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**Project Narrative: Improving Drought Tolerance and Aflatoxin Resistance in Maize; Education, Extension, and Translational Breeding via Altered Lipid Metabolism**

**Introduction**

Challenges of increasing population, decreasing land availability and quality, and demand for biobased products require continuing to increase our food, feed, forage and feedstock supply in a safe and sustainable manner while simultaneously decreasing agricultural inputs. Yields of crops such as maize (*Zea mays* L., corn) have dramatically increased in the US to meet these needs with the adaptation of modern breeding methods (Fig. 1). It has been estimated that over 50% of yield increases in maize can be attributed to plant genetic improvement, and the pace of this improvement continues to grow with enhanced scientific knowledge and techniques (Duvick, 1999, Troyer, 2006). Recently, maize improvement progress in much of the United States (US) has benefitted from a shift from public and small company breeding to increased large private company breeding in recent years. These larger companies have been able to quickly invest in and incorporate basic scientific discoveries from DNA sequencing, molecular biology, information technology and advanced statistics, into strategies for trait discovery and molecular breeding (Cooper et al., 2004). Plant breeding education as well as maize breeding in the Southern US have not yet incorporated or benefited from these innovations (Fig. 1).

Much of the Southern and South Central US have warmer and even subtropical climates that differ from the temperate Northern US. Differences in climate and pests (in addition to soils and photoperiod) provide a different set of opportunities and challenges for sustainable maize production in the South. Many of these challenges have yet to be adequately addressed by private industry, at least partly because they often can be antithetical needs of the temperate North. For example, Northern hybrids require increased field dry-down to minimize the post harvest use of natural gas. Thus, lines have been selected for loose husks with no ear droop (ears dry with open tip exposed to the sky). These same traits predispose the crop to pre-harvest fungal contamination in the hotter and drier South where dry down is generally not a problem (Betran and Isakeit 2004 , G. Odvody pers. com). In the research component of this proposal we integrate modern molecular breeding methods and reverse genetics screens for improving Southern US maize adaptation through plant breeding. The research foci of this proposal are directly linked with ongoing needs and projects in education and extension discussed later.

Two of the major stress induced limitations to maize production in the US, and especially in the South, are drought tolerance and high levels of seed contamination with carcinogenic mycotoxins, especially aflatoxin (Betran and Isakeit, 2004). Drought in maize has been shown to reduce yield by 15-30% and can even lead to complete failure (Campos et al. 2004; Edmeades et
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Drought additionally makes maize more susceptible to contamination with pre-harvest aflatoxins (Betran and Isakeit, 2004). Contamination with carcinogenic aflatoxins, produced by the seed-infecting fungus *Aspergillus flavus* Link:FR, is a serious food and feed safety hazard to humans, poultry and livestock. Limitations to drought stress and aflatoxin contamination are related and can likely be improved through breeding maize to better handle stress. This will ultimately lead to a safer, more economically and environmentally sustainable food and feed supply.

Of the Southern US, Texas is the largest producer of maize, and 13th in the US overall (USDA-NASS, 2009). Texas has a great diversity of environments under maize production which vary greatly in moisture, temperature and soil type. Within Texas’s extremely diverse environments, locations with high average levels of drought, heat and aflatoxin stress, such as College Station and Weslaco, make Texas an ideal state for evaluating abiotic and biotic stress tolerance in maize. Across Texas the maize acreage is approximately half rainfed (with minimal supplemental irrigation) and half under full irrigation (mostly in the panhandle/ high-plains). Fully irrigated acreage averages double the yields of rainfed counties (USDA-NASS). For increased conservation of natural resources and economic and environmental sustainability, maize must be improved to use less water while producing ever greater yields. This can be achieved by a combination of phenotypic selection under droughty conditions and molecular assisted breeding.

Drought stress may occur at various stages in maize growth and development (seedling, tasseling, silking, grain fill, etc.). Depending on the intensity, length and duration of stress numerous adaptive phenotypes such as deeper roots, lower transpiration rate, and hydraulic lift (nocturnal water transport between soil layers: Richard and Caldwell, 1987; Wan et al. 2000), can play a role in reducing drought stress (Collins et al., 2008). However, difficulty in controlling the diversity of specific drought conditions makes it extremely difficult to identify broad adaptive phenotypes for improvement; hence the small levels of success many phenotypic breeders have experienced. To minimize yield loss, researchers have found that the most critical time to manage drought surrounds one week before flowering, and through soft dough (Grant et al. 1989; Rhoads and Bennett, 1990). Therefore maintaining drought stress during this important period is needed for viewing variation for selection.

Modern US commercial hybrids and some tropical populations have improved significantly in grain yield, which is believed to be due in large part to selection for yield under stress (Tollenaar and Lee, 2002). For drought stress, this has specifically meant maintaining low anthesis silking interval (ASI) and low barrenness which appears to have a large impact on drought tolerance (Campos et al. 2006). Disappointingly, many investigators have found that molecular and breeding approaches for introgressing drought tolerance often have unexpected tradeoffs. Thus a plant that is conservative in its use of resources is ideal for a poor environment but poor in an ideal environment (Collins et al. 2008). Instead, we desire an adaptive quantitative trait locus (QTL) rather than a constitutive one thus it is critical we continue to investigate and select against tradeoffs during improvement (Collins et al. 2008). To improve maize for economically and environmentally sustainable yields we are most interested in conducting phenotypic selection under reduced irrigation regimes, especially during flowering. This may only marginally increase the overall production of maize in drought prone areas in a good year, but it will greatly increase the stability of yield under stress conditions.

It is well known that drought stress during grain fill predisposes maize to a greater risk of accumulating significantly higher levels of aflatoxins (Betran and Isakeit, 2004). Therefore,
improving drought tolerance in maize is not only expected to reduce the cost and environmental impact of irrigation, but can also lower the food and feed safety threat from aflatoxin.

While aflatoxicosis is rarely acute in humans or animals, aflatoxin is a potent chronic liver carcinogen. Levels of aflatoxin B1 as low as 20ppb are considered hazardous and thus regulated in interstate commerce. In Texas where mandatory aflatoxin testing is required, these carcinogens are on the forefront of many producers minds. Loss liability in the state from mycotoxins (mostly aflatoxin) was $13 million in 2008 (USDA-RMA, 2009), the highest reported in the nation. Because aflatoxin contamination is not regularly reported in states outside of Texas, the threat from this toxin in the rest of the US is unclear. In the reported instances where grain has been tested in the rest of the US, pre-harvest aflatoxins have reached action levels even in the temperate Midwest (Lillehoj et al. 1976; Osweiler, 2005). Global climate change and increased awareness are making aflatoxin an increasingly national issue across commodities. The increased frequency of high stress (hot and dry) years make the diverse climates of Texas ideal research locations to improve both drought and aflatoxin resistance.

**Working Research Hypotheses:**
1) As demonstrated by knock-out mutants, the lipoxigenase (LOX) gene family mediates quantitative variation for both drought tolerance and aflatoxin resistance in maize (*Zea mays* L.).
2) Natural allelic variants at LOX gene family members may be identified that condition improved phenotypes for drought tolerance and/or aflatoxin resistance without the use of transgenic events.
3) We can improve the overall drought tolerance and/or aflatoxin resistance in maize by pyramiding elite LOX alleles with improved germplasm selected through other phenotypic breeding methods.

**Research: combination of LOX genes and phenotypic breeding to improve drought tolerance and aflatoxin accumulation**

Previous research on natural lipoxygenase (LOX) family (non-GMO) mutants in the Kolomiets’ lab has shown extraordinary impacts on maize resistance to mycotoxins contamination, drought tolerance, stalk rotting pathogens, and nematodes. To conclusively establish LOX functions, 13 separate LOX genes have been cloned in maize (Kolomiets et al. 2004). By using a non-transgenic, *Mutator* transposon tagging approach, we have identified insertional knock-out mutants in 11 LOX genes. In the course of the last six years of back-crossing, mutant alleles have been incorporated into several inbred lines to generate near-isogenic lines (NILs) at BC₆ stage suitable for functional analysis.

Deletion of the *ZmLOX4* gene resulted in a dramatic increase in seedling (Fig. 2) and mature plant drought tolerance.

![Figure 2. Zmlox4 mutants are more tolerant to drought stress as compared to near-isogenic wild type plants. The picture shows that lox4 mutants recover after severe drought stress (14 days of no watering) and renewed normal growth after watering was resumed. Picture was taken 6 days after resumption of watering.](image)
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(not shown). Additionally, multi-year, multi-location field studies conclusively established that a few mutants, notably in the ZmLOX5 gene, accumulate 5-fold less aflatoxin in all four of the genetic backgrounds that were tested (Fig. 3). Importantly, these mutants did not show any detectable pleiotropic deterioration of other agronomic traits demonstrating the practicality of modification of lipid metabolism for maize improvement.

Lab-based drought-stress analysis indicated that Zmlox4 mutants are significantly more tolerant to drought stress as compared to their NIL WT (Fig. 2). In these experiments, 3-week-old Zmlox4 mutant and WT seedlings were not watered for 14 days to impose severe drought stress conditions. At this time point, the leaves of all the WT plants curled and eventually dried completely, whereas the leaves of the mutants showed minimal symptoms of wilting. The Zmlox4 mutants displayed even more impressive phenotype after resumption of normal watering regime. After both genotypes were watered every day for the next six consecutive days, Zmlox4 mutants resumed their normal growth whereas nearly 90% of wild-types were unable to recover (Fig. 2). Also, Zmlox4 mutants displayed more robust growth compared to WT under drought field conditions (unpublished data, currently undergoing more extensive testing in the field). Of significance to this proposal, Zmlox4 mutants showed no statistical difference in the levels of aflatoxin accumulation, but testing has occurred in a very limited number of genotypes.

Most of the known hosts for mycotoxigenic fungi are oil-rich crops such as maize, peanuts, cotton and various tree nuts, which points to an important role that plant lipids may have in the host-seed-pathogen interactions. Indeed, fatty acids and their metabolic products, oxylipins, have been implicated in susceptibility to mycotoxin accumulation (Gao and Kolomiets, 2008). Most oxylipins in plants are produced by lipoxygenases (LOX) that catalyze oxygenation of polyunsaturated fatty acids. Because oxylipins produced by LOX induce production of aflatoxin, we proposed a hypothesis that specific LOXs are “hijacked” and utilized by Aspergillus to induce biosynthesis of aflatoxins and hence, these LOX genes are susceptibility factors in maize plants (Gao and Kolomiets, 2008). If this hypothesis correct, then by shutting down certain LOX genes, accumulation of toxins should be reduced. Indeed, this hypothesis is strongly supported by our recently published findings that inactivation of the ZmLOX3 gene resulted not only in 200-fold reduced levels of mycotoxin fumonisin, but also increased resistance to stalk and root rots, anthracnose and southern leaf blights (Gao et al., 2007). Because lox3 mutants displayed increased susceptibility to aflatoxin accumulation (Gao et al., 2009), we focus our molecular breeding effort on two other genes, mutation of which increased drought tolerance (ZmLOX4) and reduced aflatoxin levels (ZmLOX5) but did not lead to any detectable deterioration of other agronomic traits or plant morphology (see details below). Figure 3A shows that lox5 mutants accumulate up to 5-fold lower levels of aflatoxins not only in the susceptible lines FR2128 and T714 but also in the line T772, a Texas standard for aflatoxin.

Figure 3. Accumulation of aflatoxins in Zmlox5 mutants and near-isogenic wild types is significantly reduced in four genetic backgrounds, B73, T714, FR2128 and T772. (A) NILs tested in a 2006 aflatoxin field test. (B) NILs with different mutant alleles were tested in 2007 and showed variability.
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resistance (Betran et al. 2002). Importantly, independent lox5 mutant alleles, e.g. lox5-1, lox5-2 and lox5-3 (Fig. 3), have similar but statistically different effects on aflatoxin levels providing strong genetic evidence that the effect is indeed due of disruption of this gene. This suggests two testable hypotheses: A) genetic background impacts the effects of mutant lox alleles (Fig 2A), and B) different alleles at ZmLOX4 and ZmLOX5 will have different impacts on drought tolerance and aflatoxin resistance respectively.

Synergistic breeding activities:
Over 70 finished inbred lines have recently been developed through the Texas A&M / Texas AgriLife breeding program. These lines are very diverse but have been selected under minimal irrigation conditions around Texas for yield. Many of these lines are based on temperate x tropical germplasm crosses, and some have been selected for improved aflatoxin resistance and/or specialty traits such as improved composition (QPM) and color (white, red, or blue food maize). Co-operation Dr. Wenwei Xu’s AgriLife maize breeding program in the irrigated area of Lubbock, TX has provided further access to finished inbred materials selected under drought conditions. Currently, a set of 60 Texas AgriLife lines per se are being tested in the field for performance under full vs. limited irrigation, for flag leaf wax content (as a potential predictor of drought and heat stress), and for seedling drought tolerance using a novel assay in the greenhouse. Soil moisture differences are being monitored in the field using a series of probes (Decagon devices) (Fig. 4) to quantify moisture differences between full and limited irrigation. This information is being collected to formally test lines per se with superior drought and heat tolerance. The experiment will be repeated next summer using testcross hybrids. To demonstrate the critical mass of faculty experiences in plant breeding, drought and heat stress at Texas A&M it should be mentioned that all of these techniques and assays arose out of communication with the plant breeding or soil hydropedology faculty working on other crops (cotton, wheat, cowpea, sorghum) who are having success in using them as breeding and screening tools.

In addition to the lines mentioned above, Mayfield (KM), Isakeit (TI), Murray (SM), along with others, under the support of the Texas Corn Producers, are conducting pedigree based breeding using only phenotypic selection to develop aflatoxin resistant lines. These are currently being evaluated in two testcrosses (commercial transgenic stiff-stalk and non-stiff-stalk lines) for combining ability and inoculated aflatoxin resistance under limited irrigation. A major limitation in all aflatoxin breeding experiments is the cost and time associated with measurement of the toxins. With the material from this summer we will be evaluating Near Infrared Spectroscopy (NIRS) as a less costly method for detecting A. flavus and/or aflatoxin using at least two separate platforms in cooperation with the USDA Engineering unit in Kansas (F. Dowell and T. Pearson) and William Rooney’s Texas AgriLife Sorghum breeding program.
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Results on the use of NIRs from recently published studies look promising but it is not yet clear how this will work in diverse breeding material (Wicklow et al., 2008; Fernández-Ibañez et al., 2009). Additionally this summer, in cooperation with the USDA- Corn Host Plant Resistance Research Unit in Mississippi (W.P. Williams and M. Warburton) we are screening the USDA-NPGS maize diversity panel in testcrosses with Va35 to perform a candidate gene association mapping study. The study proposed below will be complementary to but differ from Va35 experiment in testers used (Tx772-\textit{lox}4 and \textit{Tx772-\textit{lox}5} mutants), scope (drought in addition to aflatoxin) and depth (open architecture screen of capturing all alleles at two genes rather than a few SNPs at many genes). It is expected that results will differ because of the use of different testers (\textit{Zmlox4} and \textit{Zmlox5}, being recessive will allow us to better evaluate the value of the association lines.)

Both drought and aflatoxin resistance involve complex genetic \textit{x} environmental interactions making improvement a challenge. We believe the limitations that conventional plant breeding has faced in reducing drought stress and aflatoxins can be minimized and perhaps overcome with translational genomics resources, but do not expect this to be a panacea for such important problems. It is widely expected that breeding superior cultivars for increased tolerance to drought and reduced aflatoxin will be assisted by identifying and incorporating genes/alleles responsible for resistance and, conversely, deleting the susceptibility genes using robust molecular markers. Our published and preliminary data on the maize lipoxygenase (LOX) family of genes provides a strong testable hypothesis for improvement of drought and aflatoxin tolerance.

The objective of this proposal is to integrate breakthrough quality discoveries from fundamental research and associative genetics principles with traditional breeding with the outcomes of improving maize germplasm and further expand and integrate diverse activities in drought and aflatoxin occurring in research, education and extension.

\textbf{Education:}

It is clear to any faculty currently training students in the field of plant breeding that the demand by private industry alone is much greater than the supply of students. However, to increase the numbers of students trained, difficulties exist in both identifying undergraduates appropriate for the rigors of graduate school and identifying funds to support these students. Furthermore, the growing knowledge gap between applied plant breeding, basic sciences and applied extension creates difficulty cross training students in these fields (Gepts and Hancock, 2006). In this proposal, we address this knowledge base by combining the approaches and unique expertise of each of the PIs to train students in cross-disciplinary sciences across phenotypic plant breeding, molecular biology, applied pathology, associative genetics and extension.

\textit{Class integration for distance education}

The field of plant breeding is rapidly changing, at least in the private sector, to reflect the advances and the dramatic decrease in cost of collecting high-throughput genetic, -omic, and phenotypic data (Cooper et al., 2004). Current educational materials for plant breeding students do not reflect this transformative difference. A major goal of this project is to integrate two graduate level classes “Molecular Plant Pathology” (MK) and “Quantitative Genetics in Plant Breeding” (SM) to better reflect current methods of molecular breeding. Additionally, these two classes will be made available online through web-based distance education. In the Spring of 2009 SCM taught a similar class for the first time integrating knowledge of advanced molecular breeding techniques (association mapping, marker assisted selection, genomic selection, etc.). A
class website was designed which included a Wiki (Parker and Chao, 2007) for students to translate their learning experiences in basic and applied molecular breeding papers into practical application to their future careers in plant breeding. Because many different concepts need to be integrated for modern plant breeders, and programs at many schools are often small, a need is anticipated for graduate level breeding classes to be made available as distance education. The large contingent of plant breeders associated with Texas A&M and Texas AgriLife research are currently developing an online distance masters degree to facilitate distance education by students outside of Texas A&M (C.W. Smith, pers. com.).

Undergraduate internships

The need for opportunities and hands-on experience which can attract undergraduate students in the excitement and science of plant breeding can not be overlooked. Limited opportunities exist to attract undergraduates from non-landgrant (and especially minority serving) institutions. This is, in part, because there is no systematic program to contact and directly connect with these students. Additionally, the student worker wages typically received are less competitive than working at home outside of their field (due of the cost of living expenses). Finally, when helping with only routine tasks, these students do not get to provide input or initiative that fits their interests or helps them to learn. Texas A&M Plant Breeding faculty directly addressed these issues this Fall by creating an undergraduate internship for students outside of Texas A&M to rotate through breeding programs. The student who rotated through the maize breeding program this summer (SM, KM - 2009), was a biology major from a small community college in Houston. In her exit interview she said has changed her mind about working outside and would now like to be a plant breeder working in rice. Much like this students experience, the opportunity to explore lab and field work, research and extension are powerful tools to show students the fun and excitement in plant breeding and plant sciences. In this proposed project we would like to further expand this program. Assigning a graduate student mentor to undergraduate interns and developing a specific project in applied field, lab, or extension research that they will be required to lead. These projects will culminate in a paper which they will write and will be posted on the appropriate web site.

Study abroad to CIMMYT

While much of the US has had access to (currently) plentiful irrigation and inputs, this is not necessarily true in the developing world. Genetic improvements for biotic and abiotic stress resistance for traits such as drought and aflatoxin can be extremely important for self sufficiency in the developing world. We believe it is important for undergraduate and graduate students to be aware of opportunities and challenges facing breeders globally. One of the premier research institutes in drought and increasingly on aflatoxin is the International Maize and Wheat Research Institute (CIMMYT) in Mexico. The Texas A&M Soil and Crop Sciences Department interacts extensively with CIMMYT for both research and education. For the last three years there has been a study abroad trip for undergraduate and graduate students to visit CIMMYT to learn firsthand about plant breeding in the developing world (led by Texas A&M Plant Breeder - S. Hague). Support of this program has been generously supported personally by Dr. Norman Borlaug for the last few years but this funding is unlikely to continue. Furthermore, this opportunity is currently limited to students specifically enrolled at Texas A&M. Continuation and expansion of this trip would greatly benefit the education of students, and will allow both organizations to further collaborative research, education, and extension objectives.
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Extension:
As in education, there is a lack of coordination with extension on issues relating to stress tolerance, specifically drought and aflatoxin, also there is a lack of programming that has been developed. Because Texas requires mandatory testing of maize for aflatoxins (to our knowledge, the only state to do so) producers are moderately educated and very concerned about the problem that exists. However, many of the producers feel helpless in dealing with this problem. Furthermore, it is widely recognized that decreasing water tables throughout Texas threaten the sustainability of crop production during drought, yet we find little programming related to these issues. Because extension tends to be state specific, Texas is an ideal case study for first dealing with these issues in maize. From this project, lessons applicable to other states can be developed. In addition, students can be cross trained in extension. While many plant breeding students are believed to understand and support the mission of extension, there is no formal training in these issues. Currently TI, one of three extension plant pathologists across the state, is evaluating a-toxigenic strains to determine their usefulness in high-aflatoxin risk environments (most rainfed areas of Texas). This research will represent an additional tool for producers to reduce crop contamination and increase food safety.

Integration of research, education and extension through the web:
Historically, the field of plant breeding has not been communicating all of the excitement, opportunities, challenges, and success stories to the public or to other fields such as molecular biology and genetics. This is changing with diverse recent initiatives such as the plant breeding coordinating committee (PBCC - [http://cuke.hort.ncsu.edu/gpb/pr/pbccmain.html](http://cuke.hort.ncsu.edu/gpb/pr/pbccmain.html)), Iowa State University’s “I am an agronomist” campaign ([http://www.agron.iastate.edu/](http://www.agron.iastate.edu/)), the University of Wisconsin’s plant breeding YouTube channel ([http://www.youtube.com/user/wiscplantbreeding](http://www.youtube.com/user/wiscplantbreeding)), the UCDavis Plant Breeding Academy ([http://pba.ucdavis.edu/](http://pba.ucdavis.edu/)) along with many others. However, there is still no clear and coordinated effort to link and communicate research in plant breeding for drought and aflatoxin. A major integrative factor in this proposal is developing a website to increase the coherence in drought and aflatoxin research and serve as a clearinghouse for this information. Texas A&M has many researchers engaged in these joint problems across disciplines and is a natural lead for this. Creating a virtual community to connect plant breeding with drought and aflatoxin research, education, and extension would benefit the public, producers, educators, and researcher in the US and the world.

Rationale and Significance
The joint problems of drought and aflatoxin are serious and expanding problems for growers and researchers across the US and World. A new generation of scientists and educators able to integrate discoveries from basic science will be needed to solve these extremely complex problems. We are interested in pursuing this project to synthesize what is known, to expand on and centralize research activities already occurring, to educate the next generation of agricultural scientists, and to inform growers, educators, researchers, and the general public as to what is known and can be done concerning these issues. Because Texas has long experienced these problems, researchers, educators, and producers have vested interest in supporting activities, and providing excellent case studies. We ultimately view this project as a way to integrate and expand the agricultural knowledge system (research, education, extension) relating to the overlapping issues of abiotic drought tolerance and biotic aflatoxin contamination in maize.
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Relationship of objectives to program priorities:

The objectives of this project are directly related to 2009 program priorities. All education, training, research, and extension will originate or be conducted within an academic setting, Texas A&M University. Multiple students will be trained through course work and/or field experience in these issues (including two graduate students and five undergraduates specifically supported by this project). The outputs of this project: trained graduate students, distance based classes, undergraduates with knowledge in plant breeding, improved germplasm, research and review publications, and a website linking these topics. These outputs will help to build expertise in plant breeding combined with basic research. All of these outputs will focus on germplasm enhancement in maize within the same framework for both 1) abiotic stress tolerance: drought tolerance and 2) biotic stress resistance: *A. flavus* and aflatoxin resistance.

Outputs:

1) Two graduate students (specifically trained under this program) able to integrate molecular and field data and familiar with making objective data-driven decisions for plant breeding sciences. 2) A distance/ web-based education class integrating recent discoveries in plant breeding, molecular biology, quantitative genetics and applied pathology. 3) Students educated in the challenges of plant breeding, drought, aflatoxin, and agriculture in the developing world. 4) Elite alleles at LOX genes evaluated across a variety of genetic backgrounds. 5) Multiple scientific research publications authored by students including review articles. 6) Popular press and website communications at both the educational and extension levels. 7) Students with knowledge in extension programming.

Improvement of sustainability in U.S. agriculture and food systems:

This project addresses three main issues which serve to improve the sustainability of U.S. agriculture and food systems: 1) Hypothesis driven translational research that will result in maize germplasm improved for stress tolerance. These new lines will reduce mycotoxins contamination, improve the plant response to water stress and will allow for the extension of the areas where maize can be grown. This will result in significant cost reductions, in terms of water resources and yield. Additionally molecular markers developed from superior alleles will be made available for rapid conversion of other germplasm. 2) The training and education of future plant breeders who can address current and future challenges in sustainable agricultural production. Two graduate students and five undergraduate students will be specifically trained under this project and associated objectives will result in increased educational opportunities and materials. 3) An increased awareness of stress issues for researchers, educators, extension agents, producers and the general public. Awareness of the opportunities and challenges in drought and aflatoxin will increase best management practices and attention to these issues.

Approach

Because this project integrates the three components of the agricultural knowledge system: research, education, and extension, some components may be addressed simultaneously while others are addressed sequentially. Through collaboration between the PIs and their students these objectives will be integrated. Please see the timeline (Table 1 under project management) for a more detailed view of project objectives and deliverables.
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Research approaches:

R1. Natural allelic diversity in the ZmLOX4 and ZmLOX5 genes will be identified by sequencing these family members in the USDA/NPGS maize association panel and Texas breeding material. As demonstrated by mutant alleles, specific LOX gene family members mediate quantitative variation for either drought tolerance or aflatoxin resistance in maize. While the mutants provide dramatic and conclusive evidence of importance of the ZmLOX4 and ZmLOX5 genes, there are often concerns about mutant usefulness in plant breeding. For instance, a possible unintended pleiotropic effect may include a loss of yield or quality (which has not yet been observed in the genetic backgrounds tested) or epistatic effects minimizing the usefulness in certain genetic backgrounds. We additionally hypothesize that there are other natural allelic variants at these LOX loci which may provide equivalent or better phenotypes than what has been observed in mutant studies. Our first step to test this is to survey this natural variation within two sets of material: 400 inbred lines assembled by the USDA that maximize genetic diversity in maize, and a set of 70 inbred lines developed by the Texas AgriLife maize breeding programs which represent additional sub-tropical adapted material. The 400 USDA-NPGS lines include 300 lines, developed as a community resource panel by E. Buckler and M. Goodman to capture the maximum diversity in maize (Flint-Garcia et al. 2005) and an additional 100 diverse lines selected by USDA-ARS-Corn Host Plant Resistance Research in Mississippi for Southern adaptation (P. Williams, pers. com.). All 400 USDA-NPGS lines are currently being grown in College Station, TX to increase seed, evaluate adaptation, and obtain DNA. Of these 400 USDA-NPGS lines, 312 will be selected to be sequenced along with 70 Texas AgriLife research lines (total = 382). Primers will be designed to cover the promoter and coding regions of LOX4 and LOX5 genes (approximately 10,000 total basepairs / genotype). These genes will then be sequenced to look for novel allelic variation (SNPs and InDels) that can be used for association mapping, LD mapping, and marker assisted selection.

R2. Association mapping analysis will be used to determine the global significance of alleles for drought tolerance and aflatoxin resistance. To determine which ZmLOX4 and ZmLOX5 alleles are superior for drought tolerance and/or aflatoxin resistance we will use an association mapping approach with germplasm developed by the USDA as a community resource. Once seed has been increased (2009) 312 lines will be planted in two College Station isolation nurseries (2010). We will use two currently available modified Tx772 NILs as adapted pollinators. Tx772 is the most recently released Texas line for limited irrigation conditions and resistance to aflatoxin accumulation. One Tx772 NIL we will use is homozygous for the lox4 mutant null allele, and another one is homozygous for the lox5 mutant null allele. For the isolation nurseries, lines will be delayed planted based on College Station 2009 flowering time to maximize pollination nick. In the summer of years 2 and 3 (2010, 2011), these testcross hybrids will be planted in College Station, TX. The lox4 mutant testcrosses will be planted in two replicates for each of two blocks. Block one will be grown under limited irrigation to induce drought stress, block two will have full irrigation to serve as a control. For lox4 mutant testcrosses, comparisons will be made between limited and full irrigation conditions to evaluate drought tolerance. The lox5 homozygous mutant testcrosses will be planted in two replicates, 3 ears will be inoculated with A.flavus (USDA isolate #NRRL 3357 ) using the silk channel technique seven days after pollination (Betran and Isakeit, 2004; Gao et al., 2009). Aflatoxin will be evaluated using the VICAM Aflatest (VICAM, Watertown, MA) under standard procedures (Betran and Isakiet, 2004; Gao et al., 2009). Across all replicate hybrids; yield, height, days to anthesis, days to silk, husk coverage, % drooped ears, 1000 kernal weight, and grain composition (starch, oil, protein...
via NIRS) will be measured. Because Tx772-*lox4* (full-irrigation) and Tx772-*lox5* testcrosses plots will be grown in the same environments and differ only at *lox4* and *lox5* genes, comparisons between these two testcrosses will further illuminate the global value and any pleiotropic effects of these alleles. For these association lines, LD estimation, background markers, population structure, and kinship matrices have been (Buckler/Goodman) or will soon be developed (Williams/Warburton). Therefore no additional background marker collection will be needed. A mixed model association mapping analysis will be conducted using TASSEL software to test *ZmLOX4* and *ZmLOX5* for genetic value (Bradbury et al. 2007; Murray et al. 2009). Additionally, phenotypic data will be made freely available for others to test candidate genes for statistical association of measured traits.

**R3. Using both marker and phenotypic methods, we will pyramid sources of resistance and evaluate epistatic effects across varied subtropical adapted backgrounds.** Texas AgriLife research has more than 70 finished inbreds lines for a variety of traits in the pipeline to be released. Many of these lines are well adapted to the drought and heat of the south and most have been selected for aflatoxin resistance. We have selected, based on phenotypic diversity, 8 of these lines to pyramid the mutant loss of function *Zmlox4* and *Zmlox5* alleles using marker assisted backcrossing. Additionally, we will backcross in two natural novel alleles for each gene discovered under objective R1. Backcrossing will be conducted in years 3 and 4 (2011, 2012). We will begin evaluation of the *lox4, lox5* and double mutants at the BC4F2 stage, and the natural alleles (discovered under objective 1) at the BC2F2 stage. These will be evaluated with the same experimental procedures as the association mapping testcrosses from objective 2. These lines will further be advanced to the BC6F2, tested, and released as isolines. These releases will serve to transfer benefits derived from science-based knowledge to producers and consumers through combined classical breeding, basic scientific discoveries and marker assisted selection.

**Education approaches:**

*Ed1. Classes taught by the PIs will be integrated and reformatted for distance/ web-based education to fill a gap in translating basic molecular scientific discoveries into understandable concepts for breeding.* Many of the latest discoveries and methods across the varied biological disciplines – genomics, proteomics, molecular plant pathology, have yet to be integrated into plant breeding or plant pathology education. This is needed to train the next generation of plant breeders. SM currently teaches a class focused on integrating the tools of molecular quantitative genetics. MK has developed a new class in molecular plant pathology that is based on the most current literature pertaining to the understanding of molecular and biochemical basis of plant resistance or susceptibility to pathogens. Using our research objectives as a case study, MK and SM will integrate components of the two classes together and translate these for distance/ web-based education. This will be done using TAMU’s Vista/Blackboard software. Because integrated classes such as this are absent from many universities outside of Texas A&M University we will work to ensure that students from outside of will be able to access these courses.

*Ed2. Recruiting undergraduate students from off-campus for training in plant breeding through research based internships.* Each year, one student (two in year three) will be recruited from off campus with a preference towards non-land grant schools (including minority serving institutions and community colleges). Students will be actively involved with both lab and field based research as well as extension under the guidance of the PIs and graduate students in charge of this project. PIs will work with each undergraduate student to develop a specific project, based
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on their interests that they will lead. These projects will culminate in a paper which they will write and will be posted on the appropriate web site (see Integrative approach below). Financial and housing assistance will be provided. Assessment will be conducted through their writing and a brief presentation of their experiences.

**Ed3. Training students in plant breeding, plant pathology and extension through experiential learning.** Undergraduate and graduate students often do not have an appreciation for what extension involves, or real world application of the basic biology they have learned in the classroom. In this objective the extension plant pathologist (TI) will take two students per semester for full day trips to learn about extension. Students will contribute to diagnostics. Assessment will be conducted with a follow-up survey to better understand what they found memorable and what they did not understand.

**Ed3. Study abroad trip to CIMMYT.** Undergraduate and graduate students often do not have an appreciation of the developing world or the challenges they face in agriculture. Furthermore, Mexico, where CIMMYT is located the struggle with drought and increasingly aflatoxin is constant. Over the last three years the Texas A&M Department of Soil and Crop Sciences has organized a two week study abroad trip to CIMMYT and surrounding agricultural centers. Students will interact with plant breeders, pathologist, and physiologists. In this objective two PIs (SM and MK) will organize and lead the trip with the assistance of S. Hague (its current organizer). SM and other faculty in The Department of Soil and Crop Science visited CIMMYT in December to make connections and plan joint activities of which this was a major one. We will provide assistantships for to up to eight students at Texas A&M University and other institutions. This will be a formal class and assessment will be in the form of a final paper.

**Ed4. Research Graduate Assistantships.** Although many students will be reached through the above approaches, one MS and one PhD student will be specifically trained under this project across the PIs to develop competency in applied plant breeding, molecular biology, quantitative genetics, applied pathology and extension. Because they will take leadership in the research objectives their stipends have been budgeted as research which is why research is our largest project component. Once a semester, meetings will be conducted with their committee (PI’s). Assessment of success will be in the completion of this project and by their contribution to their fields.

*Extension approaches:*

**Ex1. Material developed in this program will be compared with other materials in on-farm trials with local producers, in cooperation with county extension agents.** Trials will be done in three core locations which have multi-county agent participation and will result in a greater impact for the effort. One location is Rio Farms, headquartered in Monte Alto. Rio Farms is a private, grower-funded research farm in the three-county Lower Rio Grande Valley. Another location, in the Brazos Valley, is the Impact Center, a demonstration and applied research farm which is associated with the Texas AgriLife Experiment Station in Burleson county. Finally, the Stiles farm, a Texas AgriLife Extension Center in Thrall. The Rio Farms and Impact Center locations are irrigated. Also, at the Impact Center, it is possible to inoculate plants with *Aspergillus flavus* if there is a need to increase aflatoxin pressure in the tests. All tests at these locations will have a randomized, replicated design, and will be large enough to harvest by machine. Yield and aflatoxin content will be measured and weather data will be collected for each site. All three locations have well-attended, annual field days which will be used to promote these tests. Additional tests may be arranged at other locations in commercial fields, to cover other maize
production areas of Texas, such as west Texas, the northern Blacklands and the Coastal Bend. The impact of field tests on growers will be measured by questionnaires distributed at county meetings.

**Ex2. Information generated through this research will be incorporated in the extension plant pathologist’s educational program.**

This information will be disseminated by presentations made at grower meetings, bulletins on the website (in a suitable format for distribution as printed material), and articles written for the farm press media. As previously mentioned, field tests will be publicized during field days and county agricultural tours.

**Ex3. Students will be involved in the extension programming.**

Students will participate in the setup and evaluation of field tests. They will also assist in the development of extension articles. In order to assess the effectiveness of training students, they will be required to prepare an extension bulletin related to the research.

**Integrative Approach:**

*A web-portal will be created to synthesize information on drought and aflatoxin.* Scientists and educators have a wealth of knowledge about drought and aflatoxin. Currently, there is no central web portal to communicate the body of knowledge clearly relating research scholarship to education, extension, and research in related fields. Under this objective we will most clearly link research, education and extension. The two graduate students, specifically supported under this project, will work with the PIs to identify original research publications, reviews, educational and extension material. These reviews will serve as the basis of content for the web in addition to being the students introductory dissertation chapter on the issues of drought and aflatoxin. Working with the outreach coordinator and web designer in the Department of Soil and Crop Sciences (T. Hons) and AgriLife research, we will design a site that serves as a portal for research, education and extension information on drought and aflatoxin issues. Drought and aflatoxin index pages will differ but both will have connections to the same content where appropriate. We envision the site will have three main areas. First, the content controlled site where background content will be synthesized and posted from graduate students, researchers, educators, and extension agents. These will include tutorials with simple “self-assessment” quizzes allowing visitors to help recall what they have learned. Second, an articles and research links section. This will connect very different fields of research in the areas encompassing drought and aflatoxin relevant to plant breeding. Third, a wiki component (similar to Wikipedia) to the site where registered users can contribute content and discuss issues relating to drought and aflatoxin.

Content will be solicited from researchers within and external to Texas A&M University and will ultimately include all relevant commodities. Articles and research links page content will be managed by the graduate students funded under this program. Website visits and hits will be tracked where allowed to determine impact. After the completion of the grant, website maintenance will be conducted by PIs and AgriLife Research. While obtaining a unique address might ease concerns of bias towards a single university, we balance this with ensuring there is a responsible organization for maintaining the site after the expiration of the grant.

**How results or products will be used:**

Research results will be immediately put into action. A major strength of the team is that it includes basic and applied researchers, educators, an extension specialists and a employee
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degree student. Results from experiments can immediately be translated into real world results. Producer education about the impacts of actions such as drought tolerant varieties and best management practices for aflatoxin risk minimization will be useful across the United States and the world for increasing sustainability of agriculture and improving producers profitability. Similarly, public and producer education about the importance and benefits of plant breeding will serve to elevate the field.

Maize breeding and germplasm development in the Southern US is unique from much of the temperate Northern US as there are few breeding programs. In contrast much of the material bred by private industry is selected first in the Midwest and then later evaluated in Texas and the South Central US. As such, many of the concerns that South Central Growers have (e.g. drought and aflatoxin) have not yet been adequately addressed by private industry. It is anticipated that lines developed through this program will be of immediate interest to the Southern hybrid seed industry and acquired either for direct use in hybrids or in breeding procedures to create superior proprietary inbred lines.

One of the major outputs of this work are graduate students well trained across a range of disciplines relating to plant breeding, molecular genetics, and pathology, and who are able to communicate effectively with producers and the public (extension). Recent hiring activity among private plant breeding and biotechnology companies suggest that these students will be immediately employable, quickly making a contribution to food security and US productivity.

Expected outcomes:
We anticipate three major and long-term outcomes from this project:
1) Students with enhanced understanding and translation of basic biology and applied pathology into plant breeding and experience with extension programming.
2) Increased public awareness of issues relating to drought, water use and aflatoxin.
3) Maize hybrids that use less water, can better withstand drought, are safe from mycotoxins for consumers, and can provide sustainable income for growers.

Pitfalls that may be encountered:
Both aflatoxin and drought, by nature, are stresses that are unpredictable and sporadic. Texas provides an ideal environment to evaluate these stresses yet environmental conditions can only be mediated not controlled. As can be seen from our current limited irrigation experiment in Figure 3, heavy rain can increase the amount of soil moisture and this cannot be practically prevented. Unfortunately, using drought inducing techniques such as rainout shelters, are not feasible for an experiment of this size or scope and do not mimic normal conditions. However, recent growing seasons suggest our locations will have ideal environments for drought stress and/or aflatoxin production. Artificial inoculation of *A. flavus* greatly increases the probability of identifying variation due to genetics rather than environmental “escapes” but past experience has shown that these interactions are still somewhat unpredictable.

Limitations to proposed procedures:
*Research* - Germplasm evaluation and plant breeding studies are nearly always limited by genetic by environmental interactions and epistasis. Both can mask the value of alleles and of individuals of interest. We will test advanced backcross materials in multiple environments and over multiple years, but the testcrosses due to experimental size will be limited to a single environment over two years. Similarly, we will only be evaluating the lines on two nearly
isogenic testcross parents. This will not allow us to separate general from specific combining ability for the traits of interest.

*Education* - Only a limited number of students can be directly reached with our education programming. To address this and expand the impact, teaching materials will be developed under this program and made accessible in print and web form. This will allow programming to be accessible to additional students and educators after the completion of the project.

*Extension* - Only a limited number of growers can be directly reached with our extension programming and these will all initially be in Texas. However, the materials developed under this program will be made accessible in print and web form to the world. This will allow this programming to reach individuals in other states and after the completion of this project.

**Hazards to project personnel:**

This project includes field, lab, and travel components which are all recognized to have standard hazards associated with these activities. These hazards are minimized by using common sense and proper protective equipment and procedures. A major additional hazard unique to this project is the inoculation, processing and handling of aflatoxin contaminated grain. Multiple studies have suggested that potential respiratory risks to workers exist (Dvorackova, 1990; Fischer et al., 2003). To mitigate this risk we have recently installed a ClearVue cyclone air handler with HEPA filter connected into shelling, counting and grinding equipment. This summer, in cooperation with Texas A&M Environmental Health and Safety and The Office of the Texas State Chemist we will be using personal air pumps to sample air at various steps of the program to identify risks from both particles and airborne aflatoxin. This information will be used to identify if the air cleaner is doing a sufficient job and at what stage the risk still must be mitigated. Finally, as mentioned as part of the objectives of this study we will examine the use of atoxigenic strains and NIRS which, if successful will also decrease the risk of exposure.

**Timeline:**

A more extensive timeline can be found in Table 1 of our management plan.

A timeline for the proposed activities, including the roles of each team member can be found in Table 1. A brief explanation follows:

*Research*

Currently: Increase seed in USDA association panels for increase. Make F1 hybrids between LOX mutants and Texas elite lines.

Year 1: Sequence ZmLOX4 and ZmLOX5 in USDA association panels. Finish USDA association panels seed increase in Fall nursery (Weslaco, TX). Make testcrosses in isolation fields between USDA association panels and Tx772 Zmlox4 and Tx772 Zmlox5 mutants in summer nursery (College Station, TX). Marker assisted backcrossing LOX mutants (continue) and two natural ZmLOX4 and ZmLOX5 alleles (begin) into eight TX elite adapted finished lines. Identify graduate students. Begin research review papers.

Year 2: Finish testcrosses between USDA association panels and Tx772 Zmlox4 and Tx772 Zmlox5 mutants in fall nursery. Evaluate testcrosses and early generation BC2F2 elite Zmlox4 and Zmlox5 backcrosses in summer field trials. Continue marker assisted backcrossing Zmlox4, Zmlox5 mutants and ZmLOX4 and ZmLOX5 natural alleles into elite material. Write drought/aflatoxin review articles.
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Year 3: Evaluate Tx772 Zmlox4 and Tx772 Zmlox5 mutant testcrosses and elite BC₄F₂ backcrosses in summer field trials (College Station, TX). Begin evaluation of natural ZmLOX4 and ZmLOX5 alleles in elite backgrounds. Continue marker assisted backcrossing Zmlox4, Zmlox5 mutants and ZmLOX4 and ZmLOX5 natural alleles into elite material. Write research article on natural allelic diversity found at these genes.

Year 4: Evaluate ZmLOX4 and ZmLOX5 natural allele BC₄F₂ backcrosses in summer in multiple field sites. Write resulting papers with graduate students. Write research article on testcross evaluation. Make backcrossed germplasm available and continue multi-location trials for official release process.

Education
Currently: Improving slides for courses.
Year 1: Integrate classes and move towards distance education. Undergraduate internship in summer. Study abroad to CIMMYT (summer). Begin website design and post live at the end of the year.
Year 2: Integrate classes and finish distance education organization. Two undergraduate internships in summer. Continue website design and content improvement.
Year 3: Undergraduate internship in summer. Late planting (August) for demonstration.
Year 4: Undergraduate internship in summer. Evaluate novel allele backcrosses in summer. Write resulting papers with graduate students.

Extension
Year 1: On-farm field days for producers and the public. Begin website design and post live at the end of the year.
Year 2: On-farm field days for producers and the public. Continue website design and content improvement.
Year 3: On-farm field days for producers and the public. Extension publication.
Year 4: On-farm field days for producers and the public. Extension publication.