on corn in Georgia. J. Econ. Entomol. 68, 855–856.

Widstrom, N.W., Wiseman, B.R., McMillian, W.W., 1988. Registration of six corn earworm resistant germplasm lines of maize. Crop Sci. 28, 202.

Widstrom, N.W., Zuber, M.S., 1983. Prevention and control of aflatoxin: Sources and mechanisms of genetic control in the plant. In: Diener U.L., Asquith R.L., Dickens J.W. (eds), Aflatoxin and *Aspergilus flavus* in Corn. Southern Coop. Series Bull. 279, pp. 72-76, Alabama Agric. Expt. Stn., Auburn, AL.

Wilson, D.M., McMillian, W.W., Widstrom, N.W., 1979. Field aflatoxin contamination of corn in South Georgia. J. Am. Oil Chem. Soc. 56, 798–799.

Wiseman, B.R., Snook, M.E., Isenhour, D.J., Mihm, J.A., Widstrom, N.W., 1992. Relationship between growth of corn earworm and fall armyworm (Lepidoptera:Noctuidae) and maysin concentration in corn silks. J. Econ. Entomol. 85, 2473–2477.

Zuber, M.S., Darrah, L.L., Lillehoj, E.B., Joesephson, L.M., Manwiller, A., Scott, G.E., Gudauskas, R.T., Horner, E.S., Widstrom, N.W., Thompson, D.L., Bockholt, A.J., Brewbaker, J.L., 1983. Comparison of open-pollinated maize varieties and hybrids for preharvest aflatoxin contamination in the southern United States. Plant Dis. 67, 185–187.