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Genetics of Resistance to Aflatoxin Accumulation in Corn (Zea mays)

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GENETICS OF RESISTANCE TO AFLATOXIN ACCUMULATION IN CORN (*ZEA MAYS*)

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agriculture and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The School of Plant, Environmental, and Soil Sciences

by
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B.Sc. (Ag.), Tribhuvan University, 2002
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To my parents,

Bishnu Prasad Sharma,

Madhu Devi Dhakal,

Without whom none of my achievement would be possible

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LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
CIM	Composite interval mapping
DM	Days to 50% maturity
dNTPs	deoxy Ribonucleoside Tri-Phosphates
DS	Days to 50% silking
dsDNA	double stranded Deoxyribonucleic Acid
DT	Days to 50% tasseling
EC	Ear circumference
EDTA	Ethylene Di-amine Tetra Acetic acid
EH	Ear height
EL	Ear length
GCA	General combining ability
GY	Grain yield/ plot
HAI	Hours after inoculation
HC	Husk cover
KN	Number of kernels/row
KW	100-kernel weight
LOD	Logarithm of odds
M	Molar
MAB	Marker-assisted breeding
MAS	Marker-assisted selection
min	Minute
mL	Milliliter
mM	Milli Molar
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
PH	Plant height
qPCR	quantitative PCR
QTL	Quantitative Trait Locus
RIL	Recombinant inbred line
RN	Number of row/ear
RNA	Ribonucleic Acid
RT-PCR	Reverse transcription PCR
SDS	Sodium dodecyl sulfate
sec	Second
SSH	Suppression subtraction hybridization
SSR	Simple sequence repeat
Taq	*Thermus aquaticus*
U	Unit
V	Volt

ABSTRACT

Aflatoxin accumulation in corn continues to be a major problem in all southeastern corn growing states. Development of resistant inbreds and hybrids is a sustainable approach to reduce aflatoxin contamination. Mapping of quantitative trait loci (QTL) for resistance to *Aspergillus flavus* infection and aflatoxin accumulation in maize and developing markers associated with them can be helpful to speed up the breeding program. An $F_{2:3}$ mapping population developed from the cross between Mp715 and B73 and a genetic linkage map was constructed using 136 simple sequence repeat (SSR) markers spanning the whole genome. QTL for aflatoxin resistance were identified in both years and were located on chromosomes 3, 4, 5, 8, 9, and 10 with contribution ranging from <1.0 to 9.2% individually toward resistance phenotype. A highly significant correlation was observed between husk cover and aflatoxin content in both years. A few QTL responsible for close husk cover identified in both years overlapped with the QTL region for aflatoxin resistance. Therefore, it should be possible to use markers identified in this study for selection and improvement of both traits simultaneously through MAB. A suppression subtraction hybridization (SSH) library was constructed using tissues from Mp715 and B73 to identify the differentially expressed genes in response to *Aspergillus flavus*. Three hundred genes related to various functions were identified from the library. Thirty differentially expressed genes were selected to study their expression pattern in seven maize inbreds through RT-PCR and showed differential expression at different time points after fungus inoculation. Higher expression of pathogenesis related protein-4, leucine rich repeat family protein, RNA binding protein, and ubiquitin C-terminal hydrolase in resistant inbreds (Mp715, Mp719, Mp420, and Mp313E) was confirmed by real-time qPCR. These genes may be responsible for resistance in these resistant inbreds. They were integrated into the linkage map generated in this study through

in silico mapping. The gene encoding PR4, which was highly expressed in resistant germplasm was located in bin 4.02 where a QTL for aflatoxin resistance was identified. The genes found in the QTL regions and markers linked with them would be helpful to improve resistance to aflatoxin accumulation in corn.

CHAPTER 1
INTRODUCTION

Aspergillus flavus is an endemic fungus responsible for aflatoxin accumulation in corn in southern states of the USA (Payne 1992; Widstrom 1996; Windham and Williams 1998). Aflatoxins are the secondary metabolites produced after the fungal infection and are immunosuppressive, carcinogenic, and hepatotoxic to human and domestic animals (Castegnaro and McGreogor 1998). The health hazards due to aflatoxin contamination in food have been reported for more than 50 years (Burnside et al. 1957). Aflatoxin is also classified as group 1 carcinogen by International Agency for Research on Cancer (IARC, 1993). Aflatoxin contamination has negative impact on the food and feed industries due to the need of more resources to ensure safety and quality of food and feed supply. Farmers suffer severe economic losses because the US Food and Drug Administration restrict interstate trade of feed grains containing more than 20ng/gm aflatoxin (Park and Liang 1993). Huge economic loss is associated with aflatoxin contamination in maize, peanut, and other crops which was nearly 500 million dollars annually in USA (Vardon et al. 2003). Recent studies showed huge loss (163 millon dollars) to maize farmers due to aflatoxin contamination (Wu 2006). Long term consumption of aflatoxin contaminated food has been found to be associated with liver cancer, stunted child growth, and depressed immune system (Eaton and Groopman 1993). Its occurrence is more prevalent in the tropical and sub-tropical regions and aflatoxin exposure is a health hazard worldwide (Strosnider et al. 2006). Human health is endangered by the long term exposure to aflatoxin in developing countries, where corn is the staple food (Williams et al. 2005). Many natural sources of resistance have been identified to reduce the aflatoxin

accumulation in corn (Scott and Zummo 1992; Campbell and White 1995; Williams and Windham 2001; Robertson-Hoyt et al. 2007).

Although the health and economic impacts of aflatoxin are well known and established, a sustainable and effective management to this problem is yet to be found. Recently, much emphasis has been given to the development and utilization of resistant genetic resources in breeding programs (Brown et al. 1999; Kang and Moreno 2002). Reducing the fungus infection and aflatoxin accumulation in grain could be an effective way to achieve resistance in corn. Aflatoxin accumulation begins after the colonization of the fungus in the developing kernels and continues till maturity (Thompson et al. 1983). Aflatoxin accumulated in the grain is stable and is extremely expensive to destroy during food processing. Thus, aflatoxin accumulation should be prevented at the pre-harvest stage. Weather and field conditions greatly influence *Aspergillus flavus* infection and toxin accumulation in corn. High temperature, drought, and low humidity, which is prevalent in southeastern United States, are favorable for the fungus infection and aflatoxin production (Diener et al. 1987; Payne 1998). Complex genetics and strong genotype-environment interaction pose major challenges in developing commercial hybrids resistant to *A. flavus* infection (Campbell and White 1995; Hamblin and White 2000). Most of the commercial hybrids developed to date lack sufficient field resistance to aflatoxin accumulation (Windham and Williams 1999; Abbas et al. 2002). Aflatoxin contamination drastically reduces the value of grain not only as feed but also as an export commodity (Nichols 1983).

Aflatoxin contamination continues to be a prominent research issue. Multiple strategies have been suggested for the reduction of pre-harvest accumulation of aflatoxin. Suitable agronomic practices (irrigation, plating dates, and fungicides), bio-control (atoxigenic strains), and host plant resistance have been recommended to minimize this problem (Larson 1997;

Cleveland et al. 2004). Though cultural practices are effective for the management of aflatoxin accumulation, the combination of management strategies with host plant resistance would be the most sustainable, economic, and promising approach (Shan and Williams 2014). Breeding programs for the development of resistant inbreds was initiated by USDA-ARS at Mississippi State in the late 1970s (Williams 2006). Numerous germplasm and breeding lines have been developed and evaluated using artificial inoculation techniques for aflatoxin resistance in field conditions. These experiments have established the foundation for the development and release of new inbred lines with natural resistance to aflatoxin accumulation (Windham and Williams 1998, 2002; Windham et al. 1999; Williams 2006). Resistant inbreds have been developed through consecutive selfing and selection against aflatoxin accumulation in wide range of environments. The first resistant inbred line Mp313E was released in1988 (Scott and Zummo 1988). Subsequently, many corn inbreds (Mp420, Mp715, Mp717, Mp718, and Mp719) with low level of aflatoxin accumulation have been released (Williams and Windham 2001, 2006, 2012).

Due to low heritability and high G x E interaction, development of resistant inbreds and hybrids has been challenging. Artificial inoculation and repeated field evaluation are necessary to identify resistant breeding lines (Zummo and Scott 1989). The earlier studies using these resistant lines showed that the resistance was quantitative and inherited in an additive fashion in hybrids. Most of the resistant lines are agronomically inferior and are not suitable for the production of hybrids (Brown et al. 1999). Newer breeding lines with stable resistance to aflatoxin in various environments are needed for the development of commercial hybrids. However, the resistant inbreds developed to date have originated from tropical germplasm. These tropical germplasm are tall, later in maturity, lower yielding, and highly susceptible to lodging when grown in the temperate US Corn Belt, which makes them unsuitable for hybrid

development. Some of the newest inbreds such as Mp718 and Mp719 have suitable plant stature and high resistance to aflatoxin (Mayfield et al. 2012; Williams and Windham 2012).

The availability of markers and advances in statistical procedures has aided construction of highly saturated linkage maps and QTL analysis. Genetic linkage mapping has become a regular procedure to study the quantitative traits (Zeng et al. 1999; Doerge 2002). In maize, many QTL studies have been conducted in last decades to identify QTL responsible for different morphological as well as yield attributes. Grain yield has been studied thoroughly in many mapping experiments and has identified a large number of QTL (Grohn et al. 1998; Khairallah et al. 1999; Cardinal et al .2001; Sibov et al. 2003; Lima et al. 2006; Li et al. 2007). Other morphological and yield related traits such as plant height, ear height, days to silking ear length, ear diameter, number of rows/ear, number of kernels/row, kernel weight were also studied (Liu et al. 2010; Park et al. 2014).

Although molecular mapping of QTL have been conducted, studies related to the biochemical and molecular basis of *A. flavus* resistance are limited. Due to the quantitative nature of resistance to *A flavus*, QTL with stable and large phenotypic effects are difficult to obtain in maize populations (Warburton et al. 2011). The suppression subtraction hybridization (SSH) is a powerful and popular technique to identify differentially expressed genes in closely related samples (Rebrikov et al. 2004). Many enzymes (chitinase, glucanase, and phenylanine ammonia lyase) are involved in response to *R. solani* infection in maize (Anuratha et al. 1996; Jedidah et al. 2000; Liu et al. 2009). Likewise, pathogenesis related (PR) proteins (e.g. PR10) are highly expressed in resistant corn lines in response to *A. flavus* infection (Chen et al. 2010). This indicates that genes involved in disease defense mechanisms are activated during the infection process and the differentially expressed genes in the resistant and susceptible varieties could be

detected. The identification of the genes and metabolic pathways involved in the resistance would be helpful to study the host parasite interaction and aid in development of new resistant inbreds and hybrids through marker assisted breeding.

Many efficient tools are available for the exact measurement of aflatoxin accumulation in corn kernels. Enzyme-linked immunosorbent assays (ELISA) and high performance liquid chromatography (HPLC) are most sensitive and expensive. The VICAM AflaTest is a sensitive and widely used test but it is expensive and time consuming. Conventional visual assessment methods like ear rot percentage are highly error prone. Recently a technique based on quantitative PCR (qPCR) has been developed and used for the rapid quantification of fungal biomass in grain (Mideros et al. 2009). A very strong correlation was observed between aflatoxin accumulation and fungal biomass measured by qPCR assay, which was similar to the studies using GFP-producing isolates in cotton (Rajasekaran et al. 2008). The qPCR assay was used to identify the fungus and to determine the fungal biomass and its correlation to toxins such as aflatoxin and deoxynivalenol (Nicolaisen et al. 2009; Demeke et al. 2010; Atoui et al. 2011; Horevaj et al. 2011). These newly developed qPCR techniques can provide an efficient method to quantify fungal biomass in breeding program for identification of resistant lines (Mideros et al. 2009).

Introgression of resistant quantitative trait loci (QTL) to susceptible, adapted, and agronomically superior lines through marker assisted breeding (MAB) is considered as a potentially effective method to improve resistance to aflatoxin accumulation. Mapping studies in corn (Paul et al. 2003; Brooks et al. 2005; Bello 2007; Warburton et al. 2009, 2011, 2015; Mayfield 2011) have identified many QTL in different chromosomal regions but no consistent QTL with large phenotypic effect (>20%) have been identified to date. However, if QTL with

moderately large effect in different environments from different resistant sources could be identified, it would be helpful for pyramiding of these QTL through markers closely linked to the resistant QTL. It is assumed that the identified QTL from resistant donors will behave consistently in different genetic backgrounds. Recently, some stable QTL across multiple environments and genetic backgrounds have been identified (Willcox et al. 2013). However, the lack of molecular markers linked to resistant QTL in these genotypes is hindering incorporation of the resistance into lines with commercially acceptable genetic background. Therefore, this dissertation research project was undertaken to understand the genetics of resistance to aflatoxin accumulation in corn and the specific objectives are as follows:

- To identify QTL for resistance to aflatoxin accumulation in maize inbreds.
- To identify QTL for diverse morphological traits in an $F_{2:3}$ mapping population of maize.
- To identify differentially expressed genes in response to *A. flavus* infection in maize using suppression subtraction hybridization (SSH).

CHAPTER 2
LITERATURE REVIEW

2.1 *Aspergillus flavus*

The genus *Aspergillus* was first reported by P.A. Micheli, a priest and mycologist, in 1729 AD. The name *Aspergillus* was given due to the resemblance of its conidiophore to an aspergillum, a device used to sprinkle holy water (Bennett and Klich 1992). *Aspergillus* is a diverse genus of fungi and contains more than 200 species (Samson 1992). It has been classified many times on the basis of its morphological characteristics. The species *Aspergillus flavus* was described as an asexual species that produces only asexual spores (conidia) and overwintering fruiting bodies called sclerotia (Amaike and Keller 2011). *Aspergillus flavus* is a widely distributed soil borne opportunistic fungus, which spends most of the time as a saprophyte in the soil (Scheidegger and Payne 2003). It can cause diseases in maize, cotton, peanut, humans, and domestic animals. In maize, it is the cause of ear rot disease, which is most prevalent in warm and dry environments. It produces a polyketide-derived mutagenic and extremely carcinogenic secondary metabolite known as aflatoxin along with other compounds such as cyclopiazonic acid and aflatrem (Zhang et al. 2004; Georgianna et al. 2010). The first recorded outbreak of a disease due to *A. flavus* was Turkey X disease in poultry in England, which was due to the consumption of aflatoxin contaminated feed (Forgacs and Carll 1962). Inhalation and/or consumption of a high amount of aflatoxin for long periods of time cause aflatoxicosis in both humans and domestic animals. It is a serious health threat in the developing countries of Africa and Asia. Many people died in Kenya due to the consumption of aflatoxin contaminated corn (Lewis et al. 2005; Yu et al. 2008). Aflatoxicosis also caused death in dogs in USA due to the contamination of dog food with A. *flavus* in 1998 and 2005-2006 (Garland and Reagor 2001; Stennske et al. 2006). Aflatoxin is accumulated in the kernels of maize and aflatoxin B1 is the most potent

carcinogen found in nature. Long-term exposure to aflatoxin can cause liver cancer resulting in cirrhosis in humans. Aflatoxin accumulation has been a major problem in corn production in southeastern states and recently in mid-western states, when drought prevails during the grain filling stage (Anderson et al. 1975; Zuber et al. 1976). Hot and dry period at grain filling stage is conducive for high aflatoxin production and accumulation (Pyane 1992). Different species of *Aspergillus* attack many crops and they vary widely in their potential to infect and accumulate aflatoxin. *A. flavus* strain NRRL 3357 is widely used for field evaluation due to its ability to produce a high level of aflatoxin in maize grains (Windham and Williams 1998).

2.1.1 Disease cycle and epidemiology

Aspergillus flavus is an opportunistic fungus, which attacks different agricultural crops like corn, peanut, tree nuts and cotton. The fungus resides in soil as conidia and sclerotia which germinate to form mycelia which grow saprophytically. Conidia are carried by wind or insects to host tissues where they germinate and infect (Amaike and Keller 2011). Although it is distributed over the diverse climatic zones, it is mainly concentrated in warm and dry region between the tropics (Klich 2007). The fungus produces sclerotia that can survive in extreme environmental condition in soil. This is the main reason for the higher population of this fungus in hot and drought conditions (Wicklow et al. 1993; Payne 1998). The conidia are dispersed through air and infect above-ground crops such as corn and tree nuts. Likewise, conidia cause infection in peanut and cotton seed (Horn and Pitt 1997; Cotty 2001). In corn, the fungus infects silk and kernels. Kernels are most susceptible during milk to dough stages. Infection and colonization of the fungus is higher not only in hot and dry weather but also in insect and bird damaged ears, which provides easy access for the fungus to infect host tissues. Increased infection in corn was observed due to the attack of corn borer and silk-feeding beetles (Horn and

Pitt 1997; Payne 1998). Although aflatoxin contamination starts in the field (pre-harvest), it is more problematic during post-harvest condition due to high moisture, improper storage condition, and insect attack (Cotty 1997). The outbreak of the disease in Kenya was primarily associated with the post-harvest contamination (Stenske et al. 2006).

2.1.2 Genomics and regulation of secondary metabolism in *Aspergillus flavus*

The discovery and analysis of parasexual cycle in *A. flavus* provided the way for the identification of linkage groups, gene order, and centromere location (Scheidegger and Payne 2003). Classical genetic studies identified more than 30 genes in 8 linkage groups and all the loci involved in aflatoxin biosynthesis were recessive except *afl-1*. The absence of crossing over between these loci suggested the clustering of aflatoxin synthesis genes in the genome (Scheideger and Payne 2003). The whole genome of *A. flavus* has been sequenced and released by the J. Craig Venter Institute (www.aspergillusflavus.org, NCBI). The genome size of *A. flavus* is about 37Mb with eight chromosomes and 12,000 functional genes (Chang et al. 2010; Payne et al. 2006).

Secondary metabolites are complex compounds biosynthetically derived from primary metabolites which are not essential for the normal growth, development, and reproduction of host plant. These compounds are diverse in nature having beneficial antibiotic as well as potent carcinogenic and immunosuppressant activities (Yu and Keller 2005). The secondary metabolite producing genes are generally clustered in the genome and many enzymatic genes and transcriptional factors are responsible for the production of single secondary metabolite synthesis (Hoffmeister and Keller 2007; Turner 2010). Aflatoxins are polyketide synthase-derived toxins synthesized in large gene cluster through complex metabolic process, which is located near the telomere of chromosome 3 of *A. flavus* (Amaike and Keller 2011). Around 21 genes were known

to encode biosynthetic enzymes such as fatty acid synthase, polyketide synthase, monooxygenease, reductase, dehydrogenase, methyltransferase, esterase, an oxidase, and an esterase. A binuclear zinc cluster, *aflR*, is found responsible for the regulation of the transcription of the aflatoxin biosynthetic genes. Overexpression of *aflR* increases the aflatoxin production and upregulates other genes in aflatoxin biosynthesis gene cluster. Bio-synthesis of secondary metabolites is responsive to external environmental conditions like carbon and nitrogen source, temperature, light, and pH (Yu and Keller 2005). They are regulated by Cys_2 His_2 zinc finger protein and gene expression on aflatoxin biosynthesis clusters are altered by this transcription factor, which is dependent on the medium /environment like carbon/nitrogen source.

2.2 Factors affecting *A. flavus* infection and aflatoxin accumulation

Different biotic and abiotic factors influence *A. flavus* infection and subsequent accumulation of aflatoxin in corn. The infection of fungus and accumulation of the toxin is high in maize crops grown under dry conditions (Dienner et al. 1987; Payne 1998). The aflatoxin accumulation in preharvest grain is prevalent when the crop is exposed to disease development by heat, drought, insect damage, and other stresses. Drought tolerant varieties produce lower amount of aflatoxin compared to resistant control. In addition to this, gene expression studies in response to biotic and abiotic stresses revealed that disease resistance related genes are upregulated by stress and vice versa (Chen et al. 2004). Development of varieties and hybrids with resistance to disease, insects, and environmental stresses could be the most economical and sustainable means for minimizing the *A. flavus* infection and aflatoxin accumulation (Lillehoj et al. 1978; Jones and Duncan 1981; Widstorm 1996; Payne 1992).

Kernel (physical barrier, wax, and cutin layer) and endosperm (proteins) characteristics are also important for the resistance to *A. flavus* infection and aflatoxin production. Wax, cutin

layer, and different proteins inside the grain are important for resistance (Chen et al. 2002). Kernel pericarp wax in GT-MAS:gk was found to be associated with the resistance to *A. flavus* infection and aflatoxin production. It showed a unique band during chromatography analysis as compared with susceptible lines. The whole wax showed higher amount of phenol-like compounds in resistant genotypes that suppresses the *in-vitro* growth of *A. flavus* (Guo et al. 1995; Gembeh et al. 2001). The differences in kernel proteins were found between resistant and susceptible germplasm. Two proteins of 28 kDa and 100 kDA found in resistant inbred Tex6 inhibited the fungus growth and subsequent aflatoxin accumulation. Likewise, trypsin inhibitors were highly expressed in resistant germplasm and less expressed or absent in susceptible germplasm. These types of resistant proteins could be used as markers by plant breeders for the development of resistant inbreds through genetic engineering (Chen et al. 1998, 1999).

Insect damage to ear and silk facilitates the infection of fungus. Aflatoxin accumulation increased significantly when southwestern corn borer was applied in the ear of susceptible lines (Williams et al. 2002). High thrips population and dry condition after pollination showed increased fungal infection and aflatoxin accumulation (Munkvold 2003a; Battilani et al. 2008; Parson and Munkvold 2010). The silks and wounds caused by insects are the primary infection site for the airborne conidia where they colonize and infect the developing kernels (Munkvold 2003a; Duncan and Howard 2010). Bt-hybrids showed low aflatoxin accumulation due to the resistance to borer. Early planting of the hybrids resistant to both borer and fungus can be useful to reduce aflatoxin accumulation in the region where it is endemic especially in southern states (Parson and Munkvold 2010).

2.3 Quantification of fungal biomass and toxins

The aflatoxin accumulated in the corn grain is generally used to measure the resistance of the germplasm. It is determined by immunocapture aflatoxin determination (VICAM AflaTest). This is very sensitive and widely used method for aflatoxin determination (Windham and Williams 1998).

qPCR assays have been developed to estimate fungal biomass in maize and other crops (Mideros et al. 2009; Nicolaisen et al. 2009; Horevaj et al. 2011). These assays are relatively fast, economical, and can be applied to large number of genotypes. The results showed high positive correlation between fungal biomass and aflatoxin concentration in the maize kernels. These studies suggested that fungal biomass estimated by qPCR can be used to infer the concentration of aflatoxin (Mideros et al. 2009). The real-time q-PCR assays were successfully used to quantify the fungal biomass (*Fusarium* and *Aspergillus sp*), which corresponded well to the mycotoxin data from the field in cereals (Nicolaisen et al. 2009; Demeke et al. 2010; Horevaj et al. 2011; Atoui et al. 2012). Near infrared reflectance spectroscopy (NIRS) was found to be highly accurate and faster in detecting the infection and identification of the fungus species in maize kernels (Tallada et al. 2011).

2.4 Gene expression and proteomic studies

Several gene expression and proteomic studies have been conducted recently to study the differentially expressed genes in response to *A. flavus* infection. Several candidate genes and resistance proteins were identified on the basis of differential expression. These studies identified differentially expressed proteins in resistant versus susceptible germplasm (Guo et al. 1995; Huang et al. 1997; Chen et al. 2002; Kelly et al. 2009; Pechanova et al. 2011; Kelly et al. 2012).

Validation of these identified proteins in resistant germplasm has been done on gene-by-gene basis. Resistance related candidate genes like chloroplast precursor, trypsin inhibitor, and chitinase gene family have been validated (Warburton et al. 2012; Mylroie et al. 2013). Likewise, transgenic and mutant lines were also used for the study and validation of the genes like trypsin inhibitors, PR10, and lipoxygenase (Christensen et al. 2013). The higher expressions of some enzymes in infected corn kernels like aldolase reductase (Guo et al. 1995), pericarp wax component (Gembech et al. 2001), and peroxiredoxin (PER1) were associated with the lower aflatoxin accumulation (Chen et al. 2006).

Stress related genes were also found associated with aflatoxin accumulation. Study of 94 resistant related genes in 9 different germplasm showed differential expression pattern of the genes (Jiang et al. 2011). The germplasm were clustered in two groups with resistant germplasm highly expressed stress related genes. In contrast, susceptible germplasm had lower expression but higher aflatoxin accumulation. Heat shock protein 21, protein kinase, mitogen activated protein kinase (MAP- kinase), gluatathione reductase, and leucine-rich repeat proteins (LRR-protein), which are related to stress and disease resistance, were highly expressed in resistant germplasm (Jiang et al. 2011). The resistant inbred (MP313E) exhibited significantly higher expression of resistance genes, selected from microarray study and validated by qPCR, as compared to susceptible parent. Among these genes, seven were found in the QTL regions identified by various studies. RNA regulators, molecular chaperones, and detoxification proteins were highly expressed in the resistant parent (Kelly et al. 2012: Shan and Williams 2014).

Many proteomic studies showed higher expression of pathogenesis related proteins (PR protein) in resistant varieties compared to susceptible ones. Pathogenesis related protein (PR10) expressed five times higher 7-22 days after pollination (DAP) upon infection by *A. flavus* in

resistant varieties (Chen et al. 2006). Many proteins involved in cellular metabolism, transportation, and related to response were expressed in the developing cob in the resistant cultivars (Pechanova et al. 2010). Pathogenesis related proteins were generally expressed after biotic stress. Differential expression of many proteins during different growth periods showed that resistant inbreds depend on constitutive defenses and susceptible inbreds were dependent on inducible defenses (Pechanova et al. 2011).

Microarray study between resistant (Mp313E) and susceptible (Va35) inbreds (48 hrs after inoculation) revealed that 112 genes were up regulated in resistant lines (Kelly et al. 2009). These expressed genes can be useful for the development of molecular markers to understand this complex interaction and introgression of resistance to hybrids. Higher accumulation of the toxin in susceptible varieties was due to the early initiation of the aflatoxin and increased fungal activities after infection. The activities of fungus were reduced drastically in resistant lines (Mp313E) 7 days after inoculation. The gene expression study showed that aflatoxin production was initiated earlier in susceptible genotypes (Va35) compared to resistant genotypes (Ankala et al. 2011). Genome wide expression study involving the resistant and susceptible maize inbred lines with and without *A. flavus* inoculation revealed eight highly expressed genes that were mapped to previously identified QTL regions (Kelly et al. 2012).

The role of PR10 in aflatoxin resistance was shown by RNAi gene silencing technology (Chen et al. 2010). RNAi-silenced mature kernels produced significant increase in aflatoxin concentration compared to non-silenced control. Proteomic analysis showed significant reduction in PR10 protein in silenced kernel compared to control. The negative correlation between PR10 and kernel aflatoxin indicated the major role of PR10 in aflatoxin resistance.

2.5 Fungus inoculation techniques and efficiency

Different artificial inoculation techniques have been developed and used to evaluate the maize germplasm for resistance to *A. flavus* (Windham and Williams 2012). Artificial inoculation is slow and expensive but widely used due to its reliability as compared to visual screening (percentage ear rot), which is easier but not highly correlated with toxin in the grain (Windham and Williams 1998). These can be broadly classified as non-wounding and wounding methods. Non-wounding methods are easier and similar to natural infection process and useful to identify the resistant germplasm due to tight husk cover, kernel characteristics like wax or thick pericarp. However, wounding techniques are more efficient and consistent in infection and subsequent accumulation of the toxin (Windham and Williams 2007). Field evaluation of hybrids developed from resistant and susceptible inbreds showed that wounding methods (side-needle and modified-pinbar) produced significantly higher levels of infection and aflatoxin accumulation in susceptible hybrids than non-wounding techniques (spray) in both years. Likewise, pathogenicity and virulence of *A. niger* was higher when conidia were applied inside the husk of the ears by wounding (Windham and Williams 2012).

2.6 Genetic variation for resistance to aflatoxin accumulation

The amount of aflatoxin accumulated in the grain is the most common way to measure the resistance in corn (Windham and Williams 1998). Many resistant inbred lines such as Mp313E, and Mp717, with suitable agronomic characteristics have been released (Williams and Windham 2001, 2006). Their performance as inbred lines and in crosses showed higher level of resistance against aflatoxin accumulation and can be used in hybrid development. Mp716 exhibited lower aflatoxin accumulation because of its resistance against southwestern corn borer (Williams et al. 2003). Tex6 is an unreleased line with considerable resistance to aflatoxin and

the resistance was associated with additive genetic effect in crosses with B73 and Mo17 (Hamblin and White 2000).

Recently two resistant inbreds Mp718 and Mp719 were developed and released by USDA-ARS, Mississippi State (Williams and Windham 2012). Both lines are 2 week earlier than Mp715 in mid-silking and found similar with respect to aflatoxin accumulation. Aflatoxin accumulation was significantly lower in hybrids between these resistant and other susceptible lines (Williams and Windham 2012). Resistant inbred lines (Mp313E and Mo18) exhibited excellent general combining ability for reducing the aflatoxin accumulation when crossed with other inbreds. These results suggest that these resistant inbred lines can be useful for development of resistant hybrids (Gardner et al. 2007; Williams et al. 2011).

2.7 QTL mapping for resistance to aflatoxin

The variations in phenotypes can be explained by combined action of many genes with small effects or polygenes, which are largely influenced by environmental factors. Therefore, the breeding for the quantitative traits is a difficult, time consuming and labor-intensive process (Xu 2012). Molecular markers have revolutionized the modern breeding program in plants and animals. The detection of quantitative trait loci (QTL) for complex traits and their utility through marker-assisted selection is the major function of molecular markers. There are many developments and improvement in analysis and software package since the first article on QTL mapping was published. The effect and location of the genes responsible for the quantitative traits are determined by marker based genetic analysis (Xu 2012).

The basic concept was presented by Sax more than 70 years ago (Sax 1923). The QTL mapping became more interesting and important when the mapping studies in corn and tomato identified some markers with large phenotypic variance for the complex characters (Tanksley

1993). As a consequence of this, numerous studies have been conducted for mapping and identification of QTL for quantitative traits such as yield, starch and oil content, quality, maturity, resistance to biotic and abiotic stress in corn and many other crop species (Lee 1995). These identified QTL or chromosome regions can be utilized for the improvement of the characters of interest through marker assisted breeding. Marker assisted breeding (MAB) is very efficient in pyramiding of the genes and speeding up the variety development process. Different kinds of molecular markers (RFLP, RAPD, AFLP, and SSR) are available for mapping QTL in different crop species. The SSR markers are widely used for mapping the QTL for resistance to aflatoxin accumulation (Paul et al. 2003; Brooks et al. 2005; Warburton et al. 2009, 2011). QTL mapping or Linkage disequilibrium mapping has become a standard procedure to study the quantitative traits as it helps to identify QTL number, position, and genetic effect (Zeng et al. 1999; Doerge 2002).

The QTL for complex quantitative characters like insect resistance were mapped in corn. Three QTL resistant to corn borer are found in chromosomes 1, 3, and 8 (bins 1.02, 3.05, and 8.05, respectively) that explained more than 75% of the genotypic variance in an RIL mapping population (Ordas et al. 2010). Meta- analysis of QTL for the resistance to three major ear rot in corn showed 29 meta QTL in chromosome 1 to 8. Among them, six MQTLs located in chromosome 3 and 4 can be utilized for improving resistance through MAS. It revealed that resistance QTLs were clustered in same chromosome (Xiang et al. 2010). Mapping population developed from Mp313E and Va35 exhibited a major QTL resistant to aflatoxin on the long arm of chromosome 4 of Mp313E (Davis et al. 1999). Both parental sources contributed to lowering aflatoxin accumulation. Some QTL associated with the lower accumulation of the aflatoxin in corn were identified and mapped in a BC_1S_1 population [(Tex 6 x B73) x B73] and Tex6 x B73

$F_{2:3}$ populations. Composite interval mapping with multiple regressions showed many markers associated with lower aflatoxin production and identified QTL on chromosome 5 in backcross population and on chromosomes 3, 4, and 10 in $F_{2:3}$ populations (Paul et al. 2003). These independent studies indicated consistency in resistant QTL locations on the same location of the chromosome 4.

Mapping new sources resistance to the aflatoxin accumulation in corn was carried out in different mapping population in recent years. Till now no QTL with major effect (> 20%) has been identified. Same QTL were not identified in different years and locations within the same mapping population. Identification of QTL with large and consistent phenotypic effect has been a consistent challenge for corn breeders (Paul et al. 2003; Brooks et al. 2005; Robertson-Hoyt et al. 2007; Warburton et al. 2009, 2011). In $F_{2:3}$ mapping population of Mp715x T173, QTL with large phenotypic effects were identified in different years on chromosomes 1, 3, 5, and 10 and QTL with minor effects were detected on chromosomes 4 and 9. The individual QTL with largest effect explained about 18.5% phenotypic variance and multiple QTL model explains up to 45.7% of the phenotypic variation. Some previously identified QTL (Paul et al. 2003; Brooks et al. 2005) were found on the same chromosome and location in this experiment (Warburton et al. 2011). Paul et al. (2003) found that marker bnlg1666 was associated with lower accumulation of aflatoxin. The mapping study involving Mp313E identified a QTL(*alf3*) associated with the marker bnlg371 located on chromosome 2, which contributed up to 18% of the phenotypic variation (Brooks et al. 2005) and the marker bnlg2291 linked with the locus *alf5* explained 8 to 18 % phenotypic variance.

Mapping population derived from Mp717 x NC300 identified many QTL on all chromosomes except 4, 6, and 9. Both parents contributed to resistance to aflatoxin accumulation

and no major QTL were detected. Mp717 could be a novel source of aflatoxin resistance because many loci were distinct from the previously mapped QTL (Warburton et al. 2009). Some QTLs are consistently identified using recombinant inbred lines (RILs) and near-isogenic lines (NILs). QTL in chromosome 4 (4.08 and 4.09), 8 (8.02 and 8.03) and 10 (10.06 and 10.07) were identified in RIL population developed from B73 x CML322. The resistance QTL in chromosome 4 (4.08) was confirmed using NILs. The QTL in chromosome 4 were also identified in different mapping experiments and largest QTL identified in 4.08 can be a good candidate for further study (Mideros et al. 2014). In addition to these chromosomal regions, a major QTL was identified on chromosome 5 and expressed stably in different environments using an RIL population. This QTL was identified in the narrow region of chromosome (59.7-63.6 cM) in different locations and could be suitable for the further mapping and breeding purposes (Yin et al. 2014). The inconsistency and small phenotypic variance explained by the QTL for aflatoxin accumulation hinder their utilization in crop breeding program. It requires identification and utilization of multiple QTL identified for specific environment. But the marker assisted selection in combination with phenotypic selection should be more efficient and effective for improving the quantitative traits like aflatoxin accumulation and resistance (Warburton et al. 2011).

The identification of QTL with additive effects in different environments shows the possibility of the development of markers and pyramiding of these QTL for the introgression and development of stable resistant source (Willcox et al. 2013). Till now, mapping studies are conducted on different resistance sources with the assumption that these QTL behave similarly in different genetic backgrounds. So the study of resistance using same resistance source using different susceptible varieties are necessary to study the behavior of QTL in different genetic

backgrounds and stability. Recent study involving Mp313E with susceptible parent (Va35) and comparison of QTL with different susceptible parents identified QTL in the same chromosomal regions. Willcox et al. (2013) recently identified five QTL in same genetic locations using Mp313E as resistant source in different genetic backgrounds. This shows the stability of QTL from resistance sources in different backgrounds and the possibility of utilization of these QTL in future breeding programs through marker-assisted breeding. The identification of stable QTL in different environment and diverse genetic backgrounds are extremely useful for the improvement of the quantitative traits like aflatoxin accumulation.

2.8 QTL mapping for other morphological traits

Various studies has been conducted in recent decade to identify QTL responsible for different morphological as well as yield attributes in corn using diverse mapping populations. Large number of QTL has been identified for the grain yield (Grohn et al. 1998; Khairallah et al. 1999; Cardinal et al .2001; Sibov et al. 2003; Lima et al. 2006; Li et al. 2007). Other morphological and yield related traits such as plant height, ear height, days to silking, ear length, ear diameter, number of rows/ear, number of kernels/row, kernel weight were also studied in above mentioned as well as in other recent studies (Liu et al. 2010; Park et al. 2014). The genetic correlation among these traits was also observed in these studies indicating clustering of QTL for various traits on some genomic regions.

2.9 Climate change impacts on aflatoxin infection and contamination

The climatic condition during maturity and harvest highly determines the aflatoxin contamination in corn. Hot and dry spells during grains filling stage are favorable for *A. flavus* infection. The change in weather patterns may result to acute aflatoxicosis in hot and arid regions (Cotty and Jamie- Garcia 2007). Contamination with aflatoxin in corn occurs in both preharvest

(development phase) and post-harvest phase and the effect of weather on infection and toxin accumulation is different in these two phase (Cotty 2001). The growing crop is usually resistant to *A. flavus* infections unless the climatic condition favors fungus growth. The climatic condition affects the host conditions and crops can be highly contaminated (Cotty and Lee 1990; Doster and Michailides 1999). Crop damage due to hails and wounds by mammals, insects, and birds increases the chances of infection and aflatoxin contamination and specific crop/insect combination were found to have relationship to aflatoxin contamination. Corn borer in maize and pink bollworm in cotton are some example of this relationship and their survival between cropping season, dispersal, and crop infestation is largely dependent on climatic condition (Russel et al. 1976; Sommer et al. 1986; Williams et al. 2002). The changes in weather pattern influences the crop management, crop rotations, and harvesting. Heavy rainfall during harvesting and delaying in drying of grains provide favorable environment for fungus infection and toxin production. They are predominantly found in warm region and changes in climatic condition results in higher fluctuation in crop infection and toxin contamination (Shearer et al. 1992; Bock et al. 2004). The toxin producing capacity differs with geographical regions but *A. flavus* is very complex as different isolates from the same field can produce different amount of aflatoxin. As it is the most important causal agent in corn, it is very difficult to predict the effect of climate change on its ability of toxin production (Joffe 1969; Cotty 1997). The endemic occurrence of the fungus along with erratic weather pattern has further increases the possibility of higher infection and toxin contamination in crop and may restrict the corn growing area (Cotty and Jamie-Garcia 2007). The recent occurrence of lethal aflatoxicosis in Kenya is the example of the influence of climate change on aflatoxin infection and contamination (Lewis et al. 2005). As it is not possible to predict the climatic condition and its subsequent impact on aflatoxin production,

the use of adaptive, early maturing and resistant varieties, cultural practices, proper harvesting, storage, and changes in cropping patterns may be the suitable and sustainable way to cope with this perennial problems.

2.10. Morphological traits indirectly associated with aflatoxin resistance

Many morphological traits related to ear were known to have some relationship with *A. flavus* infection and aflatoxin accumulation in grain. Air, rain, and insects are the main vectors for the dispersal of fungus spores to target host tissues. In corn, fungal spores are carried to silk and ears through air and insect vectors. So, insect resistance is considered as an alternative for reducing the infection and aflatoxin accumulation (Warburton and Williams 2014). Higher accumulation of aflatoxin occurs in insect damaged ears as it provides the entry point for the establishment and colonization of fungus (Widstrom 1979; McMillian et al. 1980; Setamou et al. 1998; Windham and Williams 1999; Widstrom et al. 2003; Williams et al. 2005). These results showed the possibility of reducing the aflatoxin accumulation through development of insect resistant lines by conventional breeding methods (Warburton and Williams 2014). However, transgenic efforts to reduce aflatoxin accumulation through incorporation of Bt genes for insect resistance has not been very successful (Odvody and Chilcut 2002; Williams et al. 2002). Traits like husk tightness, kernel hardness, and non-upright ears are also linked to the reduction of aflatoxin resistance (Odvody et al. 1997; Betrán and Isakeit 2004). The tight husk prevents the easy entrance of fungus to the developing kernels through silk. Non-erect ears with tight husk not only restrict the fungus entry but also restrain the entry of rain in the maturing ears. It helps to keep the ears dry during growth and maturity period making it less conducible for fungus infection and colonization (Lillehoj et al. 1983; Barry et al. 1986). Germplasm with harder kernels and drought resistance also exhibited significantly lower amount of aflatoxin (Guo et al.

2008). Early maturity is also useful for reducing aflatoxin accumulation but it depends on the location and weather of the growing locations (Moreno and Kang 1999; Mideros et al. 2012). Resistance to insect and drought are highly effective for reducing the *A. flavus* infection than other traits. Manipulation of these morphological traits should be considered as auxillary approach for the development of sustainable resistance to aflatoxin contamination and accumulation (Warburton and Williams 2014). Flowering time was strongly correlated to aflatoxin accumulations; early flowering hybrids accumulated more aflatoxins because most of them are short and open husk types to ensure maturity in short time frame (Betrán et al. 2004).

2.11 Management of aflatoxin accumulation

2.11.1 Pre-harvest Management

The cultural practices for the reduction of *A. flavus* infection and aflatoxin accumulation are related to the disease epidemiology and disease cycle. The efficient way to achieve this is to alter the production environment, which favors the crop growth and suppress the fungal infections. The most commonly used strategies are tillage, fertilizer application, plant population management, crop rotation, planting date, and irrigation. As sclerotia of the fungi survive on crop residues and soils, crop rotation and residue management has been investigated as strategies for the disease management. The tillage and crop residue management were shown to have positive effect on reducing the infection but the results are not consistent and convincing.

Aflatoxin contamination in corn depends on host susceptibility, environmental conditions, activity of vectors like corn borer, and aphid. Early planting in temperate areas can significantly reduce the risk of *A. flavus* infection. Early planting with adequate irrigation lowers infection and aflatoxin accumulation as drought stress is known to increase the incidence of disease (Munkvold 2003b). Nutrient and weed stresses are also found responsible for the

increased level of infection and application of higher amount of nitrogenous fertilizer significantly reduced the aflatoxin accumulation.

2.11.2 Postharvest management

Although *A. flavus* infection starts in the field before harvesting, appropriate time of harvesting and postharvest activities are also equally important for the management of aflatoxin accumulation. Earlier harvesting reduced aflatoxin accumulation (Jones and Duncan 1981). Keeping the crops in the field for longer time increases the probability of fungus infection as it dry slowly and insect damage may occur in the field. Higher moisture along with insect damage favors the growth and development of fungus. Close monitoring of environmental/weather condition as well as field condition (insect damage and infection rate) are extremely important. If there is less infection and insect damage, then the crop can be allowed to dry in the field to reduce moisture level in desirable range. The physical damage during harvesting and transportation also result in infection and subsequent aflatoxin accumulation (Munkvold 2003b).

The reduction of moisture content in grain after harvesting can significantly reduce the infection by fungus. The process is highly successful and effective at 4^{o}-15^{o}C and low relative humidity. Highly infected crop with higher moisture content should be dried as quickly as possible to reduce the risk of infection and growth of fungus during storage (Wilcke and Morey 1995).

Fungus development can take place during storage of the grains due to moisture variability inside the grains and outer environment. The storage and postharvest handling procedures are important for the management of fungus infection. Open structures are generally used for the storage of the grains in developing countries where moisture and temperature of the grains cannot be controlled. The fluctuation in grain moisture and insect attack during storage in

the developing countries due to improper storage conditions are the major cause for toxin accumulation (Hell et al. 2000). Cleaning of storage facility and structures are also equally important before grain storage. Storage temperature is the most important factor for the management of fungus infection. Storage of grains between 1-4^{o}C is highly effective as the fungal activity is extremely low at this temperature. Close monitoring is necessary to reduce the infection during storage as moisture conditions inside the stored grains are unpredictable (Munkvold 2003a, b).

2.11.3 Biological control of *A. flavus* infection

The utilization of nontoxigenic strain of *A. flavus* has been emerging as a means of sustainable management strategy to reduce aflatoxin accumulation. The competition, exclusion, or inhibition between the toxic and atoxigenic strains for nutrients is the main idea behind this approach (Amaike and Keller 2005). The successful application of these biocontrol agents has been demonstrated in corn, peanuts (Abbas et al. 2006; Dorner 2010), and cotton (Cotty and Lee 1990). Since then, many experiments were conducted in large scale to demonstrate the commercial utilization of atoxigenic strain for the disease management (Cotty and Bayman 1993; Cotty and Bhatnagar 1994). Currently, two commercial biological control agents using atoxigenic strains are registered by US Environmental protection Agency. Afla-guard is recommended for the use in corn and peanuts and produced by Syngenta, whereas AF36 (Arizona Cotton Research and Protection Council, Phonenix, AZ) is recommended for cotton (Dorner 2010). Both strains lack some genes in aflatoxin biosynthesis gene clusters so that they are not capable of producing aflatoxin after host infection.

CHAPTER 3
MATERIALS AND METHODS

3.1. Molecular mapping of QTLs for resistance to aflatoxin accumulation and agronomic traits

3.1.1 Selection of parents

Two genetically diverse parents, B73 and Mp715, were selected for the development of a mapping population. B73 is the most popular inbred used as parent for the production of commercial hybrids (Darrah and Zuber 1986). It has many desirable agronomic characteristics such as early maturity, short height, and wide adaptability. It has yellow kernels and red cobs. It is highly susceptible to *A. flavus* (Campbell et al. 1993; Campbell and White 1995; Naidoo et al. 2002) and its genome sequence is publicly available (www.maizegdb.org). Mp715 is an aflatoxin resistant germplasm released by USDA-ARS and the Mississippi Agricultural and Forestry Experiment Station in 1999. It was developed from Tuxpan by continued selfing for eight generations and selecting for lower aflatoxin accumulation in ears with artificial inoculation techniques. It is tall and late in maturity compared to B73. It has yellow kernels and white cobs. The lodging problem caused by weak root system of this inbred, has limited its use in breeding program (Williams and Windham 2001).

3.1.2 Development of mapping population

An $F_{2:3}$ population was used for the mapping of quantitative trait loci (QTL). Hybrids from the cross (B73 x MP715) were self -pollinated to produce F_2 population. Each F_2 plant was self-pollinated to produce enough F_3 seeds for field evaluation. A total of 235 $F_{2:3}$ families were used in the replicated field trials to evaluate *A. flavus* infection and aflatoxin accumulation.

The field experiment was conducted in Randomized Complete Block Design (RCBD) with three replications in the summer of 2013 and 2014 at the Louisiana State University

AgCenter Central Research Station in Baton Rouge, LA (30°20'51" N, 91°10'14" W). The plantings were done in the second week of April in both years. The parents and 235 $F_{2:3}$ lines were planted in single row of 5.0 m long in each replication. The plant to plant distance was maintained at 25 cm after final thinning. Twenty plants/family were finally maintained. Individual lines were randomly distributed within the replication. Weeding was done regularly to keep the field clean and to reduce the weed-crop competition for nutrition and other inputs. Standard crop management practices were followed to maximize the growth and yield of the crop. Fertilization and frequent irrigation were provided depending on the requirement of the crop and weather conditions.

3.1.3 Preparation of the fungus culture

Aspergillus flavus strain (NRRL 3357) was used for the field inoculation and evaluation because of its ability to produce high level of aflatoxin in corn kernel. This strain is widely used in field evaluation studies. Fungal inoculum, obtained from the ARS Culture Collection, Bacterial Foodborne Pathogens and Mycology Research Unit, USDA-ARS, Peoria, IL, was grown overnight in potato dextrose broth and then grown on the sterile corn-cob grits (size 2040, Grit-O-Cobs, The Andersons Co., Maumee, OH) in 500 ml flask. Each flask contained 100 ml sterilized distilled water, 50 gm grits, and incubated at 28° C for 3 weeks. Conidia from each flask was washed from the grits with 500 ml sterilized water containing 20 drops of Tween 20/liter and filtered through four layers of cheese cloth. The concentration of conidia was measured with hemacytometer and stored at 4° C.

3.1.4 Field inoculation, sample preparation, and aflatoxin determination

Primary ear in each plant was inoculated with side needle technique 2-weeks after mid-silk (silk emergence in 50% plants in each line). The fungal inoculum was diluted to the concentration of $3x10^8$ conidia /ml and 3.4 mL of fungal suspension was injected into each ear through the husk. The primary ears from the standing plants were harvested at maturity [approximately 60 days after sliking (DS)]. Each inoculated ear was hand shelled and dried for 1 week in oven (38^oC) to reduce the moisture content of the grains. The grains from each $F_{2:3}$ line from each replication were bulked and mixed properly. A representative sample of 50 g from each line along with parents and hybrids was taken and kept in a small plastic bag separately. Samples were ground with Romer mill (Romer Industries Inc., Union, MO). The VICAM AflaTest (VICAM, Watertown, MA) was used to determine the aflatoxin concentration in 50g ground sample from each plot. The aflatoxin concentration was measured as parts per billion (ppb). This test is highly efficient and effective to measure the smallest amount of toxin up to 1 ppb.

3.1.5 Selection of simple sequence repeat (SSR) primers

Simple sequence repeat (SSR) primers were used for the construction of linkage map and subsequently used for the mapping of quantitative trait loci for the resistance to aflatoxin accumulation. The SSR primers and their locations on maize genome are available on public data base. The maize genome is divided into 100 bins (10 chromosomes) and markers were selected from each bin from the maize genome database (www.maizegdb.org). A total of 562 SSR markers were selected with uniform distribution over the whole corn genome. The sequence and bin position of the SSR markers are provided in the Appendix 1.Other linkage maps were also surveyed for the selection of potential markers for our research.

3.1.6 Phenotyping

The important morphological and yield traits were recorded at different growth and developmental stages of the crop. Tasseling days, silking days, plant height, ear height, maturity date, husk cover, ear length, ear circumference, number of rows/ear, number of grains/rows, 100-kernel weight, and grain yield/plot were recorded. The measurements of these traits were done on five randomly selected plants from each family in each replication. All the primary ears were harvested after complete maturity and were kept separately for the measurement of other traits and aflatoxin quantification. The ear and yield related traits were measured from the harvested ears in the laboratory. The following morphological traits were measured.

- Plant height (PH): Plant height (cm) was measured from the ground level up to the point where the branching of tassel starts.
- Days to 50% tasseling (DT): Number of days from sowing to emergence of tassels in 50% of the plants.
- Days to 50% Silking (DS): Number of days from sowing to emergence of silks in 50% of the plants.
- Days to 50% maturity (DM): Number of days from sowing to maturity in 50% plants.
- Ear height (EH): Ear height (cm) was measured form ground level up to the primary ear.
- Husk cover (HC): It was visually scored on a scale of 1-5 following CIMMYT protocol. Score 1 was for tightly closed ears and score of 5 was given for the ears with open husks.
- Ear length (EL): Length (cm) of the harvested primary ear.

- Ear circumference (EC): Circumference (cm) was measured in the middle part of the each ear.
- Number of row/ear (RN): Number of rows in each ear of the randomly selected plants.
- Number of kernels/row (KN): Number of kernels per each row for the selected ears.
- 100-kernel weight (KW): weight (gm) of 100 kernels.
- Grain yield/ plot (GY): Grain yield (gm) was measured for each family after shelling all harvested ears in each replication.

3.1.7 Polymorphic survey and genotyping

Leaf tissues were collected from each plant from each $F_{2:3}$ family and stored at-80^{o} C. The tissues from each $F_{2:3}$ family was bulked (10 plants/family) and DNA was extracted using the CTAB method (Saghai-Maroof et al. 1984). The quality of DNA was checked by running an agarose gel and quantification was done using NanoDrop spectrophotomere (model ND-1000 Thermo Fischer scientific, Wilmington, USA). The DNA was diluted to the desired concentration (50 ng/µl) for genotyping. The stock DNA was stored at -80^{o}C to protect the DNA from degradation. All the primers were surveyed to identify polymorphism between parents. The polymorphic markers were used for genotyping the $F_{2:3}$ population. The SSR markers were amplified by polymerase chain reaction (PCR) using the following thermal cycling parameters: 95^{o}C for 5 min; 95^{o}C for 45 sec, 55^{o}C for 45sec, 72^{o}C for 1min, 35cycles; 72^{o}C for 5 min. Each PCR reaction was conducted in 25µl volume with the following composition: water: 11.7 µl, 10X buffer: 2.5 µl, 25mM magnesium chloride: 2.5 µl, forward primer (50 ng/µl): 1.25µl, reverse primer (50 ng/µl): 1.25 µl, dNTPs (2mM): 2.5 µl, Taq polymerase: 0.3 µl, and DNA (50 ng/µl): 3 µl. The PCR products from both parents and lines for each marker were separated was separated on a 4.5 % W/V of super fine resolution (SFR) agarose (Amresco) gels and visualized

under ultraviolet light after ethidium bromide staining. The gels were run using 1X TAE buffer at 150V for 2-3 hours depending on the markers. Scoring was done according the SSR protocol of Maize Mapping Project (www. maizegdb.org).

3.1.8 Statistical analysis

Phenotypic data for each trait were averaged for each family, parents, and hybrid in each replication for statistical analysis. Histograms and frequency distribution for the morphological traits were analyzed using Microsoft Excel. All the statistical analyses were performed using SAS 9.3 (SAS Institute Inc., Cary, NC, 2010). Analysis of variance (ANOVA) was performed to determine the significant difference among the families for these traits using mixed model (PROC MIXED). Descriptive statistics (mean, standard deviation, range, coefficient of variation, maximum and minimum value) were obtained using PROC MEANS. Aflatoxin data was natural log-transformed to reduce non-normality before analysis. The correlation between aflatoxin accumulation and different morphological traits was calculated using PROC CORR. Broad-sense heritability for each year was calculated using variance component from ANOVA (expected mean square) following Hallauer and Miranda (1981). The genotypes (families), replications, and years were considered as random. The heritability on the basis of family for each trait over years was estimated using the formula as below;

Broad sense heritability (h^2) = $\frac{\sigma^2_g}{\sigma^2_g + \frac{\sigma^2_{ge}}{n} + \frac{\sigma^2_e}{(n \times r)}}$

Where,

σ^2_g =genetic variance,

σ^2_{ge}=genotype x environment variance

σ^2 = random error variance

n=number of years

r=replications

3.1.9 Linkage map and QTL analysis

The lines with more missing data were removed from the analysis. Genotypic data from 191 $F_{2:3}$ lines and 136 SSR markers were finally used for constructing linkage map using MAPMAKER/EXP 3.0 (Lander et al. 1987). Map distances were calculated using Kosambi function (Kosambi 1941). The arrangement of markers on each linkage group (chromosome) was determined on the basis of LOD values.

QTL analysis was done for individual year data as well as for the data pooled over both years. The means of each morphological trait of $F_{2:3}$ families were used for QTL analysis using Win-QTL Cartographer v 2.5 (Wang et al. 2012a). Composite interval mapping (CIM) was performed using genome wide error (α) =0.05 and LOD threshold of 2.5. The highest LOD score was used to indicate QTLs in the linkage map of each chromosome. Backward regression method with standard model (model 6) and window size of 10 cM were used for QTL analysis. The marker interval, map position, additive and dominance component, LOD value and the phenotypic variance (R^2) explained by each QTL were obtained from the CIM analysis. The QTLs were named using an abbreviated trait name and chromosome number. For example, QTL for aflatoxin accumulation located on chromosome 1 was indicated by *qAFL1*.

3.2 Identification of differentially expressed genes in response to the infection of *A. flavus* through suppression subtractive hybridization (SSH)

3.2.1 Inoculation and sampling

Two varieties B73 (susceptible) and MP715 (Resistant) were used to identify the differentially expressed genes in response to *A. flavus* infection by suppression subtraction

hybridization (SSH). Previously described procedure was followed for the fungus inoculum preparation and inoculation. Inoculated and non-inoculated ears from both inbreds were collected at 2 different time points (2 and 3 weeks after inoculation). The harvested developing ears were immediately frozen in liquid nitrogen. The kernels around the point of inoculation (that were not wounded and free from fungus) are carefully separated and stored in -80^{o} C for further use in the experiment.

3.2.2 RNA isolation and SSH-cDNA library construction

Total RNA from the inoculated ears of both parents were extracted from both inbred at two different times (2 weeks and 3 weeks after inoculation). Due to high starch in the developing maize kernels, it was extremely difficult to isolate RNA as the homogenate formed a sticky mixture and solidified. It could not be separated during centrifugation. To solve this problem, a modified SDS/Trizol RNA extraction was followed (Wang et al. 2012b). The solidification of the homogenate was prevented using the alkaline Tris buffer (pH 9.0) and β-mercaptoethanol. This step reduced the solidification due to the absence of detergent in the extraction buffer. Only after this step, highly concentrated (20%) SDS solution was added to the suspended mixture to facilitate cell lysis and dissolution of starch. RNA quality and integrity of total RNA was checked in 1.0 % SFR Agarose gel using 1X TAE buffer. RNA quantification was done by using Nano Drop spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The RNA was treated with *DNase*I and stored at -80^{o}C for future use.

Messenger RNA was isolated using PolyTract mRNA isolation kit (Promega, Madison, USA) following the guidelines of the manufacturer. Two micrograms of poly (A+) mRNA was used for the synthesis of double stranded DNA. Suppression subtraction hybridization (SSH) was carried out using the cDNA subtraction kit (Clontech, Wisconsin, USA) following the instruction

of the manufacturer. Forward SSH library was prepared using inoculated cDNA (resistant line Mp715) as tester and inoculated cDNA (susceptible line B73) as driver. The subtracted cDNA fragments were cloned into pGEM-T Easy Vector (Promega, Madison, USA) and transformed to DH5α cells of *E. coli*. These transformed cells were grown on LB agar medium containing 100mg/L ampicilin, 1mM IPTG and 80 mg/L X-gal in 37^{o} C overnight. The positive colonies were selected and grown in LB medium containing ampicilin (50mg/L) in 96-well plate for 5 hr at 37^{o} C and stored in -80^{o} C with 30% glycerol

3.2.3 Sequencing and classification of the differentially expressed clones

Four hundred eighty clones were randomly selected and sequenced. Sequencing of these selected cDNAs was done using the vector specific primer (T7 and SP6) at the University of Washington High Throughput Genomics Unit. The blastx program in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify the sequences in non-redundant database that are homologous to the sequences obtained from the SSH (Altschul et al. 1997). The E-value was examined to determine whether these matches are significant (E-value less than 10^{-3}). These genes were classified in different functional categories on the basis of their predicted functions. NCBI, gene ontology (http://geneontology.org/), UniProt (http://www.uniprot.org), and related literature were used as guidelines for the classification of these genes on different categories.

3.2.4 Differential screening of subtracted cDNA library

The selected clones were grown overnight at 37^{o} C with gentle stirring in LB medium for the isolation of cDNA inserts. The plasmid DNA was isolated following plasmid DNA isolation protocol adopted from Qiagen (Valencia, CA, USA. These cDNA clones were amplified using

vector specific primers (T7 and SP6) flanking both sides of the inserts. Each PCR reaction was conducted in volume of 25 µl with the following composition: nuclease free water: 11.8 µl, 10X buffer: 2.5 µl, magnesium chloride (25mM): 2.5 µl, dNTPs (2.0 mM): 2.5 µl, T7 and SP6 primer (50 ng/ µl): 1.25 µl each, Promega Taq (5U/ µl): 0.2 µl, template DNA (50 ng/µl): 3.0 µl. PCR was performed according to the following thermal cycling parameters: 95^{o}C for 4 min; 95^{o}C for 45 sec, 55^{o}C for 45 sec, 72^{o}C for 1 min, 35 cycles; 72 ^{o}C for 5 min final extension. The PCR products were electrophoresed on 1.2% agarose gel to confirm the quality and size of inserts.

The PCR products containing cDNA inserts were concentrated using Speed Vac at 4^{o}C for half hour. The concentrated products were denatured with 0.6M NaOH solution and used for blotting. Five microliters of concentrated PCR product of each clone were blotted in duplicate to Hybond-N^{+} (Amersham Biosciences Corp., Piscataway, NJ). Each blot consisted of 48 samples (46 clones, actin, and negative control). After blotting, the membranes were allowed to air dry at room temperature. The air dried membranes were neutralized with Tris-HCl (1.5M NaCl, 0.5M Tris-HCl, pH 8.0) for 5 min. The membranes were rinsed with SSC Tris-HCl solution (2x SSC, 0.2 M Tris-HCl, pH8.0) for 30 sec with gentle shaking. Membranes are removed from the solution and allowed to drain the remaining solution. The membranes were carefully wrapped using plastic wrap and cross-linked with ultraviolet light. The blots were exposed to UV light for 1 min (30 sec x 2). cDNA probes were prepared from the inoculated and uninoculated RNA using iScript cDNA synthesis kit (Bio-Rad, CA, USA). The procedure for probe labelling, prehybridization, hybridization, membrane washing, and detection were performed following the protocol of the manufacturer (Amersham ECL Direct Nucleic Acid Labelling And Detection Systems, Cat # RPN 3000, GE Healthcare, Bukinghamshire, UK). After overnight exposure, the

X-ray films were developed and air dried in dark room. The differentially expressed genes were selected on the basis of signal strength of the clones with different probes on the film.

3.2.5 Validation of the differentially expressed genes in a set of germplasm with varying level of aflatoxin resistance

The maize germplasm with different degree of resistance and susceptibility were selected for the field experiment. Seven germplasm were used for this experiment. Out of seven, five (Mp715, Mp719, Mp420, Mp313E and Mo18W) are resistant and two (B73 and Va35) are susceptible to *Aspergillus flavus* (Williams 2006; Gardner et al. 2007).

3.2.5.1 Field evaluation

The selected germplasm were planted in 2013 and 2014 at the Louisiana State University AgCenter Central Research Farm, Baton Rouge, in second week of April. The trial was conducted in randomized complete block (RCB) design with three replications. Plot size was maintained as 5 meter row with 20 plants for each germplasm. Plant-to-plant spacing was maintained at 25 cm after final thinning of plants. Irrigation was provided when necessary. All other agronomic practices like weeding, thinning, and fertilizer application were performed according to the recommended practices to obtain the maximum yield. The fungal inoculation was done after two weeks of mid-silking of the germplasm using side needle technique. The primary ears were inoculated with 3.4 ml fungus inoculum containing $3x10^8$ conidia/ml.

Important morphological traits for these germplasm were measured and evaluated. Tasseling date, silking date, plant height, ear height, maturity date, husk cover, ear length, ear circumference, number of rows, numbers of grains/rows, 100-kernel weight, and grain yield/plot were measured in field and laboratory. After maturity, all ears were harvested and kept separately to avoid mixing. All ear and yield related traits were measured in laboratory. Five

randomly selected plants from each line in each replication were used for collection of data. After measuring all the traits, the ears were hand shelled and bulked for each replication separately for yield and 100-kernel weight measurement. Fifty gram sample was taken from the bulked grain for aflatoxin quantification.

The sample for each germplasm were ground and kept in plastic bags. The VICAM AflaTest (VICAM, Watertown, MA) was used to determine the aflatoxin concentration from each sample. The procedure for the determination of aflatoxin concentration was followed as per the guidelines provided by the VICAM (VICAM, Watertown, MA) and the aflatoxin content was measured as part per billion (ppb).

3.2.5.2 Evaluation of the selected genes by semi-quantitative reverse transcriptase PCR (RT-PCR)

The tissues were collected from each germplasm from control and inoculated plants with fungus. To study the gene expression at different time points after inoculation, the developing ears from each germplasm were harvested 24 and 48 hours after inoculation along with control and kept in liquid nitrogen immediately. The developing kernels were then separated and kept in -80^{o}C for RNA isolation and gene expression studies. The total RNA was isolated from twenty one samples from seven germplasm following the same procedure as described earlier. Two micrograms of total RNA from sample was used for first-strand cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad, CA, USA). The reaction was performed in 20µl reaction using 4µl 5X iScript reaction mix, 1µl reverse transcriptase, 7µl nuclease-free water, and 8µl RNA template. The complete reaction mix was incubated in thermal cycler using the following conditions: 5 min at 25^{o}C, 30 min at 42^{o}C, and 5 min for 85^{o}C. The differentially expressed genes were selected from inoculated Mp715-B73 SSH library. Forty gene specific RT-PCR primers were designed according to their transcript sequences using Primer 3.0 software. Each

RT-PCR reaction was conducted in 25 µl volume [water: 9.6-11.3 µl; Promega Flexi green buffer: 5.0 µl, magnesium chloride (25 mM): 2.5-4.0 µl, Forward primer (50 ng/µl): 1.25 µl, Reverse primer (50 ng/µl): 1.25 µl, dNTPs (2 mM): 2.5 µl, Promega Taq (5U/µl): 0.2 µl and template cDNA: 1.0-1.2 µl]. The selected primers were amplified using the following thermal cycling parameters: 95^{o}C for 5 min; 95^{o}C for 45 sec; 55^{o}C for 45 sec; 72^{o}C for 1 min, 35-42 cycles, and final extension at 72^{o}C for 5 min. *Actin1* gene was used as internal control for the normalization of gene expression. Equal amount of PCR reaction (20 µl) was loaded for gel electrophoresis. The PCR products were run in 2.0% agarose gel with ethidium bromide in 1X TAE buffer at 120 V for 1 hr. The gels were visualized using Kodak Gel Logic 200 Imaging system (Kodak Co., Rochester, NY, USA).

3.2.5.3 Validation of the selected genes in germplasm by quantitative real-time PCR (qPCR)

Total RNA isolated from different germplasm at different time point and control was used for real-time RT-PCR analysis. Based on the metabolic functions and the result of RT-PCR, six important genes were selected for qPCR analysis. The cDNA was synthesized using 2µg total RNA using iScript cDNA synthesis Kit (BioRAD, CA, USA) as described before. One microliter of cDNA was used for quantitative analysis of the gene expression. The reaction condition consisted following steps: 95^{o} C for 3 min; 40 cycles of 95^{o} C for 30 sec, 60^{o} C for 30 followed by denaturation stage. The experiment was carried out in triplicate for each genes used for the quantitative expression study. Data acquisition for the amplification plot was performed at the extension step and final denaturation step continuously. Reactions were conducted in MyiQ BioRad Single color Real-time PCR Detection system. The quantitative expression data were collected and analyzed using associated software with the MyiQ BioRad Single color Real-time

PCR Detection system. Comparative CT method ($2^{-\Delta\Delta CT}$) was used for gene expression analysis (Livak and Schmittgen, 2001).

All the phenotypic and aflatoxin concentration data were averaged for each germplasm for each replication. Aflatoxin data was natural log-transformed before the analysis to reduce non-normality. The descriptive statistics, ANOVA, and correlations were computed using SAS 9.3 (SAS Institute Inc., Cary, NC, 2010).

3.3 *In silico* mapping and co-localization of differentially expressed genes in linkage map

Differentially expressed gene sequences from SSH library were used for in *silico* mapping and co-localization of QTLs for aflatoxin accumulation on the linkage map. The gene sequences were submitted to Maize GDB Blast (http://maizegdb.org/popcorn/search/sequence_search/home.php?a=BLAST_UI) to search for the highest similarity against B73 Ref Gen v2. The bin position and physical location of these genes were obtained using the blast search on the database. The physical location of linked markers with QTLs was searched by using locus lookup tool against the reference genome in Maize GDB (Andorf et al. 2010). With the information of the physical location of genes and linked markers, the expressed genes were placed on the linkage map developed from the $F_{2:3}$ mapping population of the cross B73 x Mp715. Co-localization was inferred using the information about the location of expressed genes and QTLs in the linkage map.

CHAPTER 4
RESULTS

4.1. Phenotypic characterization of the $F_{2:3}$ mapping population

Mean, range, coefficient of variation, and heritability values for twelve agronomic traits (PH, DT, DS, DM, EH, HC, EL, EC, RN, KN, KW, and GY) were estimated from the pooled data of both years (Table 4.1). Phenotypic values for each trait for individual year and analysis of variance (ANOVA) for these traits are provided in Appendices 2 and 3, respectively.

Table 4.1 Descriptive statistics and heritability for the morphological traits estimated from the pooled data (2012 and 2013) of the parents and $F_{2:3}$ mapping population derived from the B73 x Mp715 cross.

Trait	B73	Mp715	$F_{2:3}$			
	Mean	Mean	Mean	Range	CV[#]	Heritability
Plant height (cm)	160.3	188.8	190.9	88-222	6.3	0.69
Days to 50% tasseling	62	73	71	55-131	3.4	0.68
Days to 50 % silking	64	75	73	42-85	4.0	0.58
Days to 50 % maturity	105	116	110	86-130	4.0	0.62
Ear height (cm)	61.8	84.1	85.5	53-117	8.0	0.48
Husk cover rating	3.0	1.3	1.9	1-5	46.2	0.34
Ear length (cm)	12.6	17.7	14.6	7.3-20.0	12.8	0.66
Ear circumference (cm)	11.7	12.4	11.9	7.0-17.3	7.1	0.23
Number of rows/ear	13	13	14	9-20	7.9	0.45
Number of kernels/row	16	23	28	6-46	16.5	0.23
100-kernel weight (gm)	16.6	22.2	19.0	9.0-26.0	12.3	0.30
Grain yield (gm/plot)	96.3	54.2	277.3	13-1006	40.1	0.28

[#] CV, Coefficient of variation

The analysis of variance showed that genotype (G) x Year (Y) interaction was non-significant for the traits, PH, DM, HC, EL, and RN, whereas it was significant for DT, DS, EH, EC, KN, KW, and KY. Genotype source was significant for 7 traits (DT, DS, DM, HC, EL, EC,

and KW). Low coefficient of variation was noticed for most of the traits except HC and GY. Heritability estimates were low for HC, EC, KW, KN, and GY whereas moderate estimates were obtained for PH, DT, DS, DM, EH, EL, and RN (Table 4.1).

Phenotypic variation for plant height revealed normal distribution with similar range for the $F_{2:3}$ mapping population in both years (Figure 4.1). B73 was around 30 cm shorter in stature compared to Mp715. The average PH of the $F_{2:3}$ mapping population was similar to Mp715 in both years.

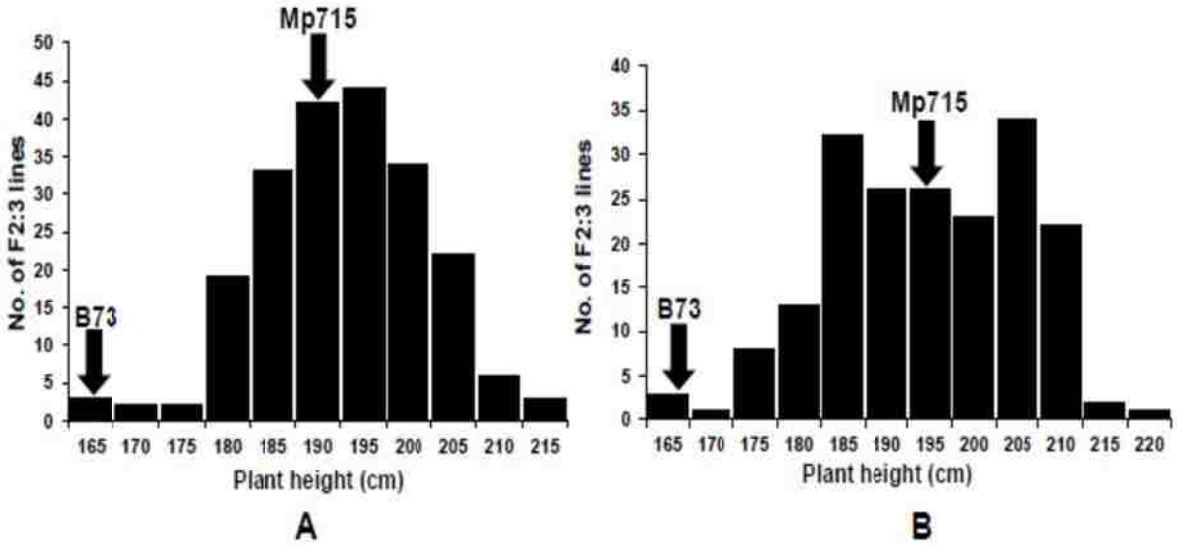

Figure 4.1 Frequency distribution of $F_{2:3}$ mapping population for plant height in 2012 (A) and 2013 (B). Arrows indicate mean values for the parents, B73 and Mp715.

B73 exhibited earlier tasseling in both years (Table 4.1 and Figure 4.2). The number of days to tasseling was similar for B73 in both years. The DT of $F_{2:3}$ mapping population was similar to Mp715, which was 10 days later in tasseling compared to B73. The distribution for DT was slightly skewed in both years towards Mp715.

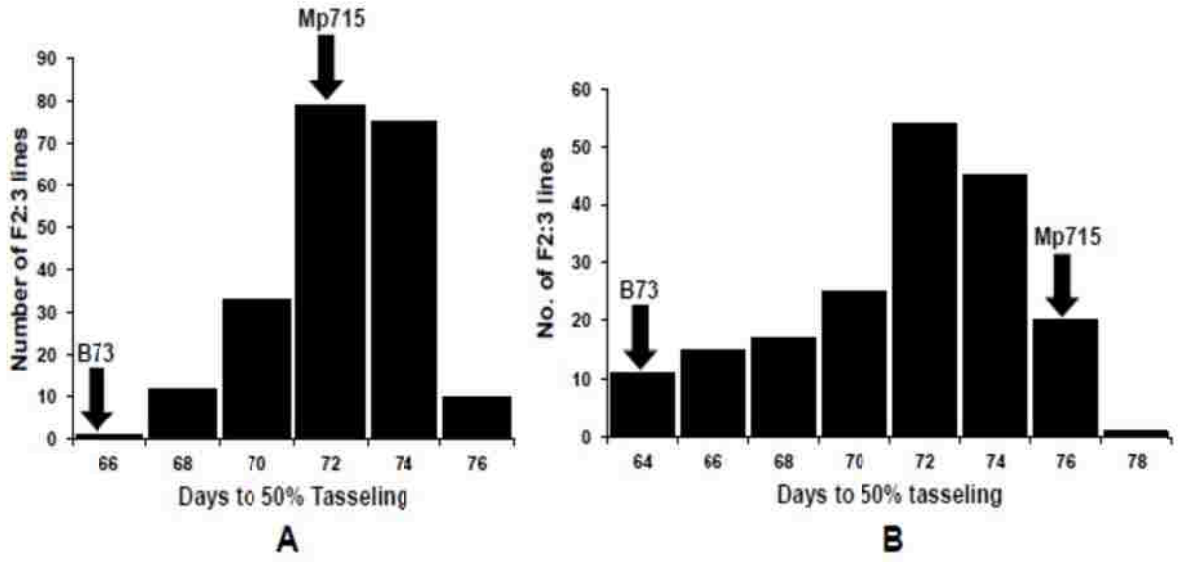

Figure 4.2 Frequency distribution of $F_{2:3}$ mapping population for days to tasseling in 2012 (A) and 2013 (B). Arrows indicate mean values for the parents, B73 and Mp715.

The days to silking (DS) followed the similar pattern as the days to tasseling (Table 4.1 and Figure 4.3). Mp715 took 10 more days for silking compared to B73, which exhibited silking in 64 days. The mean DS for $F_{2:3}$ mapping population was 73 days. The range was higher among the families in both years. The distribution was highly skewed in 2013 compared to 2012.

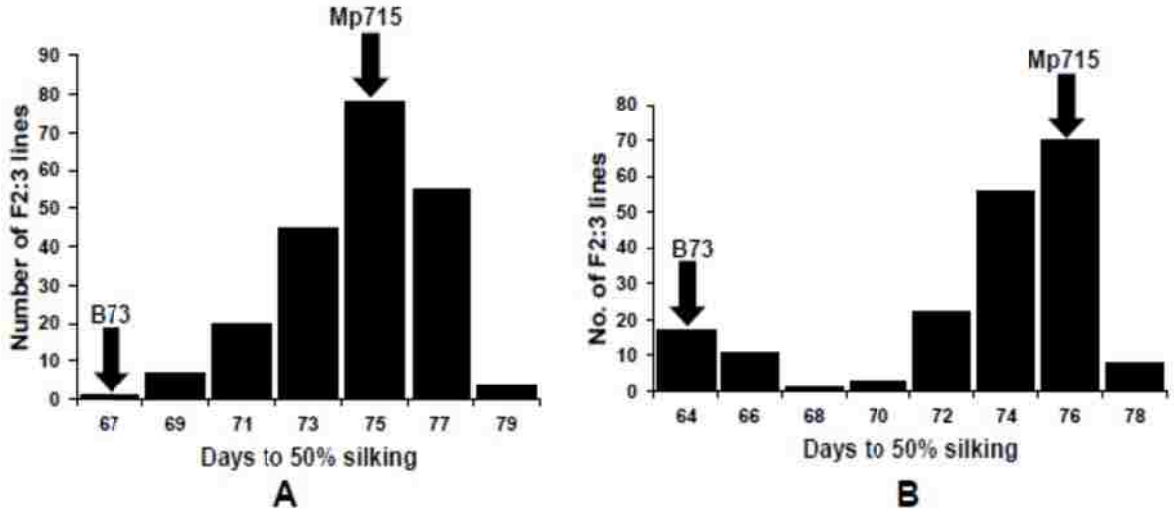

Figure 4.3 Frequency distribution of $F_{2:3}$ mapping population for days to silking in 2012 (A) and 2013 (B). Arrows indicate mean values for the parents, B73 and Mp715.

The susceptible parent B73 matured earlier than Mp715 in both years with an average of 105 days (Table 4.1 and Figure 4.4). The mean DM of $F_{2:3}$ mapping population was lower than Mp715. The frequency distribution for this trait was normal and several $F_{2:3}$ families fell outside the range of both parents indicating transgressive segregation.

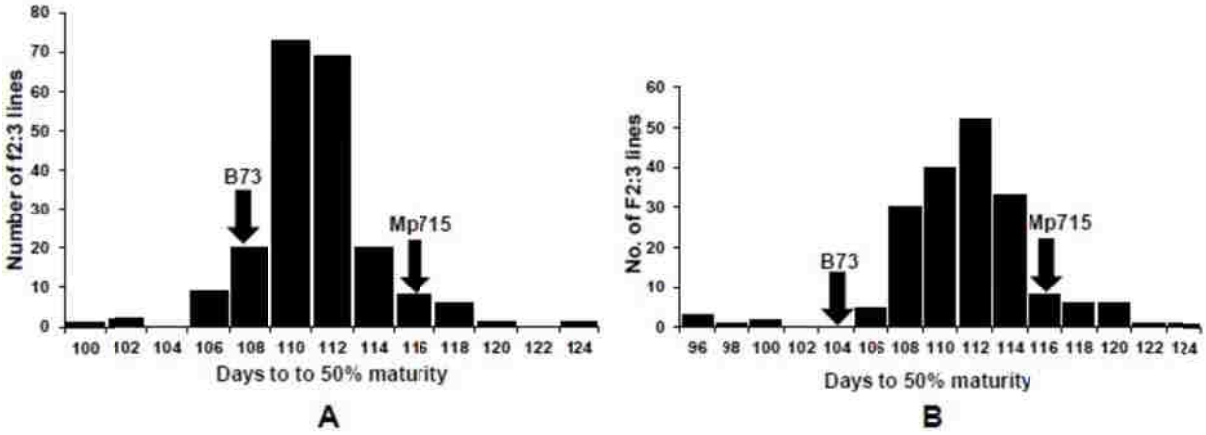

Figure 4.4 Frequency distribution of $F_{2:3}$ mapping population for days to maturity in 2012 (A) and 2013 (B). Arrows indicate mean values for the parents, B73 and Mp715.

The ear height of B73 was lower than Mp715 (Table 4.1 and Figure 4.5). Mp715 was taller and it was reflected in its higher ear height than B73. The average EH of $F_{2:3}$ mapping population was similar to Mp715 in both years.

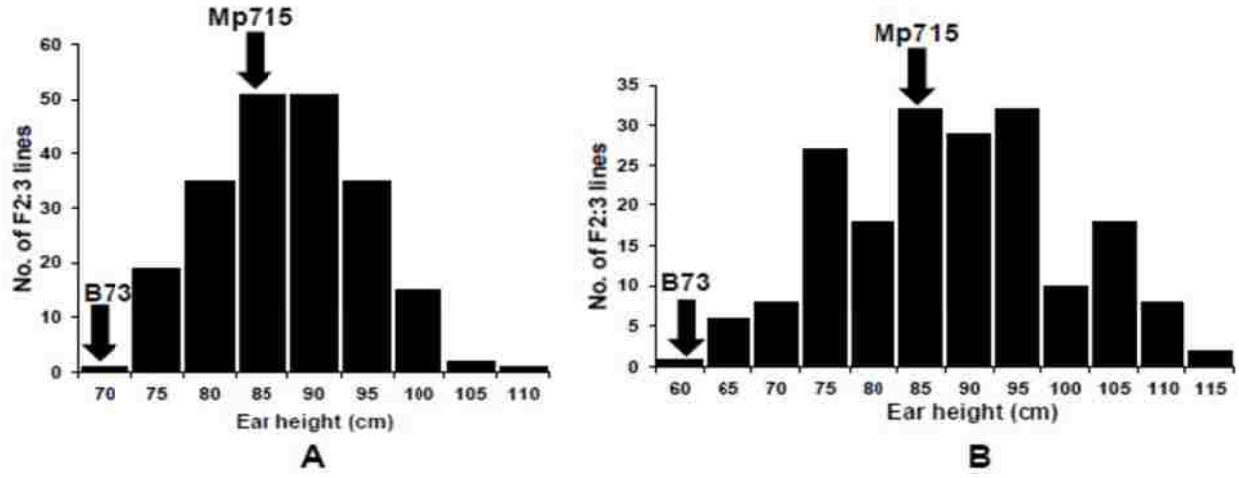

Figure 4.5 Frequency distribution of $F_{2:3}$ mapping population for ear height in 2012 (A) and 2013 (B). Arrows indicate mean values for the parents, B73 and Mp715.

The visual difference was observed for the husk cover (HC) for both parents. B73 had open husk cover, which was reflected in higher husk cover rating (Table 4.1 and Figure 4.6). Mp715 had lower rating as compared to B73 because ears were tightly covered with husk in Mp715. The HC rating ranged from 1 (completely closed) to 5 (completely open) in the $F_{2:3}$ mapping families and the mean rating for $F_{2:3}$ mapping population similar to Mp715 (score 1.5) in 2012 but was slightly higher in 2013 (score 2.3) (Appendix 2).

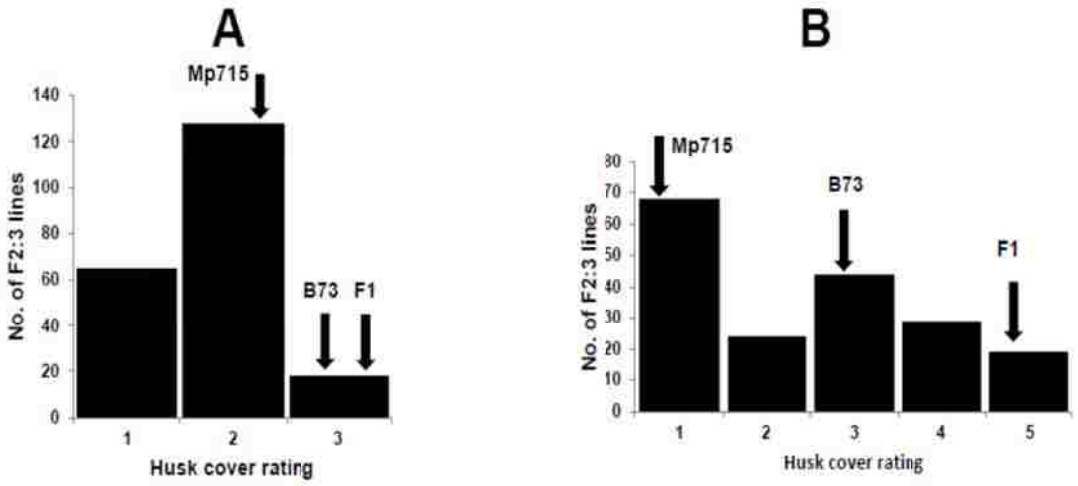

Figure 4.6 Frequency distribution of $F_{2:3}$ mapping population husk cover rating in 2012 (A) and 2013 (B). Arrows indicate mean values for the parents, B73 and Mp715.

The average ear length (EL) was lower in B73 compared to Mp715 (Table 4.1 and Figure 4.7). Mp715 recorded higher EL in both years. The mean EL for $F_{2:3}$ mapping population was similar to B73 but lower compared to Mp715. The ear length of most of the families lied between two parents and the distribution was normal. The range for this trait was higher in both years.

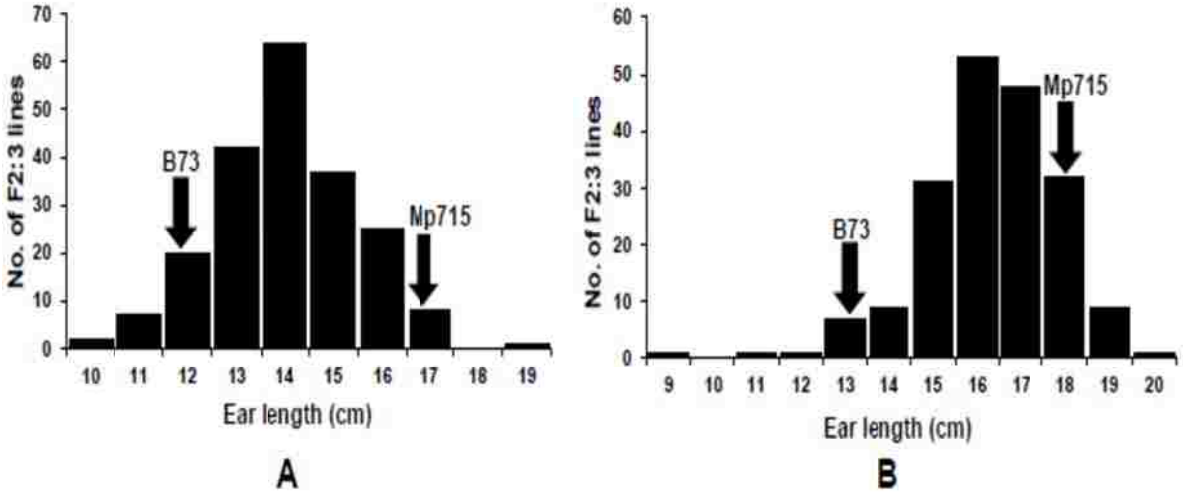

Figure 4.7 Frequency distribution of $F_{2:3}$ mapping population for the ear length (cm) in 2012 (A) and 2013 (B). Arrows indicate mean values for the parents, B73 and Mp715.

The average ear circumference (EC) of both parents was somewhat similar (Table 4.1 and Figure 4.8). The overall mean of EC for $F_{2:3}$ mapping population was slightly lower than Mp715 in both years. The trait had wide range and was normally distributed in $F_{2:3}$ mapping population.

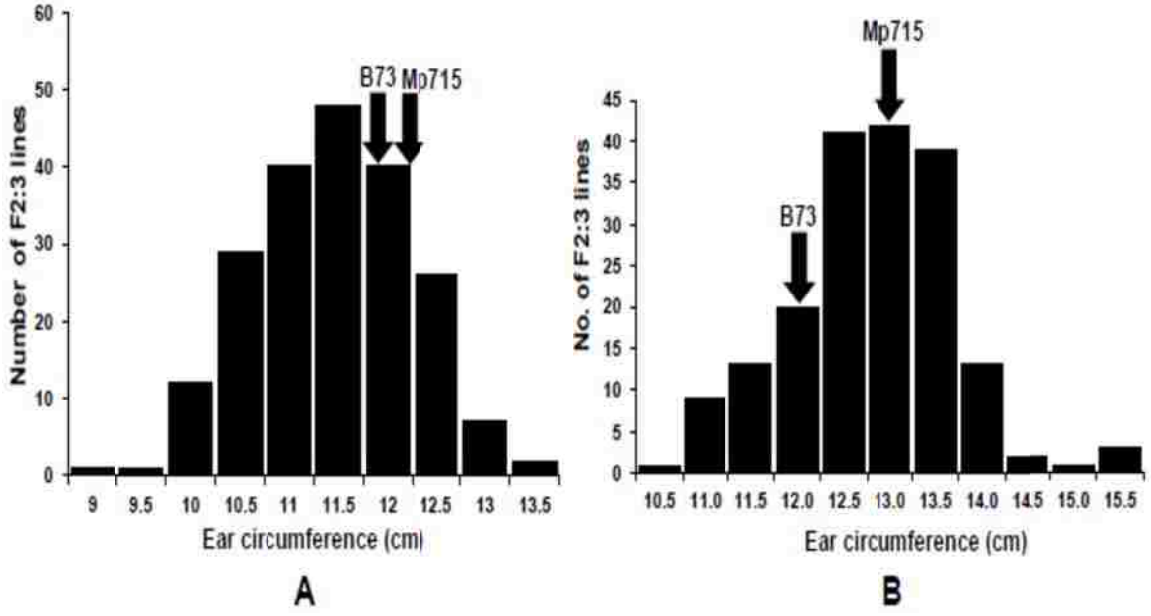

Figure 4.8 Frequency distribution of $F_{2:3}$ mapping population for the ear circumference in 2012 (A) and 2013 (B). Arrows indicate mean values for the parents, B73 and Mp715.

Minimal difference was observed in the number of rows/ear (RN) between parents (Table 4.1 and Figure 4.9). But the variation among the progeny lines was higher, which was reflected by the higher range for this trait.

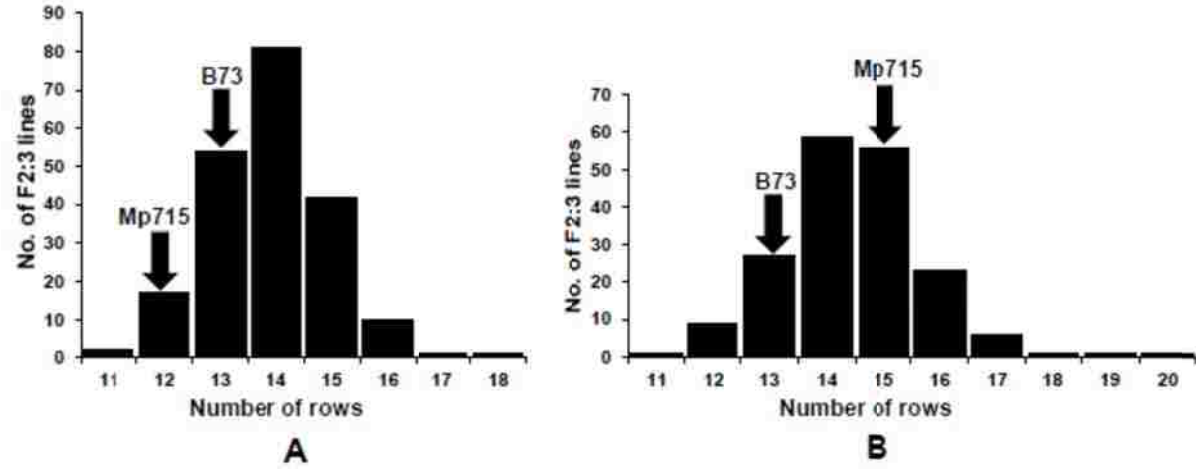

Figure 4.9 Frequency distribution of $F_{2:3}$ mapping population for the number of rows/ear in 2012 (A) and 2013 (B). Arrows indicate mean values for the parents, B73 and Mp715.

The average number of kernels/row (KN) was lower in B73 compared to Mp715 in both years (Table 4.1 and Figure 4.10). The average KN in $F_{2:3}$ mapping population was higher than both parents. Higher range observed for this trait in both seasons indicated higher genetic variability in the mapping population.

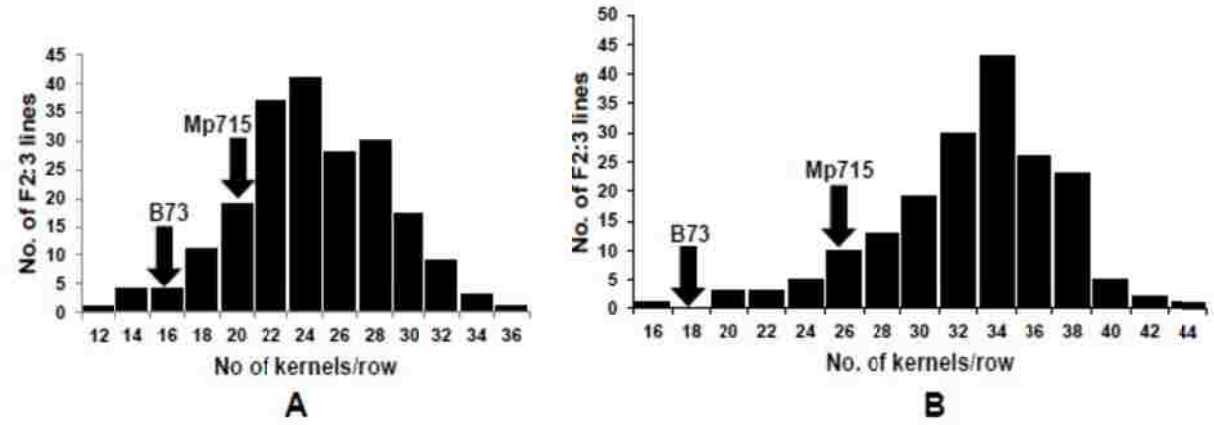

Figure 4.10 Frequency distribution of $F_{2:3}$ mapping population for the number of kernels/row in 2012 (A) and 2013 (B). Arrows indicate mean values for the parents, B73 and Mp715.

The mean 100-kernel weight (KW) in B73 was lower than Mp715 in both years (Table 4.1 and Figure 4.11). The mean KW of the $F_{2:3}$ mapping population was lower than both parents in 2012. The range for KW in the $F_{2:3}$ mapping population was high in both years and the distribution of this trait was nearly normal in both years.

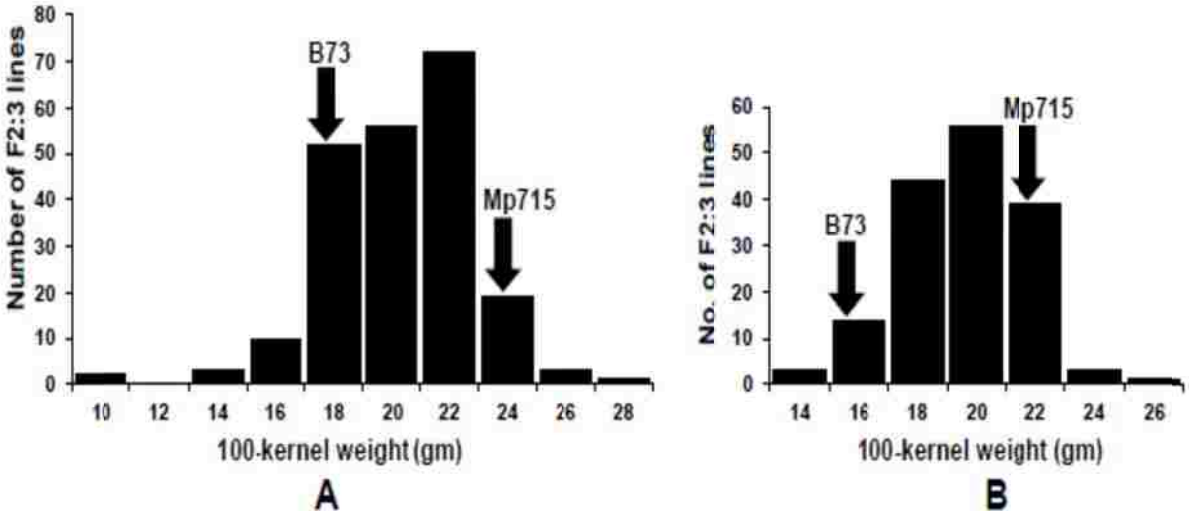

Figure 4.11 Frequency distribution of $F_{2:3}$ mapping population for the 100-kernel weight in 2012 (A) and 2013 (B). Arrows indicate mean values for the parents, B73 and Mp715.

The average grain yield/ plot (GY) was higher in B73 as compared to Mp715 in both years (Table 4.1 and Figure 4.12). The average grain yield of $F_{2:3}$ mapping population was higher than both parents. The maximum GY was 549 gm in 2012 among the $F_{2:3}$ f mapping population but it was higher (1006.0 gm) in 2013. The range for GY was higher in 2013 compared to 2012.

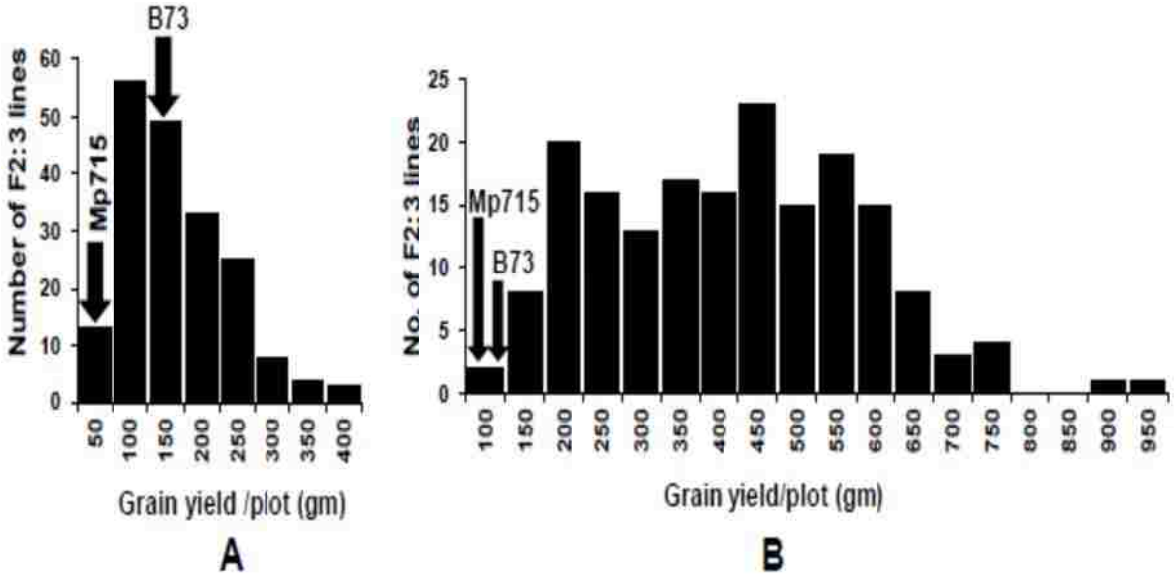

Figure 4.12 Frequency distribution of $F_{2:3}$ mapping population for the grain yield (gm/plot) in 2012 (A) and 2013 (B). Arrows indicate mean values for the parents, B73 and Mp715.

4.2 Aflatoxin accumulation in corn

Higher difference in aflatoxin content in grain was observed between parents and $F_{2:3}$ families in both years (Table 4.2, Figures 4.13 and 4.14). The susceptible parent B73 recorded higher aflatoxin concentration (2446.7 and 2866.7 ng g^{-1} in 2012 and 2013, respectively) compared to the resistant parent Mp715 (38. 7 and 323.3 ng g^{-1} in 2012 and 2013, respectively). Overall, aflatoxin accumulation in parents and was lower in 2012 as compared to 2013. The mean aflatoxin content of $F_{2:3}$ families was higher than Mp715 but lower than B73 in 2012 but it was lower than Mp715 in 2013.The raw aflatoxin concentration was highly skewed among the $F_{2:3}$ families ranging from 1.5- 10,000 and 0.0- 11,600 ng g^{-1} in 2012 and 2013, respectively.

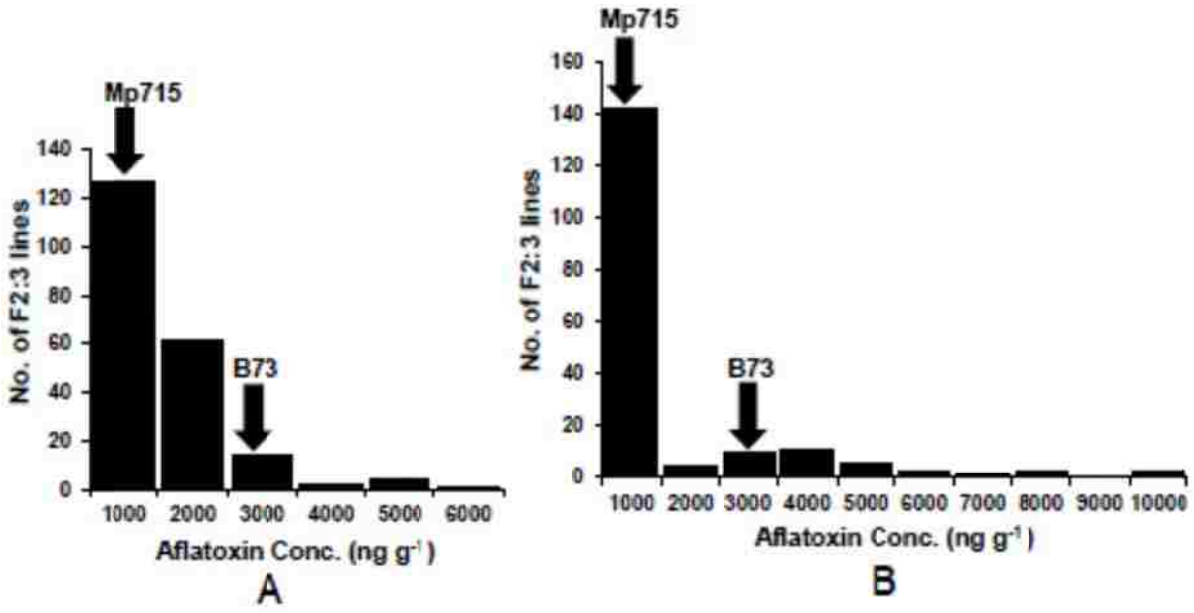

Figure 4.13 Frequency distribution of $F_{2:3}$ mapping population for the original aflatoxin concentration (ng g^{-1}) values in 2012 (A) and 2013 (B). Arrows indicate mean values for the parents, B73 and Mp715.

The distribution of raw aflatoxin among the $F_{2:3}$ families was skewed in both years but was normal after log transformation (Figures 4.13 and 4.14). Analysis of variance showed the highly significant differences among $F_{2:3}$ families for aflatoxin accumulation in both years as well as across the years (Table 4.3). Highly significant genotype x year interaction was also observed for aflatoxin accumulation. Heritability estimate for aflatoxin content was moderately low.

Table 4.2 Mean for aflatoxin (log-transformed) of parents, and $F_{2:3}$ lines and heritability derived from B73 x Mp715 cross taken in 2012, 2013, and across years.

Genotype	**2012**	**2013**	**Across year**
B73	7.75	7.88	7.81
Mp715	3.54	5.70	4.62
$F_{2:3}$	6.21	5.21	5.68
Range	0.4-9.2	0.0-9.4	0.0-9.4
CV (%)	22.50	43.60	14.37
Heritability (H^2)	-	-	0.35

Table 4.3 Sources of variation and their *F* values for aflatoxin (log-transformed) of $F_{2:3}$ families, generated from B73 x Mp715, taken in 2012, 2013, and across years.

Source of variation	2012	2013	Across year
Replication	2.03**	4.05*	10.39**
Genotype ($F_{2:3}$ lines)	2.08**	42.95**	7.44**
Year	-	-	318.22**
Genotype ($F_{2:3}$ lines) x Environment(Year) interaction	-	-	11.63**

*Significant at the 0.05 probability level; **Significant at the 0.01 probability level;
NS Non-significant

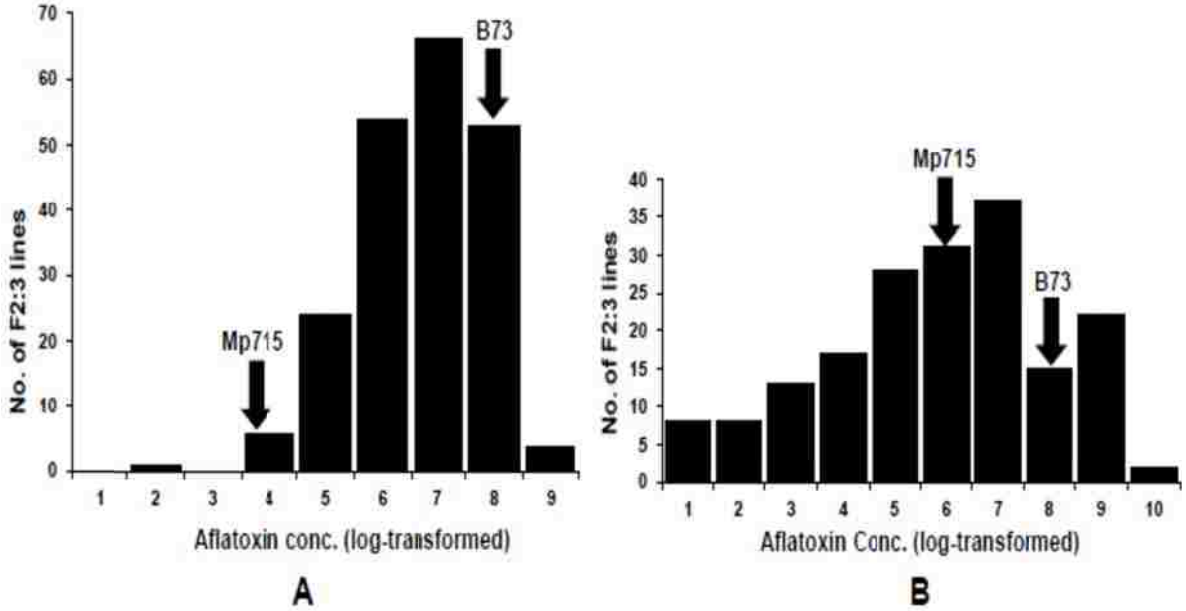

Figure 4.14 Frequency distribution of $F_{2:3}$ mapping population for the natural log–transformed aflatoxin concentration in 2012 (A) and 2013 (B). Arrows indicate mean values for the parents, B73 and Mp715.

4.3 Phenotypic correlation among morphological traits

The phenotypic correlations among morphological traits were calculated using Pearson's correlation coefficients (Table 4.4). Highly significant positive correlation was observed between aflatoxin accumulation and husk cover rating in both years. The important ear related traits such as ear circumference and number of rows/ear were positively correlated to aflatoxin accumulation in 2013.

Table 4.4 Pearson's correlation coefficients among phenotypic traits for $F_{2:3}$ lines developed from B73 x Mp715 cross in 2012 (normal font) and 2013 (bold font).

	AF	**HC**	**EL**	**EC**	**GR**	**RN**	**GY**	**KW**
AF	1	**0.43****	**0.15**	**0.24****	**0.15**	**0.24****	**0.16**	**0.20**
HC	0.41**	1	**0.28**	**0.19**	**0.27**	**0.25**	**0.24**	**0.19**
EL	0.00	0.22	1	**0.39****	**0.53****	**0.25****	**0.42****	**0.34****
EC	-0.09	0.14	0.53**	1	**0.37****	**0.53****	**0.32****	**0.45****
GR	-0.01	0.17	0.50	0.52**	1	**0.22***	**0.54****	**0.20***
RN	-0.02	-0.02	0.10	0.36**	0.16*	1	**0.22***	**0.18***
GY	0.003	0.11	0.12	0.38**	0.34**	0.087	1	**0.18***
KW	-0.06	0.17	0.39	0.41**	0.19	0.02	0.05	1

*Significant at the 0.05 probability level; **Significant at the 0.01 probability level
AF, Aflatoxin concentration (natural log-transformed); HC, husk cover rating (1-5 scale); EL, Ear length; EC, Ear circumference; GR, Grains/rows; RN, Number of rows/ear; GY, Grain yield/plot); KW, 100 kernel weight.

Ear circumference was significantly and positively correlated to ear length in both years. Ear length was positively correlated with number of grains/row, number of rows/ear, grain yield /plot and 100-kernel weight in 2013. Ear circumference was positively correlated with number of grains/row, number of row/ear, and grain yield/plot, and 100-kernel weight in both years. Number of grains/row was positively correlated with number of rows and grain yield in both years and 100-kernel weight only in 2013. Likewise, significant positive correlations were observed between number of rows/year with grain yield and 100-kernel weight in 2013. Grain yield was significantly correlated with 100- kernel weight in 2013.

4.4 Genotyping and linkage map construction

A total of 562 SSR markers distributed over the whole maize genome were selected from the publically available maize genome database (www.maizegdb.org). Polymorphic survey

between parents showed high polymorphism (Figure 4.15) and 306 polymorphic loci (55%) were identified during initial screening (Table 4.5).

Table 4.5 Results from SSR marker polymorphism survey between parents (B73 and Mp715) for linkage map construction.

Chr	Polymorphic	Monomorphic	Null	Unamplified	Total	Polymorphism (%)
1	35	11	3	1	50	70.0
2	27	12	3	4	46	58.7
3	31	19	2	6	58	53.5
4	38	24	5	5	72	52.8
5	38	24	5	5	72	52.8
6	23	15	5	2	45	51.1
7	27	17	0	2	46	58.7
8	32	29	4	2	67	47.8
9	32	12	6	12	62	51.6
10	23	19	1	1	44	52.3
Total	**306**	**182**	**34**	**40**	**562**	**54.5**

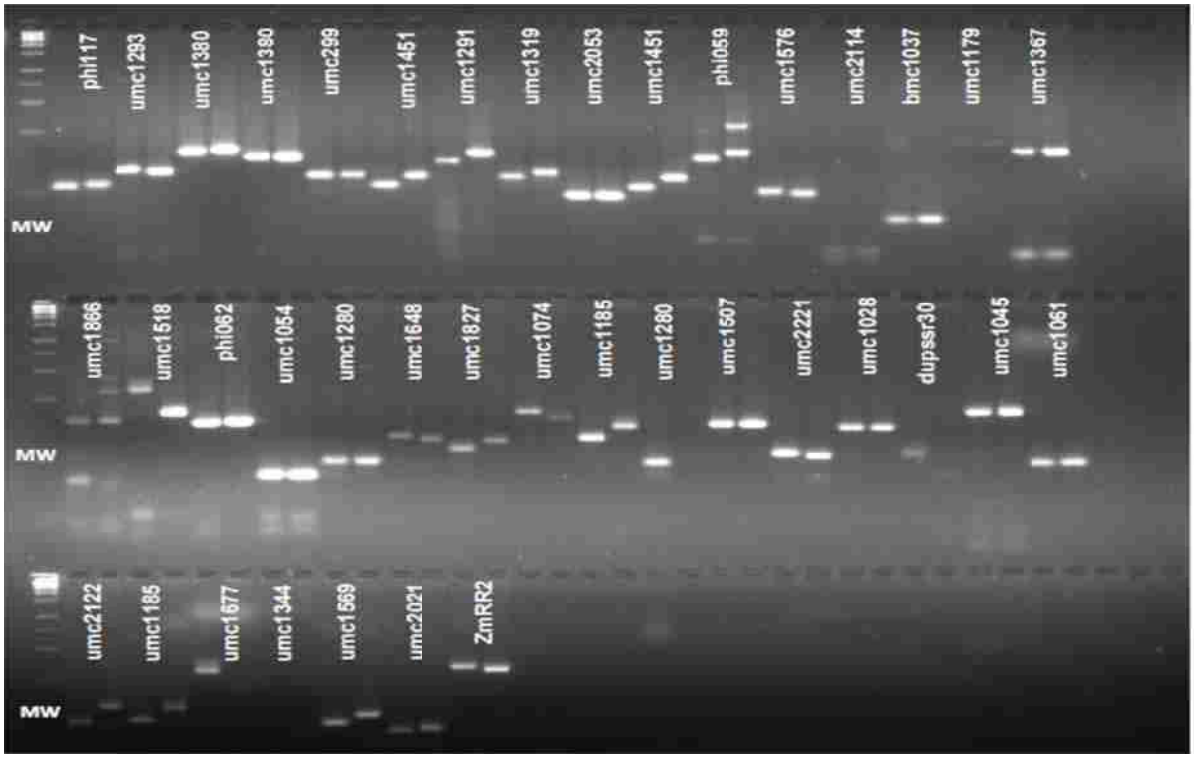

Figure 4.15 A representative agarose gel showing polymorphism survey between two parents for SSR markers of chromosome 10. For each marker, first lane is B73 and second lane is Mp715. MW=Hi-Lo DNA Marker (Minnesota Molecular Inc, MN).

A linkage map was constructed using 136 polymorphic markers, which are distributed along the 10 chromosomes (Table 4.6). The total map length was 2921.1 cM with average loci interval of 21.6 cM Markers in each chromosome were resolved in to single linkage group except linkage group 5.

Table 4.6 Number of SSR markers mapped on each chromosome and chromosome length (cM) in the $F_{2:3}$ mapping population.

Chr No.	No. of markers	Chr. Length (cM)	Marker interval (cM)
1	17	371.2	21.8
2	14	289.9	20.7
3	16	290.8	18.2
4	14	307.6	23.7
5	14	274.7	19.6
6	10	236.4	23.6
7	18	366.6	20.4
8	11	267.9	24.4
9	10	245.3	24.5
10	12	271.0	22.6
Total	**136**	**2921.1**	**21.6**

4.5 Mapping of QTL for resistance to aflatoxin accumulation

Whole genome scan using composite interval mapping (CIM) revealed 4 and 8 QTL for aflatoxin content in 2012 and 2013, respectively (Table 4.7 and Figure 4.16). Four QTL identified in 2012 were on chromosomes 3 (bin 3.08), 4 (bin 4.01), 5 (bin 5.04), and 9 (bin 9.02). The phenotypic variances explained by these QTL were small to moderate with a range of 4 to 9%. In case of the QTL on chromosome 3 and 4, aflatoxin reducing alleles were derived from the resistant parent Mp715, whereas B73 alleles were desirable for the QTL on chromosomes 5 and 9. The QTL identified in 2013 were on chromosomes 4 (bins 4.02, 4.09), 8 (bins 8.05 and 8.09), 9 (bin 9.06), and 10 (bins 10.03, 10.04, and 10.07). The range of phenotypic variances accounted

by each QTL ranged from <1 to 9 %. The Mp715 alleles were responsible for enhancing resistance in QTL identified in bins 9.06 and 10.07 whereas B73 contributed resistance alleles in rest of the QTL.

Table 4.7 Quantitative trait loci for aflatoxin resistance detected in the $F_{2:3}$ mapping population developed from the cross B73 x MP715.

Year	Chr	QTL	Closest marker	Position/Bin	LOD	Gene effect		Gene action	R^2	SDA
						A	D			
2012	3	*qAFL3*	bmc1536	240/3.08	2.50	0.37	-0.37	D	9.2	MP715
	4	*qAFL4-1*	umc2150	37.9/4.01	2.85	0.07	0.55	OD	3.6	MP715
	5	*qAFL5*	bmc1208	112.2/5.04	3.00	-0.58	-0.03	A	7.6	B73
	9	*qAFL9-1*	bmc1626	33.0/9.02	2.55	-0.47	-0.18	PD	3.5	B73
2013	4	*qAFL4-2*	umc1509	84.3/4.02	4.02	-0.08	-3.95	OD	3.8	B73
	4	*qAFL4-3*	umc1503	289.8/4.09	3.02	-0.43	-1.08	OD	<1.0	B73
	8	*qAFL8-1*	bmc1782	101.2/8.05	3.92	-0.87	-0.91	D	<1.0	B73
	8	*qAFL8-2*	phi233376	264.5/8.09	2.75	-0.14	1.32	OD	2.9	B73
	9	*qAFL9-2*	umc1733	107.0/9.06	2.53	0.72	-0.26	PD	4.8	MP715
	10	*qAFL10-*	umc1381	108.0/10.03	2.64	-0.09	-1.82	OD	5.0	B73
	10	*qAFL10-*	umc1827	131.2/10.04	3.91	-1.30	0.29	A	8.8	B73
	10	*qAFL10-*	bmc1518	251.1/10.07	3.22	0.15	-1.27	OD	4.1	MP715
Average	1	*qAFL1-1*	dupssr26	118.3/1.04	3.92	0.76	0.10	A	5.2	MP715
	1	*qAFL1-2*	umc1689	136.3/1.05	2.67	-0.38	0.35	D	7.3	B73
	1	*qAFL1-3*	umc2223	291.3/1.10	3.33	0.29	0.29	D	<1.0	MP715
	2	*qAFL2*	bmc2277	63.2/2.02	2.51	-0.38	0.04	A	3.7	B73
	4	*qAFL4-1*	umc2150	31.6/4.02	2.58	-0.39	0.45	D	8.7	B73
	4	*qAFL4-4*	umc1088	126.2/4.05	4.72	0.69	-0.17	PD	10.0	MP715
	7	*qAFL7-1*	umc1799	338.2/7.06	3.33	0.42	0.40	D	<1.0	MP715
	8	*qAFL8-1*	bmc1782	101.2/8.05	2.50	-0.30	-0.34	D	<1.0	B73
	10	*qAFL10-*	bmc1074	172.2/10.05	6.44	-0.58	-0.21	PD	6.0	B73

A, additive effect; D, dominance effect; OD, over dominance effect; SDA, Source of desirable allele; R^2, percentage of phenotypic variation explained by QTL

Combined analysis for aflatoxin (log-transformed) showed QTL on chromosomes 1 (bins 1.04, 1.05, and 1.10), 2 (bin 2.02), 4 (bins 4.02 and 4.05), 7 (bin 7.06), 8 (bin 8.05), and 10 (bin 10.05). Three QTL with small phenotypic effect were identified in chromosome 1, but were not detected in individual year analysis. Likewise, QTL on chromosomes 3 and 5 appeared only in

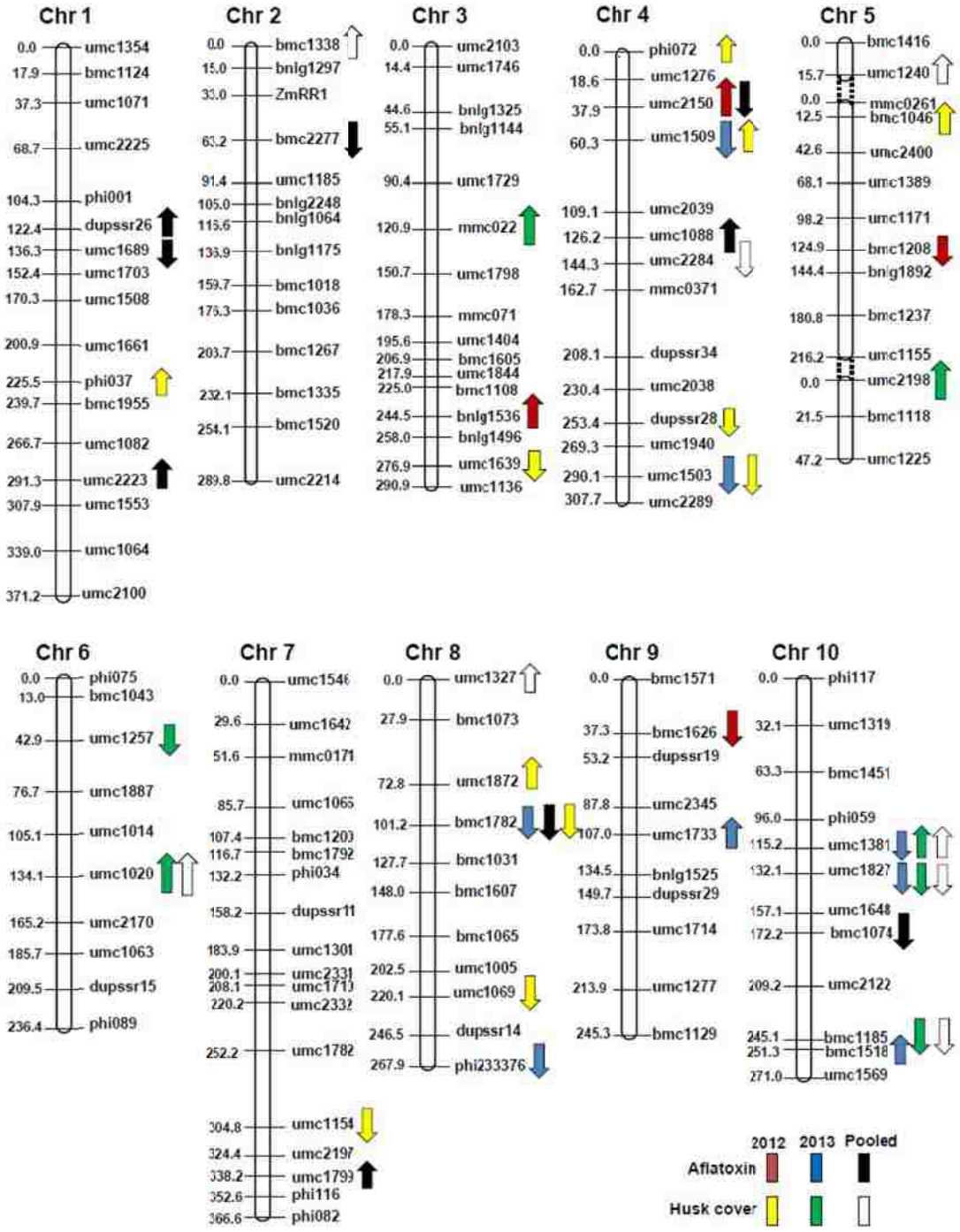

Figure 4.16 QTLs associated with aflatoxin resistance (measured as log-transformed value) and husk cover rating in an $F_{2:3}$ mapping population derived from B73 x Mp715. Red, blue, and black bars represent QTLs for aflatoxin resistance in 2012, 2013, and combined analysis. Yellow, green, and white arrows indicate QTLs for husk cover in 2012, 2013, and combined analysis. The arrows pointing to the top and bottom indicate increasing effect on phenotypic values of B73 allele and Mp715 allele, respectively.

2012 and QTL on chromosome 8 appeared only in 2013. The QTL identified on chromosome 9 were detected in both years but were in different genomic locations. A QTL with the largest phenotypic variance (10.0%) was identified on chromosome 4 (bin 4.05) in pooled analysis. The desirable alleles responsible for increasing aflatoxin resistance were from Mp715 in case of QTL on chromosome 1, 3, 4, and 7 in combined year analysis.

Two different QTLs identified on chromosome 4 were located next to each other in bins 4.01 and 4.02 in 2012 and 2013, respectively. QTL with moderate effect (7.6%) was identified on chromosome 5 (bin 5.04) in 2012. QTL with small effects were identified on chromosome 8 (bins 8.05 and 8.09) in 2013. Three QTLs with moderate phenotypic effects were identified in the bin interval 10.0310.07 on chromosome 10 in 2013. Among them, Mp715 allele of the *qAFL10-3* was responsible for increasing aflatoxin resistance.

4.6 Mapping of QTLs for husk cover

Composite interval mapping for husk cover rating revealed 10, 7, and 8 QTL in 2012, 2013, and combined analysis, respectively (Table 4.8 and Figure 4.16). The phenotypic variance explained by these QTL in both years ranged from <1 to 13%.

Four QTL were located on chromosome 4 (bins 4.01, 4.02, 4.08 and 4.09) in 2012. QTL with desirable alleles from Mp715 were located in bins 1.08, 4.01, 4.02, 5.03, and 8.02 whereas B73 alleles reduced husk cover rating in the rest. The QTL located in bins 4.09 and 8.02 explained 13% and 9% of phenotypic variation but rests were with minor effects. Seven QTL detected in 2013 were on chromosomes 3 (bin 3.05), 5 (bin 5.06), 6 (bins 6.03 and 6.05) and 10 (bins 10.03, 10.04, and 10.05). The QTL on chromosome 6 (bin 6.05) was detected in both 2013 and combined year analysis with 7.4 % and 5.5% phenotypic variance, respectively. These QTL were derived from resistant parent. Three QTL on chromosome 10 were identified in 2013 and in

combined analysis. The QTL (*qHC10-1*) with larger phenotypic effect in 2013 and combined analysis was located in chromosome 10 (10.03) and was derived from Mp715. Both husk cover QTL (*qHC10-1, qHC10-2*) overlapped with the QTL for reduced aflatoxin accumulation identified in 2013. Mp715 allele increased the aflatoxin content in case of both QTL whereas B73 and Mp715 alleles increased husk cover rating at the QTL located in bins 10.03 and 10.04, respectively.

Table 4.8 Quantitative trait loci for husk cover detected in the $F_{2:3}$ mapping population developed from the cross B73 x MP715.

Year	Chr	QTL	Nearest marker	Position/Bin	LOD	Gene effect		Gene	R^2	SDA
						A	D			
2012	1	*qHC1-2*	phi037	225.5/1.08	2.74	0.18	0.16	D	<1.0	Mp715
	4	*qHC4-1*	phi072	8.0/4.01	3.11	0.06	0.35	OD	<1.0	Mp715
	4	*qHC4-2*	umc1509	84.3/4.02	17.4	0.01	1.06	OD	2.7	Mp715
	4	*qHC4-4*	dupssr28	253.1/4.08	3.06	-0.07	0.28	OD	5.6	B73
	4	*qHC4-5*	umc1503	288.0/4.09	3.76	-0.18	0.28	D	13.4	B73
	5	*qHC5-2*	bmc1046	10.0/5.03	2.75	0.22	0.17	PD	<1.0	Mp715
	7	*qHC7*	umc1154	304.8/7.05	3.39	-0.12	0.22	OD	7.3	B73
	8	*qHC8-2*	umc1872	71.9/8.02	3.25	0.23	-0.14	PD	9.4	Mp715
	8	*qHC8-3*	bmc1782	103.2/8.05	2.65	-0.07	0.27	OD	5.4	B73
	8	*qHC8-4*	umc1069	220.1/8.08	2.51	-0.02	0.26	OD	3.0	B73
2013	3	*qHC3*	mmc022	120.9/3.05	3.36	0.24	0.58	OD	<1.0	Mp715
	5	*qHC5-3*	umc2198	4.0/5.06	4.01	0.37	0.63	OD	<1.0	Mp715
	6	*qHC6-1*	umc1257	35.0/6.03	2.55	-0.44	-0.37	A	2.9	B73
	6	*qHC6-2*	umc1020	143.1/6.05	2.78	0.56	-0.11	OD	7.4	Mp715
	10	*qHC10-1*	umc1381	115.0/10.03	3.99	0.57	-0.58	D	12.4	Mp715
	10	*qHC10-2*	umc1827	132.1/10.04	4.38	-0.87	-0.01	A	6.8	B73
	10	*qHC10-3*	bmc1185	236.2/10.05	2.91	-0.65	0.13	PD	9.2	B73
Average	2	*qHC2*	bmc1338	4.0/2.01	6.88	0.20	-0.58	OD	15.5	Mp715
	4	*qHC4-3*	umc2284	156.3/4.06	3.44	-0.20	-0.50	OD	1.6	B73
	5	*qHC5-1*	umc1240	9.0/5.00	2.50	0.31	0.08	PD	2.9	Mp715
	6	*qHC6-2*	umc1020	134.1/6.05	2.75	0.26	-0.10	PD	5.5	Mp715
	8	*qHC8-1*	umc1327	0.0/8.01	3.46	0.30	-0.03	A	5.8	Mp715
	10	*qHC10-1*	umc1381	114.0/10.03	3.78	0.33	-0.31	D	11.4	Mp715
	10	*qHC10-2*	umc1827	136.1/10.04	3.44	-0.47	-0.12	PD	4.6	B73
	10	*qHC10-3*	bmc1185	247.1/10.05	2.83	-0.31	0.11	PD	6.3	B73

A, additive effect; D, dominance effect; OD, over dominance effect; SDA, Source of desirable allele; R^2, percentage of phenotypic variation explained by QTL

4.7 Mapping of QTL for Agronomic Traits

4.7.1 Plant height

Two QTL for plant height with 14.2 % and 12.0% phenotypic variance were identified on chromosome 6 in 2012 (Table 4.9). In 2013, 5 QTL were identified on chromosomes 2, 4, 8, and 10. The phenotypic variance explained by these individual QTL were less than 10%. The QTL with largest effect in this year was identified on chromosome 10. Combined analysis identified 3 QTL on chromosomes 2, 4, and 6. Each QTL had phenotypic variance less than 5% and the largest with 3.2% on chromosome 4. One QTL in chromosome 6 was identified in combined analysis, which was near by the QTL identified in 2012. One QTL was flanked by markers umc2170-dupssr15 and other QTL was nearby umc1020 on chromosome 6.

Table 4.9 Quantitative trait loci for plant height detected in the $F_{2:3}$ mapping population developed from the cross B73 x MP715.

Year	Chr	Closest marker	Position/Bin	LOD	Gene effect		Gene action	R^2	DPE
					A	D			
2012	6	umc2170	165.1/6.06	2.68	5.49	-9.61	OD	14.2	MP715
	6	dupssr15	209.5/6.06	3.4	-6.62	10.54	OD	12.0	B73
2013	2	zmRR1	49.0/2.00	82.77	95.44	94.47	D	3.6	MP715
	4	umc1503	290.1/4.09	2.76	4.29	-26.77	D	4.8	MP715
	4	umc2289	307.1/4.11	3.8	28.88	-3.82	A	7.7	MP715
	8	umc1069	220.1/8.08	2.87	-1.72	25.78	OD	3.1	B73
	10	umc1827	131.2/10.04	7.53	-42.21	9.13	PD	16.7	B73
Overall	2	bmc1335	224.7/2.08	3.30	-0.02	-6.59	OD	1.5	B73
	4	phi072	0.0/4.01	4.64	3.93	2.55	PD	3.2	MP715
	6	umc1020	148/6.05	3.39	3.88	3.65	D	1.6	MP715

A, additive effect; D, dominance effect; DPE, direction of phenotypic effect
R^2 indicates the percentage of phenotypic variation explained by the QTL.

4.7.2 Tasseling days

Four QTL were identified on chromosomes 1, 4, 8, and 10 in 2012 (Table 4.10). QTLs on chromosome 1, 4, and 8 accounted for <10 % phenotypic variance and the QTL with large effect

(11.6%) was in bin10.03 between umc1381-umc1827. In 2013, six QTL were identified on chromosomes 5, 8, 9, and 10. Two QTLs were identified on chromosome 9 and 10. The QTL with largest phenotypic variance (52.4%) was detected on chromosome 5 (5.02) and the smallest one on chromosome 8 (8.08). Other QTL with higher phenotypic variance (21.4%) was located on chromosome 10 (10.04). The QTL on chromosome 10 with large phenotypic effect was consistently found in both years. Six QTL were identified on chromosomes 2, 4, 9, and 10 in combined analysis. One QTL with similar phenotypic effect was found in the same genomic region on chromosome 10 (10.04) in 2013. The QTL with large phenotypic effects were found consistently in bin10.04 in both 2012 and 2013 but not identified in combined analysis.

Table 4.10 Quantitative trait loci for days to tasseling detected in the $F_{2:3}$ mapping population developed from the cross B73 x MP715.

Year	Chr	Closest marker	Position/Bin	LOD	Gene effect		Gene action	R^2	DPE
					A	D			
2012	1	umc1661	201.9/1.07	3.95	0.94	0.41	PD	3.3	MP715
	4	umc1509	59.9/4.02	2.58	0.64	-0.29	PD	6.2	MP715
	8	bmc1031	127.7/8.06	3.09	-0.34	0.75	OD	6.0	B73
	10	umc1827	124.2/10.04	4.14	-0.38	1.40	OD	11.6	B73
2013	5	bmc1046	22.5/5.02	3.39	-12.14	12.35	D	52.4	B73
	8	umc1069	220.1/8.08	4.98	-0.80	12.93	OD	5.8	B73
	9	dupssr19	53.2/9.02	3.67	10.59	-2.73	PD	7.6	MP715
	9	umc1714	190.8/9.08	109.42	-34.85	35.45	D	8.0	B73
	10	umc1827	132.1/10.04	8.71	-15.89	5.82	PD	21.4	B73
	10	bmc1074	170.1/10.05	4.18	11.39	2.18	A	5.4	MP715
Overall	2	bnlg1297	25.0/2.02	2.60	0.79	-0.78	D	9.7	MP715
	4	umc1940	289.3/4.09	4.51	1.46	0.22	A	5.3	MP715
	4	umc2289	316.7/4.11	3.03	-1.21	0.30	OD	11.1	B73
	9	umc1277	213.9/9.08	2.53	0.41	-0.93	OD	5.4	MP715
	10	umc1381	121.2/10.03	4.41	-0.12	1.69	OD	8.1	B73
	10	bmc1074	184.2/10.05	2.55	1.14	0.63	PD	3.0	MP715

A, additive effect; D, dominance effect; DPE, direction of phenotypic effect
R^2 indicates the percentage of phenotypic variation explained by the QTL.

4.7.3 Silking days

Few QTL were identified for silking days in 2012 compared to 2013 (Table 4.11). In 2012, only two QTLs were identified in bins 3.01 and 3.05, which accounted for 9.0% and 2.8% of phenotypic variance, respectively. Altogether, six QTL were identified in 2013 on chromosomes 4 (4.11), 8 (8.08), 9 (9.02 and 9.08) and 10 (2 QTLs in 10.04). The QTL on chromosome 4, 8, 9, and 10 were with small effects explaining less than 10% phenotypic variance. One QTL in chromosome 9 (9.08) accounted for 58.3% of phenotypic variance. Other QTL on chromosome 10 linked with umc1827 accounted for higher phenotypic variance (17.2%). Combined analysis resulted in four QTL located on chromosomes 7, 8, and 10. In 2013 and combined analysis, QTLs with larger effects were identified in a genomic region flanked by marker umc1827 and bmc1074 on chromosome 10. However, the phenotypic effects of these QTLs were smaller in combined analysis compared to 2013.

Table 4.11 Quantitative trait loci for days to silking detected in the $F_{2:3}$ mapping population developed from the cross B73 x MP715.

Year	Chr	Closest marker	Positon/Bin	LOD	Gene effect		Gene action	R^2	DPE
					A	D			
2012	3	umc1746	26.4/3.01	3.14	1.44	0.43	PD	9.0	MP715
	3	mmc022	110.4/3.05	2.78	0.90	0.65	PD	2.8	MP715
2013	4	umc2289	307.1/4.11	2.94	9.17	-0.74	A	5.3	MP715
	8	umc1069	220.1/8.08	3.65	0.17	11.13	OD	3.6	MP715
	9	dupssr19	53.2/9.02	2.66	8.12	-4.02	PD	6.0	MP715
	9	umc1714	190.8/9.08	96.31	-35.86	35.37	D	58.3	B73
	10	umc1827	131.2/10.04	7.55	-14.91	4.71	PD	17.2	B73
	10	umc1648	167.1/10.04	6.18	14.44	3.68	PD	7.9	MP715
	3	umc1729	104.4/3.03	6.04	2.89	2.84	D	3.2	MP715
Overall	7	umc1782	270.2/7.04	3.65	0.00	-4.64	A	4.7	MP715
	8	dupssr14	261.5/8.09	3.01	0.18	1.79	OD	2.1	MP715
	10	umc1827	144.1/10.04	2.84	0.30	2.20	OD	6.4	MP715
	10	bmc1074	190.2/10.05	4.41	1.75	1.54	D	4.4	MP715

A, additive effect; D, dominance effect; DPE, direction of phenotypic effect
R^2 indicates the percentage of phenotypic variation explained by the QTL.

4.7.4 Maturity days

More QTL were detected in 2012 compared to 2013 (Table 4.12). Four of five QTLs identified on chromosomes 1, 3, 4, and 9 in 2012 were minor in nature with phenotypic variances <10 % with the exception of the QTL on chromosome 1. The major QTL located in 1.08 explained 54.4% of phenotypic variance. In 2013, four QTLs were identified on chromosomes 4, 5, and 10. QTL with large effect (60.3% of phenotypic variance) was identified on chromosome 5. Small QTL were identified on chromosome four in both years but they were in different genomic locations. Other large QTL with 19.9% phenotypic variance was identified on chromosome 10 in 2013, but not in 2012 or combined analysis. Combined analysis revealed four QTL with small effects on chromosome 1, 3, 4, and 9. The marker umc1404 on chromosome 3

Table 4.12 Quantitative trait loci for days to maturity detected in the $F_{2:3}$ mapping population developed from the cross B73 x MP715.

Year	Chr	Closest marker	Position/Bin	LOD	Gene effect		Gene action	R^2	DPE
					A	D			
2012	1	phi037	218.9/1.08	2.93	1.65	-1.01	PD	54.4	MP715
	3	umc1404	197.6/3.07	2.85	-0.42	-1.36	OD	2.5	B73
	4	phi072	2.0/4.01	3.1	-0.30	1.59	OD	3.5	B73
	4	umc2039	113.1/4.03	3.14	-0.52	1.34	OD	6.9	B73
	9	dupssr29	154.7/9.08	2.73	-1.11	-0.11	A	5.7	B73
2013	4	umc2289	307.1/4.11	3.57	16.28	-1.49	A	7.1	MP715
	5	bmc1046	23.5/5.02	119.04	-54.62	55.48	D	60.3	B73
	10	umc1827	131.2/10.04	8.68	-26.48	5.17	A	19.9	B73
	10	bmc1074	172.1/10.05	3.3	23.42	3.63	A	4.0	MP715
Overall	1	umc1661	224.9/10.07	3.09	1.79	-0.03	A	5.9	MP715
	3	umc1404	195.6/3.07	3.73	-0.22	-1.58	OD	1.5	B73
	4	dupssr28	245.4/4.08	2.99	-1.33	-0.38	PD	5.3	B73
	9	dupssr29	142.5/9.07	2.87	-1.09	1.07	D	9.5	B73

A, additive effect; D, dominance effect; DPE, direction of phenotypic effect
R^2 indicates the percentage of phenotypic variation explained by the QTL.

was linked with QTL in both 2012 and combined analysis. The largest QTL in chromosome 1 was near phi0037 in both 2012 and combined analysis. Likewise, QTL region linked with dupssr29 on chromosome 9 was identified in 2013 and combined analysis.

4.7.5 Ear height

Composite interval mapping identified 2 and 7 QTL in 2012 and 2013, respectively (Table 4.13). All the QTLs identified were minor QTL, which accounted for less than 10% of the phenotypic variance. In 2012, the QTL with largest effect was located in bin7.04 and was very close to marker umc2332. It explained 7.7% of the phenotypic variance. In 2013, more QTL were identified. Two QTL were on chromosome 4. The QTL in bin 4.03 accounted for 9.4% phenotypic variance.

Table 4.13 Quantitative trait loci for ear height detected in the $F_{2:3}$ mapping population developed from the cross B73 x MP715.

Year	Chr	Closest marker	Position/Bin	LOD	Gene effect		Gene action	R2	DPE
					A	D			
2012	7	umc2332	220.2/7.04	2.87	-4.95	0.99	A	7.7	B73
	9	bmc1626	36.0/9.03	4.4	4.82	3.11	PD	2.4	MP715
2013	4	umc2039	109.1/4.03	3.94	13.65	-8.31	PD	9.4	MP715
	4	umc2284	139.2/4.06	2.53	0.09	17.59	OD	5.4	MP715
	7	umc2332	220.2/7.04	2.56	-18.65	-4.86	PD	1.3	B73
	8	umc1005	202.5/8.08	3.84	-16.45	-5.68	PD	3.6	B73
	8	umc1069	220.1/8.08	4.44	7.43	18.00	OD	1.6	MP715
	9	bmc1626	37.0/9.03	3.88	-15.48	8.69	PD	9.8	B73
	9	dupssr19	53.2/9.02	5.68	20.99	-5.20	PD	11.7	MP715
Overall	2	umc1335	242.1/2.08	3.02	-3.60	-2.01	PD	4.3	B73
	4	umc1276	18.6/4.01	3.02	2.89	-2.18	PD	8.2	MP715
	4	umc1509	60.3/4.02	2.75	-1.76	3.19	OD	6.2	B73
	6	umc1257	42.9/6.03	2.92	-2.98	-1.78	PD	2.4	B73

A, additive effect; D, dominance effect; DPE, direction of phenotypic effect
R^2 indicates the percentage of phenotypic variation explained by the QTL.

Other QTL with smaller effect were identified in bin4.06, bin7.04, and bin 8.08. One QTL with 11.7 % phenotypic variance was identified in chromosome 9. Combined analysis identified 4 QTL on chromosomes 2, 4, and 6 and the QTL with largest effect (8.2 %) was located on chromosome 4. The other QTL in bin 4.03 was linked to marker umc1509 and the same marker was also flanked by the QTL identified in 2012 and combined analysis. Some common genomic regions were identified using common markers flanking the QTL identified in both years as well as in combined analysis.

4.7.6 Ear length

Eight QTL for ear length were identified on chromosomes 1, 2, 4, 5, 6, 7, 9, and 10 in 2012 (Table 4.14). The QTL with large phenotypic effects on chromosomes 1, 2, 4, and 5 accounted for 42.5%, 19.3%, 23.9%, and 39.3% phenotypic variance, respectively. The remaining QTLs were with small effects. Four QTL were detected on chromosomes 5, 8, and 10 in 2013 and on chromosomes 1, 4, and 7 in combined analysis. QTL identified in bin 4.07 and bin 7.04 accounted for higher phenotypic variance (>10.0%) while the remaining two were with small effect. Same marker dupssr34 in bin 4.07 was linked with QTL in both 2012 and combined analysis but with different phenotypic effect.

Table 4.14 Quantitative trait loci for ear length detected in the $F_{2:3}$ mapping population developed from the cross B73 x MP715.

Year	Chr	Closest marker	Position/Bin	LOD	Gene effect		Gene action	R^2	DPE
					A	D			
2012	1	bmc1955	236.5/1.08	5.9	-1.41	1.81	OD	42.5	B73
	2	bmc1267	191.3/2.07	3.51	-1.41	1.00	PD	19.3	B73
	4	dupssr34	212.1/4.07	6.39	-1.59	1.54	D	23.9	B73
	5	bmc1046	15.5/5.02	3.13	0.85	1.45	OD	39.3	MP715
	6	dupssr15	209.5/6.06	3.71	-0.57	1.52	OD	5.3	B73
	7	umc1301	187.9/7.03	2.61	-0.32	1.24	OD	4.7	B73
	9	bmc1571	0.0/9.04	3.19	-1.27	-0.04	A	5.5	B73
	10	phi117	7.0/10.04	3.88	1.19	1.00	D	2.5	MP715
2013	5	bmc1046	23.5/5.02	48.65	-7.49	8.65	D	57.4	B73
	8	umc1069	220.1/8.08	3.35	-0.33	2.42	OD	4.3	B73
	10	umc1827	132.1/10.04	5.83	-2.99	0.76	PD	13.2	B73
	10	bmc1074	166.1/10.05	4.27	2.76	0.24	A	7.3	MP715
Overall	1	phi037	225.5/1.08	2.64	-0.17	1.00	OD	3.9	B73
	4	umc1276	21.6/4.01	3.68	-0.49	1.06	OD	7.5	B73
	4	dupssr34	202.7/4.07	2.93	-0.57	1.06	OD	10.6	B73
	7	umc2332	229.2/7.04	3.04	-0.90	0.41	PD	12.4	B73

A, additive effect; D, dominance effect; DPE, direction of phenotypic effect
R^2 indicates the percentage of phenotypic variation explained by the QTL.

4.7.7 Ear circumference

Many QTL were identified for ear circumference in 2012 compared to 2013 (Table 4.15). They were located on each chromosome except 5 and 10. Two QTL were identified on chromosome 4 and the remaining chromosomes had one QTL with varying phenotypic effects. Larger QTLs (>10% phenotypic effect) were detected on chromosomes 1, 2, 4, 7, and 9. In 2013, QTLs were identified on chromosomes 1, 4, and 8 with one QTL each and three QTL on chromosome 10. All QTLs except the one on chromosome 10 were with small effects.

Combined analysis identified 5 QTL on chromosomes 1, 2, 3, 4, and 7. Only one QTL on chromosome 7 accounted for higher phenotypic variance (18.7%) in 2012. The QTL identified in

bin 7.04 in the combined analysis was with similar phenotypic variance (18.6%). The marker dupssr34 was linked with QTL on chromosome 4 in both 2012 and combined analysis. Likewise, QTL were identified on chromosome 1 in 2012 (bin1.10), 2013 (bin1.12) and across the year (bin 1.08) analysis.

Table 4.15 Quantitative trait loci for ear circumference detected in the $F_{2:3}$ mapping population developed from the cross B73 x MP715.

Year	Chr	Closest marker	Position/Bin	LOD	Gene effect		Gene action	R^2	DPE
					A	D			
2012	1	bmc2223	287.7/1.10	3.91	-1.01	1.12	D	33.8	B73
	2	bmc1267	191.3/2.07	3.2	-0.99	0.96	D	19.9	B73
	3	mmc022	120.4/3.05	3.17	-0.29	1.03	OD	4.9	B73
	4	dupssr34	188.7/4.07	56.78	-5.34	6.06	D	18.1	B73
	4	umc1503	293.1/4.09	2.59	-0.19	1.08	OD	3.9	B73
	7	umc1710	214.1/7.04	2.51	-1.07	0.68	PD	18.7	B73
	8	umc1327	7.0/8.01	4.06	1.07	1.05	D	1.3	MP715
	9	bmc1571	8.0/9.04	2.94	-0.71	0.85	D	12.7	B73
2013	1	umc2100	364.0/1.12	3.27	1.58	0.21	A	6.6	MP715
	4	umc2289	306.1/4.11	3.09	1.70	-0.46	PD	7.6	MP715
	8	umc1069	220.1/8.08	3.16	-0.12	1.85	OD	3.5	B73
	10	umc1827	132.1/10.04	6.43	-2.42	0.65	PD	14.3	B73
	10	bmc1074	171.1/10.05	5.09	2.24	0.22	A	7.4	MP715
	10	umc2122	189.2/10.06	75.38	6.33	6.49	D	4.1	MP715
Overall	1	phi037	228.5/1.08	2.63	0.02	0.74	OD	2.1	MP715
	2	bmc1520	272.1/2.09	3.18	0.65	0.43	PD	2.9	MP715
	3	bnlg1496	258.0/3.09	3.55	0.76	-0.30	PD	9.7	MP715
	4	dupssr34	208.1/4.07	2.99	-0.39	0.65	OD	8.6	B73
	7	umc2332	229.2/7.04	4.36	-0.75	0.38	PD	18.6	B73

A, additive effect; D, dominance effect; DPE, direction of phenotypic effect
R^2 indicates the percentage of phenotypic variation explained by the QTL.

4.7.8 Number of rows/ear

Only two QTL on chromosomes 7 and 10 were responsible for number of rows/ear in 2012 (Table 4.16). Both QTL were smaller and accounted for 2.9% and 4.6% phenotypic

variance, respectively. In contrast, six QTL were identified in 2013 on chromosomes 4 (bin 4.11), 5 (bin5.02), 8 (bin8.08), 9 (bin 9.02) and 10 (10.04 and 10.05). The QTL region linked with marker bmc1046 on chromosome 5 accounted for large phenotypic variance (51%), but was not identified either in 2012 or combined analysis. Another QTL with large phenotypic effect (15%) was identified on chromosome 10 (10.05). Combined analysis over the years identified only one minor QTL on chromosome 10 (10.05), which accounted 6% of the total phenotypic variance. One QTL linked with marker bmc1074 on chromosome 10 was identified in 2012, 2013, and as well as in combined analysis but it was minor in nature. Another major QTL in chromosome 10 was identified in 2013 but not in 2013 and in combined analysis.

Table 4.16 Quantitative trait loci for number of rows/ear detected in the $F_{2:3}$ mapping population developed from the cross B73 x MP715.

Year	Chr	Closest marker	Position/Bin	LOD	Gene effect		Gene action	R^2	DPE
					A	D			
2012	7	umc1301	183.9/7.03	3.66	0.15	0.85	OD	2.9	MP715
	10	bmc1074	186.2/10.05	2.77	-0.66	-0.37	PD	4.6	B73
2013	4	umc2289	307.1/4.11	2.69	1.78	-0.30	A	5.6	MP715
	5	bmc1046	23.5/5.02	2.6	-2.04	2.16	D	51.3	B73
	8	umc1069	220.1/8.08	3.69	-0.26	2.19	OD	4.7	B73
	9	dupssr19	53.2/9.02	2.58	1.85	-0.46	PD	5.3	MP715
	10	umc1827	132.1/10.04	6.42	-2.91	0.90	OD	14.8	B73
Overall	10	bmc1074	172.2/10.05	2.56	-0.35	0.34	D	5.6	B73

A, additive effect; D, dominance effect; DPE, direction of phenotypic effect
R2 indicates the percentage of phenotypic variation explained by the QTL.

4.7.9 Number of kernels/row

Many QTL were identified for number of kernels/row in both years 2012, 2013, and combined analysis (Table 4.17). Five QTL were identified on 5 different chromosomes in 2012. QTLs located on chromosomes 3 (3.06), 4 (4.07) and 8 (8.09) accounted for > 10% of the phenotypic variance. The largest QTL was located in chromosome 8 (8.09) with 20.6% of the

phenotypic variance. Other two QTL on chromosomes 5 (5.07) and 7 (7.02) were smaller and each accounted for < 10% of the phenotypic variance.

Six QTL were identified in 2013 on chromosome 4(4.06), 5 (5.06), 8 (8.08 and 8.09), and10 (10.04). QTL on chromosomes 4 and 10 linked to umc1827 were larger with larger phenotypic variance 58.5% and 15.0%, respectively. Rest of the QTL was with less than 5% phenotypic effect. Combined analysis over the years identified large number of QTL. One QTL with small effect was identified on chromosome 2 (2.03) but no QTL was identified on

Table 4.17 Quantitative trait loci for number of kernels/row detected in the $F_{2:3}$ mapping population developed from the cross B73 x MP715.

Year	Chr	Closest marker	Position/Bin	LOD	Gene effect		Gene action	R^2	DPE
					A	D			
2012	3	umc1798	147.9/3.06	2.58	-1.75	2.17	OD	11.5	B73
	4	dupssr34	208.1/4.07	8.46	-2.19	3.33	OD	23.0	B73
	5	bmc1118	21.5/5.07	2.63	-0.53	2.00	OD	4.6	B73
	7	phi034	137.2/7.02	4.75	0.18	3.37	OD	6.8	MP715
	8	dupssr14	241.1/8.09	4.34	-2.34	2.06	D	20.6	B73
2013	4	mmc0371	181.7/4.06	19.08	-15.37	13.39	D	58.5	B73
	5	umc2198	0.0/5.06	5.42	0.71	6.40	OD	2.5	MP715
	8	umc1069	220.1/8.08	4.39	0.36	5.86	OD	3.5	MP715
	8	phi233371	264.5/8.09	3.2	-0.71	5.69	OD	4.3	B73
	10	umc1827	132.1/10.04	6.5	-6.90	2.00	PD	15.0	B73
	10	umc1648	163.1/10.04	2.55	4.55	-0.09	A	4.9	MP715
Overall	2	umc1185	91.2/2.03	3.12	-0.54	1.57	OD	4.9	B73
	4	dupssr34	208.1/4.07	7.45	-2.02	2.33	D	19.0	B73
	5	bmc1416	8.0/5.00	4.03	-0.59	2.83	OD	5.7	B73
	7	phi034	124.7/7.02	3.14	-0.23	2.16	OD	3.5	B73
	8	dupssr14	246.5/8.09	8.89	-2.27	2.20	D	23.6	B73
	10	umc1381	115.0/10.03	2.55	1.55	-0.54	PD	5.7	MP715
	10	umc1827	132.1/10.04	2.70	-1.72	0.39	PD	5.5	B73

A, additive effect; D, dominance effect; DPE, direction of phenotypic effect
R^2 indicates the percentage of phenotypic variation explained by the QTL.

chromosome 2 in 2012 and 2013. QTL regions in chromosomes 5 and 8 were identified in 2012, 2013 as well as in combined analysis. A minor QTL on chromosome 5 flanked by markers umc2198 and bmc1118 was identified in both years. One QTL on chromosome 8 appeared consistent in both years and in combined analysis. This QTL region was flanked by the same markers dupssr14 and phi233374. The effect was higher in 2012 (20.6%) and combined analysis (23.6%) but was very small in 2013 (4.3%). The genomic regions in chromosome 5, 8, and 10 were consistently identified. Furthermore, one QTL in chromosome 8 appeared consistently over the years and in combined analysis.

4.7.10 100-kernel weight

More QTL were identified for 100-kernel weight in 2012 compared to 2013 (Table 4.18). They were located on chromosomes 1, 2, 6, and 10. The QTL in bins 10.05 and 2.03 accounted for higher phenotypic variance but was not identified in 2013 or combined analysis. The remaining were minor QTL that accounted<10% of the total phenotypic variance. Only three QTL were detected on chromosomes 4, 9, and 10 in 2013. One small effect QTL was identified on chromosome 4 flanked by common markers umc1503-umc2289 in 2013 and in combined analysis. Likewise, the QTL region on chromosome 10, flanked by markers umc1827 and umc1648, in 2012 and 2013 may be the same QTL. Combined analysis revealed large number of QTLs on chromosomes 1, 3, 4, 5, 6, 7, and 8. Two QTL on chromosome 1 were flanked by markers umc1082 and umc2223 in 2012 and combined analysis. QTL with large phenotypic effect (9.3% and 12.9% in 2012 and combined analysis, respectively) were located in the same genomic segment. Similarly, QTL region on chromosome 10, identified in 2012and 2013, was linked to markers umc1648 and umc1827, respectively. Since both markers were in the same bin of chromosome 10 and 25 cM apart from each other, the two QTLs may be the same.

Table 4.18 Quantitative trait loci for 100 kernel weight detected in the $F_{2:3}$ mapping population developed from the cross B73 x MP715.

Year	Chr	Closest marker	Position/Bin	LOD	Gene effect		Gene action	R^2	DPE
					A	D			
2012	1	umc2223	282.7/1.10	2.62	-1.46	0.74	PD	9.3	B73
	2	umc1185	96.4/2.03	2.73	-1.16	1.64	OD	18.2	B73
	6	umc1257	61.9/6.03	5.15	1.72	1.55	D	6.2	MP715
	10	umc1648	165.1/10.04	2.83	-0.55	1.79	OD	6.5	B73
	10	bmc1074	190.2/10.05	3.66	-2.02	1.89	D	54.0	B73
2013	4	umc2289	305.1/4.11	3.13	3.01	0.33	A	5.6	MP715
	9	umc1714	187.8/9.08	2.68	-2.77	2.26	D	10.9	B73
	10	umc1827	132.1/10.04	6.97	-3.92	1.47	PD	17.6	B73
Overall	1	dupssr26	126.4/1.04	5.18	-1.36	1.09	D	32.9	B73
	1	bmc1955	257.7/1.08	3.13	-1.09	0.69	PD	14.1	B73
	1	umc1082	276.7/1.09	3.04	-1.06	0.74	PD	12.9	B73
	3	umc1978	150.7/3.06	4.39	-0.90	1.46	OD	13.4	B73
	4	umc1503	296.1/4.09	3.83	0.27	1.52	OD	3.9	MP715
	5	umc1155	211.8/5.05	2.70	-1.10	0.47	PD	10.7	B73
	6	umc1887	78.7/6.03	5.11	0.89	0.63	PD	4.6	MP715
	7	mmc0171	64.6/7.00	2.88	-0.44	1.41	OD	9.6	B73
	8	bmc1782	94.8/8.02	2.75	-0.47	1.04	OD	7.0	B73

A, additive effect; D, dominance effect; DPE, direction of phenotypic effect
R^2 indicates the percentage of phenotypic variation explained by the QTL.

4.7.11 Grain yield

Grain yield is the most important trait in every crop and large number of QTL were identified in 2012, 2013, and combined analysis (Table 4.19) and many of these QTL were consistent over years. Five QTL were identified on chromosome 5, 8, and 10 with small phenotypic effects in 2012. Two QTL on chromosome 5 between markers bmc1208 and umc1155 might be same as they were located in the same region. In 2013, six QTLs were identified and new QTL were identified on chromosomes 3, and 6 in addition to 5, 8, and 10. One QTL with large phenotypic effect (58.9%) was identified in chromosome 8 but was not

identified in 2012 and combined analysis. The QTL region identified on chromosome 10 in 2013 was also identified in combined analysis and was linked to marker umc1827. The phenotypic variance explained by this QTL was higher in 2013 (14.2%) than combined analysis (7.8%).

Table 4.19 Quantitative trait loci for grain yield detected in the $F_{2:3}$ mapping population developed from the cross B73 x MP715.

Year	Chr	Closest marker	Position/Bin	LOD	Gene effect		Gene action	R^2	DPE
					A	D			
GY12	5	bmc1208	130.9/5.04	2.56	-4.13	41.59	OD	4.2	MP715
	5	umc1155	214.8/5.05	2.7	-9.97	39.37	OD	7.3	MP715
	5	umc2198	1.0/5.06	3.11	-24.28	40.12	OD	8.8	MP715
	8	dupssr14	256.5/8.09	3.24	-1.97	59.32	OD	4.1	MP715
	10	phi117	0.0/10.00	2.95	-1.83	38.56	OD	3.2	MP715
GY13	3	mmc022	123.9/3.05	3.8	-30.15	126.34	OD	7.4	MP715
	5	bmc1237	180.8/5.05	2.97	-67.25	-6.49	A	4.7	MP715
	6	umc1020	151.1/6.05	2.68	79.57	49.73	PD	4.1	B73
	8	umc1069	234.1/8.08	5.56	-24.07	181.06	OD	58.9	MP715
	8	phi233371	260.5/8.09	7.61	-8.46	194.20	OD	7.6	MP715
	10	umc1827	132.1/10.04	3.97	-96.77	67.91	PD	14.2	MP715
Overall	3	mmc022	127.9/3.05	3.41	-14.70	83.72	OD	6.6	MP715
	5	bmc1416	1.0/5.00	2.91	-24.18	45.59	OD	7.3	MP715
	5	umc1155	200.8/5.05	2.78	-37.09	63.49	OD	18.7	MP715
	7	dupssr11	164.2/7.02	4.36	-24.76	75.63	OD	16.8	MP715
	8	dupssr14	237.1/8.08	3.99	-33.47	80.04	OD	55.0	MP715
	8	phi233376	260.5/8.09	6.88	-14.99	111.58	OD	10.3	MP715
	10	umc1827	132.1/10.04	3.14	-31.27	39.77	OD	7.8	MP715

A, additive effect; D, dominance effect; DPE, direction of phenotypic effect
R^2 indicates the percentage of phenotypic variation explained by the QTL.

The QTL on chromosome 5 and 8 appeared consistently in 2012, 2013, and in combined analysis. The QTL on chromosome 5 was flanked by markers bmc1237 and umc1155. The phenotypic variance accounted by this QTL was 7.3%, 4.7%, and 18.7% in 2012, 2013, and in combined analysis, respectively. Likewise, a QTL on chromosome 8 flanked by markers dupssr14 and phi233376 was consistently identified in both years. The phenotypic effect

accounted by this QTL was small in 2012, 2013, but higher in combined analysis. Although there was variation in phenotypic effect in different years, these two QTL on chromosomes 5 and 8 were consistent in both years.

4.8 Identification of Differentially Expressed Genes Using SSH Technique

4.8.1 Phenotypic characterization of maize germplasm

Aflatoxin accumulation in maize germplasm was measured in both 2013 and 2014. Highly significant differences among these germplasm were observed (Table 4.20). Analysis of variance showed no difference for replication, and year for aflatoxin accumulation.

Table 4.20 Mean aflatoxin (log transformed) and raw aflatoxin (ng g^{-1}) accumulation in different maize germplasm in 2013, 2014, and across the years.

Germplasm	Aflatoxin (log-transformed)			Aflatoxin (ng g-1)		
	2013	2014	Across year	2013	2014	Across year
B73	7.13^{ab}	7.45^{a}	7.29^{a}	1347^{b}	1780^{a}	1563^{b}
Mp715	4.15^{c}	4.98^{b}	4.57^{c}	74^{c}	162^{c}	118^{d}
Mp313E	4.18^{c}	4.72^{b}	4.45^{c}	67^{c}	118^{c}	93^{d}
Mo18W	6.35^{b}	7.02^{a}	6.69^{b}	590^{c}	1133^{b}	862^{c}
Va35	7.85^{a}	7.62^{a}	7.73^{a}	2633^{a}	2067^{a}	2350^{a}
Mp420	4.73^{c}	4.82^{b}	4.78^{c}	135^{c}	133^{c}	134^{d}
Mp719	4.56^{c}	4.16^{b}	4.36^{c}	113^{c}	73^{c}	93^{d}
Grand Mean	5.56	5.82	5.69	709	781	745
SE	0.33	0.26	0.20	222	152	145
CV (%)	25.8	27.2	24.8	109.2	137.2	121.4

***** Values with same letter in the column did not differ significantly at P=0.05.

Va35 recorded the highest amount of aflatoxin in both years followed by B73. Both lines were highly susceptible to *A. flavus* infection. Resistant germplasm like Mp715 and Mp313E showed the least amount of aflatoxin accumulation in both years. Mp420 and Mp719 also showed lower aflatoxin accumulation. The resistant reaction of the line Mo18W was moderate

because it showed relatively higher aflatoxin accumulation compared with the resistant germplasm.

Table 4.21 Sources of variation and their *F* values for aflatoxin (log-transformed) raw aflatoxin (ng g^{-1}) and husk cover rating among germplasm grown in 2013, 2014, and across years.

Source	Aflatoxin (log-transformed)			Aflatoxin (ng g^{-1})			Husk cover (1-5)		
	2013	2014	Pooled	2013	2014	Pooled	2013	2014	Pooled
Var	21.21^{**}	30.43^{**}	52.03^{**}	18.97^{**}	32.44^{**}	38.36^{**}	6.35^{**}	18.73^{**}	15.7^{**}
Rep	0.57^{NS}	0.41^{NS}	2.84^{NS}	0.85^{NS}	1.21^{NS}	0.43^{NS}	3.13^{*}	1.64^{NS}	1.30^{NS}
Year	-	-	0.93^{NS}	-	-	1.29^{NS}	-	-	3.91^{*}
Var*Year	-	-	1.13^{NS}	-	-	0.87^{NS}	-	-	0.70^{NS}

Similarly, husk cover, which was positively correlated with aflatoxin accumulation, was found highly significant among germplasm in both years and across the year (Table 4.21). Mean husk cover rating of these germplasm in both years is presented in Table 4.22. The highly susceptible varieties like B73 and Va35 showed the highest husk cover rating in both years.

Table 4.22 Mean husk cover rating (1-5 scale) in different maize germplasm in 2013, 2014, and across the years.

Germplasm	2013	2014	Across year
B73	4^{a}	3^{b}	3.3^{b}
Mp715	1^{c}	1^{d}	1.0^{e}
Mp313E	2^{bc}	1^{d}	1.3^{de}
Mo18W	3^{ab}	2^{cd}	2.3^{c}
Va35	4^{a}	5^{a}	4.7^{a}
Mp420	2^{bc}	1^{d}	1.3^{de}
Mp719	2^{bc}	2^{bc}	2.0^{dc}
Grand Mean	2.4	2.1	2.3
SE	0.49	0.34	0.33
CV (%)	63.5	59.0	69.6

* Values with same letter in the column did not differ significantly at P=0.05.

Resistant germplasm Mp715 showed lowest husk cover rating. The husk cover rating in other resistant germplasm such as Mp313E and Mp420 was also low. Mo18 showed intermediate

husk cover rating which was a moderately resistant germplasm as evidenced from the data on aflatoxin accumulation (Table 4.21).

Significant difference was observed for all measured agronomical attributes among these germplasm in both years as well as across the year (Table 4.23; Appendices 4-6). Mean comparison for pooled analysis for these traits revealed that Mp715 took the maximum number of days for silking (82 DAS) followed by Mo18W. Va35 was the earliest among these germplasm.

Table 4.23 Mean value of different morphological and yield attributes in different maize germplasm (Averaged across 2013 and 2014).

Germplasm	PH	EH	SD	EL	EC	RN	GR	KW	GY
B73	167^{c}	87^{d}	67^{d}	14.0^{c}	13.3^{ab}	12^{ab}	16^{ab}	17.5^{d}	123.2^{b}
Mp715	206^{ab}	118^{a}	82^{a}	15.3^{bc}	12.7^{b}	12^{b}	20^{a}	20.5^{bc}	78.5^{c}
Mp313E	215^{a}	113^{a}	76^{b}	15.9^{bc}	12.8^{b}	12^{b}	19^{a}	21.3^{ab}	133.7^{b}
Mo18W	195^{b}	98^{c}	81^{a}	21.0^{a}	14.0^{a}	12^{ab}	18^{a}	19.8^{c}	225.7^{a}
Va35	116^{d}	55^{e}	64^{e}	15.3^{bc}	13.0^{ab}	12^{ab}	13^{b}	21.8^{a}	215.7^{a}
Mp420	197^{ab}	106^{b}	73^{c}	16.2^{bc}	13.7^{ab}	13^{ab}	18^{a}	17.9^{d}	132.5^{b}
Mp719	188^{b}	89^{d}	73^{c}	16.3^{b}	13.4^{ab}	13^{a}	19^{a}	18.2^{d}	128.2^{b}
G Mean	183	95	74	16.3	13.3	12	18	19.6	148.2
SE	6.0	2.0	0.6	0.7	0.4	0.53	1.4	0.4	6.8
CV (%)	19.4	21.3	9.0	16.2	7.2	9.9	23.0	9.4	35.0

*Values with same letter in the column did not differ significantly at 0.05.
PH, Plant height; EH, Ear height; SD; Silking days; EL, Ear length; EC, Ear circumference; RN, Number of rows/ear; GR, Grains/ rows; KW, 100 kernel weight; GY, Grain yield/plot.

Ear length was the highest in Mo18W followed by Mp719 and Mp420. Highest number of grains/row was observed in Mp719 followed by Mp715 and Mp313E, which were statistically similar. Highest 100 kernel weight was observed in Va35 (21.8gm) followed by Mp313E and Mp715. Mo18W recorded the highest grain yield/plot (225.7 gm).

The relationship between morphological traits in corn germplasm was studied using Pearson's correlation coefficients (Table 4.24). There was significant positive correlation between aflatoxin accumulation and husk cover rating in these germplasm. Germplasm with open husk like B73 and Va35 were highly susceptible to *A. flavus* and aflatoxin accumulation. Similar relationship was also observed for $F_{2:3}$ mapping population in our field experiment in 2012 and 2013. Highly significant negative correlations were observed between number of kernels/row with aflatoxin accumulation and husk cover in 2013 but not in 2014. Likewise, significant positive correlation between ear circumference and ear length was observed in 2014 but not in 2013.

Table 4.24 Pearson's correlation coefficients among phenotypic traits in maize germplasm in 2013 (normal font) and 2014 (bold font).

	AF	HC	EL	EC	RN	KR	KW	GY
AF	1	**0.77***	**0.04**	**0.04**	**0.27**	**-0.28**	**-0.04**	**0.69**
HC	0.96**	1	**-0.37**	**-0.14**	**0.44**	**-0.51**	**0.12**	**0.67**
EL	0.09	0.04	1	**0.86***	**0.34**	**0.38**	**0.00**	**0.38**
EC	0.34	0.28	-0.30	1	**0.66**	**-0.04**	**-0.30**	**0.47**
RN	-0.39	-0.19	-0.51	0.17	1	**-0.45**	**-0.23**	**0.61**
KR	-0.91**	-0.80*	-0.10	-0.07	0.50	1	**0.23**	**-0.24**
KW	0.17	0.08	0.26	-0.75	-0.52	-0.53	1	**0.35**
GY	0.61	0.59	0.75	-0.09	-0.54	-0.53	0.26	1

*Significant at the 0.05 probability level; **Significant at the 0.01 probability level
AF, Aflatoxin concentration (natural log-transformed); HC, husk cover rating (1-5 scale); EL, Ear length; EC, Ear circumference; RN, Number of rows/ear KR, Kernel number/row; KW, 100 kernel weight; GY, Grain yield/plot).

4.8.2 SSH library construction, sequence assembly and EST annotation

To identify the *Aspergillus flavus* induced genes in maize, SSH experiment was performed using *A. flavus* resistant Mp715 and susceptible B73 maize genotypes. Four hundred eighty clones were randomly selected from the SSH-cDNA library and used for Sanger sequencing. For further confirmation of the quality of inserts, these transformed cells were

grown overnight in LB medium containing antibiotic ampicillin with gentle agitation. The plasmid DNA was isolated and amplified with vector specific primers (SP6 and T7). These amplified clones were separated in agarose gel electrophoresis and visualized with Gel Logic 2000 Imaging System (Kodak). The clones with single insert were used for sequencing and further bioinformatics analysis. The PCR amplification of randomly selected clones was shown in Figure 4.17.

Figure 4.17 Amplification of randomly selected clones from suppression subtraction hybridization (SSH) cDNA library. Lane 1 to 21: randomly selected clones. MW: Hi-Lo DNA Marker (Minnesota Molecular Inc, MN)

The vector sequence, repetitive sequence, and extremely short read sequence (less than 80bp) were removed and remaining high quality sequences were used for similarity search and functional annotation. The selected sequences were used for homology search against the GeneBank nonredundant protein database using BLASTX in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). When many sequences showed similarities to same protein, these sequences were considered as single gene. Altogether 300 unique genes were identified from homology search (Appendix 7). These sequences showed high similarities to the nucleotide sequences from maize and sorghum and represented the genes of different biological functions. These differentially expressed genes were then classified into 15 different classes on the basis of their biological functions (Figure 4.18). The groups with the highest number of genes

were metabolism (18.3%), protein regulation/function (12.3%) and protein synthesis (11.7%). The other groups represented in the library were genes related to signal transduction (7.3%), stress response (7.3%), and resistance proteins (8%).

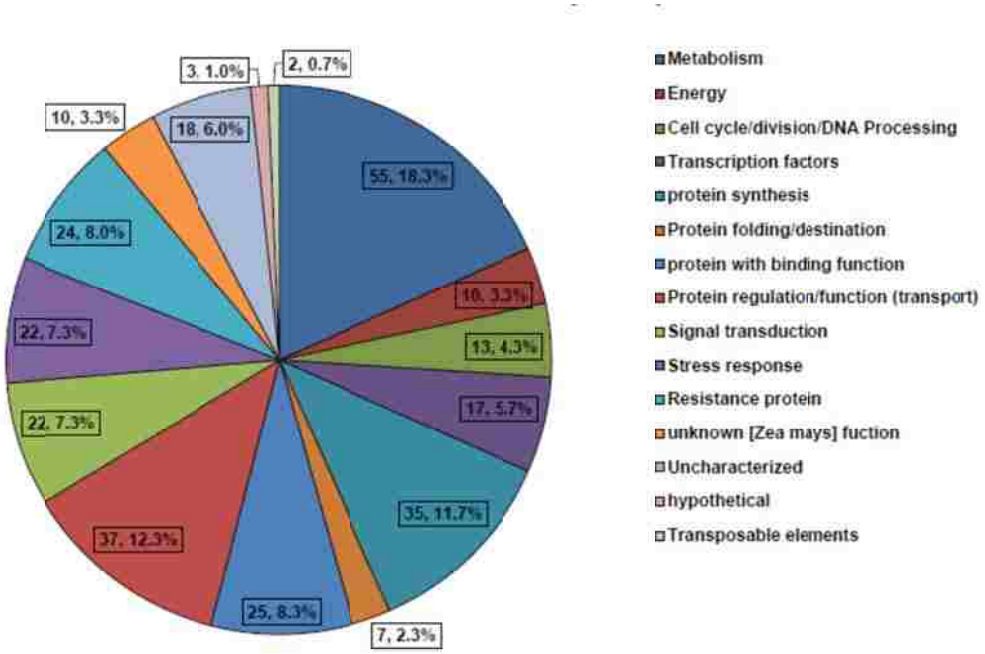

Figure 4.18 Functional categories of differentially expressed genes in SSH library. The number and percentage of unigenes in each functional class was listed in the pie chart.

4.8.3 Reverse Northern to select the differentially expressed genes

Differentially expressed genes were identified using labeled probe of tester (Mp715) or driver (B73) cDNA. Reverse Northern hybridization was performed to select the differentially expressed clones from the 300 selected unique cDNA clones and detail information about these genes are provided in Appendix 7. A representative hybridization blots for differential screening of the genes with different probes is shown in the Figure 4.19. The analysis of the blots showed the strong hybridization signals for most of the clones when probed from *A. flavus* inoculated Mp715 cDNA whereas hybridization signal was weak or absent when probed with inoculated B73 cDNA. Since Mp715 is a resistant line, the clones with strong hybridization signals with

Mp715 were considered as the good candidate genes expressed in response to resistance to fungus infection. Identification and characterization of highly expressed/up-regulated genes would be helpful for achieving resistance to aflatoxin through introgression and genetic manipulation. These differentially expressed genes involved in various biological functions were further described as below.

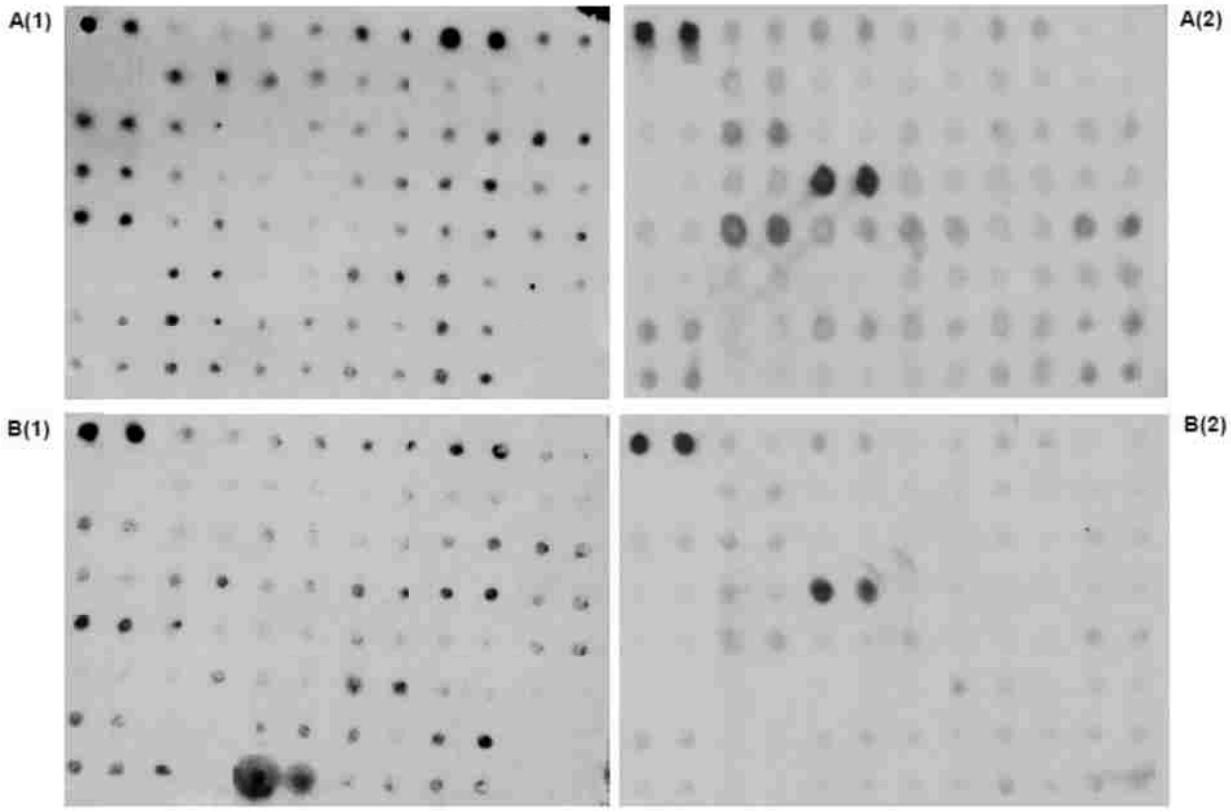

Figure 4.19 Representative differential screening of cDNA clones from Mp715-B73 *Aspergillus flavus* infected forward suppression subtractive hybridization (SSH) library. Each clone was replicated twice on reverse Northern blots and the membranes were hybridized with labeled probes. A (1) and A (2) the clones from library hybridized with inoculated Mp715 probe. B (1) and B (2) the clones from library hybridized with inoculated B73 probe

4.8.3.1 Genes associated with carbohydrate metabolism

Transcriptional changes in various genes involved in carbohydrate metabolism have occurred after the infection with *A. flavus* in developing corn kernels. Suppression subtraction hybridization library prepared from inoculated Mp715 and B73 has identified large number of

genes involved in synthesis and hydrolysis of starch and mobilization of sugars. Genes having high similarity with UDP-sugar pyrophospharylase (P2C01), asparagine synthetase (P2H09), transaldolase (P2H11), trehalsoe-6-phosphate synthase (P2D07), fructose-6-phosphate P4G11), phosphoglyceromutase (P4H09), xylose isomerase (P1B06), fructose-1,6-bisphosphatase (P1E10), putative oxidoreductase (P4H12), phosphofructokinase (P5A06), glutamine-dependent NAD (+) synthetase (P4G06), ADP-glucose pyrophosphorylase embryo small subunit (P5H02), shikimate kinase (P5G04), acyl-thioesterase (P4D10) and beta-galactosidase (P4E08) were highly expressed in reverse Northern hybridization experiment when inoculated Mp715 was used as a probe.

4.8.3.2 Genes associated with signal transduction pathway

Large number of genes (7.3%) were highly expressed in response to *A. flavus* infection that was related to signal transduction. Genes involved in the signal transduction pathways are mainly plant receptor protein kinases (RPK) which are responsible for the perception of pathogen signal and accelerate inducible defense. ESTs highly similar to protein kinase, mitogen activated protein kinase, CBL interacting proteins were highly expressed when probed with cDNA from inoculated resistant inbred. Transcripts highly similar to leukocyte receptor cluster membrane protein (P2E09), CBL-interacting protein kinase (P2B02), alcohol dehydrogenase (serine/threonine protein kinase (P3E11), flower specific gamma-thionin precursor (P3F04), mitogen activated protein kinase 7 (P1A08), plasma membrane associated protein (P1A01), protein tyrosine phosphatase (P3H12) were differentially expressed in SSH library.

4.8.3.3 Transcription factors

Transcription factors control the transcriptional regulation by suppressing or activating the expression of other downstream genes involved as a response of pathogen attack (Guo et al.,

2011). Many genes involved in transcriptional activities were differentially expressed in SSH library. Genes such as elongation factor 1-delta (P2G01), eukaryotic translation initiation factor 3 (P2F08), bZIP transcription factors (P2B01), zinc finger protein binding protein (P2G01), and zinc finger protein (P3C07) were some of the important transcription factors differentially expressed in our library. These were highly expressed in resistant inbred, Mp715, in reverse Northern hybridization experiment.

4.8.3.4 Genes involved in stress response

Many stress response genes were found to express in host-plant after pathogen attack (Cleveland et al., 2004; Lee et al., 2012). Many genes which were previously identified to be involved in the stress response were differentially expressed in our SSH library. Late embryogenesis protein, phosphatase, wound-induced proteins were involved in stress response. ESTs similar to late embryogenesis abundant protein (P4H06), Win1 precursor (P2B05), protein phosphatase 2C (P2B05), wound-induced protein WIN2 Precursor (P5H01), cysteine proteinase inhibitor (P3A03), Dnaj heat shock protein (P2F10), chaperone protein (P3C04), Bowman-Birk type wound induced proteinase inhibitor (P2A03), meloidogyne-induced giant cell protein (P2H09), and stromal ascorbate peroxidase (P5E06) were differentially expressed in our SSH library (Supplementary Table S1). The expressions of these genes were higher in resistant inbred Mp715 after the inoculation of the fungus which was confirmed by reverse Northern analysis.

4.8.3.5 Genes associated with disease resistance

Pathogenesis-related proteins were the mostly induced disease resistance genes in response to pathogen attack by host plant (van Loon et al., 2006; Luo et al., 2011). Large number of genes (8.0%) related to disease resistance were differentially expressed in our library. Several

genes annotated as PR-proteins, chitinase, and multidrug resistance were found in SSH library. ESTs highly similar to pathogenesis related protein such as PR10 (P2H04), PR1 (P1A05), PR4 (P1B07), PR5 (P3H05) were identified in our SSH library. Reverse Northern analysis with inoculated probe of Mp715 showed higher expression for these genes. In addition to these PR proteins, other disease resistance genes such as chitinase (P1G01), multidrug resistance protein (P4G02), vacuolar defense protein (P5B09), leucine rich repeat family (LRR) family protein (P1B05), and Barperm1 (P3C09) were also found differentially expressed in our experiment.

4.8.4 Gene expression analysis in different maize germplasm: Semi-quantitative RT-PCR

To validate the expression pattern of the genes from reverse northern analysis, semi-quantitative RT-PCR was performed for the selected genes involved in important physiological pathway such as disease resistance. Altogether thirty highly expressed genes were analyzed for semi-quantitative RT-PCR analysis at three time points (0 hr, 24 hr, and 48 hr) after *A. flavus* inoculation (Figure 4.20 and Table 4.25). These genes were screened in seven maize inbreds with different level of resistance to *A. flavus* infection (Resistant: Mp715, Mp719, Mp420, Mp313E; Moderately resistant: Mo18W; Susceptible: B73, Va35). These genes showed differential expression pattern at different time points in different resistant and susceptible germplasm. All genes were highly expressed in Mp715 and Mp719 at each time point with the exception of P1A06, P1B05, P4A06, and P5G05. Genes associated with disease resistance such as PR4 (P1B07), leucine rich repeat family protein (P1B05), and PR5 (P3C05) was highly expressed in resistant inbreds. Higher expression in LRR family protein gene (P1B05) was observed at 24 and 48 HAI in resistant inbreds as compared to control. But its expression was reduced at 48 HAI in comparison to control in susceptible inbredVa35. The expression of PR4 increased along with time in resistant inbreds but decreased in susceptible inbred, Va35.

Expression of LRR family protein was increased along with time after fungus inoculation but was extremely low on Mo18W and B73. Its expression decreased along with time in Va35. Trehalsose-6-phosphate (P1A12), a gene involved in carbohydrate metabolism and leukocyte receptor (P2E09), a gene involved in signal transduction were highly induced in resistant germplasm. Embryonic abundant protein (P4F10) which is related to stress was highly expressed in resistant inbreds as compared to susceptible (B73 and Va35).

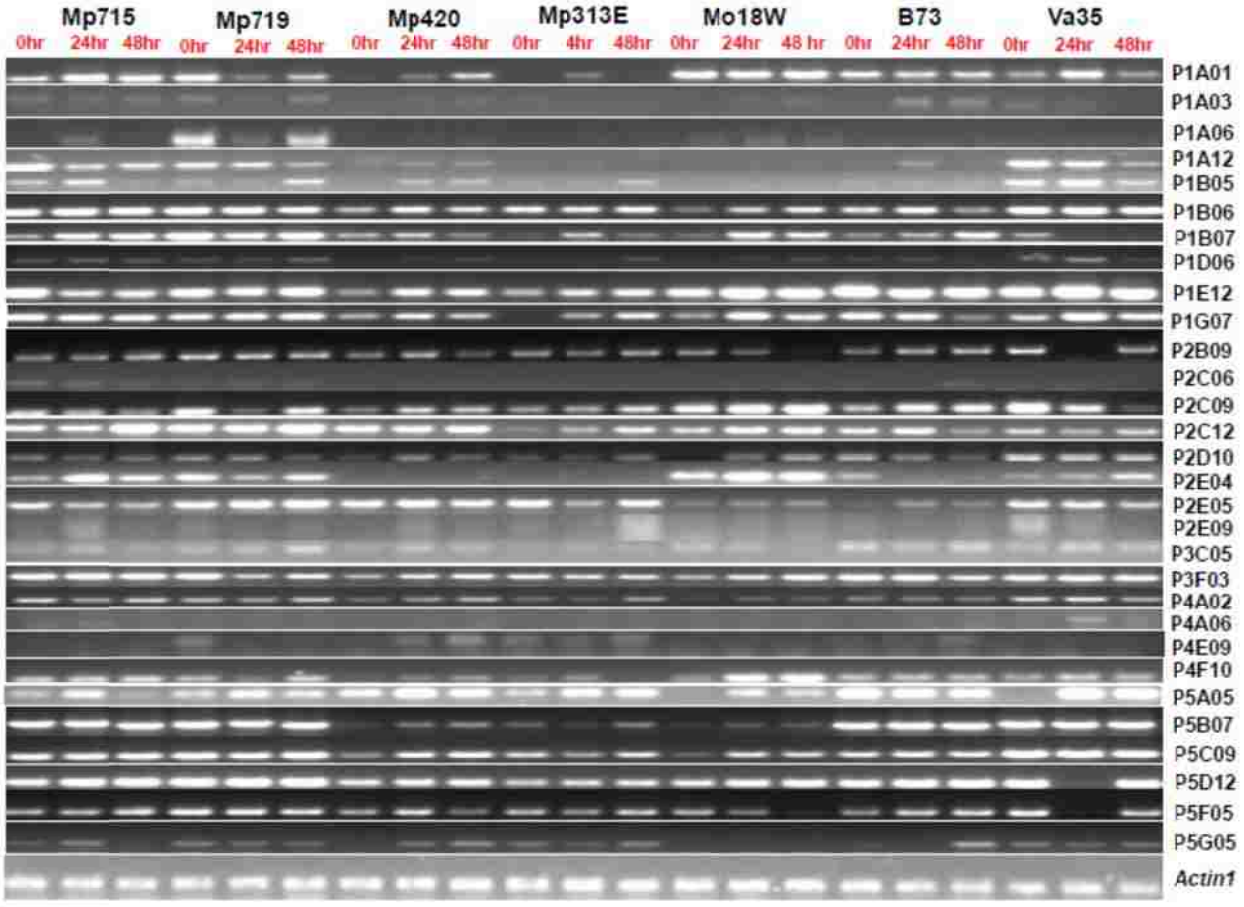

Figure 4.20 Expression pattern of the selected differentially expressed genes from the SSH library of *Zea mays* in different germplasm at different time points after *A. flavus* inoculation. The number in right side of the figure corresponds to the gene provided in the Table 4.26.

Some transcription factors also exhibited higher expression on resistant germplasm. As highly expressed genes in Mp715-B73 library (after reverse Northern hybridization) were

selected for RT-PCR, our hypothesis was that the expression pattern on these germplasm should follow the similar expression pattern. The expression analysis shows the clear differences between resistant and susceptible inbreds for these selected genes. Overall, RT-PCR analysis showed the similar pattern of reverse Northern hybridization. As the genes involved in disease resistance, stress response, and signal transduction were highly expressed all resistant inbreds as compared to susceptible, it shows the possibility of same mechanisms involved in the resistance to aflatoxin. And these genes should be the potential source for further evaluation and introgression through breeding program for the development of resistant inbreds.

Table 4.25 List of the genes used for RT-PCR screening in maize germplasm.

SN	Clone ID	Name of the gene	Sequence (5'-3')
1	P1A01	plasma membrane associated protein [*Zea mays*]	F:TCTACGTGCTCATGCTCCAC R:TTGACACCACGACCTCATGT
2	P1A03	Hypothetical protein	F:GGCATGGAATGGAACTCACT R:CAGGTGCAACATCACAGAGG
3	P1A06	uncharacterized protein LOC100191593 precursor [*Zea mays*]	F:TGACAGTCGGCAATAAGCTG R:CGCAGTTGACGAACTGGTAG
4	P1A12	trehalose-6-phosphate synthase [*Zea mays]*	F:ACCTATGCAGCGGGTATGAC R:CTCCTTCATCGACACAAGCA
5	P1B05	leucine Rich Repeat family protein	F:TGACGGACATATTGCTGCAT R:TTACCATTCGGCTTGAGGTC
6	P1B06	xylose isomerase [*Zea mays*]	F:ATAGCCAAGGAGCTGCTCAA R:CCAGGCTGTAGCCTCAGAAC
7	P1B07	pathogenesis-related protein 4	F:TGACAGTCGGCAATAAGCTG R:CGCAGTTGACGAACTGGTAG
8	P1D06	WD40 repeat-containing protein SMU1-like	F:ACCTCCTTGTCCGCACATAC R:ATGCCAGCATTTCAGAATCC
9	P1E12	metallothionein-like protein type 2	F:ACCACCACCCAGACTGTCAT R:AAGGCGATGGAGCAGATAGA
10	P1G07	nucleoside diphosphate kinase 1 [*Zea mays*]	F:CATCATCAGTCGCTTCGAGA R:CATCCAAGCTCAACTGCTCA
11	P2B09	BETL-9 protein precursor [*Zea mays*]	F:TGCTGCTGAAACTGATTTGG R:CGAGTGGTGTTCTTGCTTGA
12	P2C06	adenine nucleotide translocator [*Zea mays*]	F:TTGTCGATGTCTACCGCAAG R:AGAGGATCTGGAGCTGGTCA
13	P2C09	globulin-1 S allele precursor [*Zea mays*]	F:TCGGGTTCTTCTTCTTCCAA R:TTCACCTGCCTGTAGCTCCT
14	P2C12	eukaryotic translation initiation factor 3 subunit 7	F:AAGTCGCGGAACTTCTTCAA R:GCAACCAAGCTTGTCTCCTC
15	P2D10	activator of 90 kDa heat shock protein ATPase	F:GAACAGCTATCCTGCCCAAG R:CCAGGTGACTGAAGCTGTGA
16	P2E04	putative 23S ribosomal RNA	F:CGCCAACGTGTACCCTTACT R:GCTTGTTGCCGTAGAAGAGG
17	P2E05	cp protein	F:AAGGTGGGGAGCTACTGGTT

SN	Clone ID	Name of the gene	Sequence (5'-3')
			R:CCTGCAGTGAAATGGACCTT
18	P2E09	Leukocyte receptor cluster member-like protein	F:AAGGATGCGAAAAATGGTTG R:ACCCATGTTCCGACTGTAGC
19	P3C05	basic pathogenesis-related protein PR5	F:CGCAAATGCAAAGTTCTCAA R:AACGATCACAGGGACTCCAC
20	P3F03	S-adenosylmethionine synthetase 1 [*Zea mays*]	F:CATGTTTGGGTATGCGACTG R:CACCGTAGGTGTCGATGATG
21	P4A02	hsc70-interacting protein [*Zea mays*]	F:GTGATGCCAATGCTGCTCTA R:CTCTTGGCCTTGTCGTAAGC
22	P4A06	metallothionein	F:TCCACACAACACACAGCTCA R:CCGTCGTAGGAGGAGGAGTA
23	P4E09	chaperonin [*Zea mays*]	F:TCTTCGGCTTGGCTACAGTT R:AAACCCTGGACAAGTTCGTG
24	P4F10	embryonic abundant protein 1 [*Zea mays*]	F:CAGGTCAGGAAAGCAGGAAG R:ACTTGGACTCGTCGATGCTG
25	P5A05	cation transport regulator-like protein 2 isoform	F:TTCGTTTCCACTCCAGATCC R:CAGACGATACAGCCTCACGA
26	P5B07	60S ribosomal protein L7a [*Zea mays*]	F:CGATCGAACTTGTTGTGTGG R:AGAAGCAAAGCTGCATGGAT
27	P5C09	RNA-binding protein 25 [*Zea mays*]	F:GACTATCCCATGGACCGAGA R:ACAGGTCTGTAACCGCCAAC
28	P5D12	lipid transfer protein [*Oryza sativa*	F:TTAGCACTCGCAGGTCACAG R:CGGATGTAGCTCCCGTAGTT
29	P5F05	basic leucine zipper and W2 domain-containing protein	F:TCTGGTTGCCAAAGGGATAG R:CTCGTGCAAAAAGCATTCAA
30	P5G05	ubiquitin C-terminal hydrolase	F:GAGCAAGATTGTGTGGAGCA R: AAACAAATCAGCCGATGACC
31	-	Actin1	F:TGACCTCACCGACCACCTAA R: CCAGGGACGTGATCTCCTTG

4.8.5 Quantitative real-time RT-PCR (qPCR) for gene expression in germplasm

After the evaluation of expression pattern of selected genes through semi-quantitative RT-PCR, six important genes showing high expression and involvement in disease resistance pathway were selected for real-time qPCR to validate the expression at different point of inoculation (Table 4.26 and Figure 4.21). These selected genes were screened on the same set of inbreds. These genes showed differential quantitative expression and the change in expression (fold change) were compared with control (0 hr) in each germplasm at different time point. However, genes like PR4, LRR family protein, RNA binding protein and ubiquitin C-terminal hydrolase were highly expressed among resistant inbreds. PR4 was highly expressed (9.57 fold)

in susceptible inbred Va35 at 48 hours after inoculation (HAI) as compared with control, but extremely low in B73. In other resistant inbreds such as Mp719 and Mp420 its expression increased along with time and was 4.2 and 3.1 times higher as compared with their respective control. In contrast, PR4 exhibited highest expression in Mp715 and Mp313 at 24 HAI and decreased afterwards. Likewise, expression of gene for LRR family protein was slightly higher in B73 compared to Va35 at 48 HAI, but their expression was lower as compared with other resistant inbreds.

Table 4.26 List of genes used for real-time RT-PCR study.

Clone ID	**Name of the gene**	**Sequence (5'-3')**	**Product size(bp)**
P1MP715-B73B07-SP6	pathogenesis-related protein 4	F:TGACAGTCGGCAATAAGCTG R:CTGCTGCGGGTTGTAGAAGT	118
P1MP715-B73B05-SP6	leucine Rich Repeat family protein	F:GTACATTCGGTTGCGATGTG R:TCTGCCACCGGCTCTATACT	121
P3MP715-B73C05-SP6	basic pathogenesis-related protein PR5	F:TTTGGAAGGACCCTGTCTTG R:CGCACACCAATAGAGAGCAA	123
P5MP715-B73A05-SP6	cation transport regulator-like protein 2	F:TCGTGAGGCTGTATCGTCTG R:TCCTCCAAGTTCAAGGATCG	104
P5MP715-B73C09-SP6	RNA-binding protein 25 [*Zea mays*]	F:GGCAACCGTATTGTTCGAGT R:TCATAGCTCCGTTCCCTGTC	137
P5MP715-B73G05-SP6	ubiquitin C-terminal hydrolase	F:TCATTTGCCTTCTTGTCGTG R:TGAACCTTGCCCTATTCAGG	91
-	Actin1	F:GAAACCTTCGAATGCCCAGC R:CACACCATCACCGGAATCCA	105

Expression pattern of gene for LRR family protein (P1B05) shows the similar pattern as PR4 (P1B07) in Mp715, Mp719, and Mp420. PR4 (P1B07), PR5 (P3C05), and LRR repeat family protein gene (P1B05) responded quickly to fungus inoculation and their transcription reached to maximum at 24 HAI and decreased at 48 HAI in Mp715. But, their expression increased along with time in Mp719 and Mp420. Transcript accumulation of cation transport regulator-like protein increased along with time after fungal inoculation in resistant inbreds. Highest fold change (10.35) in expression was observed in Mp719 at 48 HAI as compared to control.

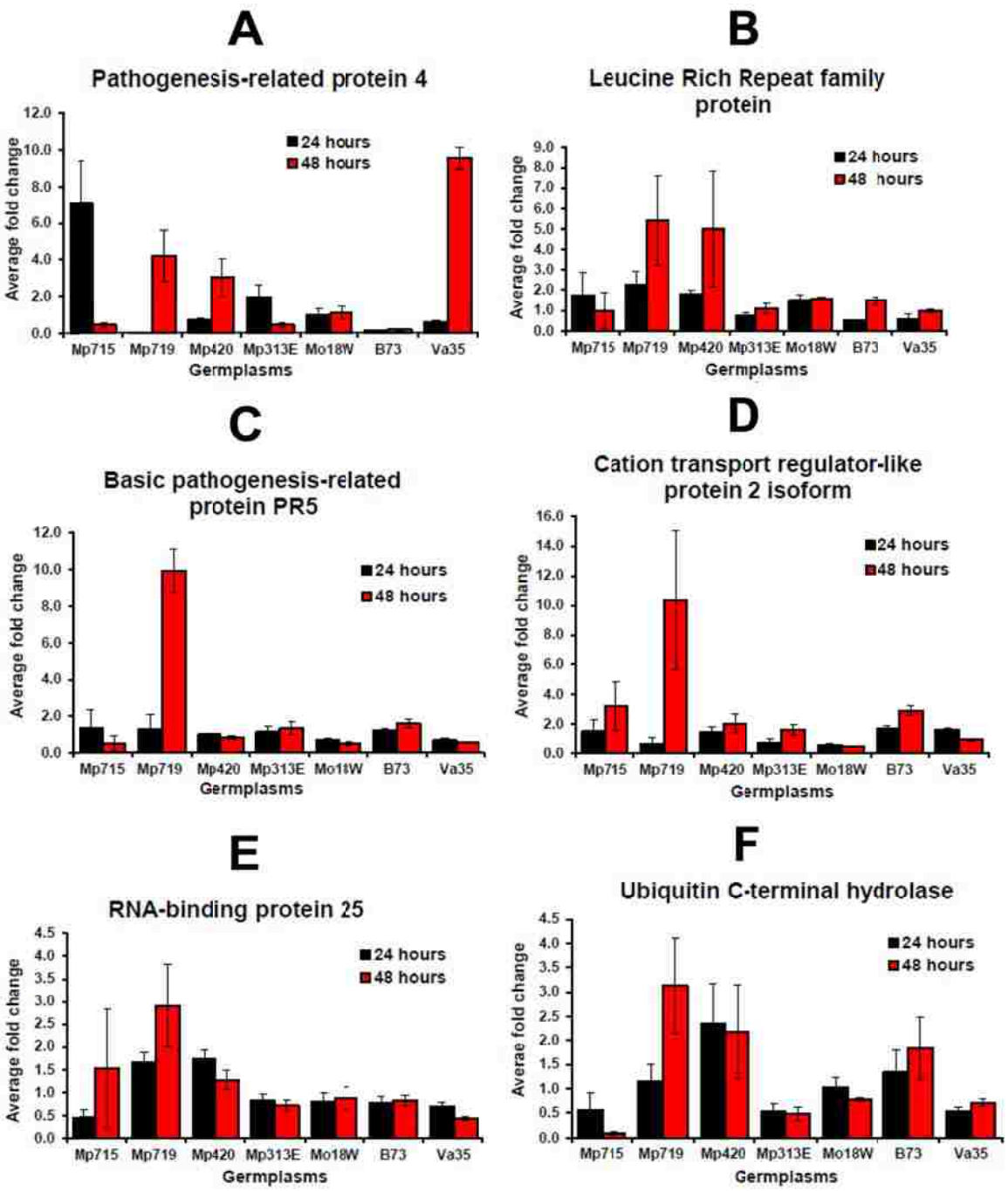

Figure 4.21 Real-time RT-PCR analysis of six genes selected from the subtracted cDNA library. Expression of these genes was evaluated at two time points (24 and 48 hours after inoculation) in different maize germplasm using *Actin* as an internal standard. The average fold change in expression was compared with control in each germplasm. (A) Pathogenesis-related protein 4 (PR 4); (B) Leucine rich repeat family protein; (C) Basic pathogenesis-related protein 5(PR5); (D) Cation transport regulator-like protein 2 isoform; (E) RNA binding protein 25; (F) Ubiquitin C-terminal hydrolase.

In contrast, its expression reduced in Va35 as time advances after inoculation. All six genes were highly expressed at 48 HAI in Mp719, and extremely higher change in their expression between 24 and 48 HAI was observed. PR4 was highly expressed in Mp313E at 24 HAI, but almost similar in 24 and 48 HAI for rest of the genes. However, not much difference in expression was observed between 24 and 48 HAI for all selected genes in Mo18W. The expression pattern of selected genes in qPCR was in agreement with the result of RT-PCR.

4.9 Mapping of differentially expressed genes found in and around QTL regions responsible for resistance to aflatoxin accumulation

The expressed genes identified from suppression subtraction hybridization (SSH) library were mapped on to the maize linkage map generated in this study. The genes with various biological functions were located in and around different bins on the chromosomes where QTLs for aflatoxin resistance were identified in our mapping population (Table 4.27). Large numbers of differentially expressed genes were identified in QTL regions on different chromosomes (Figure 4.22). Many genes are found in the region between the marker umc2150-umc1509 in chromosome 4. These two markers were linked with QTLs responsible for aflatoxin resistance in our population. So, identification and localization of resistance genes in this region will be helpful to select the resistance genes in future. One EST, P1B07, was found in this region has higher similarity to pathogenesis-related protein 4 (PR4), which was associated with disease resistance. Genes related to stress like Win1 precursor (P2B05), Wheatwin-2 (P2HO4), wound induced protein WIN2 precursor, and defense related proteins like putative vacuolar defense protein (P5B09) were identified in this chromosomal region. As their function suggest they were involved in important physiological functions like biotic and abiotic stress and defense. These genes may be important in host-plant resistance mechanism because of their location in the QTL region. Important ESTs, which were highly similar to AAA-type ATPase family protein (P1H01)

and cation transport protein chaC (P5F01), were located between marker umc1171 and bmc1208 where QTL was identified in 2012. Likewise, many differentially expressed genes identified from our library were located in chromosome 10 between the marker umc1381 and umc1827. Genes with high similarity to aspartic proteinase (P1A10) and S-adenosylmethionine synthetase1 (P3F06) were found in this region. In addition, genes highly similar to chitinase (P1G01) and nucleic acid binding protein (P4F06) were located in chromosome 10 near the linked marker umc1827.

Table 4.27 List of the expressed genes from SSH library located in and around QTLs responsible for aflatoxin resistance.

SN	Clone	Protein	Chr	Bin	Homology (%)	E-value
1	P4B09	aldehyde dehydrogenase [*Sorghum bicolor*]	1	1.03	99.0	1.302e-90
2	P4G12	pathogenesis-related protein 1 [*Zea mays*]	1	1.03	100.0	7.337e-93
3	P4D02	40S ribosomal protein S19-like	1	1.04	99.6	9.879e-112
4	P5F10	Bromodomain associated family protein	1	1.05	99.7	0
5	P2F08	eukaryotic translation initiation factor 3 subunit B	1	1.05	99.5	0
6	P1C08	peptidylprolyl isomerase, putative	1	1.06	100.0	1.665e-133
7	P5C02	60S ribosomal protein L17 [*Zea mays*]	1	1.06	97.9	4.558e-115
8	P5B08	pathogenesis-related protein 5 [*Zea mays*]	1	1.06	98.0	1.014e-91
9	P3A07	putative acyl-CoA synthetase	3	3.08	99.2	1.763e-54
10	P2C12	eukaryotic translation initiation factor 3 subunit	3	3.08	98.8	0
11	P1B04	asparagine synthetase2 [*Zea mays*]	3	3.09	98.6	3.894e-63
12	P2E08	protein phosphatase 2C	3	3.09	98.8	0
13	P1A06	uncharacterized protein LOC100191593 precursor	4	4.02	100.0	6.336e-110
14	P1B07	pathogenesis-related protein 4	4	4.02	100.0	6.336e-110
15	P2A08	barwin-like	4	4.02	100.0	6.916e-110
16	P2B01	bZIP transcription factor	4	4.02	99.0	1.270e-100

SN	Clone	Protein	Chr	Bin	Homology (%)	E-value
17	P2B05	win1 precursor [*Zea mays*]	4	4.02	100.0	1.651e-109
18	P2H04	Wheatwin-2	4	4.02	100.0	1.609e-109
19	P3A06	prohevein [*Hevea brasiliensis*]	4	4.02	100.0	1.611e-109
20	P3A11	pi1 [*Solanum lycopersicum*]	4	4.02	100.0	1.542e-109
21	P4C05	pseudo-hevein [*Hevea brasiliensis*]	4	4.02	100.0	1.662e-109
22	P4G05	win2 precursor [*Zea mays*]	4	4.02	99.5	7.691e-108
23	P5B09	putative vacuolar defense protein	4	4.02	100.0	1.606e-109
24	P5H01	Wound-induced protein WIN2 precursor	4	4.02	100.0	7.318e-110
25	P1A08	mitogen activated protein kinase 7	5	5.03	97.9	1.633e-156
26	P1D01	uncharacterized protein LOC100276217	5	5.03	97.2	0
27	P1E09	catalase isozyme B [*Zea mays*]	5	5.03	98.3	1.226e-81
28	P2A01	senescence-associated protein 12	5	5.03	100.0	2.177e-83
29	P2E06	T cytoplasm male sterility restorer factor 2	5	5.03	100.0	7.833e-98
30	P3C07	zinc finger protein-like	5	5.03	98.5	3.200e-95
31	P3A10	acetyl-coenzyme A carboxylase ACC1A	5	5.03	100.0	8.895e-157
32	P4D09	fibropellin III	5	5.03	99.2	1.758e-54
33	P4H12	putative oxidoreductase	5	5.03	99.2	1.958e-123
34	P5H04	putative MAPK [*Zea mays*]	5	5.03	97.9	3.262e-156
35	P3F01	meloidogyne-induced giant cell protein-like protein	5	5.04	100.0	5.689e-114
36	P3F11	lysine ketoglutarate reductase trans-splicing related 1	5	5.04	100.0	1.318e-55
37	P2E03	salt tolerant correlative protein	5	5.04	98.8	6.279e-79
38	P1C09	conserved hypothetical protein	5	5.04	96.1	2.595e-88
39	P1H01	AAA-type ATPase family protein	5	5.04	98.2	1.106e-74
40	P3H01	Cation transport regulator-like protein 2	5	5.04	100.0	5.537e-114
41	P5A05	cation transport regulator-like protein 2 isoform	5	5.04	100.0	5.872e-114
42	P5D10	putative cation transporter	5	5.04	100.0	5.800e-114

SN	Clone	Protein	Chr	Bin	Homology (%)	E-value
		[*Potamogeton distinctus*]				
43	P5F01	cation transport protein chaC [*Zea mays*]	5	5.04	100.0	3.082e-114
44	P3B08	vacuolar processing enzyme1 precursor [*Zea mays*]	5	5.05	100.0	1.285e-105
45	P1E05	Kinase-like protein [*Medicago truncatula*]	5	5.05	97.4	1.052e-87
46	P3D10	lammer-type protein kinase	5	5.05	100.0	9.802e-107
47	P4E10	eukaryotic translation initiation factor 2D-like	5	5.05	100.0	5.504e-154
48	P5E10	legumain	5	5.05	100.0	1.271e-105
49	P5G10	cingulinW1 precursor	8	8.04	100.0	5.375e-159
50	P2E09	Leukocyte receptor cluster member-like protein	8	8.04	100.0	2.000e-68
51	P2B09	BETL-9 protein precursor [*Zea mays*]	8	8.08	96.7	3.804e-65
52	P3C04	chaperone protein dnaj	8	8.08	92.2	8.122e-78
53	P3H04	cysteine proteinase inhibitor [*Zea mays*]	8	8.08	100.0	1.152e-140
54	P4F09	cystatin-1 precursor [*Zea mays*]	8	8.08	100.0	1.203e-140
55	P5H10	cystatin	8	8.08	99.6	1.978e-138
56	P3A09	hemolysin family calcium-binding region	9	9.02	97.7	4.250e-145
57	P3G03	ATP-dependent Clp protease proteolytic subunit 2	9	9.02	100.0	3.725e-66
58	P2D11	Cytochrome P450 family protein	9	9.03	96.8	1.621e-84
59	P3F12	60S ribosomal protein L11	9	9.03	98.1	2.601e-97
60	P4G03	elongation factor 1-alpha [*Zea mays*]	9	9.03	99.3	0
61	P5A10	protein vip1-like	9	9.03	100.0	0
62	P2F06	ubiquitin-conjugating enzyme E2 32-like	9	9.05	99.4	6.928e-178
63	P5A04	serine/threonine-protein kinase AtPK2/AtPK19-like	9	9.05	100.0	2.635e-127
64	P5C04	XK-related protein 8	9	9.05	96.2	6.053e-104
65	P4C07	histone H2A [*Zea mays*]	9	9.06	97.0	7.322e-138
66	P2G01	YT521-B-like family protein	9	9.07	99.4	0
67	P2G10	OmpA/MotB domain-containing protein	9	9.07	99.7	0
68	P3D08	acidic ribosomal protein P40 [*Zea mays*]	9	9.07	99.5	4.662e-100

SN	Clone	Protein	Chr	Bin	Homology (%)	E-value
69	P3H10	acidic ribosomal protein P40 [*Zea mays*]	9	9.07	99.5	4.511e-100
70	P2A10	diphosphonucleotide phosphatase1 [*Zea mays*]	10	10.03	100.0	3.318e-111
71	P2G12	E3 ubiquitin protein ligase UPL1	10	10.03	99.3	0
72	P1A10	aspartic proteinase [*Oryza sativa*]	10	10.03	98.3	2.57E-84
73	P3E08	2-oxoisovalerate dehydrogenase alpha subunit	10	10.03	100.0	1.739e-79
74	P3F03	S-adenosylmethionine synthetase 1 [*Zea mays*]	10	10.03	94.7	1.222e-150
75	P3F04	flower-specific gamma-thionin precursor [*Zea mays*]	10	10.03	100.0	4.275e-120
76	P4F06	nucleic acid binding protein [*Zea mays*]	10	10.04	99.6	7.689e-139
77	P1F06	RND family efflux transporter MFP subunit	10	10.04	99.3	2.490e-62
78	P1G01	chitinase [*Zea mays*]	10	10.04	93.9	1.357e-96
79	P2B12	ubiquinol-cytochrome C reductase iron-sulfur subunit	10	10.04	98.9	2.425e-132
80	P2H03	putative mitochondrial Rieske protein	10	10.04	98.9	2.598e-132
81	P2H10	cytochrome b-c1 complex subunit Rieske	10	10.04	98.9	2.576e-132
82	P3B06	putative 23S ribosomal RNA	10	10.04	99.3	1.065e-61
83	P3D04	cytochrome b-c1 complex subunit Rieske	10	10.04	98.9	2.728e-132
84	P4F02	indole-3-glycerol phosphate synthase-like	10	10.06	91.6	4.182e-170

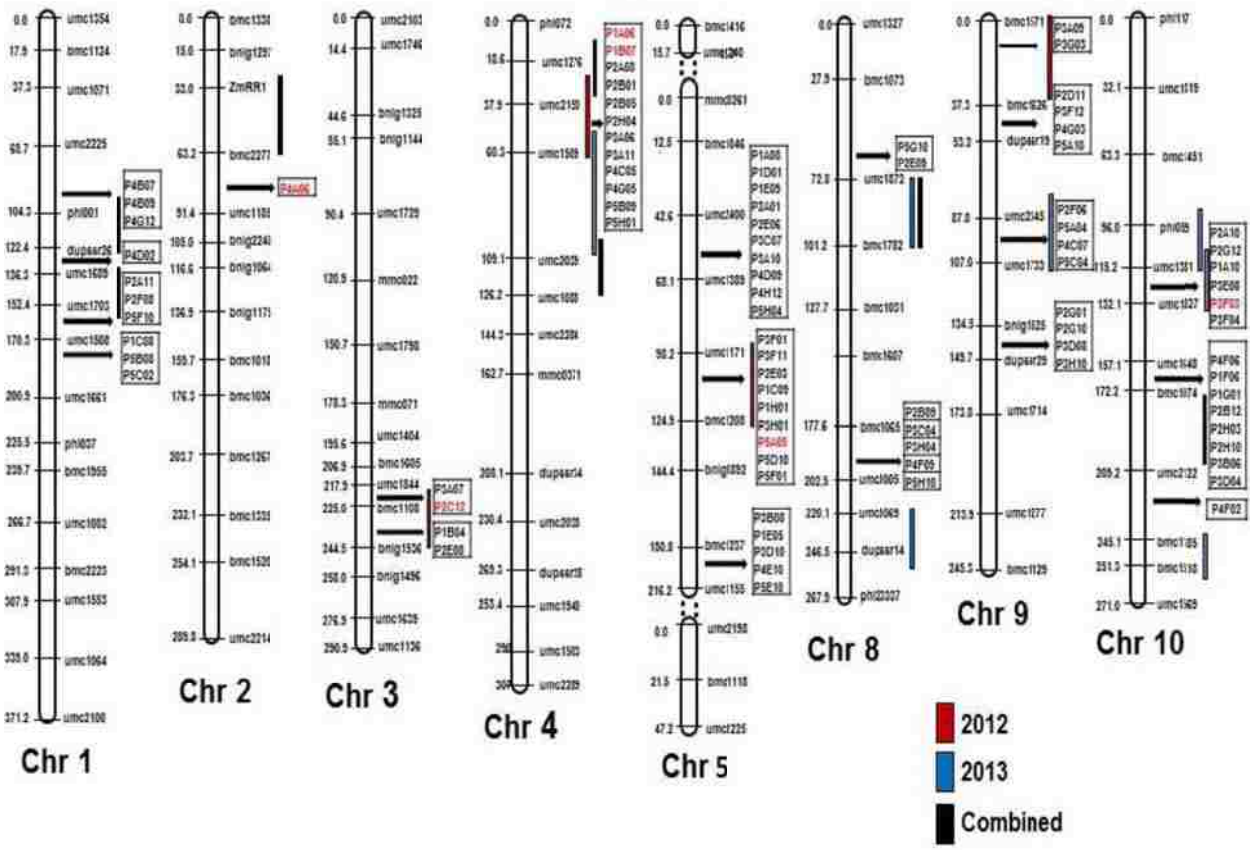

Figure 4.22 Co-localization of cDNA clones from SSH library in and around QTLs associated with aflatoxin resistance in an $F_{2:3}$ population derived from the cross B73 x Mp715. Red, blue, and black bars represent QTLs for aflatoxin resistance in 2012, 2013, and combined analysis respectively. The clones in red font were used in in real-time RT-PCR experiment.

CHAPTER 5
DISCUSSION

5.1 Genetic variation, heritability, and correlations

Many growth and yield attributes were measured in an $F_{2:3}$ mapping population evaluated in replicated field experiments for two years at the LSU AgCenter Central Research Farm, Baton Rouge. Analysis of variance showed significant difference within the mapping population for all morphological traits in both years with the exception of ear length, maturity days, tasseling days, and plant height in 2012. Pooled analysis for various traits showed the significant difference among mapping families for various traits except plant height, ear height, number of rows/ear, number of kernels/row, and grain yield. The difference in morphological traits showed the genetic variability present in the mapping population. Variation in morphological characteristics were also reported in previous experiment involving different types of mapping populations (Sibov et al. 2003; Li et al. 2007; Sabadin et al. 2008; Samayoa et al. 2014). Non-significant genotype (G) x year (Y) interaction for some traits like plant height, maturity days, husk cover, ear length, and number of rows, suggested less influence of environment and consistency in trait manifestation.

Aflatoxin accumulation in grain after artificial inoculation of *A. flavus* was the main trait of interest in our study. Raw aflatoxin and log-transformed values were highly significant in both years as well as in combined analysis. All other source of variation such as replication, year, and genotype (G) x year (E) were highly significant. Similar results were obtained from previous mapping experiments for resistance to aflatoxin accumulation (Widstrom et al. 2003; Paul et al. 2003; Busboom and White 2004; Brooks et al. 2005; Robertson. 2007; Bello 2007; Warburton et al. 2009, 2011; Mayfield 2011). Variability and inconsistent nature of this trait in different

experimental conditions such as high temperature and moisture stress during silking period are known to favor aflatoxin accumulation (Payne 1992).

High heritability was observed for traits like plant height, tasseling, days to silking, maturity, and ear length. Intermediate heritability was observed for ear height and number of rows/ear. Higher heritability for plant height and ear height was reported by Sibov et al. (2003). Lower heritability was observed for husk cover, ear circumference, 100-kernel weight, and aflatoxin accumulation. The estimated heritability for aflatoxin accumulation in our experiment was low (0.35), which is in agreement with earlier reports (Betrán et al. 2002, 2006; Warburton et al. 2009, 2011; Willcox et al. 2013). Low heritability observed for this trait suggested that multi-location testing should be done for increasing selection response.

The aflatoxin content was positively correlated with husk cover rating. Highly significant positive and consistent correlation was observed between aflatoxin accumulation and husk cover ratings in both years of field evaluation (Table 4.4). Aflatoxin accumulation was found to increase up to 200% when inoculated with southeastern corn borer (Williams et al. 2002). There were no concrete evidences to correlate the open husk characteristics with high accumulation of aflatoxin till now, but earlier study suggested the role of tight husk for the prevention of fungal spores to the ear (Barry et al. 1986). The open husk cover provides the easy access to rain water, moisture, and ear feeding insects. The insect damage of ears creates highly favorable environment for fungus establishment, growth, and infection leading to high accumulation of aflatoxin in grain. Further investigations and field evaluations are necessary to verify the relationship between open husk and aflatoxin so that it could provide an indirect means to reduce aflatoxin accumulation. Likewise, ear circumference and number of rows were positively correlated to aflatoxin accumulation but were highly significant only in 2013 (Table 4.4). Larger

ear circumference and rows number increases the ear size and ear surface area, which provides the higher surface area for the growth and development of fungus leading to more accumulation of aflatoxin. These traits with high correlation can be selected simultaneously during selection and could be used for the improving aflatoxin resistance.

5.2 QTL mapping of aflatoxin content and husk cover

High level of polymorphism observed between parents was helpful for construction of linkage map. The order and position of markers in our map was in correspondence with other maps from maize genome database and other published map (Lawrence et al. 2008; www.maizegdb.org). All chromosomes were resolved in to single linkage group except linkage group 5. There was some rearrangement on marker position in chromosome 9 which could be due to inversion or some genotyping errors. Warburton et al., (2011) also reported rearrangement of marker positions in chromosome 2 in a population derived from Mp715 x T173.

Previous mapping studies on aflatoxin resistance using different mapping population identified several QTL all over the maize genome. Our mapping experiment identified QTL with varying phenotypic effects on all chromosomes. QTL alleles from both parents were responsible for reducing aflatoxin accumulation. QTL with moderate phenotypic variance (9%) was detected in chromosome 3 (bin 3.08) only in 2012. However, past experiments identified QTL in a nearby chromosome region (bins 3.05-3.07) from resistant parents (Paul et al. 2003; Brooks et al. 2005; Willcox et al. 2013). Combined analysis identified QTL in chromosome 1 (bin 1.04), which might be the same QTL with similar effect identified in bin 1.03 by Brooks et al. (2005). These genomic regions (1.03-1.04) may be important sources of resistance as favorable allele were from resistant parent.

QTL in bin 4.02 (*qAFL4-2*) was identified in 2013 and combined analysis whereas *qAFL4-1* was identified only in 2012. Several previous studies identified QTL on chromosome 4 using diverse mapping populations that were evaluated in different environments (Brooks et al. 2005, Mayfield et al. 2011; Warburton et al. 2011; Willcox et al. 2013). The positions of previous QTL were different from our mapping study. The QTL with highest phenotypic variance (10%) was identified in 4.05 in combined analysis. The consistency of this QTL with earlier mapping studies suggests usefulness of this resistant QTL for corn improvement. One QTL with moderate effect was confirmed using near-isogenic lines in 4.08 recently (Mideros et al. 2014). This QTL was earlier identified by Paul et al. (2003). Similarly, QTL in bin 4.06 was reported in multiple mapping studies (Brooks et al. 2005; Warburton et al. 2011; Willcox et al. 2013). The genomic location of QTL (bin 4.01) identified in our experiment is different from previous mapping study and thus may be new source of resistance. The QTL on chromosome 5 identified by other researchers (Warburton et al. 2009, 2011; Yin et al. 2014) were in the nearby region of the chromosome (5.03 and 5.06) in the present study.

Most of the reported QTL for resistance to aflatoxin accumulation were time, location, and genotype specific. Stable QTL with high phenotypic effects were not identified due to the complex genetics of this trait. We have identified a QTL *qAFL8*-1, which might correspond to QTL reported by Willcox et al. (2013) and Yin et al. (2014) in bin interval 8.02-8.04. The phenotypic effects were very small and the favorable allele was contributed by the susceptible parent, B73 like previous studies (Warburton et al. 2009, 2011; Willcox et al. 2013; Yin et al. 2014). The QTL identified in bin 8.09 in our experiment may be novel source of resistance. The QTL located in bin 9.06 was reported by Warburton et al. (2011) in mapping population

involving Mp715. Although the phenotypic effect of these QTL was small, it still shows the importance of the donor line Mp715 as a resistance source.

Although QTL were identified on chromosomes 4 and 9 in both years, their locations were not consistent. The QTL identified on chromosome 4 in the adjacent regions (4.01 and 4.02 in 2012 and 2013, and combined analysis, respectively) were not reported in any of the previous studies. Fine mapping using large population can further confirm the presence of QTL in this region. The occurrence of QTL on different regions of chromosome 9 (bins 9.02 and 9.06) in both years indicated the presence of resistance genes in these regions. Significant QTL were identified on chromosome 9 in different mapping populations involving Mp313E (Willcox et al. 2013). Mp313E and Mp715 are widely used source of resistance to reduce aflatoxin (Williams and Windham 2001). Therefore, the markers associated with the QTL consistently detected in different mapping populations can be used for the selection of resistant inbreds in marker assisted breeding program.

Aflatoxin accumulation in corn can be reduced through indirect selection for morphological traits (Betrán and Isakeit 2004; Odvody et al. 1997). Bello (2007) reported that aflatoxin accumulation and secondary traits like grain texture, flowering time, and maturity were correlated. But the effect of open husk on the accumulation of aflatoxin in a genetic mapping population was not investigated. We observed highly positive significant and consistent correlation between aflatoxin concentration and husk cover rating in both years of field experiments (Table 4.5) suggesting the role of tight husk cover in lowering aflatoxin accumulation. The closed husk cover prevents easy access to moisture and ear feeding insects. The insect damage of ears creates highly favorable environment for fungus establishment, growth, and infection leading to high aflatoxin accumulation in grains. Williams et al. (2002)

reported 200% increase in aflatoxin accumulation when artificially infested with southeastern corn borer larvae. Thus, further field evaluations should be conducted to explore utilization of this morphological trait to reduce aflatoxin accumulation without compromising productivity in corn.

Most QTL alleles for reducing husk cover (close husk) were derived from the aflatoxin resistant parent, Mp715. The QTL responsible for husk cover and resistance to aflatoxin accumulation co-localized on the same region of chromosomes 4, 8, and 10. Favorable QTL alleles for both traits were from resistant parent in chromosome 4 (bin 4.01-4.02) in 2012 with small phenotypic effect. QTL for aflatoxin (*qAFL8-1*) and husk cover (*qHC8-3*) were identified in the same genomic region (bin 8.05) in 2012 and 2013, respectively. Previous mapping studies also found aflatoxin resistance QTL on chromosome 8 (Warburton et al. 2009; Yin et al. 2014) and the later study found 3 QTL in bin 8.03. Yin et al., (2014) reported three QTL responsible for aflatoxin resistance on chromosome 10 (bins 10.04 and 10.07) and largest QTL responsible for reducing husk cover (*qHC10-1*) was identified in bin 10.03 in 2013 and combined analysis. This observation provides evidence for presence of QTL responsible for both traits in the genomic region of chromosome 10 (bin interval 10.03-10.04). As these two traits were positively correlated, selection for closed husk cover offers opportunity to improve resistance to *A. flavus* infection.

5.3 QTL mapping for other traits

Several studies have been conducted to map QTL for various agronomically important traits in corn (Cardinal et al. 2001; Austin et al. 2001; Lima et al. 2006). In our study, many QTL responsible for different morphological and yield attributing traits were identified. These QTL were located all over the genome and QTL for many traits were clustered on many regions of the

genome. These genomic regions coincided with QTL identified in different mapping populations in earlier studies.

Few QTL were identified on chromosomes 2, 4, 6, 8, and 10 for plant height. Previous mapping studies identified QTL on these chromosomes but in different bins (Lima et al. 2006; Li et al. 2007; Park et al. 2014; Samayoa et al. 2014). Many QTL for ear height were identified in 2013 compared to 2012. One QTL in bin 9.03 was identified in both years. Other QTL were located in chromosomes 4, 7, 6, 8, and 9. Sibov et al. (2003) identified QTL in bins 9.03 and 7.03-7.04.

Large number of QTL responsible for days to tasseling were detected on chromosomes 1, 4, 5, 9, and 10. Samayoa et al. (2014) reported QTL in bin 1.07, which is similar to our finding. QTL were identified in bins 8.06 and 8.08 in our experiment but Samayoa et al. (2014) identified QTL in 8.05. Similarly, QTL for days to silking were identified on chromosome 8 and 10 (Samayoa et al. 2014). We identified QTL in these chromosomes but in different locations. In addition to this, QTL were identified on chromosomes 3, 9, and10 with large phenotypic effects. QTL on chromosome 10 were identified in 2013 as well as in combined analysis. These QTL may be new and can be further examined in different populations. QTL responsible for days to maturity were identified on many chromosomes. Large numbers of QTL with varying effects were identified on chromosomes 1, 3, 4, 5, 9, and 10 in both years as well as in combined analysis. The QTL for days to maturity in bin 1.07 was in similar location reported by Bello (2007).

Few QTL were identified responsible for number of kernel rows. These were located on chromosomes 4, 5, 7, 8, 9, and 10. QTL in bin 10.05 was identified in 2012, 2013, and across the year. This is in consistent with the result of Sabadin et al. (2008). Likewise, many QTL for

number of kernels per row were identified on every chromosome except chromosome 1. QTL in bin10.04 were reported earlier (Sabadin et al. 2008).

Higher number of QTL responsible to grain yield were identified in our population. They were located on chromosome 3, 5, 7, 8, and 10. Bello (2007) identified QTL in 3.06, which was nearby one QTL identified in our experiment. Samayoa et al. (2014) identified QTL for grain yield in bins 3.05 and 7.02, which was similar to our results. Sibov et al. (2003) identified QTLs on chromosome 7 and 8 but they were in different location compared to our QTL position. The stable QTL may be the strong candidate QTL for grain yield and these should be further studied in different environment and different genetic back ground for further validation of these QTL.

5.4 Identification of genes in response to *A. flavus* infection in resistant and susceptible germplasm

Suppression subtraction hybridization (SSH) library was constructed to study the differentially expressed genes after the inoculation of *A. flavus* in the developing corn kernels. Altogether 300 unique genes were identified from homology search. These sequences showed high similarities to the nucleotide sequences from maize and sorghum and represented the genes of different biological functions. The groups with the highest number of genes were related to metabolism (18.3%), protein regulation/function (12.3%), and protein synthesis (11.7%). Genes related to signal transduction (7.3%), stress response (7.3%) and resistance proteins (8%) were also present in the library (Figure 4.17).

Genes involved in carbohydrate metabolisms were known to be involved in disease resistance. Phosphofructokinase, which is associated with sugar sensing in plants and involved in resistance, is highly expressed during pathogenesis (Granot et al. 2013). Hydrolyzing enzymes like chitinase are involved in the fungal cell wall degradation (Cleveland et al. 2004; Tohidfar et al. 2005; Wang et al. 2012c). Its lysozyme activity is highly efficient in inhibiting fungal growth

through the degradation of cell wall (Collinge and Slusarenko 1987). Higher expression of chitinase was reported in fungal infected corn kernels. Higher expression of chitinase may be responsible for the reduced growth of fungus through cell wall degradation and providing resistance against fungus in resistant inbreds. Likewise, Shikimate pathway is responsible for aromatic secondary metabolism and involved in the synthesis of aromatic amino acids phenylalanine, tryptophan and tyrosine. These amino acids are involved in the synthesis of phytoalexins and involved in the resistance mechanisms (Herrmann 1995). It also provided precursors for synthesis of lignin which is responsible for basal resistance to pathogens. Luo et al. (2011) observed higher expression of genes involved in lignin biosynthesis in resistant inbred Ey125. Concentration of lignin was also found to increase after *A. flavus* infection in peanut (Liang et al. 2006).

Phytohormones are synthesized by plants which are responsible for various processes necessary for growth, reproduction, and survival. As *A. flavus* is a necrotrphic fungus, jasmonate and ethylene response pathways should be responsible for defense signaling (Glazebrook et al. 2005; Derksen et al. 2013). Dolezal et al. (2014) reported increased expression of alcohol dehydrogenase encoding *ts2* gene after infection in kernels and it was also found responsible for resistance to Northern Leaf Blight in maize (Delong et al. 1993; Wisser et al. 2011). It was involved in the jasmonate pathways in maize (Browse 2009) and its differential expression in our study further supports its role in defense signaling pathways. Under stress condition, alcohol dehydrogenase is down-regulated (Luo et al. 2010) and it suggests the susceptibility to *A. flavus* under stress condition. The increased level of Ca^{2+} in host plant in response to pathogen elicitors were responsible for increased resistance in plants through the activation of calcium dependent kinases, calcineurin B-like proteins (CBL) which induces downstream gene expression (Tena et

al. 2011; Asano et al. 2012). Differential expression of kinases, CBL-interacting protein in our SSH library indicated the increased level of Ca^{2+} in kernel after infection. Calcium mediated immunity has been recently reported on host-pathogen interaction through protein kinases and CBL-proteins (Tena et al. 2011) and Bedre et al. (2015) also reported differential expression of these transcripts in cotton.

The role of various transcription factors in host plant resistance mechanisms has been established (Singh et al. 2002). The bZIP transcription factor was differentially expressed in our study and several bZIP transcription factors as well as zinc finger proteins were induced in cotton after the inoculation with *A. flavus* in seed (Bedre et al. 2015). Expression of zinc finger transcription factors were changed because of wounding in *Arabidopsis thaliana* (Cheong et al. 2002). Wounding during inoculation of the kernels might be responsible for the differential expression of the zinc finger protein gene. The differential expression of bZIP and zinc finger proteins after fungus inoculation in various species (Cheong et al. 2002; Bedre et al. 2015) showed their involvement in host resistance response and further characterization of these transcription factors would be helpful to understand the mechanism and pathways involved in this process.

Late embryogenesis abundant protein and heat shock protein were induced after inoculation in our library. Previous studies have also reported their expression after fungus inoculation in resistant maize inbred and cotton (Jiang et al. 2011; Lee et al. 2012; Bedre et al. 2015). Heat shock proteins are important for the protection of plants from biotic and abiotic stress and induced after fungal infection and wounding (Cheong et al. 2002; Cleveland et al. 2004). Another wound inducible gene, Bowman-Birk like proteinase inhibitor, which was differentially expressed in response to fungal inoculation in our experiment, was reported in

previous experiment (Rohrmeier and Lehle 1993). The higher expression of these stress response genes in our experiment shows their importance for resistance to aflatoxin accumulation.

Genes for pathogenesis related protein-1 (PR1), PR4, PR5, and leucine rich repeat family protein known to be associated with aflatoxin resistance were identified. RT-PCR and qRT-PCR showed higher expression of these genes in resistant inbreds (Mp715 and Mp719) compared with B73. Jiang et al. (2011) studied the expression pattern of stress related genes in different maize inbred with different levels of resistance. Many antifungal defensive proteins, signal transduction genes, and stress response proteins were highly expressed in resistant germplasm, which were validated through qPCR. These genes were also represented in our SSH library and can be useful in future research for validation of its function towards aflatoxin resistance. Chen et al. (2006) reported high expression of PR10 in resistant germplasm GT-MAS: gk and susceptibility to A. *flavus* was increased by repression of maize PR10 gene by RNAi silencing (Chen et al. 2010). Kelly et al. (2012) reported the higher expression of resistance related genes in Mp313E.

Six important genes (associated with disease resistance) were selected for real- time qPCR to validate the expression at different time points of inoculation in seven inbreds. The genes associated with resistance such as PR4, LRR repeat family protein were highly expressed in resistant germplasm. Higher expression of genes related to disease resistance, stress, and signal transduction pathways were observed in resistant germplasm (Kelly et al. 2012; Asters et al. 2014; Shan and Williams 2014). Aflatoxin accumulation was low in these resistant inbreds during field evaluation (Table 4.20). Artificial inoculation and wounding in corn kernels activate the recognition and defense-response system and induce the expression of the genes related to resistance mechanisms. The defense genes mainly PR genes were expressed in resistant genotypes after fungal inoculation in response to *A. flavus* infection (Luo et al. 2011). The

proteomic studies in maize rachis also revealed the similar expression pattern of the genes in response to fungal inoculation (Pechanova et al. 2011).

The genes associated with disease resistance, stress response, and signal transduction were mapped near the QTL for aflatoxin resistance in our mapping population (Figure 4.17). Many differentially expressed genes (DEGs) were found in the region between the marker umc2150- umc1509 near a QTL on chromosome 4. A cDNA clone P1B07 with high similarity to PR4 mapped in this region. Stress related Win1 precursor (P2B05), Wheatwin-2, Wound induced protein WIN2 precursor, and defense related proteins like putative vacuolar defense protein (P5B09) were also localized in this chromosomal region. They were involved in important physiological functions like biotic and abiotic stress tolerance. Another EST (P1G01) encoding chitinase was identified on chromosome 10 in between QTL region (10.03-10.05). Warburton et al. (2012) identified the chitinase gene family in QTL and association mapping population. Our QTL mapping study identified some QTL on chromosomes 1 (1.04-1.05), 5 (5.05), and 9 (9.06), which were in agreement with the results of Kelly et al. (2012) in a population from Mp313E x B73. These were located either in the in same location or adjacent genomic region in our study.

CHAPTER 6
SUMMARY & FUTURE DIRECTIONS

6.1 Summary

As expected, the phenotypic responses for the traits in the $F_{2:3}$ mapping population developed from the cross B73 x Mp715 differed from year to year. Heritability estimates for plant height, tasseling, silking, maturity, and ear length were high whereas it was low for aflatoxin accumulation and husk cover in our mapping population. We observed significant correlations among the traits. But the most notable observation was the highly significant positive correlation between aflatoxin concentration and husk cover in both years. Tight husk reduced aflatoxin accumulation and thus this correlated trait could provide another avenue to improve resistance to aflatoxin in corn.

Since aflatoxin contamination is a major concern for corn growers in USA and other parts of the world, identification of stable and large effect QTL is needed to develop resistant inbreds and hybrids. Many QTL mapping studies have been conducted in multiple environments and multiple years (Paul et al. 2003; Brooks et al. 2005; Warburton et al. 2009, 2011) to identify stable QTL, but the QTL identified were highly inconsistent with respect to position and effect between different years, locations, and populations. This was not unexpected because several environmental factors influence the fungal infection and aflatoxin accumulation in field environment. For a highly environmentally influenced trait like aflatoxin accumulation, use of $F_{2:3}$ or backcross population used in earlier studies with the exception of Yin et al. (2014) might be responsible for inaccurate phenotyping leading to identification of QTL with little stability over years or locations. Therefore, use of recombinant inbred line (RIL) and introgression line populations involving resistant inbred may be useful to identify stable QTL. Even though minor QTL are identified using introgression lines, it would provide opportunity to pyramid the minor

QTL to improve resistance to aflatoxin. Another reason for discrepancy between different studies could be due to low resolution of QTL locations in a crop with large genome. It is important to notice that many QTL identified in our study were placed to QTL located in nearby bins reported in earlier studies. This observation calls for use of high resolution linkage map for QTL mapping. Alternatively, fine mapping using introgression lines should be useful to locate the QTL with high resolution.

Despite the difficulties in assessing consistency of QTL for aflatoxin resistance between various studies, we suggest that pyramiding of QTL with moderately high phenotypic effects derived from Mp715 should be attempted for improving resistance to aflatoxin. A significant finding from our study is that QTL for both aflatoxin accumulation and husk cover were clustered at three genomic regions on chromosome 4 (bins 4.01-4.02), 8 (8.05), and 10 (10.03-10.04), where aflatoxin resistance QTL were reported in previous studies (Paul et al. 2003; Warburton et al. 2011; Mideros et al. 2014; Yin et al. 2014). Both traits can be easily pyramided using molecular markers linked to these QTL. However, relationship between these two traits should be further confirmed in other populations and environments before conducting marker-assisted selection in breeding program. Near isogenic lines for the QTL with significant contribution toward aflatoxin resistance identified in this study should be developed in suitable genetic background for understanding the QTL x Environmental interaction as well as the molecular basis of host plant resistance through cloning of the QTL loci.

Due to the lack of knowledge about host-resistance mechanisms and the molecular markers linked to aflatoxin resistance, use of molecular markers in breeding program has been difficult. Gene expression studies and their location in previously identified QTL regions will be helpful for the identification of probable candidate gene involved in the resistance. Genes

involved in stress response, signal transduction pathways, and disease resistance can be considered as important candidate genes for further validation and functional characterization. Reverse Northern hybridization using the clones obtained from a SSH library revealed the differential expression of several genes in Mp715 and B73 after inoculation with *A. flavus*. Some important genes represented in the library were genes associated with disease resistance (PR1, PR4, PR5, LRR family protein), stress (Win1precursor, protein phosphatase 2C), and signal transduction (MAPKS, serine/threonine protein kinase). Expression studies involving 30 selected genes related to important biochemical pathways in seven maize inbred lines revealed that most of the genes were highly expressed in response to fungus inoculation in resistant inbred lines compared to the susceptible inbreds. Further validation of 6 genes using qPCR in at different time point confirmed the pattern of expression. In general, disease resistance genes like PR4, PR5, and LRR family protein were highly expressed in resistant inbreds. The integration of these expressed genes obtained from the SSH library to our linkage map through *in-silico* mapping revealed that large number of expressed genes with various physiological functions were located in and around QTL responsible for resistance to aflatoxin. The gene PR4 , which showed higher expression in resistant inbred lines in qPCR analysis, was located near*qAFL4-1* with resistant allele derived from Mp715. This approach provides strong evidence for the locations of QTL for aflatoxin resistance. Moreover, it also provides a large number of candidate genes for future investigations to elucidate the host plant resistance mechanism associated with *A. flavus* infection.

6.2 Future directions

Use of host plant resistance is an effective alternative approach to combat the aflatoxin problem using resistant inbreds and hybrids with adaptation to diverse climatic condition and desirable agronomic traits. Although many germplasm resistant to *A. flavus* have been identified and released in US, marker-assisted selection to accelerate development of aflatoxin resistant hybrids is not yet practiced by corn breeders. Following future investigations may be conducted to accelerate the use of molecular markers in breeding for aflatoxin resistance.

1. Mapping experiment should be conducted in diverse mapping population and environments to identify the stable QTL.
2. The PCR based markers linked to these stable QTL with large phenotypic effects should be developed for marker-assisted breeding (MAB).
3. The expression, stability, and contribution of these QTL in reducing aflatoxin accumulation should be studied in hybrids developed in diverse genetic backgrounds.
4. Near isogenic lines (NIL) should be developed and evaluated in diverse environmental condition for multiple years for validating the QTL identified in this study.
5. Large number of differentially expressed genes identified from the SSH library were co-localized in the QTL region for resistance to aflatoxin resistance. These potential candidate genes should be further studied for understanding the molecular mechanisms associated with aflatoxin resistance.

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APPENDICES

Appendix 1 List of polymorphic SSR markers used in the construction of the linkage map.

Name of the oligo	Sequence (5' to 3')	Bin
umc1354-F	GATCAGCCCGTTCAGCAAGTT	1.00
umc1354-R	GAGTGGAGGCGGAGGATCTG	1.00
bmc1124-F	TCTTCATCTCTCTATCAAACTGACA	1.00
bmc1124-R	TGGCACATCCACAAGAACAT	1.00
umc1071-F	AGGAAGACACGAGAGACACCGTAG	1.01
umc1071-R	GTGGTTGTCGAGTTCGTCGTATT	1.01
umc2225-F	TCGGCTGACATAATAAAACCATAGC	1.02
umc2225-R	ATGCGAATTTTACCGGGTTTTT	1.02
phi001-F	TGACGGACGTGGATCGCTTCAC	1.03
phi001-R	AGCAGGCAGCAGGTCAGCAGCG	1.03
dupssr26-F	GTCGGAGCACTCCAAGAC	1.04
dupssr26-R	CTTCTCGCTCATCAGCTTAAA	1.04
umc1689-F	GAGGCGGAGGAGGAACACAG	1.05
umc1689-R	GAACGAGTAGGGCAGCGTCAG	1.05
umc1703-F	ATTTTCTTGCTCACGTTCACTTCC	1.05
umc1703-R	TAACGGCAGCATTACATTTCTTGA	1.05
umc1508-F	GGTTCTTGGTTTGGGCCTTAGTAT	1.06
umc1508-R	GAGGCCACTAGTTGACCTTTTTCA	1.06
umc1661-F	ACGAGACTCCCTCCTCTCCTCTC	1.07
umc1661-R	GGAGTAAACTGTTGAAAGGCCCAT	1.07
phi037-F	CCCAGCTCCTGTTGTCGGCTCAGAC	1.08
phi037-R	TCCAGATCCGCCGCACCTCACGTCA	1.08
umc1955-F	GCCAAGGTGGGTCTGGCTAT	1.08
umc1955-R	ACCACCTTGTCCGTATCCTTCAC	1.08
umc1082-F	CCGACCATGCATAAGGTCTAGG	1.09
umc1082-R	GCCTGCATAGAGAGGTGGTATGAT	1.09
umc2223-R	ACTTCTGCAGAGCGAGCAGG	1.10
umc2223-F	TTTTGGGACTGAAGAAGAAGATCG	1.10
umc1553-F	TGAATGGAAGAGAAGGGAAATCTG	1.11
umc1553-R	GCTCTGTACATCCTTAGCGACACA	1.11
umc1064-F	GTGGGTTTTGTCTGTAGGGTGGTA	1.11
umc1064-R	TCCATCCACTCGACTTAAGAGTCC	1.11
umc2100-F	AAAGGCATTATGCTCACGTTGATT	1.12
umc2100-R	TGACGTGCAAACAACCTTCATTAC	1.12
bmc1338-F	GTGCAGAATGCAGGCAATAG	2.01
bmc1338-R	GCAAATGTTTTCACACACACG	2.01

Name of the oligo	Sequence (5' to 3')	Bin
bnlg1297-F	TCTCGATCGCTCCGATCTAT	2.02
bnlg1297-R	GACTCAACTCCAAAAGGCGA	2.02
ZmRR1-F	CTGCGTGTGGTGCTATGTTAGG	2.00
ZmRR1-R	GACATTGACACCTGATGTTA	2.00
bmc2277-F	TTACGGTACCAATTCGCTCC	2.02
bmc2277-R	GACGACGCCATTTTCTGATT	2.02
umc1185-F	AGTAAAAGAGGCAAGGACTACGGC	2.03
umc1185-R	GCGGCGATATATACGAGGTTGT	2.03
bnlg2248-F	CCACCACATCCGTTACATCA	2.02
bnlg2248-R	ACTTTGACACCGGCGAATAC	2.02
bnlg1064-F	CTGGTCCGAGATGATGGC	2.03
bnlg1064-R	TCCATTTCTGCATCTGCAAC	2.03
bnlg1175-F	ACTTGCACGGTCTCGCTTAT	2.03
bnlg1175-R	GCACTCCATCGCTATCTTCC	2.03
bmc1018-F	CGAGGTTAGCACCGACAAAT	2.04
bmc1018-R	CGAGTAAATGCTCTGTGCCA	2.04
bmc1036-F	GGGAGTATGGTAGGGAACCC	2.06
bmc1036-R	AAACCCTTGGAGCATACCCT	2.06
bmc1267-F	AAATCTGTGCTGTGCTGTGG	2.07
bmc1267-R	TGTCGAGTGGTCCTACGATG	2.07
bmc1335-F	GAAGGTTGCTCTTCCACTGG	2.08
bmc1335-R	TGGTTTGTGCAAGTGTCACC	2.08
bmc1520-F	TCCTCTTGCTCTCCATGTCC	2.09
bmc1520-R	ACAGCTGCGTAGCTTCTTCC	2.09
umc2214-F	ACCCCCTGATTCTCTCTTACGTTT	2.10
umc2214-R	CTGGATGAGGAGGAAGAATACGAG	2.10
umc2103-F	CCTATCCATGCTTGAGGTGTCG	3.00
umc2103-R	GTCCAGGAGGTTGTCGTCCA	3.00
umc1746-F	ACACGAGCATCCTACATCCTCCTA	3.01
umc1746-R	ACCTTGCCTGTCCTTCTTTCTCTT	3.01
bnlg1325-F	CTAAATGCGCAGCAGTAGCA	3.03
bnlg1325-R	TGCTCTGCAACAACTTGAGG	3.03
bnlg1144-F	TACTCGTCGTGTGGCGTTAG	3.03
bnlg1144-R	AGCCGAGGCTATCTAACGGT	3.03
umc1729-F	GTCGTACCACACCAGCCACA	3.03
umc1729-R	TTCACTTCCACTTGTTGAACTTGC	3.03
mmc0022-F	AGGTGTTGTTTTTGTTCGCT	3.05
mmc0022-R	TGCTTGTTTAAGCTCATTATT	3.05
bmc1798-F	AAGTTGGTGGTGCCAAGAAG	3.06

Name of the oligo	Sequence (5' to 3')	Bin
bmc1798-R	AAAAGGTCCACGTGAACAGG	3.06
mmc071-F	TTACGGACAAGACGCTACTAC	3.08
mmc071-R	ATACGTTTCGGCCAATCTCCT	3.08
umc1404-F	GTTGGCCTTCCTCTCTACCCC	3.07
umc1404-R	CCAGTTCATCAGGTCATCAACCTT	3.07
bmc1605-F	TCCTGCCCCCTTTGTTTTC	3.07
bmc1605-R	CACCTCTGAACCCCTGTGTT	3.07
umc1844-F	GGCATGGGTCTCTCATAAAGTCAT	3.08
umc1844-R	CGACGTATATGGCTGAGAACCCTA	3.08
bmc1108-F	GGATTCCTTTATGACGGGGT	3.08
bmc1108-R	AGTAACAACCAAGGCATCGG	3.08
bnlg1536-F	CAAAAAAAAAATATGTATACGGGG	3.09
bnlg1536-R	ATGCACGAGCTTTTGGAGTT	3.09
bnlg1496-F	CTGGGCAGACAGCAACAGTA	3.09
bnlg1496-R	AGCCAAAGACATGATGGTCC	3.09
umc1639-F	CTAGCCAGCCCCCATTCTTC	3.10
umc1639-R	GCAAGGAGTAGGGAGGACGTG	3.10
umc1136-F	CTCTCGTCTCATCACCTTTCCCT	3.10
umc1136-R	CTGCATACAGACATCCAACCAAAG	3.10
phi072-F	ACCGTGCATGATTAATTTCTCCAGCCTT	4.01
phi072-R	GACAGCGCGCAAATGGATTGAACT	4.01
umc1276-F	ACGCAATTATTACTGCCACACGTC	4.01
umc1276-R	CTACCTTGTTCCTAGGGCCGTCTA	4.01
umc2150-F	GTTGTTCACTTTCCAAAACCCTTG	4.01
umc2150-R	GCCTTGTGCTTCTTGGAGTGTT	4.01
umc1509-F	CTTTCTGCAGATTCACCGTTTCTT	4.02
umc1509-R	TTGGTTCTTTTGACCATAGACAAGC	4.02
umc2039-F	CATCTCCTACCAGCTCACCCC	4.03
umc2039-R	GCTCGGGGTAGTAGTGTTCTCCTT	4.03
umc1088-F	TCATCCTCCTAGCTCCTCTACTCG	4.05
umc1088-R	AAAACAGTCAGCAGAACCCACTTT	4.05
umc2284-F	CGTTCTTCCTTTTTCCTCTTCGTC	4.06
umc2284-R	ATCTATAGGAGACGGTACGGGGAC	4.06
mmc0371-F	CCACGCACCTCTTGTAAC	4.06
mmc0371-R	GGGAACCTACAGCTTGGT	4.06
dupssr34-F	TCAGTGCTTTCATTGTAACGA	4.07
dupssr34-R	ATAAACATCTTGCCAGCAAA	4.07
umc2038-F	ACAGAAACCAATGCATGTGATGAG	4.07
umc2038-R	TGCATGGTTGCTTCAGCAGT	4.07

Name of the oligo	Sequence (5' to 3')	Bin
dupssr28-F	GAAGGAAGCCTTTGTTACAAGT	4.08
dupssr28-R	CTGGAGTGCTGGTCTTGTTAT	4.08
umc1940-F	AACAACAAATGGGATCTCCGTTAC	4.09
umc1940-R	CCATCTGCTGAGGGCTTATCTG	4.09
umc1503-F	TTCATGACACACAAACCACAGATG	4.09
umc1503-R	GCACCCTAGCAGACTACAACATCC	4.09
umc2289-F	GGCTCCGATTCACTTGATGC	4.11
umc2289-R	CAGCACCACCCAGTTAACCAC	4.11
umc1416-F	AGATGAATGTTGGGGTCAACAAGT	5.00
umc1416-R	CTTGTCAGCCACAGAAGTGCC	5.00
umc1240-F	GCAGCAGGTATTGGAAGTCGTAGT	5.00
umc1240-R	CTGGTCCCTCAGGAAATCCAT	5.00
mmc0261-F	TAGTAATGCTTAACGAAAGCAA	5.02
mmc0262-R	CGCTTACGCTTACTACTAGCTT	5.02
bmc1046-F	TGAGCCGAAGCTAACCTCTC	5.03
bmc1046-R	GATGCAAAGGAGGTTCAGGA	5.03
umc2400-F	TTTGGTGAAAGTGAAACCAAAGGT	5.02
umc2400-R	CTAGCTAGCTTCCTTCCTCCTCG	5.02
umc1389-F	AAAACACAACGCTGGACATCAAC	5.03
umc1389-R	GGTCGTTTTGCTTAGCCCATTTTA	5.03
umc1171-F	ACGTACTACAGATAATGGGCGACG	5.04
umc1171-R	CGCCGTACCCATGAGTATAATGTAA	5.04
bmc1208-F	GCTGTGATGGTGAGACGAGA	5.04
bmc1208-R	GCAGGCACTACTAAAACCGC	5.04
bnlg1892-F	ACGCCATCACTCTCGCTC	5.04
bnlg1892-R	TGGCATCCATCAATCCAAC	5.04
bmc1237-F	TGGCGCGATTTTCTTCATAT	5.05
bmc1237-R	AAAGAGCAACCTTCAACGGA	5.05
umc1155F	TCTTTTATTGTGCCCGTTGAGATT	5.05
umc1155R	CCTGAGGGTGATTTGTCTGTCTCT	5.05
umc2198-F	CTCTTCACTCGCTTCTCCCAGA	5.06
umc2198-R	AGCCCAGAGAAGGGAAGCAG	5.06
bmc1118-F	CAGAGTTGATGAACTGAAAAAGG	5.07
bmc1118-R	CTCTTGCTTCCCCCCTAATC	5.07
umc1225-F	CTAGCTCCGTGTGAGTGAGTGAGT	5.08
umc1225-R	TTCCTTCTTTCTTTCCTGTGCAAC	5.08
phi075-F	GGAGGAGCTCACCGGCGCATAA	6.00
phi075-R	AAAGGTTACTGGACAAATATGCGTAACTCA	6.00
bmc1043-F	TTTGCTCTAAGGTCCCCATG	6.00

Name of the oligo	Sequence (5' to 3')	Bin
bmc1043-R	CATACCCACATCCCGGATAA	6.00
umc1257-F	CAACGGAAGTGGCTGTAGAGTTTT	6.03
umc1257-R	ACAGAGCATGTCAGGTATTTGCAG	6.03
umc1887-F	CTTGCCATTTTAATTTGGACGTTT	6.03
umc1887-R	CGAAGTTGCCCAAATAGCTACAGT	6.03
umc1014-F	GAAAGTCGATCGAGAGACCCTG	6.04
umc1014-R	CCCTCTCTTCACCCCTTCCTT	6.04
umc1020-F	CCTGGAGAGCCACTACAAGGAA	6.05
umc1020-R	TCAGCCTGAGCTCACATCATCT	6.05
umc2170-F	CACTGCAAGCCTCTACAGACAATG	6.06
umc2170-R	GAGAGTTCTCCAGGCGAGGTG	6.06
umc1063-F	AGGCCACTGAGCAGGTGAAG	6.07
umc1063-R	GTGATGGTAGAGGAGTCCTTGGTG	6.07
dupssr15-F	GAAGTCGATCCATCCACC	6.06
dupssr15-R	GGGGTAGTGGAGATAACTAGTG	6.06
phi089-F	GAATTGGGAACCAGACCACCCAA	6.08
phi089-R	ATTTCCATGGACCATGCCTCGTG	6.08
umc1546-F	CTGGTCTTGGCCTTGGACTTCT	7.00
umc1546-R	GTCACAGCAAAGTCATCCTCCTCT	7.00
umc1642-F	CACTACAGCGCCTGTAACTGCC	7.00
umc1642-R	CATGAGCTAAGCAAGAGGGGTATG	7.00
mmc0171-F	AATCCTACTTGCTGCCAAAGC	7.00
mmc0171-R	CTTTGAGCTTTTTGTGTGGAC	7.00
umc1066-F	ATGGAGCACGTCATCTCAATGG	7.01
umc1066-R	AGCAGCAGCAACGTCTATGACACT	7.01
bmc1200-F	CGTCCTCGTTGTTATTCCGT	7.02
bmc1200-R	GTTCCCTCTCTCCCTCCCTC	7.02
bmc1792-F	CGGGAATGAATAAGCCAAGA	7.02
bmc1792-R	GCGCTCCTTCACCTTCTTTA	7.02
phi034-F	TAGCGACAGGATGGCCTCTTCT	7.02
phi034-R	GGGGAGCACGCCTTCGTTCT	7.02
dupssr11-F	AGGCAAGGCTTTCTTCATAC	7.02
dupssr11-R	CGGACGACGACTGTGTTC	7.02
umc1301-F	AACAGTCAAGCTCACTTTCCGC	7.03
umc1301-R	CATCCATAAGCTGAAGGAGTGAGG	7.03
umc2331-F	CGGTGAGTGAGTGAGTGAGTGAGT	7.04
umc2331-R	AAGAACTGCAAAAAGGTACCCACA	7.04
umc1710-F	ACTTTGCAACTACCGTACATGGGT	7.04
umc1710-R	TTCGACTGCACGTGAAAATCTATC	7.04

Name of the oligo	Sequence (5' to 3')	Bin
umc2332-F	GTCGGAGAAGGAGCTACTGAGCTA	7.04
umc2332-R	CACAGGTACGTCTGGATGCTGT	7.04
umc1782-F	CGTCAACTACCTGGCGAAGAA	7.04
umc1782-R	TCGCATACCATGATCACTAGCTTC	7.04
umc1154-F	CCACCACAAGACAAGACAAGAATG	7.05
umc1154-R	CCTGATCGATCTCATCGTCGT	7.05
umc2197-F	CGACCTCTTTGCTGTCTCATTTTT	7.05
umc2197-R	CAAGCAATTTCCCCATCTCATACT	7.05
umc1799-F	GTGATGAATAATGTCCCCAATTCC	7.06
umc1799-R	GGACAGATGTCTGGAGATTGCTTT	7.06
phi116-F	GCATACGGCCATGGATGGGA	7.06
phi116-R	TCCCTGCCGGGACTCCTG	7.06
phi082-F	CACAGCACAGGCAGTTCG	7.05
phi082-R	CGCGGCAAAAGATCTTGAACACCT	7.05
umc1327-F	AGGGTTTTGCTCTTGGAATCTCTC	8.01
umc1327-R	GAGGAAGGAGGAGGTCGTATCGT	8.01
bmc1073-F	TCGATCTAAGTATTGTAAACGTACG	8.01
bmc1073-R	GTATTTGGAGGCGCCATAGA	8.01
umc1872-F	CTTTTGTGATGTCTGCAATATGCC	8.02
umc1872-R	TTAGTAGGTGCATTGGATGCTCAA	8.02
bmc1782-F	CGATGCTCCGCTAGGAATAG	8.05
bmc1782-R	TGTGTTGGAAATTGACCCAA	8.05
bmc1031-F	AATCGGTGAGGCTTCACAAC	8.06
bmc1031-R	ATGCCTACCTACCACCATGC	8.06
umc1607-F	ACTAATTTTCGGTAGTCGTGTGCG	8.07
umc1607-R	GGAAAGAGAGAGGCTGTAGGTGGT	8.07
bmc1065-F	TGATGCTCGTTGCTTACCTG	8.07
bmc1065-R	TTGCCTCTCGTCTTCCAACT	8.07
umc1005-F	TTTGATCACAGACTTATCCCTGTT	8.08
umc1005-R	CTAATGACGAACCCCTAAAAGGT	8.08
umc1069-F	AGAGAATCCCCAAGCAAACAAAC	8.08
umc1069-R	CTTCATCGGAGCCATGGTGT	8.08
dupssr14-F	AGCAGGTACCACAATGGAG	8.09
dupssr14-R	GTGTACATCAAGGTCCAGATTT	8.09
phi233376-F	CCGGCAGTCGATTACTCC	8.09
phi233376-R	CGAGACCAAGAGAACCCTCA	8.09
umc1571-F	GCACTTCATAACCTCTCTGCAGGT	9.04
umc1571-R	CACCGAGGAGCACGACAGTATTAT	9.04
bmc1626-F	TTAAATCCAGAGTGTCCCCG	9.03

Name of the oligo	Sequence (5' to 3')	Bin
bmc1626-R	TTCTGGATGGTTGCACACAT	9.03
dupssr19-F	GCTGAAGGACTAAAGAAACCG	9.02
dupssr19-R	CCTCCAAGGTTGGTACTGTC	9.02
umc2345-F	AAAAAGAGCAGCGGAACGTG	9.06
umc2345-R	GTCGTGCTGGCTACTCTGCTG	9.06
umc1733-F	ACACCCAACCTCCCACTGTAAAA	9.06
umc1733-R	GATTGGGATTGGGATTGGAAAT	9.06
bnlg1525-F	AGGAATTGCGAGTCTTCCAA	9.07
bnlg1525-R	CAACCCCCAAAATGAACAAA	9.07
dupssr29-F	CAGCGAATACTGAATAACGC	9.07
dupssr29-R	TGTTGGATGAGCACTGAAC	9.07
umc1714-F	CAAGGGCTCTTGCTCTTGAACTAA	9.08
umc1714-R	CGACGACCTTAATTGTGTTCCTTT	9.08
umc1277-F	TTTGAGAACGGAAGCAAGTACTCC	9.08
umc1277-R	ACCAACCAACCACTCCCTTTTTAG	9.08
bmc1129-F	GAGAGTATGCTACTCGCCGC	9.08
bmc1129-R	GACGAGTTTGGAGTGCCATT	9.08
phi117-F	ATCGGATCGGCTGCCGTCAAA	10.00
phi117-R	AGACACGACGGTGTGTCCATC	10.00
umc1319-F	TGAGAGCCACCTTCTTGAGCTACT	10.01
umc1319-R	TTCCTTGAAGGCGAAGGTAGGTAT	10.01
bmc1451-F	TGATCGATGGCTCAATCAGT	10.02
bmc1451-R	ATCTGGAACACCGTCGTCTC	10.02
phi059-F	AAGCTAATTAAGGCCGGTCATCCC	10.02
phi059-R	TCCGTGTACTCGGCGGACTC	10.02
umc1381-F	CTCTAGCTACGAGCCTACGAGCA	10.03
umc1381-R	CCGTCGAGTCAACTAGAGAAAGGA	10.03
umc1827-F	GCAAGTCAGGGAGTCCAAGAGAG	10.04
umc1827-R	CCACCTCACAGGTGTTCTACGAC	10.04
umc1648-F	CTGCAGTACGTGAGCCTGTACG	10.04
umc1648-R	GCTTGAGCTGTGAGGAAGTTTTG	10.04
bmc1074-F	CATGCTAATAGCCTACCGGG	10.05
bmc1074-R	TTTCCCCCTGATTCGTTATG	10.05
umc2122-F	TTGACAAGCTAGTGTGCAACTGTG	10.06
umc2122-R	TGAAAGCCCACTGGACAAACTAAT	10.06
bmc1185-F	CGGTCCAGGCAGGTTAATTA	10.05
bmc1185-R	GACTCGAGGACACCGATTTC	10.05

Appendix 2 Descriptive statistics for various morphological traits in parents and $F_{2:3}$ lines derived from the cross B73 x Mp715.

	2012					**2013**				
Trait	**B73**	**Mp715**	**F2:3**			**B73**	**Mp715**	**F2:3**		
	Mean	**Mean**	**Mean**	**Range**	**CV[@]**	**Mean**	**Mean**	**Mean**	**Range**	**CV[@]**
Plant height (cm)	160.9	187.7	189.9	100-222	7.9	159.6	190.0	191.9	88-220	7.5
Days to 50% tasseling	61	71	72	55-82	4.6	62	76	71	61-131	7.8
Days to 50 % silking	64	74	74	55-85	4.9	65	76	72	42-80	5.8
Days to 50 % maturity	107	115	110	86-130	5.2	103	116	111	92-125	4.2
Ear height (cm)	63.8	85.0	85.09	55-117	13.1	59.8	83.2	86.0	53-116	12.6
Husk cover rating	3.0	1.7	1.5	1-5	44.0	3.0	1.0	2.3	1-5	61.2
Ear length (cm)	12.4	17.3	13.5	7.3-20.0	16.9	12.8	18.2	15.9	8.0-19.9	11.0
Ear circumference (cm)	11.6	11.6	11.2	7.0-15.7	10.2	11.7	13.1	12.6	9.0-17.3	8.0
Number of rows/ear	13	12	13	9-19	10.0	13	15	14	10-20	10.3
Number of kernels/row	16	20	24	6-42	24.9	17	25	32	13-46	17.2
100-kernel weight (gm)	17.9	23.1	13.43	9-19	10.0	15.3	21.3	18.7	12-26	12.4
Grain yield (gm/plot)	101.7	47.3	163.7	13-549	58.2	91.0	61.0	390.4	28-1006	52.3

[@] CV, Coefficient of variation

Appendix 3 Sources of variation and *F* values for various morphological traits in $F_{2:3}$ population derived from the cross B73 x Mp715.

Source of variation	2012	2013	Across year
Plant height			
Replication	1.25^{*}	2.01^{NS}	3.49^{**}
Genotype	1.26^{NS}	5.55^{**}	0.41^{NS}
Year	-	-	7.91^{**}
Genotype x Year	-	-	1.14^{NS}
Days to 50% tasseling			
Replication	96.2^{**}	39.41^{**}	4.62^{**}
Genotype ($F_{2:3}$ lines)	1.26^{NS}	85.85^{**}	40.1^{**}
Year	-	-	59.03^{**}
Genotype x Year	-	-	1.85^{**}
Days to 50% silking			
Replication	82.66^{**}	25.87^{**}	4.39^{**}
Genotype ($F_{2:3}$ lines)	1.22^{*}	11.98**	27.62^{**}
Year	-	-	65.5^{**}
Genotype x Year	-	-	1.63^{**}
Days to 50% maturity (DM)			
Replication	305.9^{**}	11.74^{**}	2.39^{**}
Genotype ($F_{2:3}$ lines)	1.14^{NS}	7.57^{**}	96.18^{**}
Year	-	-	0.14^{NS}
Genotype x Year	-	-	0.9^{NS}
Ear height (EH)			
Replication	2.41^{NS}	15.67^{**}	6.26^{**}
Genotype ($F_{2:3}$ lines)	1.28^{**}	90.03^{**}	1.58^{NS}
Year	-	-	4.37^{**}
Genotype x Year	-	-	1.37^{**}
Husk cover rating			
Replication	11.75^{**}	2.52^{NS}	223.16^{**}
Genotype ($F_{2:3}$ lines)	1.44^{**}	8.45^{**}	3.82^{**}
Year	-	-	7.11^{*}
Genotype x Year	-	-	2.32^{NS}
Ear length			
Replication	80.6^{**}	0.78^{NS}	1.59^{**}
Genotype ($F_{2:3}$ lines)	1.18^{NS}	2.61^{**}	48.72^{**}
Year	-	-	305.20^{**}
Genotype x Year	-	-	1.03^{NS}

Source of variation	2012	2013	Across year
Ear circumference			
Replication	25.92**	8.02**	3.43**
Genotype ($F_{2:3}$ lines)	1.66**	3.21**	18.55**
Year	-	-	609.94**
Genotype x Year	-	-	1.29*
Number of rows/ear (NRE)			
Replication	9.59**	0.001NS	3.91**
Genotype ($F_{2:3}$ lines)	2.07**	3.65**	2.30NS
Year	-	-	97.18**
Genotype x Year	-	-	1.20NS
Number of kernels/row (NKR)			
Replication	1.19NS	2.98NS	3.19**
Genotype ($F_{2:3}$ lines)	1.55**	4.50**	1.23NS
Year	-	-	717.09**
Genotype x Year	-	-	1.49**
100-kernel weight			
Replication	39.78**	28.15**	3.06**
Genotype ($F_{2:3}$ lines)	1.69**	8.03**	25.64**
Year	-	-	16.75**
Genotype x Year	-	-	1.5**
Grain yield/plot			
Replication	23.85**	1.75 NS	4.99**
Genotype ($F_{2:3}$ lines)	6.25**	4.00**	0.44NS
Year	-	-	975.07**
Genotype x Year	-	-	2.73**

*Significant at the 0.05 probability level; **Significant at the 0.01 probability level;
NS Nonsignificant

Appendix 4 Mean value of different morphological and yield attributing characters in different maize germplasm in 2013 and 2014.

2013

Germplasm	PH	EH	SD	EL	EC	RN	KPR	KW	GY
B73	170^{d}	86^{c}	66^{e}	13.7^{c}	13.7^{a}	13^{ab}	16^{ab}	17.8^{d}	117.3^{b}
Mp715	211^{ab}	119^{a}	83^{a}	15.3^{bc}	13.3^{a}	12^{ab}	19^{a}	20.6^{bc}	75.7^{c}
Mp313E	218^{a}	115^{a}	77^{b}	16.2^{bc}	12.7^{a}	13^{ab}	20^{a}	21.5^{ab}	133.3^{b}
Mo18W	200^{bc}	95^{c}	81^{a}	20.3^{a}	13.3^{a}	11^{b}	17^{ab}	19.7^{c}	230.0^{a}
Va35	118^{e}	59^{d}	63^{f}	16.7^{b}	13.3^{a}	12^{ab}	11^{b}	22.1^{a}	205.0^{a}
Mp420	217^{a}	105^{b}	74^{c}	14.7^{bc}	13.6^{a}	13^{ab}	21^{a}	17.0^{d}	137.0^{b}
Mp719	189^{c}	91^{c}	72^{d}	17.3^{b}	13.5^{a}	14^{a}	21^{a}	17.5^{d}	131.7^{b}
GM	189	96	74	16.3	13.4	12	18	19.4	147.1
SE	4	3	0.7	0.9	0.5	0.71	2.5	0.4	10.0
CV (%)	18.2	20.5	9.8	15.4	6.5	10.0	27.1	10.4	35.7

2014

Germplasm	PH	EH	SD	EL	EC	RN	GR	KW	GY
B73	164^{b}	87^{d}	67^{d}	14.3^{c}	13.0^{bc}	12^{ab}	16^{abc}	17.1^{f}	129.0^{b}
Mp715	200^{ab}	117^{a}	81^{a}	15.3^{bc}	12.0^{c}	11^{ab}	21^{a}	20.4^{bc}	81.3^{c}
Mp313E	212^{a}	111^{ab}	76^{b}	15.7^{bc}	12.8^{bc}	11^{ab}	18^{abc}	21.2^{ab}	134.0^{b}
Mo18W	190^{ab}	100^{c}	81^{a}	21.7^{a}	14.7^{a}	13^{a}	20^{ab}	19.9^{cd}	221.3^{a}
Va35	113^{c}	51^{e}	64^{e}	14.0^{c}	12.6^{bc}	13^{a}	15^{c}	21.5^{a}	226.3^{a}
Mp420	177^{ab}	108^{b}	73^{c}	17.7^{b}	13.9^{ab}	13^{a}	15^{bc}	18.7^{e}	128.0^{ab}
Mp719	186^{ab}	88^{d}	74^{bc}	15.3^{bc}	13.3^{abc}	13^{a}	18^{abc}	18.8^{de}	124.7^{b}
GM	177	95	74	16.3	13.2	12	18	19.7	149.2
SE	14	2	0.7	0.9	0.5	0.6	1.4	0.4	6.8
CV (%)	20.5	22.6	8.4	17.3	7.9	9.7	18.4	8.5	35.1

***** values with same letter in the column did not differ significantly at P=0.05.
PH, Plant height; EH, Ear height; SD; Silking days; EL, Ear length; EC, Ear circumference; RN, Number of rows/ear; GR, Grains/ rows; KW, 100 kernel weight; GY, Grain yield/plot.

Appendix 5 Sources of variation and F values for various morphological traits in maize germplasm

Source of variation	2013	2014	Across years
Plant height			
Var	85.78**	5.57**	28.72**
Rep	2.72NS	0.94NS	6.07**
Year			2.45NS
Var*Year			1.51
Ear height			
Var	49.86**	110.98**	145.47**
Rep	0.77NS	0.55NS	0.74NS
Year			0.17NS
Var*Year			1.37NS
Days to50% silking			
Var	76.47**	105.38**	139.32**
Rep	2.48NS	1.69NS	0.1NS
Year			3.22NS
Var*Year			1.0NS
Ear length			
Var	5.74**	8.11**	8.76**
Rep	2.64NS	0.17NS	0.0NS
Year			0.84NS
Var*Year			0.88NS
Ear circumference			
Var	0.33NS	3.59**	1.42*
Rep	1.75NS	0.01NS	0.38NS
Year			0.68NS
Var*Year			0.41NS
Number of Rows			
Var	1.37*	1.77*	1.22*
Rep	0.12NS	1.2NS	0.88NS
Year			0.17NS
Var*Year			0.55NS

Source of variation	2013	2014	Across years
Kernels /row			
Var	1.85*	2.29*	2.96*
Rep	0.61NS	3.23**	0.1NS
Year			2.5NS
Var*Year			1.34
100 kernel weight			
Var	25.39**	17.71**	22.46**
Rep	1.28NS	10.32**	0.59NS
Year			4.44*
Var*Year			0.45NS
Grain yield (gm/plot)			
Var	28.1**	62.48**	60.96**
Rep	2.43NS	2.92NS	0.17NS
Year			1.54NS
Var*Year			0.69NS

*Significant at the 0.05 probability level; **Significant at the 0.01 probability level;
NS Nonsignificant; Var, variety; Rep, replication

Appendix 6 Variation in husk cover and *A. flavus* growth in different maize germplasm. A. B73, B. Mp715, C. Mp719, D. Va35, E. Hybrid (B73 xMp715).

A **B** **C** **D** **E**

Appendix 7 List of genes obtained from the suppression subtractive hybridization experiment.

SN	Clone ID	Name of the protein	Chr	Bin	Homology (%)	e-value
1	P1A01	plasma membrane associated protein [*Zea mays*]	7	7.02	100.0	9.051e-149
2	P1A03	Hypothetical protein	1	1.08	98.6	7.295e-104
3	P1A04	hypothetical protein [*Zea mays*]	1	1.08	98.6	7.295e-104
4	P1A05	pathogenesis-related protein 1 [*Zea mays*]	1	1.03	98.0	2.449e-93
5	P1A06	uncharacterized protein LOC100191593	4	4.02	100.0	6.336e-110
6	P1A08	mitogen activated protein kinase 7	5	5.03	97.9	1.633e-156
7	P1A10	aspartic proteinase [*Oryza sativa*]	10	10.03	98.3	2.57E-84
8	P1A12	trehalose-6-phosphate synthase [*Zea mays*]	1	1.02	97.6	4.157e-137
9	P1B02	6-phosphogluconolactonase [*Oryza brachyantha*]	2	2.08	98.7	0
10	P1B03	cytosolic aconitase [*Triticum aestivum*]	2	2.07	100.0	2.838e-154
11	P1B04	asparagine synthetase2 [*Zea mays*]	3	3.09	98.6	3.894e-63
12	P1B05	leucine Rich Repeat family protein	1	1.07	98.8	4.829e-121
13	P1B06	xylose isomerase [*Zea mays*]	1	1.1	100.0	1.097e-150
14	P1B07	pathogenesis-related protein 4 [*Triticum monococcum*]	4	4.02	100.0	6.336e-110
15	P1B08	Probable non-specific lipid-transfer protein 2	2	2.08	98.8	1.076e-81
16	P1B10	zein-alpha 19C2 precursor [*Zea mays*]	7	7.02	100.0	5.546e-142
17	P1C02	anamorsin homolog [*Zea mays*]	2	2.01	100.0	1.185e-79
18	P1C05	60S ribosomal protein L5-1 [*Zea mays*]	8	8.06	100.0	1.212e-59
19	P1C08	peptidylprolyl isomerase putative [*Ricinus communis*]	1	1.06	100.0	1.665e-133
20	P1C09	conserved hypothetical protein	5	5.04	96.1	2.595e-88
21	P1C12	cytochrome b5 [*Zea mays*]	8	8.05	100.0	2.255e-29
22	P1D01	uncharacterized protein LOC100276217 [*Zea mays*]	5	5.03	97.2	0
23	P1D06	WD40 repeat-containing protein	3	3.04	98.8	1.054e-

SN	Clone ID	Name of the protein	Chr	Bin	Homology (%)	e-value
		SMU1-like				120
24	P1D11	E3 ubiquitin protein ligase UPL1	3	3.04	100.0	7.347e-116
25	P1D12	glyceraldehyde-3-phosphate dehydrogenase	6	6	98.0	3.761e-65
26	P1E05	Kinase-like protein [*Medicago truncatula*]	5	5.05	97.4	1.052e-87
27	P1E07	selenium-binding protein [*Zea mays*]	3	3.05	98.5	0
28	P1E08	aconitase2 [*Zea mays*]	4	4.04	99.6	5.454e-126
29	P1E09	catalase isozyme B [*Zea mays*]	5	5.03	98.3	1.226e-81
30	P1E10	fructose-16-bisphosphatase cytosolic [Zea mays]	8	8.03	100.0	2.177e-42
31	P1E12	metallothionein-like protein type 2 [*Zea mays*]	3	3.04	92.7	2.627e-161
32	P1F03	lipid transfer protein [*Oryza sativa* Japonica Group]	3	3.06	96.8	6.964e-81
33	P1F06	RND family efflux transporter MFP subunit	10	10.04	99.3	2.490e-62
34	P1F10	uncharacterized protein LOC100276695 [*Zea mays*]	3	3.06	97.9	5.967e-89
35	P1F11	putative methyltransferase PMT7 [*Arabidopsis thaliana*]	8	8.03	99.3	1.934e-152
36	P1F12	monodehydroascorbate reductase	2	2.06	99.4	1.575e-156
37	P1G01	chitinase [*Zea mays*]	10	10.4	93.9	1.357e-96
38	P1G02	endoplasmic oxidoreductin-1 [*Zea mays*]	1	1.1	100.0	2.177e-87
39	P1G05	Histone H2A [*Medicago truncatula*]	5	5.01	98.5	1.243e-162
40	P1G06	Spermidine synthase 1	2	2.06	100.0	4.118e-99
41	P1G07	nucleoside diphosphate kinase 1 [*Oryza sativa* Indica Group]	7	7.03	100.0	1.714e-156
42	P1G11	putative xylanase inhibitor [*Triticum aestivum*]	2	2.09	93.9	1.079e-97
43	P1H01	AAA-type ATPase family protein [*Arabidopsis thaliana*]	5	5.04	98.2	1.106e-74
44	P1H04	protein BRICK1 [*Zea mays*]	5	5.09	96.7	8.100e-79
45	P1H09	VHS domain-containing protein	8	8.03	99.6	4.299e-139
46	P1H10	putative aminoacrylate hydrolase RutD-like	6	6.07	95.5	2.992e-94

SN	Clone ID	Name of the protein	Chr	Bin	Homology (%)	e-value
47	P1H11	actin [*Zea mays*]	8	8.03	98.8	3.825e-122
48	P1H12	protein tyrosine phosphatase	1	1.09	100.0	1.048e-66
49	P2A01	senescence-associated protein 12	5	5.03	100.0	2.177e-83
50	P2A02	pathogen-related protein 10-1	1	1.03	98.4	7.741e-88
51	P2A03	Bowman-Birk type wound-induced proteinase inhibitor	3	3.04	94.2	5.984e-89
52	P2A08	barwin-like	4	4.02	100.0	6.916e-110
53	P2A09	S-adenosylmethionine synthetase	8	8.05	100.0	8.719e-177
54	P2A10	diphosphonucleotide phosphatase1 [*Zea mays*]	10	10.03	100.0	3.318e-111
55	P2A11	ATP-dependent endonuclease	3	3.05	96.4	5.079e-31
56	P2A12	ubiquilin-1 [*Zea mays*]	1	1.05	100.0	4.087e-125
57	P2B01	bZIP transcription factor	4	4.02	99.0	1.270e-100
58	P2B02	CBL-interacting protein kinase	4	4.08	99.2	1.242e-115
59	P2B03	B12D protein [*Zea mays*]	6	6.04	100.0	5.764e-129
60	P2B04	permatin precursor	3	3.06	98.1	2.787e-97
61	P2B05	win1 precursor [*Zea mays*]	4	4.02	100.0	1.651e-109
62	P2B06	chorismate mutase	1	1.07	100.0	3.058e-56
63	P2B07	transposase IS630	2	2.04	98.9	6.503e-135
64	P2B09	BETL-9 protein precursor [*Zea mays*]	8	8.08	96.7	3.804e-65
65	P2B10	Cancer-related nucleoside-triphosphatase homolog	1	1.01	100.0	7.711e-78
66	P2B12	ubiquinol-cytochrome C reductase iron-sulfur subunit	10	10.04	98.9	2.425e-132
67	P2C01	UDP-sugar pyrophospharylase	6	6.02	99.6	4.292e-140
68	P2C02	hydrolase	5	5	98.9	1.701e-84
69	P2C04	ubiquitin [*Oryza sativa*]				
70	P2C05	unknown [*Zea mays*]	2	2.08	99.2	2.712e-122
71	P2C06	adenine nucleotide translocator [*Zea*	5	5.06	97.9	3.670e-86

SN	Clone ID	Name of the protein	Chr	Bin	Homology (%)	e-value
		mays]				
72	P2C08	Polynucleotidyl transferase	1	1.01	100.0	2.177e-78
73	P2C09	globulin-1 S allele precursor [*Zea mays*]	1	1.09	99.0	7.855e-144
74	P2C10	40S ribosomal protein S21	5	5	93.8	2.852e-67
75	P2C11	transmembrane protein 205-like	3	3.05	99.0	5.697e-99
76	P2C12	eukaryotic translation initiation factor 3 subunit 7	3	3.08	98.8	0
77	P2D01	26S proteasome non-ATPase regulatory subunit 12-like	5	5.09	99.4	3.643e-76
78	P2D04	Cathepsin O	2	2.06	99.3	5.840e-134
79	P2D05	PDIL2-2 - Zea mays protein disulfide isomerase	3	3.04	99.5	3.623e-101
80	P2D07	trehalose-6-phosphate synthase [*Zea mays*]	1	1.02	97.6	9.536e-137
81	P2D09	non-specific lipid-transfer protein 2	2	2.08	98.8	3.642e-81
82	P2D10	activator of 90 kDa heat shock protein ATPase	4	4.11	94.0	1.577e-99
83	P2D11	Cytochrome P450 family protein	9	9.03	96.8	1.621e-84
84	P2D12	putative Na+-dependent transporter	6	6.07	99.5	2.538e-107
85	P2E01	ATP-dependent zinc metalloprotease FTSH	6	6.01	99.7	6.983e-153
86	P2E03	salt tolerant correlative protein	5	5.04	98.8	6.279e-79
87	P2E04	putative 23S ribosomal RNA	3	3.05	97.5	1.313e-70
88	P2E05	cp protein [*Celosia cristata*]	6	6.05	99.5	1.315e-90
89	P2E06	T cytoplasm male sterility restorer factor 2	5	5.03	100.0	7.833e-98
90	P2E07	non-ribosomal peptide synthetase	7	7.02	100.0	6.256e-74
91	P2E08	protein phosphatase 2C	3	3.09	98.8	0
92	P2E09	Leukocyte receptor cluster member-like protein	8	8.04	100.0	2.000e-68
93	P2E10	voltage-dependent anion channel protein1a [Zea mays]	2	2.06	98.9	0
94	P2E12	peptidase M61 domain-containing protein	6	6.05	100.0	5.128e-164
95	P2F03	acyl-CoA-binding domain-containing protein 6	2	2	99.4	2.591e-157
96	P2F05	vicilin-like embryo storage protein [*Zea mays*]	1	1.09	97.8	0

SN	Clone ID	Name of the protein	Chr	Bin	Homology (%)	e-value
97	P2F06	ubiquitin-conjugating enzyme E2 32-like	9	9.05	99.4	6.928e-178
98	P2F07	60S ribosomal protein L12	4	4.06	98.5	7.856e-93
99	P2F08	eukaryotic translation initiation factor 3 subunit B-like	1	1.05	99.5	0
100	P2F09	Aminoacyl-tRNA synthetase	7	7.02	100.0	6.472e-77
101	P2F10	DnaJ heat shock protein	1	1.08-1.09	100.0	5.742e-119
102	P2F11	12-dihydroxy-3-keto-5-methylthiopentene dioxygenase 2	1	1.02	99.2	7.832e-58
103	P2G01	YT521-B-like family protein	9	9.07	99.4	0
104	P2G02	transmembrane receptor protein serine/threonine kinase	4	4.09	100.0	1.202e-135
105	P2G04	elongation factor 1-delta 1 [*Zea mays*]	7	7.04	97.7	1.380e-55
106	P2G05	nodulin-like protein	2	2.04	99.3	2.064e-133
107	P2G06	WD-repeat protein 57 [*Zea mays*]	9	9.04	99.3	6.883e-133
108	P2G07	zinc finger protein binding protein	2	2.05	99.7	1.213e-145
109	P2G08	NUDIX domain-containing protein	3	3.04	100.0	2.919e-67
110	P2G09	ACR255Cp [*Ashbya gossypii* ATCC 10895]	5	5	93.8	2.884e-67
111	P2G10	OmpA/MotB domain-containing protein	9	9.07	99.7	0
112	P2G12	E3 ubiquitin protein ligase UPL1	10	10.03	99.3	0
113	P2H01	BTB/POZ domain-containing protein At2g46260	4	4.09	100.0	8.427e-177
114	P2H03	putative mitochondrial Rieske protein	10	10.04	98.9	2.598e-132
115	P2H04	Wheatwin-2	4	4.02	100.0	1.609e-109
116	P2H05	component of cytosolic 80S ribosome and 40S small subunit	5	5	93.8	2.888e-67
117	P2H07	dihydrodipicolinate synthetase	1	1.09	97.1	2.258e-58
118	P2H08	bromo adjacent homology domain-containing 1 protein	9	9.04	97.4	3.607e-86
119	P2H09	asparagine synthetase2 [*Zea mays*]	5	5.04	100.0	5.776e-114
120	P2H10	cytochrome b-c1 complex subunit Rieske	10	10.04	98.9	2.576e-132

SN	Clone ID	Name of the protein	Chr	Bin	Homology (%)	e-value
121	P2H11	transaldolase 2 [*Zea mays*]	8	8.06	98.9	5.476e-134
122	P3A01	casein kinase II subunit beta-4 [*Zea mays*]	7	7.03	100.0	2.527e-144
123	P3A02	putative puroindoline b protein	1	1.1	96.8	7.887e-82
124	P3A03	splicing factor 3B subunit 1-like isoform 1	4	4.1	98.3	1.999e-143
125	P3A06	prohevein [*Hevea brasiliensis*]	4	4.02	100.0	1.611e-109
126	P3A07	putative acyl-CoA synthetase	3	3.08	99.2	1.763e-54
127	P3A08	26S proteasome regulatory particle non-ATPase subunit5	5	5.09	99.4	3.683e-76
128	P3A09	hemolysin family calcium-binding region	9	9.02	97.7	4.250e-145
129	P3A10	acetyl-coenzyme A carboxylase ACC1A	5	5.03	100.0	8.895e-157
130	P3A11	pi1 [*Solanum lycopersicum*]	4	4.02	100.0	1.542e-109
131	P3A12	phospho-2-dehydro-3-deoxyheptonate aldolase 1 [*Zea mays*]	7	7.04	99.1	1.759e-178
132	P3B02	heparanase [*Oryza sativa* Indica Group]	1	1.02	97.4	7.996e-68
133	P3B04	Acyl-protein thioesterase 2 [*Zea mays*]	3	3.03	98.3	3.264e-171
134	P3B05	putative 23S ribosomal RNA	1	1.02	99.4	1.333e-80
135	P3B06	putative 23S ribosomal RNA	10	10.04	99.3	1.065e-61
136	P3B07	heat shock protein 70	5	5.03	86.2	2.608e-142
137	P3B08	vacuolar processing enzyme1 precursor [*Zea mays*]	5	5.05	100.0	1.285e-105
138	P3B09	eukaryotic translation initiation factor 3 subunit like protein	4	4.05	99.4	7.842e-73
139	P3B11	RNA binding protein [*Arabidopsis lyrata* subsp. *lyrata*]	6	6.08	99.2	3.300e-126
140	P3C01	putative iron-sulfur cluster-binding protein	3	3.05	99.5	1.259e-105
141	P3C02	elongation factor 1 alpha	6	6.05	100.0	2.899e-72
142	P3C03	histidinol dehydrogenase chloroplastic-like	1	1.11	98.4	5.609e-154
143	P3C04	chaperone protein dnaj	8	8.08	92.2	8.122e-78

SN	Clone ID	Name of the protein	Chr	Bin	Homology (%)	e-value
144	P3C05	basic pathogenesis-related protein PR5	3	3.05	99.5	1.294e-105
145	P3C06	RNA recognition motif family protein	1	1.11	100.0	8.104e-73
146	P3C07	zinc finger protein-like	5	5.03	98.5	3.200e-95
147	P3C08	elongation factor 1-beta [*Zea mays*]	7	7.05	100.0	1.539e-58
148	P3C09	osmotin-like	3	3.06	97.3	1.661e-99
149	P3C10	putative Csa-19 [*Oryza sativa* Japonica Group]	1	1.07	96.7	9.928e-97
150	P3D02	O-methyltransferase [*Aspergillus fumigatus* Af293]	6	6.05	100.0	5.419e-164
151	P3D04	cytochrome b-c1 complex subunit Rieske	10	10.04	98.9	2.728e-132
152	P3D06	eukaryotic translation initiation factor 5A [*Zea mays*]	2	2.08	99.6	9.828e-127
153	P3D08	acidic ribosomal protein P40 [*Zea mays*]	9	9.07	99.5	4.662e-100
154	P3D09	developmentally-regulated GTP-binding protein 2	9	9.08	98.2	1.273e-105
155	P3D10	lammer-type protein kinase	5	5.05	100.0	9.802e-107
156	P3E01	ribosomal protein L3 [*Triticum aestivum*]				
157	P3E02	F14N23.18 [*Arabidopsis thaliana*]	9	9.01	100.0	2.121e-113
158	P3E03	Cation transport regulator-like protein	5	5.04	100.0	6.007e-114
159	P3E05	monothiol glutaredoxin-S11-like	2	2.05	96.4	0
160	P3E08	2-oxoisovalerate dehydrogenase alpha subunit	10	10.03	100.0	1.739e-79
161	P3E11	serine/threonine protein kinase	6	6.01	99.0	0
162	P3E12	DNA-binding protein [*Zea mays*]	3	3.04	99.4	0
163	P3F01	meloidogyne-induced giant cell protein-like protein	5	5.04	100.0	5.689e-114
164	P3F03	S-adenosylmethionine synthetase 1 [*Zea mays*]	10	10.03	94.7	1.222e-150
165	P3F04	flower-specific gamma-thionin precursor [*Zea mays*]	10	10.03	100.0	4.275e-120
166	P3F05	LOC100286358 [*Zea mays*]	6	6.04	99.2	4.548e-125
167	P3F11	lysine ketoglutarate reductase trans-splicing related 1 [*Zea mays*]	5	5.04	100.0	1.318e-55

SN	Clone ID	Name of the protein	Chr	Bin	Homology (%)	e-value
168	P3F12	60S ribosomal protein L11	9	9.03	98.1	2.601e-97
169	P3G02	mitochondrial import receptor subunit TOM5 homolog	1	1.08	98.4	2.037e-123
170	P3G03	ATP-dependent Clp protease proteolytic subunit 2	9	9.02	100.0	3.725e-66
171	P3G04	nucleolar GTP-binding protein [*Oryza sativa* Japonica Group]	9	9.01	100.0	2.135e-113
172	P3G05	putative nucleolar GTP-binding protein	9	9.01	100.0	2.126e-113
173	P3G09	uncharacterized protein LOC100217111 [*Zea mays*]	7	7.06	99.3	3.746e-66
174	P3G12	MerR-family transcriptional regulator	1	1.10	98.4	9.531e-87
175	P3H01	Cation transport regulator-like protein 2	5	5.04	100.0	5.537e-114
176	P3H04	cysteine proteinase inhibitor [*Zea mays*]	8	8.08	100.0	1.152e-140
177	P3H06	S-adenosylmethionine decarboxylase proenzyme [*Zea mays*]	2	2.04	99.3	0
178	P3H07	Metabotropic glutamate receptor	1	1.06	92.5	2.346e-33
179	P3H08	poly-gamma-glutamate synthesis protein YwtB	2	2.10	99.6	7.211e-143
180	P3H09	phosphotransferase system EIIC component	4	4.08	99.2	1.592e-124
181	P3H10	acidic ribosomal protein P40 [*Zea mays*]	9	9.07	99.5	4.511e-100
182	P3H11	ATPP2-A13 [*Zea mays*]	7	7.03	98.2	1.595e-104
183	P3H12	polyubiquitin-like protein	4	4.1	98.8	1.101e-165
184	P4A01	selenium-binding protein [*Zea mays*]	3	3.05	99.5	0
185	P4A02	hsc70-interacting protein [*Zea mays*]	4	1.11	99.7	0
186	P4A03	reticulon [*Zea mays*]	6	6.05	94.2	0
187	P4A06	metallothionein	2	2.03	90.4	4.440e-120
188	P4A07	multidrug resistance protein	7	7.04	96.6	2.582e-147
189	P4A08	eukaryotic translation initiation factor 3 subunit C-like	2	2.02	98.6	0
190	P4A10	ferredoxin-3 chloroplastic precursor	1	1.11	98.2	0

SN	Clone ID	Name of the protein	Chr	Bin	Homology (%)	e-value
		[Zea mays]				
191	P4A12	phosphatidylinositol transfer protein [*Zea mays* subsp. mays]	7	7.02	100.0	9.442e-77
192	P4B02	ornithine--oxo-acid aminotransferase [*Saccharum officinarum*]	1	1.09	95.7	2.529e-157
193	P4B07	ribosomal protein S7 [*Triticum aestivum*]	1	1.03	99.0	1.302e-90
194	P4B09	aldehyde dehydrogenase [*Sorghum bicolor*]	1	1.03	99.0	1.302e-90
195	P4B10	1-acyl-sn-glycerol-3-phosphate acyltransferase zeta precursor	2	2.06	98.1	5.860e-99
196	P4C03	methionyl aminopeptidase	4	4.08	98.3	3.572e-111
197	P4C04	30S ribosomal protein S21e	5	5	93.8	2.959e-67
198	P4C05	pseudo-hevein [*Hevea brasiliensis*]	4	4.02	100.0	1.662e-109
199	P4C07	histone H2A [*Zea mays*]	9	9.06	97.0	7.322e-138
200	P4C09	DNA binding protein putative	8	8.03	100.0	5.082e-40
201	P4C10	acyl-CoA dehydrogenase family member 10-like	2	2.08	100.0	3.848e-101
202	P4C11	uncharacterized protein LOC100279794 [*Zea mays*]	1	1.12	98.2	4.063e-160
203	P4C12	CBL-interacting serine/threonine-protein kinase 11 [*Zea mays*]	3	3.07	96.3	3.550e-56
204	P4D01	cysteine proteinase inhibitor 2 [*Zea mays*]	6	6.06	100.0	5.643e-55
205	P4D02	40S ribosomal protein S19-like	1	1.04	99.6	9.879e-112
206	P4D04	chromate transport protein	2	2.07	98.0	4.642e-120
207	P4D05	eukaryotic translation initiation factor 5 [*Zea mays*]	4	4.1	97.6	2.061e-138
208	P4D06	vesicle-associated membrane protein 725 [*Zea mays*]	1	1.11	99.3	1.235e-150
209	P4D08	translation initiation factor 4G [*Triticum aestivum*]	2	2.08	100.0	0
210	P4D09	fibropellin III	5	5.03	99.2	1.758e-54
211	P4D10	acyl-[acyl-carrier protein] thioesterase [*Triticum aestivum*]	7	7.03	100.0	6.476e-39
212	P4D11	outer mitochondrial membrane protein porin [*Zea mays*]	1	1.01	99.3	7.033e-133

SN	Clone ID	Name of the protein	Chr	Bin	Homology (%)	e-value
213	P4E01	lammer-type protein kinase [*Sorghum bicolor*]	4	4.05	100.0	1.009e-76
214	P4E07	putative phytosulfokine receptor	7	7	97.5	0
215	P4E08	beta-galactosidase-complementation protein				
216	P4E09	chaperonin [*Zea mays*]	2	2.06	99.4	6.021e-78
217	P4E10	eukaryotic translation initiation factor 2D-like	5	5.05	100.0	5.504e-154
218	P4F02	indole-3-glycerol phosphate synthase-like	10	10.06	91.6	4.182e-170
219	P4F04	ripening regulated protein DDTFR10 [*Solanum lycopersicum*]	7	7.04	96.9	6.488e-54
220	P4F06	nucleic acid binding protein [*Zea mays*]	10	10.04	99.6	7.689e-139
221	P4F07	elongation factor 1-delta 1 [*Zea mays*]	7	7.04	97.7	1.391e-55
222	P4F08	inositol-tetrakisphosphate 1-kinase 3 [*Zea mays*]	1	1.1	98.5	3.331e-58
223	P4F09	cystatin-1 precursor [*Zea mays*]	8	8.08	100.0	1.203e-140
224	P4F10	embryonic abundant protein 1 [*Zea mays*]	6	6.05	93.6	1.633e-91
225	P4F11	small subunit ribosomal protein S21e [*Arabidopsis thaliana*]	5	5	93.8	2.789e-67
226	P4F12	IgGFc-binding protein-like	7	7.05	94.1	8.105e-89
227	P4G02	multi-drug resistance protein [*Arabidopsis thaliana*]				
228	P4G03	elongation factor 1-alpha [*Zea mays*]	9	9.03	99.3	0
229	P4G05	win2 precursor [*Zea mays*]	4	4.02	99.5	7.691e-108
230	P4G06	glutamine-dependent NAD(+) synthetase-like	2	2.06	99.0	6.571e-44
231	P4G08	peptidylprolyl isomerase putative	1	1.06	100.0	2.067e-133
232	P4G10	mitogen activated protein kinase 7	5	5.03	97.9	3.244e-156
233	P4G11	pyrophosphate--fructose-6-phosphate 1-phosphotransferase	9	9	100.0	4.404e-110
234	P4G12	pathogenesis-related protein 1 [*Zea mays*]	1	1.03	100.0	7.337e-93
235	P4H01	40S ribosomal protein S21 [*Zea*	5	5	93.3	1.285e-65

SN	Clone ID	Name of the protein	Chr	Bin	Homology (%)	e-value
		mays]				
236	P4H05	transglutaminase-like enzyme protein	1	1.01	99.0	3.570e-101
237	P4H06	late embryogenesis abundant protein D-34 [*Zea mays*]	1	1.10	97.9	4.698e-85
238	P4H07	G-rich sequence factor 1-like isoform 2	6	6.08	100.0	8.902e-177
239	P4H08	RNA binding protein	6	6.08	99.2	3.413e-126
240	P4H09	phosphoglyceromutase	10	10.01	93.5	4.144e-55
241	P4H11	Dual specificity tyrosine-phosphorylation-regulated kinase	1	1.10	96.8	3.623e-66
242	P4H12	putative oxidoreductase	5	5.03	99.2	1.958e-123
243	P5A03	cysteine-type peptidase	2	2.07	100.0	2.896e-62
244	P5A04	serine/threonine-protein kinase AtPK2/AtPK19-like	9	9.05	100.0	2.635e-127
245	P5A05	cation transport regulator-like protein 2 isoform	5	5.04	100.0	5.872e-114
246	P5A06	similarity to phosphofructokinases	9	9	100.0	4.497e-110
247	P5A08	60S ribosomal protein L19-3 [*Zea mays*]	5		100.0	9.922e-69
248	P5A09	putative aminopeptidase [*Saccharomonospora cyanea* NA-134]	3	3.05	99.5	1.252e-105
249	P5A10	protein vip1-like	9	9.03	100.0	0
250	P5B03	Transketolase [*Medicago truncatula*]	2	2.05	99.4	0
251	P5B07	60S ribosomal protein L7a [*Zea mays*]	7	7.03	95.1	1.244e-120
252	P5B08	pathogenesis-related protein 5 [*Zea mays*]	1	1.06	98.0	1.014e-91
253	P5B09	putative vacuolar defense protein [*Triticum aestivum*]	4	4.02	100.0	1.606e-109
254	P5B11	probable serine incorporator-like	5	5.08	99.5	0
255	P5C01	keratinocyte-associated protein 2-like	3	3.04	98.7	3.304e-146
256	P5C02	60S ribosomal protein L17 [*Zea mays*]	1	1.06	97.9	4.558e-115
257	P5C03	S-adenosylmethionine decarboxylase 3	2	2.04	99.3	0

SN	Clone ID	Name of the protein	Chr	Bin	Homology (%)	e-value
258	P5C04	XK-related protein 8 [*Bos taurus*]	9	9.05	96.2	6.053e-104
259	P5C05	zeamatin-like	3	3.06	97.3	1.683e-99
260	P5C09	RNA-binding protein 25 [*Zea mays*]	5	5.04	100.0	1.251e-45
261	P5C10	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	1	1.10	99.0	9.971e-92
262	P5D01	S-adenosylmethionine decarboxylase 3 [*Sorghum bicolor*]	1	2.04	99.3	0
263	P5D03	chordin [*Homo sapiens*]	4	4.09	99.2	8.276e-129
264	P5D04	C-1-tetrahydrofolate synthase cytoplasmic [Zea mays]	8	8.05	99.3	2.312e-68
265	P5D05	membrane protein [*Zea mays*]	7	7.04	99.4	2.271e-73
266	P5D07	phosphoglyceromutase	10	10.01	93.5	4.988e-55
267	P5D10	putative cation transporter [*Potamogeton distinctus*]	5	5.04	100.0	5.800e-114
268	P5D11	alpha/beta hydrolase fold protein	7	7.05	94.7	7.422e-93
269	P5D12	lipid transfer protein [*Oryza sativa*]	2	2.08	98.8	3.417e-81
270	P5E03	mps one binder kinase activator-like 1A [*Zea mays*]	10	10.03	100.0	1.414e-50
271	P5E04	glycyl-tRNA synthetase 1 mitochondrial-like	4	4.08	100.0	0
272	P5E06	stromal ascorbate peroxidase	2	2.04	99.1	3.032e-47
273	P5E08	ubiquitin-fold modifier 1 [*Zea mays*]	3	3.05	99.5	1.686e-99
274	P5E09	putative RNA-binding protein RBP37 [*Oryza sativa* Japonica Group]	6	6	100.0	1.423e-30
275	P5E10	legumain	5	5.05	100.0	1.271e-105
276	P5E11	ataxin-3 [*Zea mays*]	3	3.06	100.0	0
277	P5F01	cation transport protein chaC [*Zea mays*]	5	5.04	100.0	3.082e-114
278	P5F02	aspartic proteinase	6	6.07	98.5	5.717e-129
279	P5F05	basic leucine zipper and W2 domain-containing protein	2	2.05	100.0	6.019e-121
280	P5F08	L3 ribosomal protein partial	2	2.06	100.0	6.006e-99
281	P5F10	Bromodomain associated family protein expressed	1	1.05	99.7	0
282	P5F11	60S ribosomal protein L36-2 [*Zea mays*]	6	6.05	100.0	9.533e-102

SN	Clone ID	Name of the protein	Chr	Bin	Homology (%)	e-value
283	P5F12	anoctamin-10-like	3	3.07	99.2	4.567e-60
284	P5G04	shikimate kinase [*Zea mays*]	4	4.08	97.2	1.377e-60
285	P5G05	ubiquitin C-terminal hydrolase	6	6.05	80.3	1.171e-37
286	P5G06	Cytochrome c peroxidase	2	2.04	98.2	1.412e-45
287	P5G07	transposase IS4 family				
288	P5G10	cingulinW1 precursor	8	8.04	100.0	5.375e-159
289	P5G11	metalloprotease m41 ftsh	1	1.12	98.2	4.097e-160
290	P5G12	vacuolar protein sorting protein 25 [*Zea mays*]	8	8.06	96.4	6.052e-44
291	P5H01	Wound-induced protein WIN2 precursor	4	4.02	100.0	7.318e-110
292	P5H02	ADP-glucose pyrophosphorylase embryo small subunit [*Zea mays*]	2	2.06	99.5	3.473e-91
293	P5H04	putative MAPK [*Zea mays*]	5	5.03	97.9	3.262e-156
294	P5H05	hypothetical myo-inositol catabolismprotein iolB	7	7.02	100.0	6.180e-74
295	P5H06	CCR4 associated factor 1-related protein [*Medicago truncatula*]	2	2	96.7	2.787e-92
296	P5H07	B12D protein [*Ipomoea batatas*]	6	6.04	100.0	5.717e-129
297	P5H09	60S ribosomal protein L13-2 [*Zea mays*]	6	6.05	97.5	1.570e-134
298	P5H10	cystatin	8	8.08	99.6	1.978e-138
299	P5H11	Probable ubiquitin-conjugating enzyme E2 R521	6	6.04	97.2	1.189e-130
300	P5H12	small subunit ribosomal protein S21e	5	5	93.8	2.722e-67

VITA

Ramesh Dhakal was born in Mangalpur VDC-8, Chitwan, Nepal. He received his Bachelor's degree in Agriculture Science and Master's degree in Agronomy from the Institute of Agriculture and Animal Sciences (IAAS), Tribhuvan University, Nepal, in 2002 and 2005, respectively. He worked as Technical Officer at Nepal Agricultural Research Council (NARC). During his job at NARC, he was mainly involved in the maintenance and development of new varieties in maize, wheat, and rice. He was also involved in research, development, and dissemination of maize based technologies in mid-hills of Nepal in collaboration with CIMMYT/ HMRP from 2005 to 2010. He joined Dr. Subudhi's lab at the School of Plant, Environmental and Soil Sciences (SPESS) at Louisiana State University in summer 2010 to pursue a doctoral degree and is a candidate to graduate in December 2015.