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RESEARCH

Phenotypic and Genetic Characterization of a Maize Association Mapping Panel Developed for the Identification of New Sources of Resistance to *Aspergillus flavus* and Aflatoxin Accumulation

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ABSTRACT

Maize (*Zea mays* L.) susceptibility to ear rot by *Aspergillus flavus* (Link:Fr) and aflatoxin accumulation causes global economic and human health damage. Host plant resistance is an ideal solution, but commercial varieties lack sufficient resistance to solve the problem. Due to general lack of resistant maize germplasm, no currently available association mapping panels are expected to contain significant variation for the trait. A new association mapping panel containing the majority of aflatoxin accumulation resistant maize lines at the time of compilation is presented here along with genetic and phenotypic characterization data. Phenotypic data from testcrossed lines include aflatoxin levels, days to silking, and corn earworm [*Helicoverpa zea* (Boddie)] damage measured in seven environments and fungal biomass of kernels measured in three. In addition to identifying previously reported aflatoxin resistant lines, new resistant lines were discovered, which may be used for breeding improved germplasm. The Mexican landrace Tuxpeño is the progenitor or one main contributor for most of the resistant lines and likely the source of resistance, but a few other sources may allow additional novel resistance to be pyramided into future breeding lines. Genetic characterization of kinship, genetic diversity, and substructure analysis presented here will allow this resource to be used for association mapping of aflatoxin and identification of factors responsible for this challenging quantitative trait.

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Abbreviations: BSSS, Iowa Stiff Stalk Synthetic; CEW, corn earworm; CoP, coefficient of pedigree; G×E, genotype × environment; GBS, genotyping by sequencing; GD, genetic diversity; GGE, genotype plus genotype × environment; LD, linkage disequilibrium; MAF, minor allele frequency; PCR, polymerase chain reaction; P:H, pathogen to host ratio; qPCR, quantitative polymerase chain reaction; QTL, quantitative trait loci; SNP, single nucleotide polymorphism.

The infection of maize and other oil seed and nut crop species with *Aspergillus flavus* Link:Fr is problematic and results in high economic losses. Although *A. flavus* is a saprophyte and not strictly a pathogen, favorable conditions for its growth can cause significant rotting of maize ears and kernels before and after harvest. In addition to actual destruction, grain can become contaminated by aflatoxin, a secondary metabolite produced by *A. flavus*, which is highly toxic to many animals including humans, pets, and livestock and is therefore tightly regulated. Acute and chronic health problems are caused by aflatoxins, which are carcinogenic, immunosuppressive, and hepatotoxic in humans and animals (Castegnaro and McGregor, 1998). Furthermore, the U.S. Food and Drug Administration regulates interstate commerce of feed grain designated for unrestricted use containing more than 20 ng g^{-1} aflatoxin (Park and Liang, 1993), which leads to direct

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economic losses faced by farmers who must destroy contaminated maize grain. Reports of deaths among livestock, pets, and people around the world attributable to aflatoxin occur regularly, and many unattributed to aflatoxin likely occur. In addition, the depressed immune systems of people under long term, low level exposure of aflatoxins may have increased the severity of epidemics of acquired immunodeficiency syndrome (AIDS), malaria, tuberculosis, and other diseases as well as impaired child development in the developing world (Gong et al., 2008; Jiang et al., 2008).

The development of elite, resistant cultivars would be an economical and efficient way to combat *A. flavus* and aflatoxin contamination of maize. Genetic variation is known to exist for aflatoxin accumulation and *A. flavus* resistance in maize, and stably resistant breeding lines have been developed. However, transfer of resistance to elite cultivars has been hampered by low heritability, caused by high genotype × environment (G×E) interaction, a highly quantitative nature, and difficulty in accurate phenotyping, particularly at the very low levels at which aflatoxins can be problematical. Heritability estimates in past trials have ranged from 0 to 0.6, with a median around 0.3 (Busboom and White, 2004; Brooks et al., 2005; Hamblin and White, 2000). Only a few resistant breeding lines have been developed that show consistent suppression of aflatoxin accumulation over a range of environments; most lines show variable resistance at high levels of fungal pressure. Quantitative trait loci (QTL) mapping studies have identified dozens of potential QTL for aflatoxin, *A. flavus*, and/or ear rot resistance (Widstrom et al., 2003; Paul et al., 2003; Busboom and White, 2004; Brooks et al., 2005; Robertson-Hoyt et al., 2007; Warburton et al., 2009; 2011; Mayfield et al., 2011; Willcox et al., 2012), and some of these have been identified in multiple studies and/or from more than one donor line. Unfortunately, no single QTL has been identified that can explain a majority of the phenotypic variation within a study (major gene resistance) or a significant amount of the variation in every study. However, because at least one QTL with a moderately large, repeatable, additive effect has been found from each resistance source mapped to date, it is hypothesized that the development of markers from within the largest QTL from multiple different resistance sources will allow the possibility of pyramiding these genes with larger additive effects into elite breeding lines and thus finally allow this trait to be more easily manipulated in practical breeding programs.

The main difficulty in detecting QTL that express resistance in multiple environments is the high G×E interaction variance that aflatoxin and *A. flavus* resistance displays. High levels of aflatoxin production depend on many factors including the environment (especially temperatures, soil moisture levels, and relative humidity), the genotype of the host plant under study, and the genotype of the fungus (Bayman and Cotty, 1991) in addition to potential interactions between factors. The ability to more quickly and accurately quantify the amount of fungus and toxin in each maize ear could allow sufficient statistical power to study the interaction of these factors and identify the most resistant lines and alleles. New resistant germplasm, phenotyping methodologies, and techniques for identifying genes or markers for resistance are needed to improve efforts to breed for resistance.

To aid in the identification of new *A. flavus* and aflatoxin accumulation resistant genotypes and resistance genes, a panel of 300 diverse inbred lines has been assembled for association mapping of aflatoxin accumulation resistance. The lines include most public maize lines known to have higher levels of resistance to aflatoxin contamination or *A. flavus* infection, lines that had been developed for resistance against other biotic and abiotic stresses, and lines that represent as much of the diversity present in maize that could be accurately phenotyped in a common trial in the southern United States. The objectives of the present study were to phenotype testcrosses of these maize lines for aflatoxin accumulation resistance and related traits (including a new method of precise measurement of fungal biomass in seeds), to identify new sources of aflatoxin accumulation resistance within the panel for future use as donor lines of newly identified resistance genes, and to determine the genetic relationships and substructure present within the panel to lay the foundation for future breeding and association mapping efforts.

MATERIALS AND METHODS

Selection of the Panel

The aflatoxin association mapping panel consists of 300 diverse inbred lines, which represent most of the publicly available sources of *A. flavus* and aflatoxin accumulation resistance known at the time that the panel was assembled as well as many well-characterized inbred lines from previously published association mapping studies (Flint-Garcia et al., 2005; Setter et al., 2011) (Supplemental Table S1). Lines were provided by Jose Luis Araus (the International Maize and Wheat Improvement Center, CIMMYT), Mark Millard (USDA-ARS, Ames, IA), Baozhu Guo (USDA-ARS, Tifton, GA), and the authors. The 300 lines were chosen from a larger panel of 467 lines, which were grown during the summer of 2008 at Mississippi State University, Starkville, MS. Lines highly related by pedigree were removed from the panel (leaving only one representative of each related group) as were lines that did not set sufficient seed in this location to maintain the lines and produce testcrosses. The 300 selected lines were crossed to Va35, a susceptible, southern adapted inbred line of the non-stiff stalk heterotic pattern, in Homestead, FL, during the winter of 2008/2009. Testcrosses, rather than inbred lines, were phenotyped because when ears of inbred lines are artificially inoculated with *A. flavus*, the disease pressure is generally too great and the ear is completely rotted, leaving nothing to phenotype. Maize is also grown commercially as a hybrid, so testcrosses would have the most relevance. Furthermore, the range in adaptation and phenology in the

panel was very high, and there was no one environment where all inbred lines would have grown equally well. Some would always have been poorly adapted, but by crossing to a common tester, the range of flowering time was reduced and all hybrids were able to grow and flower in Texas and Mississippi. However, because Va35 is derived from non-stiff stalk germplasm, more heterotic response is expected in crosses of this tester to unrelated lines, such as those from the stiff stalk group than in its crosses to related non-stiff stalk germplasm.

Field Phenotyping

The 300 testcrosses were grown in College Station, TX, in 2009 and 2010, Lubbock, TX, in 2009 and 2010, Raymond, MS, in 2009, one Starkville, MS, site in 2009, and two sites within Starkville in 2010 (a and b). In this study, each location–year combination is referred to as an environment, giving a total of seven environments. In each environment, the testcrosses were planted in single-row plots thinned to between 15 and 20 plants, in a randomized complete block design with three replications. Standard production practices were followed at each location, and supplemental irrigation was applied as needed. Traits measured on the testcrossed lines include alflatoxin levels via the Vicam AflaTest (VICAM), as described below, fungal biomass levels via quantitative polymerase chain reaction (qPCR) (in Starkville 2010b and College Station 2009 and 2010 only and as described below), corn earworm (CEW), damage by *Helicoverpa zea* (Boddie), using the rating scale developed by Widstrom (1967), and maturity (days after planting to 50% silking).

Primary ears of the plants in each plot were inoculated with a 3.4-mL suspension of 3×10^8 conidia of *A. flavus* strain NRRL 3357 (American Type Culture Collection number 200026) using the side-needle technique 7 d after mid silk (50% of the plants in the plot had silks showing) (Zummo and Scott, 1989; Windham and Williams, 1998). Inoculum was prepared by incubating the fungus on sterile corn cob grits or autoclaved corn. Conidia were washed and concentrations of conidia were determined with a hemacytometer and adjusted with sterile distilled water to 9×10^7 conidia mL^{-1}. Up to 10 inoculated ears per plot were harvested at maturity, approximately 60 d after mid silk. The ears were dried and shelled, and bulked grain samples were ground using a Romer mill (Romer Industries, Inc.). The VICAM AflaTest was used to determine aflatoxin concentration in 50-g samples of ground grain from each plot, according to manufacturer's instructions.

Quantification of Fungal Biomass

The DNA was extracted from *A. flavus* infected, dried, and ground maize kernel samples using a modified cetyl trimethylammonium bromide protocol as described by Mideros et al. (2009). The DNA was quantified using a BMG Fluorostar Omega (BMG Labtech) following the manufacturer's protocols. *Aspergillus flavus* specific primers designed by Mideros et al. (2009) for the internal transcribed spacer 1 (ITS1) were used to estimate the amount of pathogen DNA present in the sample using primer pair AF2 (forward primer 5′-ATCATTACCGAGTGTAGGGTTCCT-3′ and reverse primer 5′-GCCGAAGCAACTAAGGTACAGTAAA-3′). This was compared to the amount of host DNA present in the sample as estimated using qPCR with maize primers Zmt3 (forward primer 5′-ATCATTACCGAGTGTAGGGTTCCT-3′ and reverse 5′-GCCGAAGCAACTAAGGTACAGTAAA-3′) from the maize α-tubulin sequence obtained from GenBank (X73980.1). Each *A. flavus* or maize qPCR reaction was prepared separately in 10 μL volumes with 1x SYBR green PCR (polymerase chain reaction) Master Mix (Applied Biosystems), 2 μL of sample template DNA (10 ng g^{-1}), and primers at 10 μM. Polymerase chain reaction conditions were 95°C for 10 s and 45 cycles 95°C for 10 s, 59°C for 5 s, and 72°C for 10 s. A multiplex reaction to estimate the amount of host and pathogen DNA in a single tube was not possible using the primers in this study (Mideros et al., 2009). For DNA quantification, separate standard curves were derived for each set of primers. The pathogen to host ratio (P:H) and the fungal biomass in the infected maize samples was calculated according to Williams et al. (2011).

Phenotypic Data Analysis

There was a strong relationship between the variance and mean for aflatoxin level data, and this relationship could not be removed, even when data were adjusted via the general linear model plus logarithm transformation or by spatial analysis models (data not shown). Therefore, we used proc GLIMMIX from the SAS statistical software package (SAS Institute, 2009) to calculate least squared means of aflatoxin levels within each environment using a generalized linear mixed model (GLMM) (McCullagh and Nelder, 1989) assuming a Poisson distribution and a logarithm link function. Fungal biomass, CEW, and maturity data within individual environments were analyzed by a mixed model using the procedure MIXED from SAS. For each trait, a pooled analysis across environments was calculated using the available information to estimate heritabilities; aflatoxin values were logarithm transformed and other traits were not. Broad-sense heritability was calculated on a line mean-basis according to Holland et al. (2003). The data for the 2009 Raymond, MS, field site was not included in the final analysis because an unusually active hurricane season caused 25% missing data due to lodging for this environment. In addition, far outlying data points in the aflatoxin levels for the 2010 Lubbock data set that were likely due to postharvest infection of the grain before analysis (2.6% of the data) and outlying data points in the 2010 College Station data set due to soil fertility problems in some of the entries for one of the replications (1.8% of the data) were removed and treated as missing data points. Least squared means for aflatoxin levels for each genotype–environment combination were also modeled using a genotype plus genotype × environment (GGE) additive–multiplicative model, and the biplot for the first two principal axes was drawn according to Yan et al. (2000).

Genetic and phenotypic correlation coefficients were calculated between aflatoxin level and maturity (days to silking), CEW, and fungal biomass on the basis of standardized per plot information using a multivariate mixed model, similar to that proposed by Wisser et al. (2011) and Holland (2006). Four different models were considered: (i) no adjustment for population structure or relationships between genotypes, (ii) accounting for population substructure using the matrix of estimated membership probabilities from each genotype to each structure group from Supplemental Table S2 as covariates (Q adjustment), (iii) accounting for genetic relationships between all pairs of entries using a similarity matrix (Supplemental Table S3) (K

adjustment), and (vi) accounting for both population structure and relationship between genotypes (Q plus K adjustment). The proposed models were similar to those presented by Wisser et al. (2011) but were adapted to a multi-environment trial (seven environments) where each trait was measured in each environment. ASReml-R v.3 software (Butler et al., 2009; R Core Team, 2012) was used to calculate correlation estimates. For fungal biomass, the models involving the structure subpopulation covariates (Q and Q plus K) did not converge. A second K matrix was calculated using only 1415 single nucleotide polymorphisms (SNPs) with a lower (<5%) missing data rate, but this did not resolve the problem. It may have been caused by the very low total variance for the trait, which caused genetic and/or genotypic by environment variance components to be estimated as zero or negative, violating the properties of the covariance matrix results, which must be positive definite.

Pedigrees

Pedigree information was compiled from multiple sources (Brewbaker, 2007; CIMMYT, 1998; Gerdes et al., 1993; Kim, 1994; Flint-Garcia et al., 2005; Holley and Goodman, 1988; Llorente et al., 2004; Mayfield et al., 2012; Scott and Zummo, 1990, 1992; Williams and Davis, 1984, 2000; Williams and Windham, 1998, 2001, 2006, 2012; author's breeding programs; and USDA-ARS National Genetic Resources Program [http://www.ars-grin.gov/npgs/acc/acc_queries.html; accessed 1 Dec. 2011]) (Supplemental Table S1). Many of the lines included in this panel were derived from genetically broad based populations and pools, which were generally created by mixing a very wide range and number of inbred lines, synthetic varieties, open pollinated varieties, and landrace varieties. Because the genetic variation partitioned between populations is much smaller than that partitioned within them (Warburton et al., 2002), any two lines drawn at random from the same population may actually be as different as two lines drawn from unrelated populations. Therefore, for lines derived from these wide-based breeding populations and for sources of unknown origin, the parental contribution was considered zero. Coefficients of pedigree (CoPs) between all pairs of genotypes in the study were calculated using SAS (SAS Institute, 2009) procedure Inbreed.

Genotyping by Sequencing

Genotyping of the 300 entries in the panel was done via genotyping by sequencing (GBS) according to Elshire et al (2011). A total of 13,197 SNPs with a minor allele frequency (MAF) greater than 1% were called in the panel, from which a data set consisting of 2000 SNPs with a low missing data rate (<7.5%) and low frequency imbalance between the two alleles (MAF > 0.25) was extracted for genetic diversity and structure analysis. These markers covered all 10 chromosomes and had an average of 6.3% missing data. TASSEL 3.0.1 (Bradbury et al., 2007) was used to create a matrix of kinship coefficients between each pair of entries with the 2000 SNP data set. PowerMarker 3.25 (Liu and Muse, 2005) was used to calculate the shared allele distances between each pair of entries in the study, and these were used to create a neighbor joining dendrogram of the 300 entries. PowerMarker 3.25 was also used to calculate diversity statistics on the 13,197 SNPs from the GBS data. TASSEL 3.0.1 was used to calculate the linkage disequilibrium (LD) between

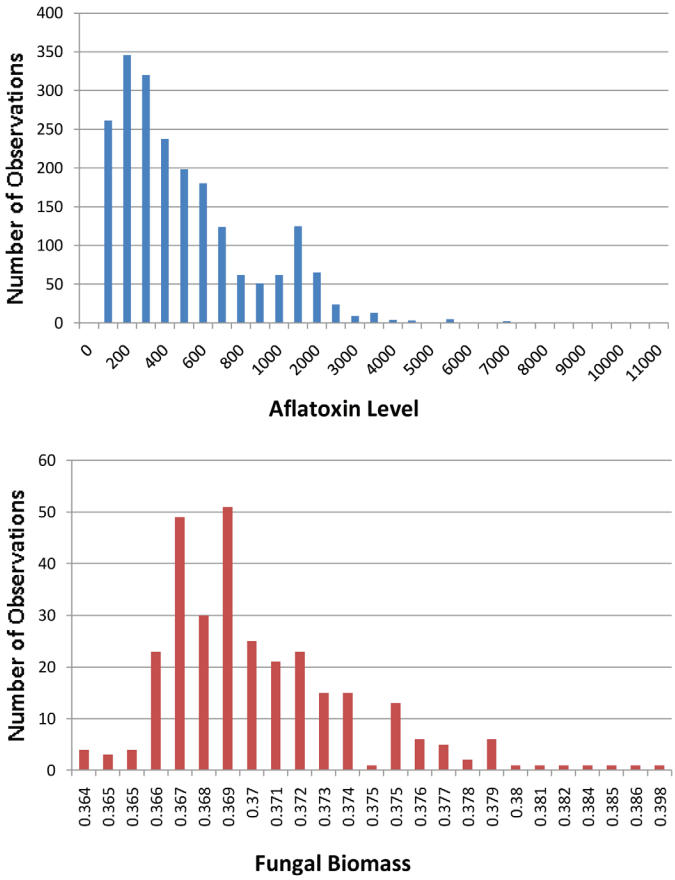

Figure 1. Distribution of line means across environments. Top: Least squared mean transformed aflatoxin levels (in ng g^{-1}) in the panel of 300 individuals over seven environments averaged over three replications per environment. Bottom: Fungal biomass means measured as pathogen to host ratios via quantitative polymerase chain reaction over three environments.

the 13,197 SNPs in this panel. Structure 2.2 (Pritchard et al., 2000) was used to estimate population substructure existing within the panel of 300 lines using the subset of 2000 SNPs and 100,000 replications for burn-in and 200,000 replications for the analysis and assuming admixture and correlated allele frequencies between subclusters. Populations were then assigned to each group for which they had an ancestry proportion Q_{jk} greater than 51.0%; if a population did not show an ancestry proportion higher than this value, it was assigned to the mixed group.

RESULTS AND DISCUSSION

Traits

A substantial range of variation for aflatoxin levels, maturity, and earworm damage ratings but a small range in fungal biomass levels were identified in this panel of lines (Supplemental Table S1; Fig. 1) and across environments (Table 1). Means of all traits were distributed over a wide range, and for aflatoxin levels, they varied from a low of 44.6 ng g^{-1} (Mp715) to a high of 1196.5 ng g^{-1} (B97) (Supplemental Table S1). Because all testcrosses in this study were wounded and directly inoculated with fungal spores during field phenotyping, thus overcoming any possible

Table 1. Means and variance of traits measured in the study in each environment (year–location combination).

	Aflatoxin (ng g^{-1})		Maturity (days after planting)		CEW† (1–5 scale)		qPCR (P:H ratio)‡	
Environment	Mean	Variance	Mean	Variance	Mean	Variance	Mean	Variance
Starkville a in 2010	209.9	110.7	64.1	2.6	2.47	0.96	–	–
Starkville b in 2010	386.1	189.0	66.7	3.3	2.52	1.32	0.336	0.005
College Station in 2010	957.6	1215.4	69.8	5.1	0.05	0.25	–	–
Lubbock in 2010	692.4	382.1	67.8	3.2	4.58	1.39	–	–
Starkville in 2009	344.2	156.1	61.0	2.3	4.02	0.94	–	–
College Station in 2009	933.1	616.8	85.9	3.0	4.33	1.53	0.376	0.015
Lubbock in 2009	357.9	123.1	79.2	3.8	4.67	1.34	–	–
Overall averages	550.6	1120.8	70.6	6.5	3.24	0.78	0.370	0.011
H^2 accession§	0.87	0.02	0.94	0.004	0.88	0.02	–	–
H^2 plot	0.38	0.08	0.58	0.08	0.42	0.09	–	–

†CEW, corn earworm.

‡qPCR, quantitative polymerase chain reaction; P:H, pathogen to host ratio.

§H^2, broad-sense heritability.

avoidance or nonkernel resistance in the plants, even the lowest values of aflatoxin accumulation are above legal limits of aflatoxin in farmer's grain. Such high levels of aflatoxin are generally not observed under natural infection conditions. Analysis of variance of aflatoxin levels showed that there was significant variation between replications within each field, between fields, and across years (data not shown). Broad-sense or mean-basis heritability for aflatoxin levels on a plot basis was 0.383 and on an accession mean basis was 0.87; other traits were higher (Table 1). Aflatoxin levels were particularly high in both Texas sites in 2010 and in College Station in 2009. Means for maturity were particularly high in the two Texas sites in 2009, where very late maturing corn was seen. Corn ear worm damage was also high in all sites in 2009 and in Lubbock in 2010. Considering how entries were chosen, it should be assumed that genetic variation exists for other traits in this panel, including drought tolerance and resistance to other biotic stresses, and therefore the panel will also be useful as an association mapping resource for these other traits.

Quantitative PCR is a very precise method of quantifying fungal load in contaminated maize kernels, much more so than ear rot scores or kernel screening methods, and is fast and highly automatable (Mideros et al., 2009). It was used to measure the P:H, which was calculated to estimate fungal biomass in Starkville 2010b and College Station in 2009 and 2010. Ratios above 0.5 were seen only where amplification of the maize primers failed to amplify, probably due to polymorphisms in the sequence of the priming sites. This occurred with 18 out of 2700 observations, and these data points were removed from the data set. The P:H values were 0.336 in Starkville 2010b, 0.376 in College Station in 2009, and 0.369 in College Station in 2010 and averaged 0.370 (Table 1). The lowest values appear to represent the amount of fungus present in all inoculated samples, because the fungus is injected into the immature ear and plant resistance mechanisms to prevent initial fungal infection were bypassed. The low P:H levels in many of the lines indicated that the spread of the fungus within the ear was minimal while higher P:H values indicate the more susceptible plants, where fungal biomass had actively increased.

Genotype × Environment and Correlation Analyses

Two mega-environments were identified with a GGE biplot analysis run to study the relationship between each environment and each genotype. One was the two College Station trials and the other represented the two Lubbock and three Starkville environments (although the two Starkville fields in 2010 were slightly outlying in this second cluster) (Supplemental Fig. S1). Only nine genotypes were specifically influenced by environment, seven of which (AAP-209, CI3A, CI03, A679, B97, CML77, and Tzi9) did badly only in the mega-environment formed by College Station, and three (P39, CML228 and Yu796-NS) did badly only in the other mega-environment. This is encouraging for the possibilities of breeding lines that are stably resistant over more than one location. Maturity was significantly correlated with aflatoxin accumulation resistance, and earlier maturing varieties had lower levels of aflatoxin (Table 2). This often happens when corn plants experience a late season drought (Payne, 1992). Depending on the timing of drought or heat stress during the growing season, differently maturing varieties may avoid some of the worst of the fungal growth season, and maturity should be considered as a covariate when mapping aflatoxin resistance factors. Although the genetic correlation between aflatoxin and maturity decreased slightly when relationships between entries were taking into account, the phenotypic correlation increased (Table 2). Genetic correlation between CEW damage and aflatoxin levels was significant (Table 2) and slightly decreased by accounting for structure subpopulation. Particularly high genetic

Table 2. Genetic and phenotypic correlation coefficients and standard errors between aflatoxin levels and the other traits measured in this study: days to female flowering (SILK), corn earworm (CEW) damage, and fungal biomass (quantitative polymerase chain reaction [qPCR]). All correlations are significant at $p = 0.05$.

			Multivariate mixed model			
Trait	Type†	No model	No adjustment	Q adjustment	K adjustment	Q plus K adjustment
SILK	gen		−0.585 ± 0.070	−0.564 ± 0.069	−0.401 ± 0.094	−0.394 ± 0.096
	phen	−0.515	−0.050 ± 0.016	−0.047 ± 0.016	−0.116 ± 0.034	−0.115 ± 0.034
CEW damage	gen		0.599 ± 0.080	0.499 ± 0.103	0.542 ± 0.096	0.552 ± 0.091
	phen	0.380	0.079 ± 0.015	0.057 ± 0.015	0.095 ± 0.034	0.095 ± 0.034
qPCR	gen		0.956 ± 0.134	0.892 ± 0.285	‡	
	phen	0.219	0.499 ± 0.018	0.481 ± 0.018	‡	

†gen, genetic; phen, phenotypic.

‡The model did not converge.

correlations in the northern United States and B14 subpopulation may cause this (data not shown); this subpopulation may contain germplasm particularly susceptible to both CEW and aflatoxin contamination. Corn ear worm feeding was generally low in this study. Fungal biomass was highly correlated to grain aflatoxin content (Table 2).

Germplasm

Original Sources of Aflatoxin and Aspergillus flavus Resistance in Maize

Germplasm developed or known to be resistant to aflatoxin accumulation tended to perform well, as expected, and lines not previously documented as resistant, including diverse southern adapted U.S. inbreds and CIMMYT derived inbreds, also displayed aflatoxin accumulation resistance (Supplemental Table S1). Many of the lines that contributed the most resistance to the testcrosses will likely be valuable sources of new breeding germplasm and/or resistance genes. Twenty-two lines constituting the most resistant hybrids were not significantly different from the hybrid of the most resistant line, Mp715, which was developed by the USDA ARS Corn Host Plant Resistance Research Unit at Mississippi State University. These 22 lines included inbreds from CIMMYT, Mississippi, North Carolina, Thailand, South Carolina, Texas, Hawaii, and the International Institute for Tropical Agriculture (IITA). They include germplasm selected for aflatoxin accumulation resistance (i.e., Mp715, Mp313E, and Tx772), lines not selected for resistance but previously known to perform well in aflatoxin trials (i.e., CML277, CML322, and CML348), and many lines not previously known to display resistance. The best third of the lines in the panel (those that were within two least significant distance units from the best line) were dominated by these sources but also included three lines from South Africa, one from Pioneer, and four inbreds selected from traditional landrace varieties from the United States (Supplemental Table S1).

Looking at the origins of the landrace (or traditional farmer's) populations from which these 22 inbred lines were originally derived, there appear to be only a few possible sources of aflatoxin accumulation resistance. Most if not all of them are tropical sources, where landraces probably evolved via natural and farmer selection but limited breeder selection. A germplasm source that contributed to the pedigrees of many lines found to be resistant in this study is Tuxpeño, a Mexican landrace. Additional sources of resistance may be Tuxpan, the Caribbean (possibly the Caribbean Insect Resistance composite), Argentina, and Colombia. Seven inbreds derive from diverse germplasm mixtures and it is impossible to tell where resistance might have come from, but in three of the six cases, Tuxpeño germplasm is reported in the list of original populations in the pedigrees, and Argentina and/or the Caribbean appear as well. The other seven mixed pedigrees are not as specific and do not list landraces, only countries, most of which are tropical. Tuxpeño is a Mexican tropical white dent race, originally collected from Tuxpan, Veracruz, Mexico. It has been useful in breeding many of the high yielding open pollinated varieties and breeding populations at CIMMYT and worldwide and is genetically quite distinct from most elite varieties (Wen et al., 2012). Tuxpan is a U.S. southern dent race with similar grain type as Tuxpeño and contains unknown tropical germplasm; one might assume from the name and morphological similarity that the tropical germplasm was probably Tuxpeño, making it not unlikely that these two resistance sources are the same or at least very related. Twenty-five of the most resistant 50 lines in the panel have Tuxpeño or Tuxpan in their pedigrees; by contrast, only four of the least resistant lines do (Supplemental Table S1).

Relatedness and Substructure Analysis

A CoP can be calculated between two individuals in a population; CoP is a measure of relatedness that calculates the probability of alleles at a locus being identical by descent (coming from a common ancestor) between the two individuals. Kinship is another measure of relatedness between each pair of entries, which can be calculated using CoP or the allele frequency of neutral molecular markers in a population of individuals. Both CoPs and markers can be used to control false positives in association mapping (Yu et al., 2005). The majority of the CoP values between

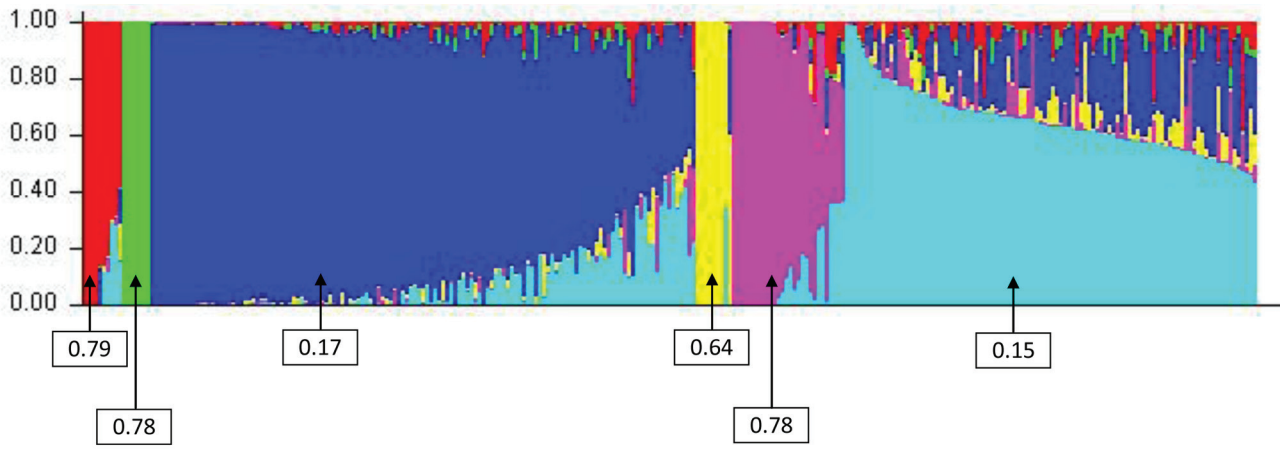

Figure 2. Structure analysis of 288 entries in the study with K set to six subpopulations. These subpopulations correspond to those listed in Supplemental Table S2 as follows: red lines correspond to "Northern/B14," green lines to "SC76," dark blue lines to "Tropical," yellow lines to "Lancaster Surecrop/C103," pink lines to "B73," and light blue lines "Misc. Temperate." Boxed numbers below each subpopulation are Wright's fixation index (F_{ST}) values.

all pairs of entries in this study were zero because of the inability to trace the pedigrees of most entries to one individual; most pedigrees ended in a genetically broad-based population instead (data not shown). Therefore, it is not recommended to use the CoPs as a measure of relationship for purposes of association mapping in this panel. The kinship coefficients estimated by SNP markers (Supplemental Table S4) will be much more reliable and complete for this purpose. However, the GBS data were unusable for 12 lines, so only 288 entries (plus some repeated entries used as controls) were fully characterized and included in this and following genetic characterization tables.

Analysis of genetic distances (the converse of the kinship) between pairs of the 288 entries indicated that the majority of the entries are fairly unrelated to each other (Supplemental Table S3). Both the mean and the median genetic diversity (GD) between pairs of entries was 0.46 and 90% fell between 0.30 and 0.50. Only 0.9% of the pairs of entries displayed a GD less than 0.2. These included the distances between four pairs of entries representing DNA from the same inbred included in the GBS study as controls (distance between these four pairs was 0), 11 pairs of North Carolina, CIMMYT, or Thai lines with identical pedigrees chosen because they were thought to have a higher probability of aflatoxin accumulation resistance, and between B73 and a B73 backcross derived North Carolina line (Supplemental Table S3). The most distantly related lines included pairs that displayed a distance of greater than 0.55; these included three pairs of entries that were between the most distantly related tropical lines vs. temperate lines. In this panel, there was very little redundancy with B73, the most commonly used temperate (Corn Belt) stiff stalk maize line, which was the first maize genome sequenced (Schnable et al., 2009). Although many lines had B73 or the Iowa Stiff Stalk Synthetic (BSSS), from which B73 was selected, in their pedigrees, only 21 lines had a genetic distance with B73 of less than 0.20. The panel includes temperate Corn Belt materials, but most lines were either more southern adapted temperate or were tropical. There was very limited redundancy with Mo17, a historically important temperate non-stiff stalk line (whose genome was also sequenced [http://www.phytozome.net/maize.php; accessed 17 Dec. 2012] but is publicly available in only limited form), as only two lines had a genetic distance with Mo17 less than 0.20. Marker data thus confirms that other than some pairs of lines related by pedigree, the majority of lines in the panel were unrelated according to the markers but closely enough related to be grown successfully under the environmental conditions of the southern United States. This is a good condition for an association mapping panel.

Structure analysis concurs with these findings. An analysis to determine the number of subpopulations using the posterior probability of the data for a given K, $\Pr(X|K)$ (Pritchard et al., 2000), indicated that probability increased for $K = 2$ up to $K = 6$ and then dropped dramatically at $K = 7$ (data not shown). Using $K = 6$, lines were placed into the subpopulation for which they had a probability of ancestry of belonging greater than 0.5; these subpopulations tended to be related by pedigree, breeding program, and/or origin (Supplemental Table S2; Fig. 2). These subpopulations were named "Tropical" (131 entries, all tropical adapted from CIMMYT, Texas A&M University, Thailand, IITA, and Mississippi and also North Carolina, lines which contained tropical germplasm selected for temperate adaptation), "Misc. Temperate" (100 entries from breeding programs in the corn belt or containing temperate [mostly non-stiff stalk] materials in their pedigrees), "B73" (24 entries, all with B73 and BSSS in their pedigrees), "Northern/B14" (10 lines from the north and/or central United States or Canada with B14 in their pedigrees), "Lancaster/C103" (nine lines from North Carolina

derived from Lancaster Surecrop via C103), and "SC76" (seven lines from North or South Carolina derived from SC76) (Fig. 2). Only 10 of the 288 lines could not be assigned to one subpopulation and were left as "mixed."

Cluster analysis classified a few lines into different clusters than the subpopulations reported by structure. Although clusters formed by the neighbor joining dendrogram of all lines were not highly delineated (Supplemental Fig. S2), the structure subpopulations can be found within them. Three clusters contained the majority of the Tropical lines. One small cluster contained all lines derived from SC76; one smaller cluster contained all lines closely related to B73 (and B73 itself). Finally, two large clusters contained most of the remaining temperate derived lines, including a subcluster of lines related to B14 and a subcluster of lines derived from C103. The mixed lines from the structure analysis were mostly grouped in one cluster with some tropical derived lines or joined another clusters. Figure 2 demonstrates that the small subpopulations are very homogenous and the two large subpopulations (tropical and temperate) are much less so. Because of their sizes and homogeneity, Wright's fixation index (F_{ST}) values and net nucleotide distances as calculated by structure are high for the four small subpopulations and lower for the two larger, indicating that the two large subpopulations are both less distinct from the other subpopulations and from an overall population from which all subpopulations are drawn than are the small subpopulations (Fig. 2). This is mostly a function of the size and variation within the two large subpopulations rather than true divergence from other populations or the overall population, based on the variation of allelic frequencies. Hansey et al. (2011) studied genetic diversity within a maize association mapping panel with a restricted phenology. The present analysis is in good agreement with Hansey et al. (2011) for those lines or groups of germplasm in common between the two studies. This substructure classification can now be used as the Q matrix to correct for population substructure in any future association analysis with this panel.

Population structure, with six assumed subpopulations and 10 mixed lines, accounted for 20.6, 2.5, 0.34, 19.1, 18.3, 15.9, 39.8, and 5.6% of the phenotypic variation of aflatoxin levels for the experiments Starkville 2010a, Starkville 2010b, College Station in 2010, Lubbock in 2010, Starkville in 2009, College Station in 2009, Lubbock in 2009, and the pooled analysis (which was significant), respectively (data not shown). Lines from the mixed temperate group and the North Carolina clusters were significantly more susceptible to aflatoxin accumulation ($p < 0.05$) than were the B73 or tropical subpopulations, or the mixed lines. While the two mixed tropical subpopulation had much lower average aflatoxin values than any other lines, they were not significantly different than the B73 subpopulation or the mixed lines. The lower aflatoxin levels in the tropical subpopulation probably reflect true resistance that evolved over time due to the greater incidence of exposure to conditions that favor the growth of *A. flavus* (including postharvest grain storage conditions) on maize in much of the tropical (and developing) world. The lower average aflatoxin values in the B73 subpopulation may actually reflect a heterotic response between the Va35 tester (a non-stiff stalk derived line) and the B73 related lines. More vigorous hybrids have greater resistance in general than those with small or no heterosis. B73 itself has been shown to be very susceptible to *A. flavus* and aflatoxin accumulation and therefore has probably not donated any measurable resistance to lines derived from it.

Genetic Diversity and Linkage Disequilibrium

Minor allele frequency among the 13,197 SNPs scored on this panel ranged from 0.01 (the lower cutoff point chosen for this data) to 0.50 (the upper maximum for this statistic) and an average of 0.16 (data not shown). Gene diversity for these biallelic SNPs ranged from 0.02 to 0.50, with a mean of 0.23, and polymorphic information content behaved similarly, ranging from 0.02 to 0.35 with a mean of 0.19 (data not shown). There is, therefore, a wide range of genetic diversity within this panel, and allelic frequencies tend to be unbalanced for the two alleles at each marker. Many are very rare, which is common in maize (Yan et al., 2009) and leads to a reduction in statistical power for samples containing these SNPs. Although other studies of SNP diversity often found fewer rare alleles, this was likely due to ascertainment bias for SNPs assayed using chip technology (Hamblin et al., 2007). Genotyping by sequencing does not suffer from ascertainment bias as greatly as SNPs from chip assays, so most SNPs and alleles are reported (unless they were null in the B73 reference sequence). Association mapping requires that LD within the genome is due only to physical proximity along the chromosome and, ideally, will not extend too far along any chromosome (Flint-Garcia et al., 2005). For the 2000 SNPs used for characterizing this study, LD existed mainly between adjacent SNPs on the chromosomes (Supplemental Fig. S3). No linkage was found between SNPs from different chromosomes, and the extent of LD along a chromosome was generally limited. There were some larger linkage blocks on chromosomes 1, 8, and 9 and more pairs of linkages at further genetic distances on chromosomes 3, 4, 6, 9, and 10. This data corresponds to LD patterns found with the 13,197 SNPs as well (data not shown). Linkage disequilibrium in this panel corresponded to what has been reported in previous studies (Remington et al., 2001; Tenaillon et al., 2001) and should not cause problems in future association mapping studies.

CONCLUSIONS

In this panel of 300 inbred maize lines, there is considerable genetic and phenotypic variation for maturity, aflatoxin, and other traits. Genotypic variation (as compared to phenotypic variation) should be high enough to make association analysis possible for genes with larger effects and also for positive selection gain. New donor lines were identified, from a limited number of sources with recent origins in the tropics. Fungal biomass measured with qPCR is a heritable trait, and genomewide association analysis of aflatoxin accumulation and fungal biomass resistance is now underway with this panel. Candidate gene association analysis for any gene sequence can be run in this panel as well, and interested parties who wish to validate that a candidate sequence is associated with aflatoxin accumulation resistance may contact the corresponding author.

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References

Bayman, P., and P.J. Cotty. 1991. Vegetative compatibility and genetic diversity in the *Aspergillus flavus* population of a single field. Can. J. Bot. 69:1707–1711. doi:10.1139/b91-216

Bradbury, P.J., Z. Zhang, D.E. Kroon, T.M. Casstevens, Y. Ramdoss, and E.S. Buckler. 2007. TASSEL: Software for association mapping of complex traits in diverse samples. Bioinformatics 23:2633–2635. doi:10.1093/bioinformatics/btm308

Brewbaker, J.L. 2007. Registration of 27 maize parental inbred lines resistant to maize mosaic virus. Crop Sci. 47:459–461. doi:10.2135/cropsci2005.11.0407

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

Busboom, K.N., and D.G. White. 2004. Inheritance of resistance to aflatoxin production and Aspergillus ear rot of corn from the cross of inbreds B73 and Oh516. Phytopathology 94:1101–1115. doi:10.1094/PHYTO.2004.94.10.1107

Butler, D.G., B.R. Cullis, A.R. Gilmour, and B.J. Gogel. 2009. ASReml-R reference manual. VSN International Ltd., Brisbane, Australia, and Queensland Department of Primary Industries and Fisheries, Brisbane, Australia.

Castegnaro, M., and D. McGregor. 1998. Carcinogenic risk assessment of mycotoxins. Rev. Med. Vet. 149:671–678.

CIMMYT. 1998. A complete listing of improved maize germplasm from ClMMYT. Maize Program Special Report. CIMMYT, Mexico, D.F.

Elshire, R.J., J.C. Glaubitz, Q. Sun, J.A. Poland, K. Kawamoto, et al. 2011. A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PLoS ONE 6(5):E19379. doi:10.1371/journal.pone.0019379

Flint-Garcia, S.A., A.C. Thuillet, J.M. Yu, G. Pressoir, S.M. Romero, S.E. Mitchell, J. Doebley, S. Kresovich, M.M. Goodman, and E.S. Buckler. 2005. Maize association population: A high resolution platform for quantitative trait locus dissection. Plant J. 44:1054–1064. doi:10.1111/j.1365-313X.2005.02591.x

Gerdes, J., C. Behr, J. Coors, and W. Tracy. 1993. Compilation of North American maize breeding germplasm. CSSA, Madison, WI.

Gong, Y.Y., P.C. Turner, A.J. Hall, and C.P. Wild. 2008. Aflatoxin exposure and impaired child growth in West Africa: An unexplored international public health burden? In: J.F. Leslie, R. Bandyopadhyay, and A. Visconti, editors, Mycotoxins. Detection methods, management, public health and agricultural trade. CABI, Oxfordshire, UK. p. 53–65.

Hamblin, A.M., and D.G. White. 2000. Inheritance of resistance to Aspergillus ear rot and aflatoxin production of corn from Tex6. Phytopathology 90:292–296. doi:10.1094/PHYTO.2000.90.3.292

Hamblin, M.T., M.L. Warburton, and E.S. Buckler. 2007. Empirical comparison of simple sequence repeats and single nucleotide polymorphisms in assessment of maize diversity and relatedness. PLoS ONE 2(12):E1367. doi:10.1371/journal.pone.0001367

Hansey, C.N., J.M. Johnson, R.S. Sekhon, S.M. Kaeppler, and N. de Leon. 2011. Genetic diversity of a maize association population with restricted phenology. Crop Sci. 51:704–715. doi:10.2135/cropsci2010.03.0178

Holland, J.B. 2006. Estimating genotypic correlations and their standard errors using multivariate restricted maximum likelihood estimation with SAS proc mixed. Crop Sci. 46:642–654. doi:10.2135/cropsci2005.0191

Holland, J., W. Nyquist, and C. Cervantes-Martinez. 2003. Estimating and interpreting heritability for plant breeding: An update. Chapter 2. Plant Breed. Rev. 22:9–112.

Holley, R.N., and M.M. Goodman. 1988. Yield potential of tropical hybrid maize derivatives. Crop Sci. 28:213–218. doi:10.2135/cropsci1988.0011183X002800020005x

Jiang, Y., P.E. Jolly, P. Preko, S.W. Jia, W.O. Ellis, T.D. Phillips, and J.H. Williams. 2008. Aflatoxin-related immune dysfunction in health and in human immunodeficiency virus disease. Clin. Dev. Immunol. 2008:790309. doi:10.1155/2008/790309

Kim, S.K. 1994. Genetics of maize tolerance of *Striga hermonthica*. Crop Sci. 34:900–907. doi:10.2135/cropsci1994.0011183X003400040012x

Liu, K., and V. Muse. 2005. PowerMarker: Integrated analysis environment for genetic marker data. Bioinformatics 21:2128–2129. doi:10.1093/bioinformatics/bti282

Llorente, C.F., F.J. Betrán, A. Bockholt, and F. Fojt. 2004. Registration of Tx772 maize. Crop Sci. 44:1036–1039. doi:10.2135/cropsci2004.1036a

Mayfield, K., F.J. Betrán, T. Isakeit, G. Odvody, S.C. Murray, W.L. Rooney, and J.C. Landivar. 2012. Registration of maize germplasm lines Tx736, Tx739, and Tx740 for reducing preharvest aflatoxin accumulation. J. Plant Reg. 6:88–94. doi:10.3198/jpr2010.12.0675crg

Mayfield, K.L., S.C. Murray, W.L. Rooney, T. Isakeit, and G.A. Odvody. 2011. Confirmation of QTL reducing aflatoxin in maize testcrosses. Crop Sci. 51:2489–2498. doi:10.2135/cropsci2011.02.0112

McCullagh, P., and J.A. Nelder. 1989. Generalized linear models. 2nd ed. Chapman and Hall, London, UK.

Mideros, S.X., G.L. Windham, W.P. Williams, and R.J. Nelson. 2009. *Aspergillus flavus* biomass in maize estimated by quantitative real-time polymerase chain reaction is strongly correlated with aflatoxin concentration. Plant Dis. 93:1163–1170. doi:10.1094/PDIS-93-11-1163

Park, D.L., and B. Liang. 1993. Perspectives on aflatoxin control for human food and animal feed. Trends Food Sci. Technol. 4:334–342. doi:10.1016/0924-2244(93)90104-I

Paul, C., G. Naidoo, A. Forbes, V. Mikkilineni, D. White, and T. Rocheford. 2003. Quantitative trait loci for low aflatoxin production in two related maize populations. Theor. Appl. Genet. 107:263–270. doi:10.1007/s00122-003-1241-0

Payne, G.A. 1992. Aflatoxin in maize. Crit. Rev. Plant Sci. 10:423–440. doi:10.1080/07352689209382320

Pritchard, J.K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945–959.

R Core Team. 2012. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Remington, D.L., J.M. Thornsberry, Y. Matsuoka, L.M. Wilson, S.R. Whitt, J. Doebley, S. Kresovich, M.M. Goodman, and E.S. Buckler. 2001. Structure of linkage disequilibrium and phenotypic associations in the maize genome. Proc. Natl. Acad. Sci. USA 98:11479–11484. doi:10.1073/pnas.201394398

Robertson-Hoyt, A., J. Betrán, G.A. Payne, D.G. White, T. Isakeit, C.M. Maragos, T.L. Molnár, and J.B. Holland. 2007. Relationships among resistances to Fusarium and Aspergillus ear rots and contamination by fumonisin and aflatoxin in maize. Phytopathology 97:311–317. doi:10.1094/PHYTO-97-3-0311

SAS Institute. 2009. SAS/STAT 9.2 user's guide. SAS Institute Inc. Cary, NC.

Schnable, P.S., D. Ware, R.S. Fulton, J.C. Stein, F. Wei, S. Pasternak, C. Liang, J. Zhang, L. Fulton, et al. 2009. The B73 maize genome: Complexity, diversity, and dynamics. Science 326:1112–1115. doi:10.1126/science.1178534

Scott, G.E., and N. Zummo. 1990. Registration of Mp313E parental line of maize. Crop Sci. 30:1378. doi:10.2135/cropsci1990.0011183X003000060080x

Scott, G.E., and N. Zummo. 1992. Registration of Mp420 germplasm line of maize. Crop Sci. 32:1296. doi:10.2135/cropsci1992.0011183X003200050054x

Setter, T.L., J. Yan, M. Warburton, J.M. Ribaut, Y. Xu, M. Sawkins, E.S. Buckler, Z. Zhang, and M. Gore. 2011. Genetic association mapping identifies single nucleotide polymorphisms in genes that affect abscisic acid levels in maize floral tissues during drought. J. Exp. Bot. 62:701–716. doi:10.1093/jxb/erq308

Tenaillon, M.I., M.C. Sawkins, A.D. Long, R.L. Gaut, J.F. Doebley, and B.S. Gaut. 2001. Patterns of DNA sequence polymorphism along chromosome 1 of maize (*Zea mays* ssp. *mays* L.). Proc. Natl. Acad. Sci. USA 98:9161–9166. doi:10.1073/pnas.151244298

Warburton, M.L., T.D. Brooks, M.D. Krakowsky, X. Shan, G.L. Windham, and W.P. Williams. 2009. Identification and mapping of new sources of resistance to aflatoxin accumulation in maize. Crop Sci. 49:1403–1408. doi:10.2135/cropsci2008.12.0696

Warburton, M.L., T.D. Brooks, G.L. Windham, and W.P. Williams. 2011. Identification of QTL contributing resistance to aflatoxin accumulation in maize. Mol. Breed. 27:491–499. doi:10.1007/s11032-010-9446-9

Warburton, M.L., X. Xianchun, J. Crossa, J. Franco, A.E. Melchinger, M. Frisch, M. Bohn, and D. Hoisington. 2002. Genetic characterization of CIMMYT inbred maize lines and open pollinated populations using large scale fingerprinting methods. Crop Sci. 42:1832–1840. doi:10.2135/cropsci2002.1832

Wen, W., J. Franco, V.H. Chavez-Tovar, J. Yan, and S. Taba. 2012. Genetic characterization of a core set of a tropical maize race Tuxpeño for further use in maize improvement. PLoS ONE 7:e32626 10.1371/journal.pone.0032626. doi:10.1371/journal.pone.0032626

Widstrom, N.W. 1967. An evaluation of methods for measuring corn earworm injury. J. Econ. Entomol. 60:791–794.

Widstrom, N.W., A. Burton, B.Z. Guo, D.M. Wilson, M.E. Snook, T.E. Cleveland, and R.E. Lynch. 2003. Control of preharvest aflatoxin contamination in maize by pyramiding QTL involved in resistance to ear-feeding insects and invasion by *Aspergillus* spp. Eur. J. Agron. 19:563–572. doi:10.1016/S1161-0301(03)00004-2

Willcox, M.C., G.L. Davis, M.L. Warburton, G.L. Windham, H.K. Abbas, J. Betrán, J.B. Holland, and W.P. Williams. 2012. Confirming QTL for aflatoxin resistance from Mp313E in different genetic backgrounds. Mol. Breed. 32:15-26.

Williams, W.P., and F.M. Davis. 1984. Registration of Mp705, Mp706, and Mp707 germplasm lines of maize. Crop Sci. 24:1217. doi:10.2135/cropsci1984.0011183X002400060062x

Williams, W.P., and F.M. Davis. 2000. Registration of maize germplasms Mp713 and Mp714. Crop Sci. 40:584.

Williams, W.P., S. Ozkan, A. Ankala, and G.L. Windham. 2011. Ear rot, aflatoxin accumulation, and fungal biomass in maize after inoculation with *Aspergillus flavus*. Field Crops Res. 120:196–200. doi:10.1016/j.fcr.2010.10.002

Williams, W.P., and G.L. Windham. 1998. Registration of root-knot nematode resistant maize germplasm lines Mp709, Mp710, Mp711, and Mp712. Crop Sci. 38:563. doi:10.2135/cropsci1998.0011183X003800020089x

Williams, W.P., and G.L. Windham. 2001. Registration of dent corn germplasm line Mp715. Crop Sci. 41:1374–1375. doi:10.2135/cropsci2001.4141374x

Williams, W.P., and G.L. Windham. 2006. Registration of maize germplasm line Mp717. Crop Sci. 46:1407. doi:10.2135/cropsci2005.09-0330

Williams, W.P., and G.L. Windham. 2012. Registration of maize germplasm lines Mp718 and Mp719. J. Plant Reg. 6:200–202. doi:10.3198/jpr2011.09.0489crg

Windham, G.L., and W.P. Williams. 1998. *Aspergillus flavus* infection and aflatoxin accumulation in resistant and susceptible maize hybrids. Plant Dis. 82:281–284. doi:10.1094/PDIS.1998.82.3.281

Wisser, R.J., J.M. Kolkman, M.E. Patzoldt, J.B. Holland, J. Yu, M. Krakowsky, R.J. Nelson, and P.J. Balint-Kurti. 2011. Multivariate analysis of maize disease resistances suggests a pleiotropic genetic basis and implicates a *GST* gene. Proc. Natl. Acad. Sci. USA 108(18):7339–7344. doi:10.1073/pnas.1011739108

Yan, J., T. Shah, M. Warburton, E. Buckler, M. McMullen, and J. Crouch. 2009. Genetic characterization and linkage disequilibrium estimation of a global maize collection using SNP markers. PLoS ONE 4:e8451. doi:10.1371/journal.pone.0008451

Yan, W., L.A. Hunt, Q. Sheng, and Z. Szlavnics. 2000. Cultivar evaluation and mega-environment investigation based on the GGE biplot. Crop Sci. 40:597–605. doi:10.2135/cropsci2000.403597x

Yu, J., G. Pressoir, W.H. Briggs, I.V. Bi, M. Yamasaki, J.F. Doebley, M.D. McMullen, B.S. Gaut, D.M. Nielsen, J.B. Holland, S. Kresovich, and E.S. Buckler. 2005. A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. Nat. Genet. 38:203–208. doi:10.1038/ng1702

Zummo, N., and G.E. Scott. 1989. Evaluation of field inoculation techniques for screening maize genotypes against kernel infection by *Aspergillus flavus* in Mississippi. Plant Dis. 72:313–316. doi:10.1094/PD-73-0313