Theor Appl Genet (2003) 107:263–270
DOI 10.1007/s00122-003-1241-0

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Quantitative trait loci for low aflatoxin production in two related maize populations

Received: 1 February 2002 / Accepted: 14 November 2002 / Published online: 1 April 2003
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Abstract Aflatoxin B_1 formed by *Aspergillus flavus* Fr:Link has been associated with animal disease and liver cancer in humans. We performed genetic studies in progenies derived from maize inbred Tex6, associated with relatively low levels of aflatoxin production, crossed with the historically important inbred B73. (Tex6xB73) × B73 BC_1S_1 and Tex6 × B73 $F_{2:3}$ mapping populations were produced and evaluated in 1996 and 1997 in Champaign, Ill. Ears were inoculated 20 to 24 days after midsilk using a pinboard method and a mixture of conidia of *A. flavus* Link:Fr. isolates. Aflatoxin B_1 levels in harvested ears were determined using an indirect competitive ELISA. Molecular markers were assayed on the populations and used to generate maps. Molecular marker – QTL associations for lower levels of aflatoxin production were determined using multiple regression (MR) and composite interval analysis with multiple regression (CIM MR). MR revealed sets of markers associated with lower aflatoxin production in 1996 and 1997, and CIM MR detected a smaller subset of loci significant in 1997. QTLs for lower aflatoxin were attributed to both Tex6 and B73 parental sources. Environment strongly influenced the detection of QTLs for lower aflatoxin production in different years. There were very few chromosome regions associated with QTLs in more than 1 year or population with MR analysis, and none with CIM MR analysis. In 1997, QTLs for lower aflatoxin were detected with CIM MR in bins 5.01-2 and 5.04-5 in the BC_1S_1 population, and in bins 3.05-6, 4.07-8 and 10.05-10.07 in the $F_{2:3}$ population. These QTL associations appear the most promising for further study.

Keywords Aflatoxin · *Aspergillus flavus* · Molecular markers · QTLs · *Zea mays* L.

Communicated by G. Wenzel

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Introduction

Aflatoxin B_1, one of the mycotoxins formed by *Aspergillus flavus* Fr: Link, is an extremely potent, naturally occurring carcinogen (Wyllie and Morehouse 1978; Squire 1981; Diener et al. 1987). Feeding maize grain with high levels of aflatoxin to animals has been associated with disease (Smith and Moss 1985). Aflatoxin B_1 has been associated with liver cancer in humans when hepatitis is also present (Anderson et al. 1975; Hsieh 1989; McGlynn et al. 1995). Aflatoxin contamination has been a chronic problem associated with maize production in the Southern US (Anderson et al. 1975; Zuber et al. 1976) and is also a problem in the Midwestern States during years with drought stress at the time of pollination and grain fill. Warm, humid conditions favor growth of the *A. flavus* fungus resulting in high ear-rot severities, while hot, dry weather favors high aflatoxin production (Payne 1992). Maize grain with levels of aflatoxin greater than 20 ng/g (ppb) cannot be legally received at grain elevators or shipped for interstate commerce in the US, and some countries will not buy grain with more than 5 or 10 ng/g of aflatoxin (Haumann 1995; FAO 1997). These limits on aflatoxin contamination can create a major

marketing problem and economic constraints (Nichols 1983).

Breeding for resistance, or more accurately kernel and plant characteristics that inhibit infection by Aspergillus ear rot and aflatoxin production, is currently considered the most effective means to control aflatoxin production. Germplasm screening studies have identified a number of public sources associated with lower levels of aflatoxin production (Thompson et al. 1984; Darrah et al. 1987; Widstrom et al. 1987; Scott and Zummo 1988, 1990; Campbell and White 1995a; Windham and Williams 1998). However, levels of aflatoxin production have not been adequate in commercial hybrids, especially in years with environmental condtions where aflatoxin production is favored (Payne 1992).

Previous research to better determine the genetic basis of tolerance to, or inhibition of, aflatoxin production has established that tolerance is quantitatively inherited, with low broad-sense heritabilities, additive gene effects generally more important than dominance gene effects (Campbell and White 1995b; Campbell et al. 1997; Hamblin and White 2000) and general combining ability (GCA) effects are usually more important than specific combining ability (SCA) effects (Zuber et al. 1978; Widstrom et al. 1984; Gardner et al. 1987; Gorman et al. 1992; Naidoo et al. 2002). Significant genotype × environment interactions have been reported in most genetic studies on aflatoxin production in corn (Payne 1992; Brown et al. 1999). These factors indicate that breeding for low levels of aflatoxin production can be severely hindered by environmental effects on the phenotype. In years when aflatoxin production is favored by environmental conditions, selection for resistance based on toxin production is feasible, but not in years when toxin production is low and separation of phenotypes is diminished.

The resistance source used in this study is Tex6, derived from an open-pollinated variety from Texas, and found to be associated with low aflatoxin production in a number of field studies (Campbell et al. 1993; Campbell and White 1995a; Hamblin and White 2000; Naidoo et al. 2002). Laboratory studies using inoculation of individual kernels confirmed Tex6 as associated with low levels of aflatoxin production (Brown et al. 1999). B73 was used as the other parent of the mapping populations because it is historically important in hybrid corn production in the US Midwest and many current commercial inbreds are related to B73. Notably, B73 is relatively intermediate in the level of resistance to aflatoxin and not highly susceptible (Campbell et al. 1993; Campbell and White 1995b; Naidoo et al. 2002).

The overall objective of this research was to determine if we could identify QTLs associated with resistance to aflatoxin production from Tex6 or B73 in two populations derived from these inbreds. QTLs initially identified in this study will need to be confirmed in other studies and progenies, and these confirmed QTLs should be suitable for marker-assisted selection (MAS). The value of MAS is high when heritability of the trait is low (Dudley 1993), which is clearly the case for aflatoxin production in maize. MAS will also facilitate pyramiding QTLs from different germplasm sources, which most-likely will be necessary to get satisfactory levels of low aflatoxin production into elite inbreds.

Materials and methods

Plant materials

Maize inbred Tex6 was crossed with inbred B73 to produce F_1 seed. A population of 176 $F_{2:3}$ families was developed by self-pollinating the F_1 and F_2 progeny. A population of 100 BC_1S_1 families was developed by backcrossing B73 to the F_1 and self-pollinating BC_1 plants. Additional families were developed for each population but were discarded due to small ears unsuitable for inoculation or evidence of outcrossing from phenotypic appearance or marker analysis.

Field plots

The field experiments were described in greater detail previously (Hamblin and White 2000). The experiments used a randomized complete-block design. Experimental units were families. For statistical analyses, year/location combinations were considered as environments. The BC_1S_1 population had two blocks (replications) and the $F_{2:3}$ population had three blocks, with one row (plot) each of 100 BC_1S_1 and 176 $F_{2:3}$ families, respectively, randomized within each block. Plots consisted of 24 kernels planted into 5.2-m rows (plus an alley of 0.5 m) spaced 0.76 m apart. Plots were planted at the University of Illinois Crop Sciences Research Farm, Urbana, Ill., in 1996 and 1997. Plants were allowed to open-pollinate before inoculations to ensure good seed set, and to provide natural kernel development conditions since pollination bags covering ears may affect fungal biomass development and aflatoxin production.

Inoculations and phenotypic observations

In 1996 and 1997, approximately 20 to 24 days after midsilk, 14–15 ears were inoculated through the husks with a spore suspension (1×10^6 conidia ml^{-1}) of four isolates of *A. flavus* (NRRL Isolates 6536, 6539, 6540, and an isolate from Illinois collected in 1988), using the pinboard inoculation technique previously described (Campbell and White 1994). The four isolates were previously found to be highly toxigenic, and a group of four was used as a safeguard in case any one isolate suddenly became non-toxigenic after lab culture. The percent of fungal colonization in the inoculated area was determined 50–60 days later and the ears harvested. We inoculated the materials on two different dates each year so that all families were inoculated within 20–24 days after the pollination period. Coefficients of variation obtained over a number of studies in our field research (Campbell and White 1994, 1995a; Hamblin and White 2000) indicate that inoculating at different dates within this range adequately compensates for the differences in maturity date among segregating families.

Aflatoxin levels were determined with an indirect competitive ELISA assay as previously described (Campbell and White 1995a). Kernels from air-dried inoculated ears within a family were bulked and ground with a Romer mill (Model 2A) to sizes below 1 mm. Ground kernels were sub-sampled and 2-g quantities were removed from the 250–500 g bulked samples. All samples were run in triplicate. The lower detection limit using this procedure is 2 ng, so samples with non-detectable aflatoxin were recorded at 2 ng/g. The aflatoxin values were collected in an Excel spreadsheet for use in statistical analysis.

SSR and RFLP analyses

DNA was isolated from a bulk of approximately 14-days leaf tissue from 20–30 seedlings of Tex6, B73, and each $F_{2:3}$ and BC_1S_1 family used a modified CTAB protocol (Mikkilineni 1997). Oligonucleotide primers for SSRs were obtained from Research Genetics, Inc. and Biosynthesis Inc. Reaction mixtures consisted of 40–100 ng of template DNA, 40 μmol each of forward and reverse primers, 0.5 mMol of $MgCl_2$ BSA 10 × PCR reaction buffer and 5 units/μl of *Taq* polymerase (Senior and Chin 1996). All reactions were run using a MJ Research PTC-100 with a 96 V-bottom well thermocycler. Different amplification protocols were used depending on the primer pair: a "Touchdown" protocol (Senior and Chin 1996), modified from the Missouri Maize Project (MMC) protocol (http://www.agron.missouri.edu), and the MMC program. Following amplification, reaction products were separated by gel electrophoresis in 4% Metaphor agarose stained with ethidium bromide, at 140 V for approximately 4 h. A Gibco BRL 50-bp ladder was included on all gels. The gels were viewed and recorded using a Stratagene Eagle Eye with a thermal printer attachment or a Kodak DC295 Digital Camera with an ultraviolet light filter attached to a Gateway 450 mhz machine-running Adobe Photoshop Professional. Of 267 SSR marker primer pairs screened, 126 were considered polymorphic between Tex6 and B73. All 126 primer pairs were assayed on the 176 $F_{2:3}$ families, and 115 of these were assayed on the 100 BC_1S_1 families.

Several RFLP markers were assayed on the populations before the marker system switched over to SSR markers. Southern hybridizations were performed using a modification of the UMC procedure (UMC 1989) and described previously (Goldman et al. 1993). *Eco*RI or *Hin*dIII (Gibco BRL, Gaithersburg, Md.) digests of genomic DNA were probed with ^{32}P-labeled genomic and cDNA clones. These clones were obtained from the University of Missouri-Columbia, and screened for polymorphism between Tex6 and B73 before use on the mapping populations.

Many SSR markers assayed on the population were eliminated for potential use in map construction and statistical analysis, by visual inspection that revealed poor and/or inconsistent quality of amplification products or banding patterns, which resulted in difficulty and poor reliability in scoring. This project involved initial use of some of the first SSR markers developed for maize, and some markers were determined more suitable than others for reliable assays on the segregating progeny. SSR amplification products or RFLP hybridization bands were given a score of 0.5 for Tex6, –0.5 for B73 and 0.0 for heterozygotes.

Phenotypic data analyses

Log_{10} transformation designed to normalize residuals was performed on the aflatoxin data with statistical analysis system (SAS) software (SAS Institute, Cary, N.C.). Variance components were estimated using the Varcomp procedure of SAS (with the method = MIVQUEO). Narrow-sense heritability was calculated as suggested by Hallauer and Miranda (1981): $h^2 = \sigma^2_g/(\sigma^2/re + \sigma^2_{ge}/e + \sigma^2_g)$. Genetic variance is σ^2_g, genotype × environment variance is σ^2_{ge} and random error variance is σ^2. Replications and environments are indicated by r and e, respectively.

The general linear models procedure (GLM) of SAS was used to analyze the effects of environments, blocks, families and the interaction between these factors. Environment effects were considered fixed, and block and family effects were considered random. Since the response of maize to inoculation by *A. flavus* in terms of fungal growth, and particularly aflatoxin production, can vary considerably in different environments (Payne 1992), environments were considered a fixed effect in this study. Specific patterns of temperature, rainfall and humidity after inoculation can greatly effect the development of fungal biomass and aflatoxin production. Evaluation of only two environments in central Illinois does not provide an adequate sampling of different environments to be able to confidently make more general statements about QTLs for resistance beyond these two environments. There have been a number of specific inbreds and hybrids that were associated with relatively low levels of aflatoxin after evaluation in just two environments that were subsequently associated with high levels of aflatoxin production upon evaluation in multiple environments (Campbell and White 1995a, b; unpublished data). Thus, consistent with this observation, the QTLs identified as associated with lower levels of aflatoxin in this study, which are strictly considered valid for just these two environments, require evaluation in other genetic studies and environments to further support their validity.

Mapping and QTL analysis

Map locations of SSR and RFLP markers were determined with JoinMap Version 2.0, a general purpose package for linkage analysis and genetic mapping (Stam 1993; Stam and van Ooijen 1995). JoinMap utility modules were used for data inspection, resulting in elimination of additional markers due to segregation distortion not apparent from the visual inspection of gels. A problem with SSR markers for maize is that some markers, which are polymorphic between two inbred parents, segregate in a dominant manner in progenies (T. Rocheford, unpublished observations; M. McMullen, personal communication). A total of 113 markers were successfully mapped on the $F_{2:3}$ population and 89 markers on the BC_1S_1 population. Thus not all of the markers assayed on the two populations were identical; however, 80 identical markers were mapped in both populations. The Haldane function was used for map construction, which assumes no interference, and makes the map compatible for use with PLABQTL software. The linkage map for the $F_{2:3}$ covered 1,489 cM with an average interval length of 13.7 cM, and for the BC_1S_1 covered 1,413 cM with an average interval length of 17.9 cM. The location of mapped SSR markers were compared to the map positions provided on the Missouri Maize Database composite map (http://www.agron.missouri.edu) and were in general agreement. Our markers mapped to the same or adjacent 'bin' in the Missouri map. The Missouri maize composite map uses 'bins' of approximately 20–30 centimorgans defined by flanking core RFLP markers to conveniently group markers and marker-trait linkage data. This size of these bins may vary in different mapping populations due to variation in recombination rates between markers. Therefore bins should be considered an approximation of a chromosome interval, designed primarily for summarization and simplification of data for comparisons.

To identify sets of markers linked with QTLs for low aflatoxin production in these populations, multiple linear regression models for resistance to aflatoxin were constructed with the PLABQTL software package (Utz and Melchinger 1996). The molecular-marker map determined by JoinMap was then used in conjunction with PLABQTL to determine the locations and effects of QTLs. PLABQTL performs simple and composite interval analysis using a fast multiple-regression procedure according to the version of PLABQTL (Haley and Knott 1992). The results are similar to those obtained with the maximum-likelihood approach implemented by other software packages such as Mapmaker/QTL 1.1, 1993 (Lander 1993). PLABQTL was used because it is regularly updated with new versions, more user-friendly and faster, and can better handle missing data than MAPMAKER/QTL or SAS programs. PLABQTL also enables accounting for other QTLs by using sets of markers as cofactors, or composite interval mapping (CIM). The CIM approach may increase the power of QTL detection and reduce the bias in the estimated positions and effects of QTLs (Jansen and Stam 1994; Utz and Melchinger 1994; Zeng 1994). We performed cross-validation analysis within the PLABQTL program (Utz et al. 2000), to provide a less-biased estimate of the proportion of genotypic variance explained by QTLs, R^2 (adj.); this value tends to be lower than that estimated by MapMakerQTL and is considered preferable (Charcosset and Gallais 1996). We used the standard F_2 option with PLABQTL for the $F_{2:3}$ population. We used the testcross option for the BC_1S_1 population and did not use one of the marker classes as instructed by the authors of the program since

Table 1A Descriptive statistics associated with 100 BC_1S_1 lines, and 176 $F_{2:3}$ lines, in 2 years (1996–1997)

Year	Trait	Mean	*SD*	CV	Skewness	Minimum	Maximum
BC_1S_1							
1996	Ear rot	48.0	12.3	25.6	–0.2	15.6	74.2
	Aflatoxin	47.8	30.8	64.5	2.6**	4.0	241.7
	Aflatoxin (log_{10})	1.6	0.3	18.7	–0.9**	0.6	2.4
1997	Ear rot	49.9	9.4	18.8	0.1	29.3	76.0
	Aflatoxin	328.7	233.5	71.0	1.6**	42.5	1,175.0
	Aflatoxin (log_{10})	2.4	0.3	12.1	–0.01	1.6	3.1
$F_{2:3}$							
1996	Ear rot	38.1	12.4	32.6	0.2	13.0	73.2
	Aflatoxin	49.6	31.5	63.5	1.7**	9.0	192.0
	Aflatoxin (log_{10})	1.6	0.3	16.0	–0.1	1.0	2.3
1997	Ear rot	47.9	11.8	24.6	0.1	22.5	86.7
	Aflatoxin	208.9	145.1	69.5	2.0**	24.0	945.0
	Aflatoxin (log_{10})	2.2	0.3	11.9	–0.1	1.4	3.0

**Significant at the 0.01 level

there is not a specific backcross option (F. Utz, personal communication).

Cofactors employed for composite interval mapping within environments were selected via stepwise multiple regression using the COV SELECTED option. We generated empirical LOD thresholds for QTL detection using permutation tests (Churchill and Doerge 1994). LOD scores of > 3.8 and > 3.18 have experiment-wise significance of $P < 0.05$ for the $F_{2:3}$ and BC_1S_1 populations, respectively, as determined by *F*-statistic transformations (Haley and Knott 1992) calculated by PLABQTL. The PLABQTL program was used to perform a simultaneous fit of all QTLs detected above a threshold of 2.5 LOD. We present in the Tables all QTLs detected above a threshold LOD of 2.5. The final model including all significant QTLs, and also QTL × QTL effects, was determined by stepwise regression using PLABQTL.

Multiple QTLs discovered within the same 20-cM interval were considered to have accounted for the same genomic region. The proportion of phenotypic variance explained by each QTL was estimated with the square of the partial correlation coefficient (R^2) by the PLABQTL program. Significant loci on the same chromosome were considered as separate QTLs if they were >50-cM apart, if markers in between had significantly lower LOD scores (Mansur et al. 1996) or if the parental origin of the resistant allele reversed from one marker to the other. For comparison between populations, if the same identical marker was significant in both populations with MR it was considered most-likely to be the same QTL. If different markers from a common bin were significant in two populations with any form of analysis, this is likely to be due either to the same QTL or to linked QTLs.

Results and discussion

Mean ear-rot ratings were similar in 1996 and in 1997 for the (Tex6xB73) × B73 BC_1S_1 population, and were higher in 1997 than 1996 for the Tex6xB73 $F_{2:3}$ population (Table 1). Mean aflatoxin levels were higher in 1997 than 1996 for both the BC_1S_1 and $F_{2:3}$ populations. The (Tex6xB73) × B73 BC_1S_1 population had higher levels of aflatoxin than the $F_{2:3}$ population in 1997 (Table 1), which is not surprising given that the backcross was made to the parent associated with higher aflatoxin levels, B73. The higher aflatoxin levels were also associated with a wider range of values among families. The weather in August and September was close to average each year. The only notable difference was a lower number of cooling days in

Table 1B Variance components and heritability for aflatoxin (log_{10}) for the BC_1S_1 and 176 $F_{2:3}$ lines

Item	BC_1S_1	$F_{2:3}$
σ^2_g	0.01	0.02
σ^2_{ge}	0.03	0.01
σ^2_e	0.13	0.16
h^2	19.1	29.3

September 1997 relative to 1996 (http://www.sws.uiuc.edu). The distribution of residuals in the two populations showed skewness for both years (Table 1). The log_{10} transformations eliminated significant skewness except for the BC_1S_1 population in 1996. The 1996 BC_1S_1 population, although still significant for skewness after transformation, was clearly improved.

Analysis of variance revealed significant differences among families in both the (Tex6xB73) × B73 BC_1S_1 and Tex6 × B73 $F_{2:3}$ populations for log_{10} aflatoxin levels. Differences among families for ear rot were not significant in the BC_1S_1 population, but there were significant differences among families for ear rot in the $F_{2:3}$ population (data not shown). Thus the data from the BC_1S_1 population was not suitable for QTL analysis. Therefore, we do not present ear-rot QTL analysis in this article because we cannot make comparisons between the two populations. The family × year interaction was significant for log_{10} aflatoxin in just the BC_1S_1 population (data not shown). Analysis for homogeneity of variance showed that the variance component for years was significantly different for both populations for log_{10} aflatoxin ($P = 0.0015$); therefore the datasets were not combined over years for QTL analysis. Consistent with these observations, narrow-sense heritabilities for levels of aflatoxin (log_{10}) were low, 29.3% in the $F_{2:3}$ population and 19.1% in the BC_1S_1 population.

We report multiple regression (MR) and composite interval analysis with multiple regression of QTL (CIM MR) methods for detecting molecular-marker associations with levels of aflatoxin (log_{10} transformed) for the individual years of 1996 and 1997. We report results for

the $\log_{10}$ transformed dataset, however, for simplicity; and we refer to this transformed data simply as aflatoxin for the remainder of the paper.

Multiple regression (MR) analysis with PLABQTL software (Utz and Melchinger 1996) revealed sets of markers associated with levels of aflatoxin in both populations in 1996 and 1997 (Table 2). For the $F_{2:3}$ population in 1996, markers were detected with MR in bins 2.02, 2.09 and 9.01 for aflatoxin. In 1996 MR analysis on the BC_1S_1 population identified markers in bins 5.07 and 8.05 for aflatoxin. For the $F_{2:3}$ population in 1997, markers were detected in bins 3.06, 4.08, 5.07, 7.04 and 10.05 for aflatoxin. For the BC_1S_1 population in 1997, markers in bins 2.00, 2.02, 5.01, 5.05, 10.05 and 10.07 were associated with aflatoxin levels.

No individual marker or bin was detected with MR in more 1 year within each population (Table 2). No common marker or bin was associated with aflatoxin in both populations in 1996. Marker Bmc1185 in bin 10.07 was detected in both populations for aflatoxin in 1997. Additionally, some markers or bins were significant in both populations but in different years. Bmc1017 in bin 2.02 was significant in the $F_{2:3}$ in 1996 and in the BC_1S_1 in 1997. Marker Bmc1346 in bin 5.07 was detected in the $F_{2:3}$ in 1997 and marker Bng1118, also in bin 5.07, was detected in BC_1S_1 in 1996.

Intervals with QTLs for aflatoxin were detected for the $F_{2:3}$ population in 1997 in the 3.05-6, 4.07-8 and 10.05-7 bin regions with CIM MR analysis (Table 3). Intervals were detected in bins 5.01-2 and 5.04-5 for the BC_1S_1 population in 1997. However CIM MR analysis did not detect QTLs in either population in 1996 at the LOD 2.5 threshold.

The detection of intervals a lower LOD value of 1.25 (data not shown) was examined for the purpose of making comparisons between years and populations. None of the QTL intervals detected in 1997 in either population at the 2.5 LOD threshold were detected in 1996 at the 1.25 LOD threshold. Intervals associated with aflatoxin in 1996 were detected in the $F_{2:3}$ in bins 2.02-03, 2.08-09 and 10.03, and for the BC_1S_1 in bin 5.07. In 1997 additional intervals were detected in bin 5.07 in the $F_{2:3}$, and in bins 2.08 and 10.05-07 in the BC_1S_1. When these intervals detected at the 1.25 LOD value are compared with those detected at the 2.5 LOD threshold, chromosome-bin regions 2.08, 5.07 and 10.05-07 were detected in more than one population, but in different years.

The amount of variation explained by individual markers associated with levels of aflatoxin with MR, tended to be relatively low in 1996 (Table 2). The partial R^2 for individual markers in 1996 for the BC_1S_1 population, ranged from 4.7 to 7.4% for aflatoxin. For the $F_{2:3}$ population in 1996, marker associations ranged from 2.1 to 2.9% for aflatoxin. In 1997 some individual markers explained greater amounts of variation. For the BC_1S_1 population in 1997, individual markers explained from 5.1 to 17.0% of the variation for aflatoxin. For the $F_{2:3}$ population in 1997, individual markers explained from 2.7 to 14.3% of the variation for aflatoxin levels.

Table 2 Significant markers associated with lower levels of aflatoxin (Log_{10}) obtained from PLABQTL multiple regression model in $F_{2:3}$ and BC_1S_1 lines derived from Tex6 × B73 over 2 years (1996–1997). Negative Regression Coefficient indicates lower aflatoxin contributed from Tex6. Positive Regression Coefficient indicates lower aflatoxin contributed from B73

	$F_{2:3}$							BC_1S_1						
Year	Marker	Bin	Reg. Coeff	SE	Part.R^2	Part.SS	Reg.SS	Marker	Bin	Reg. Coeff	SE	Part.R^2	Part.SS	Reg. SS
1996	Bmc1017	2.02	−0.06	0.03*	2.9	0.32	0.29	Bng1118	5.07	0.16	0.06**	7.4	0.63	0.52
	Bmc 1520	2.09	0.05	0.03*	2.3	0.26	0.22	Bng1666	8.05	−0.13	0.06*	4.7	0.38	0.28
	Bmc 1810	9.01	0.05	0.02	2.1	0.24	0.32							
	R^2 = 7.1 ± 3.7		LOD = 2.8	R^2 (adj.) = 5.4				R^2 = 10.4 ± 5.8		LOD = 2.37		R^2 (adj.) = 8.5		
1997	Dupssr23	3.06	0.09	0.03**	6.3	0.60	0.66	Mmc0063	2.00	0.13	0.05*	7.2	0.42	0.24
	Bmc1444	4.08	0.06	0.02*	3.6	0.34	0.30	Bmc1017	2.02	−0.12	0.05*	6.2	0.36	0.32
	Bmc1346	5.07	0.06	0.03*	2.7	0.27	0.28	Bng1143	5.01	−0.21	0.05**	16.1	1.03	0.58
	Dupssr13	7.04	−0.07	0.03*	3.0	0.28	0.05	Mmc0081	5.05	0.22	0.05**	17.0	1.10	0.97
	Bmc1185	10.07	−0.13	0.02**	14.3	1.5	1.68	Umc1038	10.05	−0.28	0.08**	10.9	0.66	0.46
								Bmc1185	10.07	−0.19	0.08*	5.1	0.30	0.10
	R^2 = 24.8 ± 5.8		LOD = 10.3		R^2 (adj.) = 22.5			R^2 = 37.1 ± 7.7		LOD = 10.05		R^2 (adj.) = 33.0		

Table 3 Quantitative trait loci (QTLs) and their epistatic effects associated with low levels of aflatoxin (Log_{10}) in BC_1S_1 and $F_{2:3}$ families from the cross of Tex6 × B73 for year 1997. Negative additive/dominance effect for lower aflatoxin from Tex6. Positive additive/dominance effect for lower aflatoxin from B73. QTL is 1 cM away from the left marker

Population	Chrom.	Position	LOD	R^2	Left marker	Bin	Right marker	Bin	Marker interval (cM)	Effect (additive/dominance)	Effects	Partial R^2
$F_{2:3}$	3	60	4.84	12.5	Phi073 (a_1)	3.05	Dupssr23	3.06	7	0.12/–0.2	0.08**	4.1
					(d_1)						–0.12*	3.2
	4	82	2.53	6.7	Bmc1784 (a_1)	4.07	Bmc1444	4.08	27	0.09/0.1	0.10**	5.8
					(d_1)						0.12	1.9
	10	102	5.19	15.1	Umc1038 (a_1)	10.07	Bmc1185	10.05	16	–0.13/–0.1	–0.13**	14.3
					(d_1)						–0.02	0.1
							$a_1 \times a_2$				0.03	0.1
							$a_1 \times a_3$				–0.03	0.3
							$a_2 \times a_3$				–0.01	0.0
												R^2 (adj.) = 20.9%
BC_1S_1	5	44	3.8	16.1	Bnlg143 (a_1)	5.01	Bnlg565	5.02	1	–0.21	–0.20**	12.1
	5	134	4.2	17.8	Bmc1287 (a_1)	5.04	Mmc0081	5.05	16.0	0.25	0.30**	17.7
					$a_1 \times a_2$						0.11	0.8
												R^2 (adj.) = 20.1%

These results are consistent with a number of QTLs with relatively small effects influencing the development of aflatoxin. However, some QTLs with effects large enough to prompt further study were revealed.

The overall multiple-regression models explained more variation for levels of aflatoxin in 1997 than 1996 (Table 2). In 1996 the adjusted R^2s were 8.5% for the BC_1S_1 and 5.4% for the $F_{2:3}$ population. In 1997 the adjusted R^2s were 33.0% for the BC_1S_1 and 22.5% for the $F_{2:3}$ population. The higher amount of variation explained in 1997 is most likely related to the higher overall levels of aflatoxin in 1997 and the greater range of values for aflatoxin among families. The greater differences among families for aflatoxin levels may represent more-accurate expression of relative genetic differences among families. In 1996 the range of overall levels of aflatoxin may not have been great enough for families, with a differing number of favorable QTL alleles to express their genetic differences to a measurable degree at the phenotypic level.

The final CIM MR model for aflatoxin explained 20.9% for the $F_{2:3}$ and 20.1% for the BC_1S_1 in 1997. The CIM MR analysis detected only one QTL region with significant dominance effects, in bin 3.05 for aflatoxin in the $F_{2:3}$ population in 1997. None of the epistatic interactions between QTLs were significant in the CIM MR model for aflatoxin for both populations in 1997.

Favorable QTLs for lower levels of aflatoxin production were contributed from both the Tex6 and B73 parental sources. Markers with a negative regression coefficient in MR and CIM MR (Tables 2, 3) indicate that the Tex6 marker allele is associated with a lower marker-class mean for aflatoxin. Markers with a positive regression coefficient indicate that lower levels of aflatoxin are associated with the B73 marker allele. Although Tex6 is considered one of the best-sources associated with lower aflatoxin levels, B73 is not extremely poor for aflatoxin levels. B73 was selected because of its historical importance and relevance to current Corn Belt commercial germplasm. Thus, it is not surprising that favorable QTLs come from both parents for a trait that appears to be under multigenic control, consistent with similar observations for other traits (Devicente and Tanksley 1993).

Different sets of QTLs for lower aflatoxin were detected in the different years with MR, and the QTLs detected with CIM MR in 1997 were not detected in 1996, even at a very low LOD level of 1.25. These results are consistent with the high levels of G × E regularly observed for aflatoxin levels on hybrids and segregating progenies (Payne 1992; Campbell and White 1994; Hamblin and White 2000). There were very few common markers or bins associated with aflatoxin levels in the two populations in the 2 years of study. Generally, QTLs with relatively small effects were identified with the exception of the BC_1S_1 in 1997 (Table 2). These results are consistent with the low narrow-sense heritabilities calculated for levels of aflatoxins in the populations, suggestive of many genes with small effects that may be influenced by the environment as the underlying genetic basis for the

production of aflatoxin. Consequently, the QTL associations reported in this initial study need to be detected in other environments and progenies for confirmation, and use in marker-assisted selection programs.

Although the two mapping populations are related, they differed in that the $F_{2:3}$ was an approximately 50%/50% Tex6/B73 genetic background, and the BC_1S_1 was an approximately 25%/75% Tex6/B73 background. The different genetic backgrounds of the progenies may affect the expression or detection of QTLs. The size of the two mapping populations were different and both were relatively small, which may also influence the detection of QTLs. Furthermore, sampling effects may influence which subsets of QTLs are detected in different random samples of the progeny from a given population (Beavis et al. 1994).

Although we did not detect the same sets of QTLs in 1996 and 1997, we do not expect QTLs for lower levels of aflatoxin production to be significant in all years, and 1996 may not have been a good year for the detection of QTLs. Nevertheless, two or three QTLs, explaining approximately 20% of the variation in a year with relatively high levels of aflatoxin (1997) is encouraging for a trait that has been very difficult to make progress with conventional breeding.

The QTL interval of bins 4.07-8 for aflatoxin in the $F_{2:3}$ population in 1997 has the favorable allele(s) associated with lower levels of aflatoxin contributed from B73 (Table 3). Research on a mapping population derived from the cross of Mp313E with Va35 has revealed a major QTL for lower levels of aflatoxin on the long arm of chromosome 4 of Mp313E (Davis et al. 1999). This provides independent data suggesting there is a gene(s) associated with lower levels of aflatoxin production in this chromosome region. We are currently investigating the relationship of the Mp313E and B73 chromosome-4L regions in backcross-derived near-isogenic lines to ascertain whether Mp313E has a more favorable allele(s) for lower levels of aflatoxin than B73.

Some of the other favorable QTLs contributed from B73, if confirmed in other studies, have the potential for crop improvement. For example, in 1997 the QTL in bin 5.04-5 explains 17.7% of the variation for aflatoxin in the BC_1S_1 population (Table 3). Favorable QTLs from B73 may not be useful for improvement of inbreds in the B73 (Stiff Stalk) × Mo17 (Lancaster) heterotic pattern; for example, Southern US or tropical germplasm. In order to develop lines associated with lower and more-stable levels of aflatoxin, it appears that it may be necessary to select favorable QTLs from different individual sources with molecular markers in order to pyramid favorable effects into elite lines. These new lines would then need to be tested for lower levels of aflatoxin in multiple environments to confirm whether the introgressed segments consistently confer lower levels of afltoxin production.

Acknowledgements This work was supported by the Multi-Crop Aflatoxin Elimination Program coordinated through the USDA-ARS National Program Staff for Food Safety and Health and an industry/commodity advisory committee. The research was conducted through a specific cooperative agreement with USDA-ARS, research grant AG-58-3620-5-148. We acknowledge the help of Sandeep Gyawali, Andy Hamblin, Dina Severn and Jeff Wong.

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