RAPID IDENTIFICATION OF ASPERGILLUS SPP. USING A PCR BASED
MELTING CURVE METHOD AND CHARACTERIZATION OF A NOVEL
CHITINASE IN INSECT RESISTANT MAIZE LINES

By

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Identification of fungal isolates is critical in studying *Aspergillus flavus* ecology and for developing methods to reduce aflatoxin contamination. In our efforts to track biocontrol applications of the atoxigenic *A. flavus* K49 (NRRL 30797), we have developed a rapid and accurate classification system for *A. flavus* based on PCR product melting temperatures (Tm). Using 18 primers and a total of 59 *Aspergilli* strains, including all 49 representatives of the Georgian peanut Vegetative Compatibility Groups (VCGs), a decision tree Tm flowchart was generated. The decision tree can classify all 59 strains using only 9 of the SSR primers and an average of 3.4 primers for each definitive classification. To confirm the effectiveness of the decision tree for strain identification, unknown samples isolated from experimental fields inoculated with various *A. flavus* strains in Stoneville, MS were analyzed. Ninety-six percent of the samples could be placed into a VCG using Tm(s) coupled with the decision tree. This dynamic system is an
excellent tool for researchers studying biodiversity of *A. flavus*. 
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CHAPTER I
INTRODUCTION

*Aspergillus* species are frequently present soil, water, decaying vegetation, and organic debris. *Aspergillus* is the most ubiquitous fungus with airborne conidia. Their spores can be distributed easily into the environment by air currents. As a result of this ubiquitous presence, people are constantly exposed to *Aspergillus* spores. Several *Aspergillus* species have been described as human pathogens.

There are several clinical demonstrations that show *Aspergillus* as a devastating pathogen. In immuno-compromised patients, it can cause invasive aspergillosis (IA), which is a life-threatening disease. Pulmonary infections are thought to arise by local tissue infection, and eventually disseminate to other deep seated organs. IA is a growing problem in hospitals because of the increase in number of patients undergoing bone marrow, solid organ transplantations, chemotherapy for cancer, or therapy with corticosteroids. IA has increased dramatically in recent years because in hospitals undergo renovations releasing pathogenic fungi into the air circulation system of central air conditioning systems (Girardin *et al.* 1994; Latge 1999).

Allergic broncho-pulmonary aspergillosis (APBA) is an uncommon but serious respiratory condition which causes chronic airway inflammation and airway damage. This condition results from continual colonization by *Aspergillus*. ABPA can cause cystic fibrosis especially in children (Nelson *et al.* 1979).
Of all aspergilli, *A. fumigatus* is the organism that was most frequently isolated from human infections (Lin et al. 1995). *A. flavus* is the second most common etiologic agent of human aspergillosis (James et al. 2000a). Other *Aspergilli* such as *A. niger*, *A. nidulans* and *A. terreus* can also be the causative agents of various infections. Some *Aspergillus* species produce mycotoxins, which are a serious problem as they have adverse effects on human health. Aflatoxins are produced by some fungal isolates from the *Aspergillus* section *Flavi* such as *A. flavus*, *A. parasiticus*, and *A. nomius*. Aflatoxins are highly carcinogenic and mutagenic secondary metabolites (Baird et al. 2006; Kumeda & Asao 1996). Another important mycotoxin is ochratoxin A (OTA); this toxin can cause renal toxicity, mutagenecity and immunotoxicity in humans and it is a possible human carcinogen. OTA is produced by *A. ochraceus* and *A. carbonarius* which belong to *Aspergillus* section *Nigri* (Esteban et al. 2006; Gonzalez-Salgado et al. 2005; Pitt 2000).

Typing techniques of *Aspergillus* isolates have been developed and improved for understanding the genetic and epidemiological relationships between environment and clinical *Aspergillus* isolates. It can help researchers to realize the fungal distribution and relatedness for determining the epidemiology of fungus. This understanding will help and design better pathogen control methods (Singh et al. 2006). Typing techniques can also give a deeper understanding of the colonization pattern of crop fields. Whether colonization of the crop fields is due to one or several isolates or if frequent re-colonization takes places can be determined by typing culture isolates from crop fields. Several phenotypic and genotypic studies have been used to distinguish among individual *Aspergillus* isolates. With the development of new typing technologies based on DNA rather than on phenotypic characteristics such as short tandem repeats, the method of
typing has changed.

In this study, we established a typing method based on the PCR-dependent reaction but analysis of the banding pattern by agarose gel was substituted for melting temperature of obtained fragments.
CHAPTER II
LITERATURE REVIEW

*Aspergillus spp.* and Maize

*Aspergillus* is an anamorphic genus consisting of about 250 recognized species. Some members of the genus produce teleomorphs (sexual states). The genus has been divided into a number of sections. *Aspergillus flavus* belongs to section *Flavi*. This section contains the major aflatoxin-producing species, *A. flavus* and *A. parasiticus* (Frisvad *et al.* 2005). In culture, *Aspergillus flavus* is fast-growing and it produces yellow-green colonies, usually 65-70 mm in diameter after 7 days of incubation in the dark at 25°C on Czapk yeast extract (Klich 1993; Klich 2002). *Aspergillus flavus* is classified into two types at morphology level called the S and L strains (Cotty 1989). The S strain produces small sclerotia (<400 μm in diameter), and it has few conidia but produces high levels of aflatoxin. The L strain produces larger sclerotia, but less aflatoxin (<400 μm in diameter) than the S strain. Some of L strains do not produce aflatoxin (Cotty 1993; Dorner 2009). However, sclerotium morphology is not a proper indicator of phylogeny because *A. flavus* consists of several major lineages (Chang *et al.* 2006) that are not fully defined. The reason is that phylogenetic analyses often do not take into account of recombination and balancing selection, and both can introduce significant phylogenetic errors (Carbone *et al.* 2007). *A. flavus* has been later divided into two groups, I and II, based on the restriction fragment length polymorphism (Geiser *et al.* 1998).
Group I contain L and R strains and produces only B aflatoxins. Group II contains only S strains that produce B and G aflatoxins. *A. parasiticus* is also widespread in the United States but less abundant than *A. flavus*. This species infects peanuts, corn and cotton seed (Horn 2005). *A. parasiticus* has a lower temperature optimum for seed invasion than *A. flavus* (Horn 2005). *A. parasiticus* also appears to be more adapted to survival in the soil and less dependent on crop infection than *A. flavus*. This is suggested by the sizable populations in a agricultural soil north of where peanuts are cultivated (Dorner et al. 1989). Fungal species other than *A. flavus* and *A. parasiticus* that occur in the United States, but have a minor impact to agriculture. *A. nomius* is often reported form dead or diseased insects (Peterson et al. 2001). *A. nomius* populations are most often found in agricultural soils from the Mississippi Delta region and in parasitized alkali bees in Wyoming (Horn 1998). *A. caelatus* is widespread in soils from the southern United States and also has been reported in insect-damaged peanuts. However, *A. caelatus* does not produce any mycotoxins (Horn et al. 1995). *A. alliaceus*, is the only species from section *Flavi* with a known sexual state, and it produces ochratoxin A (Bayman et al. 2002). Both *A. tamari* and *A. alliaceus* are isolated form soil and crops, but their distributions within United States are not defined. There are two rare species, *A. leporis* and *A. avenaceus* have also been reported: *A. leporis* is found only in rabbit dung, and *A. avenaceus*, has been reported only from soil in California (Christensen 1981).

Aflatoxin contamination of maize may be caused by several species in *Aspergillus* section *Flavi*, and these fungi have the ability to destroy crops and produce aflatoxin (Cotty 1989). *A. flavus* has been isolated form soils in all of the major biomes, but the major region is warm temperature zone (latitudes 26-35°) (Klich 2002). In United States,
aflatoxin is a chronic problem in corn in the southeastern growing area (Payne & Hagler 1983). Field contamination is associated with high temperature and drought stress but does occur in crops in more temperate climates during hot drought years (CAST, 2003). Studies of *A. flavus* densities in soil have shown that they are denser in crop soils than in forest or prairie soils (Angle & Wagner 1980; Horn 2005).

*A. flavus* infection affects crops differently. In corn, *A. flavus* causes an ear rot. In peanuts, it causes a seedling disease known as yellow mold of seedling called “aflaroot” and inhibits the root hair development in tobacco (Maren 2007). *A. flavus* also affects cotton quality by causing boll rot (Klich & Lee 1982). Aflatoxin producing *Aspergillus* of section *Flavi* also differ in morphology, physiology and ecology (Cotty 1989; Cotty 1994a). During crop development, aflatoxin is generated contaminating and it may continue to accumulated after crop maturation until the grain is harvested and ultimately consumed (Cotty 1994a). Some abiotic factors such as temperature and humidity as well as biotic factors such as insects determined the average aflatoxin-producing potential of the fungal community associated with crops (Cotty 1997). Drought stress induces a great increase in proline production in plants (Barnett & Naylor 1966), and proline has been shown to enhance aflatoxin production (Payne *et al.* 1983). Additionally, antimicrobial compound is called phytoalexins (Figure. 1), which are produced in plants, act as toxins to the attacking organisms. This compound puncture the cell wall, delay maturation, disrupt metabolism, or prevent reproduction of the pathogens and may be inhibited by drought stress (Maren 2007). This phenomenon has been shown in immature peanuts. Immature peanut plants exposed to *A. flavus* inhibited fungal growth until drought conditions stopped the production of phytoalexin (Dorner *et al.* 1989).
Phytoalexins are essential secondary metabolites produced de novo by plants in response to diverse forms of stress, including microbial attack.
*A. flavus* utilizes free saccharides and oil before using starch from crops. A previous study showed that removing lipids from cottonseed can reduce aflatoxin production up to 800-fold. *A. flavus* preferentially utilized saccharides as an initial carbon substrate followed by triglycerides. When invading non-wounded corn kernels, the fungus selectively targets the germ tissue where these materials are localized in the highest concentrations (Mellon *et al.* 2000; Mellon *et al.* 2005). In the United States, the mean economic loss from aflatoxins is estimated to be $932 million (CAST 2003). Economic loss of aflatoxin in the Mississippi was $2,000,000 for maize; $4,367,000 for Arizona cottonseed; $7,000,000 for Texas cottonseed; $25,000,000 for Georgia peanuts; $15,000,000 for Texas maize (Robens 2001).

Aflatoxins were first isolated and characterized after the death of more than 100,000 turkeys (turkey X disease) was traced to the consumption of a mold-contaminated peanut meal (Goldblatt 1969). Four major aflatoxins are called B₁, B₂, G₁ and G₂ (or AFB1, AFB2, AFB3 and AFB4) (Figure. 2) based on their fluorescence under UV light and the relative mobility on thin-layer chromatography (Turner *et al.* 2009). Aflatoxin B₁ is the most common aflatoxin, and it is a genotoxin known to be carcinogenic for human and animals (McKean *et al.* 2006; Wang & Tang 2004). To date, aflatoxin B₁ is the only mycotoxin classified as a group 1 human carcinogen by the International Agency for Research on Cancer in 2002. Over long periods of consumption of low daily doses of aflatoxins may result in chronic aflatoxicosis. Complications from aflatoxicosis include impaired food conversion, stunting in children (Gong *et al.* 2004), immune suppression, and cancer and reduced life expectancy (Cardwell *et al.* 2001; Gong *et al.* 2004; Williams *et al.* 2004). Ingestion of high concentrations of aflatoxin results in
Figure 2   Structures of Aflatoxins.

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acute aflatoxicosis, resulting in severe liver damage leading to jaundice, hepatitis and death (Williams et al. 2004). Other studies show that aflatoxin exposure can suppress immune function especially cell-mediated immune responses (Bondy 2000). More specifically, these studies on the immunotoxic effect of aflatoxin have shown that exposure to aflatoxin decreased T or B lymphocyte activity (Reddy 1987) and impaired macrophage/neutrophil effector functions (Moon 1999). For the livestock, consequences of chronic aflatoxicosis in pigs have been showed to decreased feed conversion and weight gain. In dairy cattle, milk production is decreased and in beef cattle weight gain is lowered (Newberne 1974). Ducklings may develop cholangiocarcinoma, hepatocellular carcinoma, or both when exposed to prolonged feeding with low dietary concentrations (Newberne 1974). Trout develop hepatic tumors when dietary aflatoxin levels reach a few parts per billion (Ashley et al. 1964).

Cytochrome P450 enzymes convert aflatoxins to the reactive 8, 9-expoxide form and binds to both DNA and proteins. The mechanism of DNA damage is the binding of the aflatoxin expoxide to the $N^7$ position of guanine because the reactive electrophilic expoxide can covalently interact with macromolecular nucleophilic center as well as DNA. These adducts can result in GC to TA transversion (Eaton & Gallagher 1994). The transversion alters the codon 249 of the p53 tumor suppression gene (Aguilar et al. 1993)

Outbreaks of acute aflatoxicosis are rarely reported in developed countries, but there is a greater occurrence in developing countries (Krishnamachari & Bhat 1976; Probst et al. 2007; Tandon et al. 1977). India and Kenya have reported the cases of acute aflatoxicosis. An outbreak of acute aflatoxicosis (20 cases) was reported in Makueni district, Eastern Province, and Kenya in 1981 (Ngindu 1982). Patients were clustered in
family groups that shared meals consisting of aflatoxin-contaminated maize (1,600 to 12,000 ppb). Acute hepatitis associated with consumption of moldy grains also has been reported in other areas in Africa, Western India, and Malaysia (Krishnamachari 1975a; Krishnamachari 1975b; Tzee-Cheng et al. 1991), where affected persons came from areas prone to drought and malnutrition, and unseasonable rains forced the harvest of grains before adequate drying had occurred. Typically, increased reports of jaundice and hepatitis followed within weeks of such harvests. Locally produced maize associated with this outbreak was harvested in February during peak rains, and the first illnesses were reported in Makueni district in late March and early April. The United States Food and Drug Administration (FDA) has set limits on the amount of aflatoxin allowed in various foodstuffs. Limits on aflatoxin in animal feed range from 20-300 parts per billion (ppb) and depend on the type and age of animal. In food for human consumption, the maximum amount of aflatoxin allowed is 20 ppb; in milk, less than 0.5 ppb of aflatoxin $M_1$ is allowed.

It was well known that crops contaminated with aflatoxin have a bright greenish yellow (BGYF) color and fluorescence under long-wave ultraviolet light (365 nm) (Diener et al. 1987). This fluorescence technique or method has been used to detect the contamination of aflatoxin. However, this method is unreliable because some contaminated seeds do not fluoresce under the UV light (Shotwell et al. 1981). When mature seed is exposed to specific temperatures (usually above 30°C), and high relative humidity (above 85%) after boll opening, and the seed become associated with $A. flavus$. The fungus propagates shortly after adequate moisture is introduced to the seed. Infected seeds during these conditions often do not exhibit BGYF on either linters or lint (Cotty
Health risks and economic loss of crops make the development of accurate aflatoxin testing methods a necessity. Due to the simplicity, thin layer chromatography has become the standard analysis method for screening of aflatoxins (Gilbert & Vargas 2003). Other modern analytical approaches such as high performance liquid chromatography (HPLC) and enzyme linked immunosorbent assay (ELISA) are also used. The general procedure, extraction of aflatoxin with aqueous acetonitrile or methanol, followed by clean-up of the extract solution using affinity columns. These procedures provide sensitive and selective results for a wide range of feeds and animal feed (Trucksess 2002). TLC is the oldest of the chromatographic methods, requiring less advanced and expensive equipment than other chromatographic methods. But the low accuracy has limited its use in research fields (Shetty & Bhat 1997). HPLC is a chemistry-based tool for quantifying and analyzing the amount of a chemical compound within a mixture of chemicals. It is highly versatile and easily automated for detecting aflatoxin. In addition, it is accurate, quick, and can analysis several samples at the same time. However, expensive equipments and complex procedures restrict wide application (Whitaker et al. 1996). ELISA is a recently developed method for determining aflatoxin in food and other commodities. Since it is rapid, simple and highly sensitive, the method has become popular for analyzing aflatoxins. However, due to false positives, it is essential that positive results be confirmed (Sabino et al. 1997)

**Aflatoxin Control in the Crop Field**

Control of aflatoxin contamination can be done by two ways, control the fungus or aflatoxin production. Many of factors influence growth and distribution of fungus and
aflatoxin production (Diener et al. 1987; Klich 2007; Payne & Brown 1998). Because aflatoxin is a problem in plants under stress and can be reduced by lowering plant stress. The first approach to reducing aflatoxin production is controlling the stress, such as planting at appropriate seed density, limiting insect damage, planting regionally adapted cultivars, irrigation when needed, and harvesting when crops are mature (Maren 2007). However, these approaches are not sufficient to prevent aflatoxin formation. Thus, researchers started to use chemical control, using chemical compounds to remove aflatoxin (Gourama & Bullerman 1995). *A. flavus* growth and aflatoxin biosynthesis depend on substrate, moisture, temperature, pH, aeration, and competing microflora. The growth of *A. flavus* and aflatoxin production are sometimes unavoidable. The common application is using heat. Roasting temperature range from 143°C to 149°C can reduce aflatoxin concentration in corn by 50% (Kenkel 1999). Also, ammonia, sodium hydroxide, ammonium hydroxide, sodium hypochlorite and hydrogen peroxide can remove aflatoxin (Abbas 2005). However, chemicals can protect crops against further infection, but it cannot reduce aflatoxin which has been produced before or after chemical treatments. Other approaches including using atoxigenic fungi that do not produce toxin can be used to displace the toxigenic strains (Abbas 2006). Certain atoxigenic strains have the ability to competitively exclude aflatoxin-producing strains during crop infection and thereby reduce aflatoxin contamination. In cotton, the potential for biological control of aflatoxin has been demonstrated (Cotty 1994b). A non-aflatoxigenic strain, AF36, is being used to control aflatoxin production in commercial cotton field. Another commercial product, called Afla-guard®, provides the means of introducing a competitive, non-aflatoxigenic strain of *Aspergillus flavus* into soils where peanuts are being grown. The introduced
strain competitively excludes toxigenic strains naturally present from invading developing peanuts (Lamb 2006). According to the 4-year studies, non-aflatoxigenic, indigenous \textit{A. flavus} strain K49 has shown a reduction in toxigenic isolates from 60\% of the total population to less than 20\% (Abbas 2006).

Host resistance to fungal infection is another approach to reduce aflatoxin production. Maize kernels are vulnerable to infection because fungus can infect through pollen tubes (Burow et al. 1997). \textit{Aspergillus flavus} can utilize the embryo tissue, which has a high lipid content. The fungus metabolizes the starchy endosperm, contributing to fungal metabolites found in the endosperm and aflatoxin detected in the kernel (Brown et al. 1999).

Little is known about the process of fungal invasion into maize tissue after inoculation into kernel. In inoculation of maize with GPF-tagged fungus, fungal tissue was accumulated in the pith of cubs of susceptible maize lines (Magabanua 2004). After 28 days of inoculation, fungus can be found throughout all rachis tissue (Smart 1990). According to the genetic study of maize, it has been known that resistance to \textit{A. flavus} infection and aflatoxin accumulation is a multigenic quantitative trait and that additive and dominance types of gene action are both important (Naidoo et al. 2002). As a consequence, breeding for resistance to \textit{A. flavus} or aflatoxin accumulation was considered very difficult. Therefore, the focus of the United States Department of Agriculture/Agriculture Research Service (USDA/ARS) Corn Host Plant Resistance Research Unit was to develop a resistant maize line (Mp313E) (Scott & Zummo 1990). The genetic study of Mp313E revealed that specific DNA regions called Quantitative Trait Loci (QTL), are associated with a particular phenotypic trait. These QTL are often
found on different chromosomes, which correlated to aflatoxin resistance on chromosome 2, 3 and 4 (Brooks et al. 2005, Scott & Zummo 1990).

**Chitinase Protein**

Plants do not contain an immunosystem and thus they are vulnerable to pathogens. With the purpose of protecting themselves from pathogens, plants have to develop their own defensive response. The responses are elicited during their life cycle in response to development signals and pathogen attack (Bowles 1990b). Plants respond to attack by pathogenic organisms such as fungi and can express a large number of genes encoding diverse proteins. Many of these proteins are believed to have a role in plant defense mechanism (Bol et al. 1990; Bowles 1990a; Dixon & Harrison 1990). Antimicrobial factors of proteins or peptides have been extensively investigated because such factors could be rapidly introduced into corn through genetic engineering. Several of these proteins are recognized as biochemical factors possessing antimicrobial activity to enhance disease resistance when over expressed in plants. Many of these proteins cause plant cell death or cell wall modifications at the pathogen attacking sites (Kauss 1986). Others directly react to pathogens called pathogenesis-related (PR) proteins. They can be found in wide range of organisms including plants, fungi, bacteria and animals (Matsuoka & Ohashi 1986; van Loon 1985; Van Loon & Van Strien 1999). Among pathogenesis-related proteins, the lytic enzyme chitinase has been speculated to play a crucial role in plant defense (Bowles 1990b). The protein chitinase is of particular interest because no substrates for this enzyme have been identified in plants. Chitinase catalyzes the hydrolysis of chitin, a liner homo-polymer of β-1, 4-linked N-acetylglucosame
(GlcNAc), which is commonly a component of fungal cell walls (Boller 1988; Roberts & Selitrennikoff 1988). In fungi, chitin constitutes 3-60% of the cell wall (Boller 1988). Thus, chitinase inhibits the growth of many fungi by causing lyses of hyphal tips. In combination with β-1, 3-glucanase (Broekaert et al. 1988), chitinase has been shown to accumulate around fungal hyphal (Wubben et al. 1992).

The structure and expression of chitinase genes has been studied in a variety of dicot and monocot plants (Leah et al. 1991). Three classes of chitinase have been emerged from the structure analysis of plant chitinase genes (Shin-shi et al. 1990). Class I chitinases have cysteine-rich domains at amino-terminal and a highly conserved catalytic domain. Class II chitinases lack cysteine-rich domain but have a catalytic domain which has homology to class I chitinase. Class III chitinases have no similar characteristics to class I and class II but are homologus to the acidic chitinases of cucumber and Arabidopsis (Samac et al. 1990). In 1990, chitinases in tobacco have been grouped into five major classes by both biochemical and molecular-biological techniques (Bol et al. 1990). By then, 11 families were recognized and classified for tobacco and tomato (van Loon et al. 1994). In most plants, chitinases are encoded by multigene families. These genes are differentially expressed during development and are induced by a variety of defense-related and environmental stimuli.

During the apical growth in filamentous fungi, chitin and β-1, 3-glucan fibers are synthesized in the apex of the growing hypha (Borgia & Dodge 1992). Thus, the exposed nascent chitin chains at the apex are hydrolyzed by chitinase. The enzyme activity, protein and mRNA levels of chitinase have been reported to be higher in resistant cultivars at early stage after inoculation then in susceptible cultivars (Daugrois et al. 1990;
Additionally, chitinase can be induced after ethylene treatment. This implies that ethylene is an endogenous signal for chitinase (Sieber et al. 2000). An immunocytochemical study showed that chitinases are localized in vacuoles, and the C-terminal of chitinase protein is a target sequence for vacuole localization in tobacco, bean, potato and *Arabidopsis* (Gaynor 1988; Parsons et al. 1989; Sieber et al. 2000).

*Aspergillus flavus* Diversity

Populations of *Flavi* are diverse and individuals differ greatly in phenotype, including the conidial color, sclerotium production, pigment and growth rate (Christensen & Nelson 1976; Nenoff et al. 1997). In many filamentous fungi, including *A. flavus*, physiologically distinct individual of the same species can fuse asexually to form a heterokaryon. The participants in forming stable heterokaryon are vegetatively compatible and belong to the same vegetative compatibility group (VCGs) (Horn 1996; Leslie 1996). This method provided a multi-locus phenotype for measuring genetic diversity. The formation of stable hyphal fusing is under control the *het* loci whose alleles must all be identical (Leslie 1993). The validity of using vegetative compatibility as a tool for measuring genetic diversity was indicated by two blind studies in which populations of *A. flavus* and *A. caelatus*. They were examined by using DNA fingerprinting through single nucleotide repeats (SSR) primer (McAlpin et al. 2005; McAlpin et al. 2002). In both species, population showed a nearly identical between fungal VCGs and DNA fingerprinting groups. DNA fingerprinting has also been used to describe the diversity of *A. flavus* and *A. parasiticus* populations (McAlpin et al. 1998).
Other molecular techniques, such as DNA sequencing, need long period of time for DNA preparation, PCR reaction, data analysis, and were previously limited by the huge numbers of fungal isolates and it is possible to lower the costs.

**Restriction Fragment Length Polymorphism (RFLP)**

When genomic DNA from *Aspergillus spp.* isolate is cut with a restriction enzyme, there are up to 10,000 different DNA fragments may be obtained. Obviously, these fragments cannot be distinguished because of its vast amount of fragments produced. Analyzing only large fragments (10-50 Kbp) in different isolates may show different banding patterns. Different banding patterns result from the substitution, insertion, or deletion of the restriction enzyme recognition sites. Burnie *et al.* (1992), suggests that *XhoI* and *SalI* had the highest discriminatory power with this approach. The RFLP technique has also been used for identification *A. flavus* for heterogeneous group on mitochondria DNA, but isolates from identical species could not be distinguished from each other (Moody 1990) with this method.

**Random Amplified Polymorphic DNA (RAPD)**

Based on the standard PCR reaction, a random amplified polymorphic DNA (RAPD) was developed. This approach used two primers that will anneal to genomic DNA under highly stringent conditions. The key step is each primer which has 8 to 20 base pairs combined with low annealing temperatures during PCR reactions.(van Belkum *et al.* 1993). In doing so, a primer can anneal to multiple positions in the fungal genomic DNA. After several standard PCR cycles, DNA fragments are amplified and are
determined by the number and location of their anneal sites. Unrelated isolates usually yield different banding patterns. Several reports have shown that this approach can distinguish *A. fumigatus* and *A. flavus* (Aufauvre-Brown *et al.* 1992), but the discretionary power is low (Varga & Croft 1994).

**Restriction Fragment Length Polymorphism with Hybridization**

Restriction Fragment Length Polymorphism with Hybridization technique is similar to RFLP; however, it uses a specific subset of restriction fragments for analysis. Genomic DNA is cut by restriction enzymes, and fragments are separated by agarose gel, and then transferred to nylon filter by Southern blotting. Differences in patterns between unrelated isolates can be expected because of variations in copy number and flanking regions of the target regions (Girardin *et al.* 1993). A specific probe called pAF28 was constructed for detecting *A. flavus* (McAlpin & Mannarelli 1995). This probe has been used to type clinical and environmental isolates in neonatal intensive care units (James *et al.* 2000b). For *A. terreus*, a repetitive sequence probe was used to type 12 isolates, and six patterns could be distinguished (Rath 1999). For the determination of a strain belonging to the group of *Aspergillus* section *Nigri*, three different genes were used as probes in RFLP analysis. The RFLP method is used to confirm the differences of new species with known species. Unique RFLP patterns were obtained for this isolate and it was therefore designated as novel species (de Vries *et al.* 2005). In a simpler way, the analysis is performed on one specific amplified target. This fragment is then cut by one or several restriction enzyme(s) and the obtained fragment can be separated by gel-electrophoresis. This method is often called RFLP or PCR-RFLP. Consequently, this
analysis method depends on one specific PCR amplified target. It is suitable for discriminating between species but is not sufficient to distinguish between unrelated isolations within a species (Somashekar et al. 2004).

**Amplified Fragment Length Polymorphism (AFLP)**

Amplified fragment length polymorphism (AFLP) is a PCR-based technique used in genomics research, DNA fingerprinting, and in the practice of genetic engineering. AFLP uses restriction enzymes to cut genomic DNA, followed by ligation of adaptors to the sticky ends of the restriction fragments. A subset of the restriction fragments are then amplified using primers complementary to the adaptor and part of the restriction site fragments. The amplified fragments are visualized on denaturing polyacrylamide gels either through autoradiography or fluorescence methodologies (Vos et al. 1995). In 2003, a total of 96 *A. fumigatus* isolates was examined by this technique with *Eco*RI and *Mse*I. Different fungal isolates could be separated by the same restriction enzymes (Warris et al. 2003). Using the same restriction enzyme but different adaptors, the genetic diversity of *Aspergillus* sections *Flavi* and *Nigri* was examined by AFLP. The results showed that AFLP is an excellent typing approach to differentiate *Aspergillus* spp.

**Microsatellites or Short Tandem Repeats (STR’s)**

A short tandem repeat (STR) in DNA occurs when a pattern of two or more nucleotides are repeated and the repeated sequences are directly adjacent to each other (Adair et al. 2000; Puers et al. 1993; Skowasch et al. 1992). Different isolates can be distinguished from each other based on the differences in repeat numbers. The repeat
region can be easily amplified by PCR primer. If the primer is labeled by florescent chemicals, the resolution can be increased at the electrophoresis pattern. There are 7 polymorphic microsatellite loci that have been discovered in *A. flavus* and *A. parasiticus* (Tran-Dinh & Carter 2000).

**Multilocus Sequence Typing (MLST)**

Multilocus sequence typing (MLST) is a technique commonly used in molecular biology for typing of multiple loci. This procedure characterizes the isolates of bacterial species using the DNA sequences of internal fragments of multiple (usually seven) housekeeping genes. A housekeeping gene is typically a constitutive gene that is transcribed at a relatively constant level. The housekeeping gene's products are typically needed for maintenance of the cell. Housekeeping genes are used as internal standards in quantitative polymerase chain reaction since it is generally assumed that their expression in cells is unchanged. Approximately, 450-500 bp internal fragments of each gene are used, as these can be accurately sequenced on both strands using an automated DNA sequencer. For each housekeeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the loci define the allelic profile or sequence type (ST)(Taylor 2003). Depending on the genomic housekeeping gene, MLST data has also been used to investigate the evolutionary trend for bacteria and fungi (Urwin 2003). MLST has been increasingly applied as a routine typing tool that enables international comparison of isolates. Its data have been exploited in evolutionary and population analyses that estimate recombination and mutation rates and investigate evolution relationships among bacteria belonging to the same genus.
(Urwin 2003). For this reason, in the Aspergillus section Fumigati, the MLST approach has led to the description of a new sibling species, *A. lentilus* (Balajee et al. 2005).

**Summary**

Several molecular methods have been described for typing *A. flavus* and *A. fumigatus* isolates. Typing techniques with high discriminatory power are necessary. PCR based methods required small amount of template DNA, which are easy to obtain. In the contrary, the non-PCR RFLP methods either with or without hybridization needed relatively large amount of highly purified DNA which make them time-consuming and labor intensive (Lin 1995). Several PCR methods such as AFLP, MLST and microsatellites need special equipment to separate DNA fragments with high resolution electrophoresis conditions. On the other hand, the RAPD method can be done by inexpensive equipment and it is the most widely used method for typing *Aspergillus* isolates (Lasker 2002). Furthermore, AFLP techniques is applicable to any organisms without the need for prior genome sequence information (Savelkoul 1999). Besides aspergilli, AFLP has been used with other microorganisms, including other fungi, but also in plants and animals, so does RAPD methods (McEwen 2000).

Reproducibility refers to the ability of a technique to yield the same result when a particular isolate is repeatedly tested. For large scale and longitudinal epidemiological studies, stable fingerprinting techniques are required. Methods based on band-comparison such as RAPD, RFLP and AFLP could generate non-reproducible results from variable intensity of DNA bands, which is probably due to small variations in the various steps of the procedures that may affect the final peak intensity (Bart-Delabesse 2001). Another
important concept of typing techniques is the ability to exchange data between different laboratories. Genotypes acquired from all over the world can be compared to each other, which would help researchers understand the global epidemiology of microorganisms. According to the computer technology today, digital format of data can be fully expressed in a simple way. Unfortunately, digital fingerprinting data in the shape of banding patterns is more complex and therefore difficult to exchange, so standard procedures have to be followed by all laboratories to avoid differences in banding patterns. By comparing banding patterns with each other, it is assumed that bands with the same size contain the same gene fragment. This can be misleading, because bands with the same size can consist of completely different gene fragments and thus give false identification of tested.
CHAPTER III
MATERIAL AND METHODS

**Fungal Cultures**

To obtain fresh conidia fungal spores form, a glycerol stock were spread on a PDA (Potato Dextrose Agar) plate using an inoculation loop and incubated at 28°C for 5 days. Conidia were collected by adding 5 ml 0.05% (V/V) Triton X-100 to the surface of PDA and dislodging with a glass rod, the resulting spore suspension was stored at 4°C until needed. To collect the necessary fungal tissue 50 μl of surface liquid was decanted to 100 ml potato dextrose broth and incubated at 28°C, with agitation at 250 rpm for 7 days. Fungal tissue was harvested by vacuum filtration through filter paper in the Buchner funnel, frozen in liquid nitrogen, and stored at -80°C until use.

**Fungal Genomic DNA Extraction**

This method was modified by Moller (Moller et al. 1992). Frozen tissue was ground by 2000 Geno/Grinder™ (Spex SamplePrep, LLC) under 1000 strokes per minute for 3 minutes and followed by 200 strokes per minute for 1 minute. Fungal powder was suspended in 3 ml (1 ml per gram of frozen fungal tissue) TSE buffer (10 mM Tris pH 7.6, 1 mM EDTA pH 8, 0.1 M NaCl and 1% SDS) and incubated at 37°C for one hour with gentle continuous shaking. The suspension was centrifuged at 10,000 rpm for 10 minutes and the resulting supernatant was transferred to a clean autoclaved 2-ml centrifuge tube.
An approximately equal amount of phenol was added to the supernatant and the tube was gently inverted several times until the whole liquid became clouded. The centrifuge tube which contained phenol and supernatant mixture was spun at 13,000 RPM for 15 minutes, 4°C. The top layer was extracted to a fresh new 2 ml centrifuge tube, and 500 μl of chloroform was added to the fresh tube. The tube containing the transferred liquid was inverted until a turbid liquid cloud formed. Also, this mixture was spun at 13,000 RPM for 15 minutes, 4°C. After centrifuging, the top layer that contained fungal genomic DNA was extracted to a new 2 ml centrifuge tube and 50 μl 3 M Sodium Acetate, pH 4.6, added with gently inverting. DNA precipitation was performed by adding 1 ml of 100% ethanol and the sample kept at -20°C overnight. Next day, the tube was spun at 13,000 rpm for 15 minutes, 4°C to pellet DNA. Descanted ethanol slowly and avoided disturbing the pellet. The same tube with 1 ml 70% ethanol was washed to remove salts and spun down the pellet at 13,000 RPM for 15 minutes. Descant ethanol and air dry about 10 minutes. DNA was resuspended in TE buffer (The amount depended on the size of the pellet, generally was added about 50-100 μl of TE) and resulting DNA assessed for quality. Samples below a ratio of DNA absorbance at 260 / 280 nm of 1.9 were discarded and regrown or extracted as needed.

**Known Aspergilli Strains And Primers**

To establish Tm profiles of known *Aspergillus flavus*, representative VCGs were obtained from National Peanut Research Laboratory, U.S. Department of Agriculture, Georgia, *A. flavus* strains. Additionally, *A. flavus* strains F3W4 (NRRL 30796) and K49 (NRRL 30797) were provided by the USDA-ARS Mycotoxin Research Unit, Stoneville,
Mississippi, and *A. flavus* NRRL 3357 (ATCC 200026; SRRC 167), *A. paraciticus* SU-1 (SRRC 167B; ATCC # 56775 and 201461), *A. oryzae* RIB 40 (National Research Institute of Brewing Stock Culture and ATCC-42149), *A. tamari* NRRL 20918, *A. pseudotamarii* NRRL 2420 and *A. sojae* were provided by the USDA-ARS-SRRC Food and Feed Safety Unit. Primers for PCR were selected and designed by the basis of published data (Nai-Dee 2000; Scherm *et al.* 2005) and the annealing temperature of each primer was calculated at 5°C below the primer Tm for the PCR reaction (Table 1). If the calculated was greater than 70°C, then 65°C was used for annealing temperature in the quantitative real time PCR (qRT-PCR) to avoid polymerization reaction and the formation of primer dimers.

**Quantitative Real-Time PCR**

A LightCycler® 480 (Roche) was used for qRT-PCR reaction and detecting Tm of PCR products. Amplification was conducted using LightCycler® 480 SYBR Green I Master (Roche) in a 20 μl reaction mixture containing 50 ng of fungal DNA as a template, 10 μM of each primer, and 1X of Master Mix. Cycling reactions were based upon the recommended in the LightCycler® 480 SYBR Green I Master manual (Roche) as listed on Table 2. Annealing temperature was 5°C below the calculated Tm or 65°C if this value would be above 70°C. The software provided with the real-time PCR instrument was used to acquire Tm of PCR products. The Tm detecting threshold was set as the software default and the limitation of Tm peaks of plot (fluorescence vs. temperature) was set at two peaks. All samples tested were repeated a total of three times.
Table 1 Information of Primers Including Name, Sequence, Annealing Temperature Used in This Study (forward primer listed first for each pair).

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<th>Primer name</th>
<th>Primer Sequence (5'-3')</th>
<th>Annealing temperature</th>
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</thead>
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<td>51°C</td>
</tr>
<tr>
<td></td>
<td>GGTTCGTAGGTGGATAGAG</td>
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</tr>
<tr>
<td>AFPM2</td>
<td>CCACGCTCCTCAAATACG</td>
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<tr>
<td></td>
<td>CTGGACCGAGATCAGC</td>
<td></td>
</tr>
<tr>
<td>AFPM3</td>
<td>CACCACCAGTGAGGG</td>
<td>56°C</td>
</tr>
<tr>
<td></td>
<td>CTTTTCGACCTCCGAGAC</td>
<td></td>
</tr>
<tr>
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<td>TCTTGCTATACTATCTTCACC</td>
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<tr>
<td></td>
<td>AGCGATACAGTTTTAACACC</td>
<td></td>
</tr>
<tr>
<td>AFPM5</td>
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<tr>
<td></td>
<td>TCCTACCCGAGAGGAGTCTG</td>
<td></td>
</tr>
<tr>
<td>AFPM6</td>
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<td></td>
<td>CGAAAGGCAGTTGAGAAGGC</td>
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<tr>
<td>AFPM7</td>
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</tr>
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<td>TTGAGGCTGCTGTTGGAACGC</td>
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</tr>
<tr>
<td>Nor1</td>
<td>ACGGATCACCTTAGGCAAGCAC</td>
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<td></td>
<td>CTACCAGGGGAGTTGAGATCC</td>
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</tr>
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</tr>
<tr>
<td></td>
<td>CTTTCAGGTGACCAGAACGAT</td>
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<tr>
<td>Omt1</td>
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<td>AGTTTGTTGAACGCACCCAGT</td>
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<tr>
<td>347ACT70</td>
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</tr>
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<td></td>
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<tr>
<td>327TAA4</td>
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<td>177TAA18</td>
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<td>123AC27</td>
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<td></td>
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Table 2  Protocol for Use with LightCycler® Multiwell Plate 96.

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<tr>
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<td>Quantification</td>
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<tr>
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<th>Hold (hh:mm:ss)</th>
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<td>1.5</td>
<td>None</td>
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</table>

¹ For this step, the primer annealing temperature was used as Table 1.

NOTE: The upper table is the main PCR parameters and they must be programmed by the following reaction temperatures and acquisition mode (lower).
The Computer Program

All computer manipulation process of Tm values was executed under Microsoft® Windows® XP. The program, Weka 3.4.x, was downloaded from Department of Computer and Mathematical Science, University of Waikato, New Zealand. To run Weka, Java™ 1.4 (or later version) can be obtained on website: java.com, because it is required to install before running Weka 3.4.x. Weka is a collection of machine learning algorithms for data mining tasks. The algorithms can either be applied directly to a dataset or called from developed Java code. Weka contains tools for data pre-processing, classification, regression, clustering, association rules, and visualization. It is also well-suited for developing new machine learning schemes. In order to generate the decision tree, fungal Tm profiles were inputted by following the Weka demanded data form as follows:

@RELATION (Program name)

@ATTRIBUTE (Primer name) (Tm profiles for this primer)

@ATTRIBUTE CLASS (Fungal name)

@DATA (Tm profiles of fungus), (Fungal name)

The completed fungal Tm profiles data set was listed at Table 3. The fungal identification decision tree was output as “.txt” file. Because of for slinky, the original generated fungal identification decision tree was rewritten by Microsoft® Office Visio.

Field Samples

To assess the ability of Tm profiling and the decision tree to identify unknown samples, selected samples from an ongoing field trial were examined. Unknown samples
Table 3 The Fungal Tm Data Sets.

@RELATION aspergillus
@ATTRIBUTE afpm1 {83,84}
@ATTRIBUTE afpm2 {83,87,83-87,86-83,83-86,82-86,86}
@ATTRIBUTE afpm3 {84,85,86}
@ATTRIBUTE afpm4 {83,79-84,80-84,84,80-83,81,80-82,82-84,80,85,82-86}
@ATTRIBUTE afpm5 {75,76,77,76-87,75-86}
@ATTRIBUTE afpm6 {87,88}
@ATTRIBUTE afpm7 {87,91,92}
@ATTRIBUTE 347_ACT70 {77,78}
@ATTRIBUTE 327_TAA41 {76,77,88,89,N}
@ATTRIBUTE 277_TTC32 {79,82,79-82,83,84,80-85,79-83}
@ATTRIBUTE 250_TTC23 {78-82,77-82,82,78-81,81}
@ATTRIBUTE 205_TTTC17 {78,79}
@ATTRIBUTE 123_AC27 {80,81,82,83,84,85,86,87,82-59,83-89,82-88}
@ATTRIBUTE 177_TAA18 {75,87,69-76,76,74-87}
@ATTRIBUTE 14B_TCC18 {80,80-88,79,79-87,80-89,78}
@ATTRIBUTE NOR1 {91}
@ATTRIBUTE OMT1 {82,87,88,89-93,88,94,70-86,88-93}
@ATTRIBUTE VER1 {75,87,88,89,88-93}

@ATTRIBUTE class

@DATA
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84,86,84,80-84,76,88,91,78,89,79,77-82,78,82,75,79,91,89,88,A-flavus-3357
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<td>84,83-86,84,80-84,76,88,92,78,89,82-82,78,80,75,80,91,85-89,88</td>
<td>A-flavus-F10-NPL</td>
</tr>
<tr>
<td>84,83,85,80-83,77,88,77,N,82,77-82,78,83,69-76,80,91,70-86,89</td>
<td>A-oryzae-RIB</td>
</tr>
<tr>
<td>84,83,84,80-84,76,88,92,78,89,82,77-82,79,83,75,79,91,89-93,88</td>
<td>A-sojue-NRRL-6271</td>
</tr>
<tr>
<td>83,83,84,80-84,76,88,91,78,77,84,81,78,82-59,75,79,91,88-93,88</td>
<td>A-parasiticus-Delta-J-mutant-50</td>
</tr>
<tr>
<td>83,83,84,81,75,87,91,77,76,83,81,78,82-59,75,79,87,91,88-93,87</td>
<td>A-parasiticus-Delta-J-mutant-21</td>
</tr>
<tr>
<td>83,83,84,80-84,75-86,88,91,78,77-79-83,81,78,82-88,75,78,91,88-93,88</td>
<td>A-parasiticus-162-N2-SEC</td>
</tr>
</tbody>
</table>
Table 3 (Continued)

| 84,83-86,84,80-84,76,88,92,78,89,79-82,77-82,78,82,75,79,91,89,88, A-pseudotamarii-N |
| RRL-2420 |
| -yellow |
were isolated from soil collected in October 2008 in experimental corn fields located in the Mississippi Delta (Stoneville, Mississippi). The field experiment was designed to assess the over-wintering of an atoxigenic *A. flavus*. Three separate treatments were involved; a control field, a field with the atoxigenic strain *A. flavus* K49 applied with a barley formulation, and *A. flavus* K49. Isolates of *A. flavus* were recovered from soil plated on MDRB (Modified dichloran rose Bengal) media as described by Abbas (2006). After 7 days of growth, fungal colonies were selected and streaked on 70 mm plates and incubated for 7 days. Conidia from the fungal cultures were collected by loop and inoculated into a 12 ml culture tube with 8 ml potato dextrose broth, which were then incubated at 28°C with constant shaking at 150 rpm for 7 days. The resulting fungal tissue was collected and genomic DNA extraction was performed as described above. After collection of conidia with the inoculation loop, the cultures were tested for aflatoxin-production using the ammonium hydroxide vapor exposure (AV) method (Abbas *et al.* 2004). The test was done by placing a drop of ammonium hydroxide on the cover of the 70 mm culture plates and put a 28°C incubator for 5 minutes. A color change of the medium plate to purple or reddish indicates aflatoxin production. A total of 89 fungal colonies were selected for examination, each of which was run three times.

**Plant Materials and Fed with Fall Army Worm (FAW)**

Resistant maize inbred lines Mp708, Mp496 and Mp716 and susceptible inbred line Tx601 were used for this study. All plants were grown in the Plant Science Research Center, Mississippi Agriculture and Forestry Experiment Station. The green house condition is maintained at a maximum day temperature of 33°C and minimum night
temperature of 26°C. Four to five weeks old plants in mid-whorl developmental stage were selected for fall army worm infestation. FAW (*Spodoptera frugiperda*) larvae were nursery by the USDA-ARS Insect Rearing Laboratory, Starkville, MS. FAW were reared in 30-ml plastic insect diet cups for 5 days under a photoperiod of 16:8 at 26.6°C on an artificial diet. Each plant was infested by seven to eight larvae, each weighing about 10-12 mg, placed in the whorls of the plants and plants were covered by the plexiglass chambers. After 24 h, wounded plant tissues (tissue within a 1 cm radius of insect damage site) were collected and immediately frozen in liquid nitrogen, and then stored at -80°C for later analysis.

**Preparation of Protoplast**

Leaves of appropriately treated maize plants were immersed in 0.5 M mannitol, chopped with scissors into small pieces and washed in 0.5 mannitol for two times. All tissue slices were transferred to 50 ml centrifuge tubes and immersed into enzyme mixture solution containing 2% (w/v) cellulysin (Calbiochem), and 0.4% (w/v) macerase (Calbiochem) in 0.5 M mannitol, and adjusted to pH 5.5. The preparation was incubated in 30°C for 5 h with gently shaking. Protoplasts were separated from undigested leaves by filtration through a 100-μm mesh nylon cloth and collected by centrifuge at 250g, 5 minutes. For purification, protoplasts were resuspended in 0.5 M sucrose containing 10% (w/v) ficoll and overlayed with 0.5 M sucrose containing 3% (w/v) ficoll and then with 0.5 M mannitol. The gradient solution was centrifuged at 1000g for 5 minutes. Protoplasts floated to the interface between the layers of 0.5 M sucrose containing 3% ficoll and of 0.5 M mannitol. Protoplasts were collected with a Pasteur pipette,
resuspended with 0.5 M mannitol and collected by centrifugation with 250g for 5 minutes. All solutions used for protoplasts were adjusted to pH 6.5 with 10 mM potassium phosphate.

**Preparation of Vacuoles**

Vacuoles were released from protoplast essentially as described (Bekesiova et al. 2008). All steps were done at room temperature. To digest protoplast, a solution of 0.3 M mannitol, 20 mM HEPES (adjusted to pH 8 with 1 M Tris), 1 mM EGTA, and 0.5 CHAPS was added to 0.5 to 1 ml of protoplast suspension. To purify the vacuoles, 4 ml of the crude vacuole preparation was gently mixed with 4 ml of 0.4 M mannitol containing 20% ficoll, 20 mM HEPES, pH 8.0, 1 mM EGTA, and 0.5 mM CHAPS. This suspension was overlayered with 3 ml of the same solution containing 4% ficoll and with 2 ml of solution without ficoll. The gradient was centrifuged at 100g for 20 min. The vacuoles were collected at the interface at the 0%/4% ficoll, collected by Pasteur pipette and frozen in liquid nitrogen.

**Protein Concentration**

The protein content of each sample was determined by Bradford reagent (Sigma) as followed by the manufacturer’s instructions. In brief, 1 μl of each protein sample was mixed with 1 ml of Bradford assay reagent and incubated at room temperature for 5 minutes. The protein standard curve was plotted by a serial concentration of bovine serum albumin (BSA, 1, 2, 3, 4, 5, 7, 8, and 10 μg) were separately mixed with 1 ml of Bradford assay reagent and incubate at room temperature for five minutes, then, protein
concentration was determined by the spectrophotometer (GeneQuant, GE healthcare) at OD 595 nm. The standard curve was plotted by the net OD values at 595 nm vs. the known BSA concentrations.

**SDS-PAGE**

Ten μg of total protein from each sample was mixed with 3X SDS Sample Buffer (Biolabs) and denatured by heating aliquots to 90°C for 10 min. The protein samples were loaded into SDS-PAGE for analysis. The SDS-PAGE was composed by the 12% (w/v) resolution gel and placed on top by the 4% (w/v) stacking gel. The resolution gel was made by 3.3 ml of distilled water, 4 ml of 30% acrylamide mix (acrylamide: N’, N’ – bis-methylene-acrylamide = 29:1), 1.5 M Tris, pH 8.8, 0.1 ml of 10% (w/v) SDS, 0.1 ml of 10% (w/v) ammonium persulfate and 4 μl of TEMED in a 20-ml beaker. This mixture was poured into gel cassette and left about 2 cm from the top of the front glass plate. The stacking gel composed 1.4 ml of distill water, 0.33 ml of acrylamide mix, 0.25 ml of 10 M Tris, pH 6.8, 20 μl of 10% (w/v) SDS, 20μl of 10% (w/v) ammonium persulfate and 2 μl of TEMED. The stacking gel mixture was poured overlaid on the polymerized resolution gel and a 10-well comb was inserted in to the stacking gel. Whole gel cassette and performing gel electrophoresis was supported by the Bio-Rad Mini-PROTEAN 3 CELL sets. The gel was run under constant 100 V and stop until the front line reached to the bottom of the gel.
Western Blotting

After SDS-PAGE electrophoreses, the gel was soaked in the transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% v/v methanol, pH 8.3) and the proteins were transferred to the Nitrocellulose membrane with the Owl Semi-dry Electroblotting System for 1 hr at 0.8 mA/cm². After transfer, the membrane was blocked with SuperBlock Blocking Buffer (Thermo Scientific) at 4°C, overnight. After removed blocking buffer, A 1: 2500 dilution of Anti-tobacco class I chitinase rabbit IgG primary antibody (Agrisera) was diluted in Tris-buffered saline containing 0.1% Tween-20, pH 7.4 (TBST), and then, incubated with membrane for 1 hour at 20°C. After incubation, the primary antibody solution was removed and the membrane was washed in TBST for 5 min 4 times at 20°C. A 1:250,000 solution horseradish peroxidase conjugated Fc specific anti-rabbit goat IgG (Pierce) diluted in TBST was added on the membrane and incubated for 1h 20°C. After incubation, the membrane was washed in TBST again for 5 min 4 times at 20°C. Residual TBST was removed from membrane and a working solution by mixing equal part of the Stable Peroxide and the Luminol/Enhancer from the SuperSignal West Femo kit (Pierce) was overlaid on the membrane for 5 min. The membrane was removed from working solution and placed it in a plastic wrap. The absorbent tissue was used to remove excess liquid and carefully pushed out any bubbles from between the blot and the surface of the plastic wrap. The membrane was transferred to a film cassette and exposed onto High performance chemiluminescence film (GE) in the dark room to acquire the signal onto the film. The exposure time was adjusted from 5 sec to 1 min until got the best result.
Chitin-Azure Chitinase Assay

Ten micrograms of chitin azure was added to microcentrifuge tubes containing 990 μl of 200 mM sodium phosphate buffer at pH 7.0. Ten microliters of protein sample was added, and the mixture was incubated at 37°C for 24 hr on a shaker. The mixture was then centrifuged at 16,000g for 10 min and the absorbance at 570 nm of the supernatant was determined. Samples were compared to blanks containing sample buffer without chitinase. One enzyme unit was defined as changing in the optical density at 570 nm of 1.0 in 24 h.

Maize Nucleic Acid Extraction

Maize total RNA isolation was extracted by TRI Regent® (Ambion) in accordance with the manufacturer’s recommendation and slightly modified for plant tissue RNA extraction. Frozen tissue was ground to fine powders by mortar and pestle. The homogenized sample was added 1 ml of 100 mg tissue and the sample volume should not exceed 10% of the volume of TRI Reagent®. Whole mixture including insoluble material was transferred to fresh centrifuge tube. Sometimes, if plant tissue contained a lot of protein, polysaccharide, and the whole mixture showed very sticky. To avoid this, centrifuge at 12,000xg for 10 minutes at 4°C was an option. The 1-Bromo-3-Chloro propane was added to the homogenate and shaken vigorously for to increase RNA yield. After 5 minutes incubation at room temperature, the whole mixture was centrifuge at 12,000g for 10 minutes at 4°C. The top aqueous phase was transferred to a fresh tube and added 500 μl of isopropanol per 1 ml of TRI Reagent® with vortex at moderate speed for 5 seconds and then incubated at room temperature for 5-10 minutes. To precipitate RNA,
the sample was centrifuged at 12,000g for 8 minutes at 4°C and then discarded supernatant. The pellet was washed by 75% ethanol per 1 ml TRI Reagent®. Ethanol was removed by centrifuge at 7,500g for 5 minutes and removed all residual ethanol by centrifuging again shortly and removing the ethanol that collected with a fine tip pipette.

RNA was air dried and dissolved by Ambion Nuclease-free water.

**First-Strand cDNA Synthesis**

The cDNA synthesis was done by Invitrogen™ ThermoScript™ RT-PCR system. Briefly, 1 ng of template total RNA for each sample was used. The total RNA was denatured by incubating at 65°C for 5 minutes to prevent RNA secondary structure. Oligo(dT)$_{20}$ primer for this cDNA synthesis was selected and instead of random hexamer. The master reaction mixture was prepared during RNA incubation followed by the ThermoScript™ RT-PCR system protocol. One μl (1ng/μl) of Total RNA was mixed with 1 μl Oligo(dT)$_{20}$ primer (50 μM), 10 mM dNTP mix and final adjusted the total volume to 12μl with DEPC-treated water. The tube was placed on ice and ready for use. Four μl 5x cDNA synthesis buffer, 1μl 0.1 M DTT, 1μl RNaseOUT™, 1μl DEPC-treated water and 1μl ThermoScript™ RT were added in the tube and mixed gently by pipeting. The first-strand cDNA synthesis reaction was carried out by incubating for 60 minutes at 50°C. The reaction was terminated by heating at 85°C for 5 minutes. All cDNA synthesis reactions were used for PCR immediately and stored at -20°C.

**Polymerase Chain Reaction**

The Platinum® *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) was used for
polymerase chain reaction. Two primers, (PrmF: 5’- CGCCGCAGTGCCCCTACCC – 3’ and PrmR: 5’ – TCTCCCGATGATCCGCTCTTATTA -3’) designed for amplification of maize chitinase 3’-end gene. The PCR mixture contained 10 μl of the cDNA, 1 μl each of PrmF and PrmR (10 μM) primers, 1μl of dNTP, 1μl of Platinum® Taq DNA polymerase, 5 μl of 10X PCR buffer and used distilled water to adjust final volume to 50 μl. PCR was conducted in Eppendorf PCR machine with preheat at 95°C for 10 minutes, following by 30 cycles at 95°C for 90 seconds, 60°C for 30 seconds, 72°C for 70 seconds, and for final extension was 72°C for 10 minutes.

Purification of PCR Product

The QIAquick® PCR purification kit (Qiagen) was used to purify the PCR product. The PCR product was elctrophoresed on a 1.2 % (w/v) agarose gel with 1X TAE buffer (20 mM Tris-HCl, pH 7.5, 7.5 mM sodium acetate, 0.5 mM EDTA). The gel was running under 100V for 50 minutes. The gel was stained by ethidium bromide and then soaked in water for 5 minutes to remove excess ethidium bromide. The DNA was cut by clear razor blade under UV illumination and transferred into microcentifuge tube. Three volume of QG buffer was added into 1 volume gel (mg) and incubated for 10 minutes until the gel dissolved completely. If the mixture was not yellow (orange or violet) after gel dissolved, 10 μl of 3 M sodium acetate was added, pH 5.0. One gel volume of isopropanol was added to the sample and mixed for increasing the DNA yield. The mixture was transferred to the QIAquick spin column and centrifuge for 1 minute for binding DNA. The column was washed by 0.5 ml of QG buffer to remove unbound residuals. Again, the column was washed by 0.75 ml of PE buffer, centrifuge for 1 min.
The flow-through was discarded and the spin column was centrifuged for additional 1 min at 13,000 rpm for removing residual solution contained in the binding membrane of the spin column. Finally, the spin column was placed in the 1.5 ml microcentrifuge tube and the DNA was eluted by adding 50 µl of EB buffer, centrifuged for 1 min.

**Cloning the PCR Product**

The purified PCR product was cloned to TOPO TA Cloning Kit for Sequencing (Invitrogen). According to the manufacturer’s user manual, two µl of fresh PCR product was mixed with to 1 µl of Salt Solution and 1 µl of TOPO vector and then water was added up to 6 µl, incubated at room temperature for 20 min. Added 2 µl of the cloning mixture to a vial of One Shot Chemically Competent *E. coli* and incubated on ice for 20 min. The vial was heat-shocked for 30 sec at 42°C without shaking and immediately transferred on ice. Cells were added 250 µl of room temperature S.O.C medium and incubated at 37°C for 1 hour. The aliquots containing 50 µl of cell suspension was spread on a prewarmed LB plate containing 50 µg/mL ampicillin and incubated overnight at 37°C.

**Isolation of Recombinant Plasmid**

Six colonies were picked up from the plates and transferred the single colony to 15 ml culture tube which contained 5 ml LB medium with 50 µg/mL ampicillin. Culture tubes were incubated at 37°C overnight under vigorous shaking (250 rpm). Cell pellets were collected by centrifuge at 10,000 rpm for 10 min. After descant the supernatant, cell pellets were resuspended in 250 µl Buffer P1 (QIAprep Spin Miniprep Kit, Qiagen) and
transferred to a microcentrifuge tube. Cell suspension was again mixed with 250 μl P2 Buffer by inverting the tube 4 to 6 times. Then, the cell lysate was added with 350 μl N3 Buffer and mixed immediately and thoroughly by inverting the tube for several times. The supernatant was collected to QIAprep spin column after centrifuging at 13,000 rpm for 10 min. The QIAprep spin was centrifuged for binding DNA to the affinity membrane and washed by 0.5 mL Buffer PB in order to removing trance nuclease activities because using \textit{endA}+ \textit{E. coli} strain. To get rid of the carbohydrate content from the QIAprep spin column, the volume of 0.75 mL Buffer PE was added and centrifuged by 13,000 rpm for 1 min and then centrifuge at the same condition again for removing residual wash buffer PE. At last, the QIAspin column was placed into microcentrifuge tube, the DNA was eluted by adding 50 μl of EB buffer, centrifuged for 1 min.

\textbf{Sequencing Recombinant pCR4-TOPO/Chitinase Plasmid}

DNA sequencing was done by the DNA facility, Iowa State University. According to the demands of DNA facility, the concentration should not less then 0.25 μg/μl and its amount should be over 0.5μg. Sequencing primers, T7 primer (5’-TAATACGACTCACTATAGGG-3’) were provided by DNA facility itself. Sequencing results were directly downloaded from website and analyzed by using LASERGENE software (DNASTAR).
CHAPTER IV
RESULTS

Performing Tm Calling Analysis

The melting temperature analysis was performed by using automated algorithms to find the peak areas and melting temperatures (Figure 3). Because of short sequence repeats primers used in this study, the minimum number of melting peaks was set to 2 peaks. Short sequence repeats primers potentially representing variable numbers of tandem repeat loci, primers had higher than expected chances to anneal other target sequence.

DNA Concentration and Melting Temperatures

Since I used this method to differentiate fungal strains and to ensure that the Tm values were consistent, the DNA concentration was gradually increased from 50 ng to 100 ng with the same primer, NOR1. Each representative DNA concentrations were repeated for 5 times. The result shows that the averages of Tm values are constant at 91°C and not changed (Table 4). It suggested that DNA melting temperature acquired under this board of concentration range was not influenced by DNA concentration. As a result, the personal manipulation error and varieties of DNA extraction kits and quantitative instruments will not affect the melting temperature.
Figure 3   Representative Melt Curves.

Tm was acquired after PCR reaction and calculated by software supported by Roche LightCycler 480®. Typical representation of the results of one peak (A) and two peaks (B).
Table 4  Tm Constancy Test.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Repeats (°C)</th>
<th>Average(°C)</th>
<th>SD&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 ng</td>
<td>90.98</td>
<td>91.02</td>
<td>90.91</td>
</tr>
<tr>
<td>60 ng</td>
<td>90.82</td>
<td>90.63</td>
<td>90.53</td>
</tr>
<tr>
<td>70 ng</td>
<td>90.77</td>
<td>90.62</td>
<td>90.52</td>
</tr>
<tr>
<td>80 ng</td>
<td>90.79</td>
<td>90.67</td>
<td>90.73</td>
</tr>
<tr>
<td>90 ng</td>
<td>90.79</td>
<td>90.71</td>
<td>90.84</td>
</tr>
<tr>
<td>100 ng</td>
<td>90.80</td>
<td>90.54</td>
<td>90.61</td>
</tr>
</tbody>
</table>

<sup>a</sup> Standard Deviation

NOTE:  *A. Flavus* VCG 54 with Primer NOR1.
Selection of Classification Options

As described above, Weka processed the classification tree. After inputting all fungal representative data, several different classification trees were obtained at once (Figure 4) and they can be divided into two different major trees. The first tree is classified by groups, sorting by the first SSR primer divided into two or three groups and followed by the use of another primer to classify again (Figure 4A). In the end, this procedure can eventually identify every fungal strain at the branch end of the classification tree but still needed more SSR primers. This result violates the concept of developing new identification methods, because the procedure requires more time and eas more costly. The second tree (Figure 4B) was generated by the variety of representative melting temperatures. Samples were classified by the primer that generated the variety most representative of melting temperature for each fungal isolate. Then, the second step is following by the next primer, which still presenting the most variety classification results for this group. Apparently, from the Figure 4B, it is shown that this classification procedure not only combines the advantage of Figure 4B, but if possible, some samples can be identified during classification. Thus, the classification method based on the variety of melting temperature was chosen. In other words, primers which represented less variety or common melting temperature compared with other primers will be removed. In doing so, this method can shorten identification procedure and save expenses.
Classify samples by grouping (A) and classify samples by Tm and groups (B).

Figure 4   Two Possible Classification Methods.
The *Aspergillus* spp. Identification Flowchart

After collecting all Tm values of each fungal strain versus selected primers, Tm values were input into Weka. Each VCG represented unique Tm patterns with its represented primers and every VCG can be separated independently (Figure 5). The flowchart shows that every fungal VCG can be separated by this sorting program and the similarity is 100% identical to Vegetative Compatibility Grouping methods. Also, other *Aspergillus* strains, not assigned to VCGs, were used to test through this flowchart: *A. flavus* NRRL 3357 (ATCC 20026, SRRC 167), a wild type strain that producing high level aflatoxin; *A. flavus* NRRL 30797 (K49), non-aflatoxin producing strain for biocontrol; and aflatoxin-producing *A. flavus* NRRL 30796 (F3W4). These three strains started at the first key primer, AFPM2, and then followed the different flowchart pathways depending on their individual represented Tm(s). Consequently, all of these three fungal trains lay on the different ends of each branch. Other species of *Aspergilla* such as *A. paraciticus, A. oryzae, A. tamari, A. pseudotamarii* and *A. sojie* also can be differentiated by the flowchart. Primers that were applied to discriminate VCGs by traditional fingerprinting method can be used to identify *Aspergilla* between diverse species as well through this flowchart.

Using the Decision Tree

By using a decision tree it is possible to simplify the identification system the smallest combination of primers can be used for identification. Decision trees provide a structured approach to decision making where the most informative tests are conducted
Figure 5   The VCGs Grouping Flowchart Generated by the C4.5 Algorithm Decision Tree Analysis.

Branches are labeled by melting temperature and for a selected primer.
first, and further tests run depending on the outcome of the first tests. To classify an example using a decision tree (Figure 6A), one begins at the top node and, based on the outcome of the test associated with this node, follows the appropriate branch, and continues conducting the test at the next node until reaching a leaf node. In the case that none of the choices available in the tree matches the test result, there is an implicit choice of “unknown” available at each node (as indicated by U in Figure 6B)

**Comparison of Flowchart and Ammonia Vapor (AV) Test**

Using the Tm flowchart procedure, we successfully classified five samples as *A. flavus* VCG43, 27 as *A. flavus* K49, 32 as *A. flavus* 3357 and 21 as *A. flavus* F3W4 (Table 5). The remaining 4 were classified as unknown because their Tm values were not represented in the Tm database.

**Sequences of Chitinase Gene**

As described earlier, the chitinase enzyme (Prm3) is related to fungal, bacterial and virus attack, especially for fungal attack by digesting the cell wall of fungus. According to the previously study (Shivaji, personal communication), the level of mRNA accumulation in insect resistant inbred line, Mp708, was more than in the susceptible line, Tx601, when FAW attack. Furthermore, the destination of most chitinase proteins in the vacuoles, few stay in the plant tissues. After protein modification in the Golgi apparatus, chitinase will be transferred to the vacuoles and led by its 3’-end signal pepitide. The results of comparison of chitinase mRNA sequences shows that all insect resistant inbred lines have
Figure 6   Illustrations of Identification Flowchart Based on the Melting Temperature Varieties.

Beginning at the sample pool (top), the results of each test leads to another test until one reaches an oval leaf node representing a final classification (A). An implicit choice of “unknown” (U) indicated by a dotted line which means the data of its melting temperature was not in the database (B).
Table 5   Comparison of Ammonium Vapor Test (AV) and Flowchart Methods.

<table>
<thead>
<tr>
<th>AV Test</th>
<th>Yellow&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No color change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flowchart</td>
<td>A. flavus 3357</td>
<td>A. flavus F3W4</td>
</tr>
<tr>
<td></td>
<td>62</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Yellow color appearing on the plate means this fungal isolates produces aflatoxin.
complete 3’-end sequences except Mp496 and the insect susceptible control line Tx601 lacks complete 3’-end sequences (Figure 7). This result implies that lacking of complete 3’-end sequences may decrease the ability of transportation chitinase protein affectively to the chitinase protein so that vacuoles do not contain enough chitinase protein for defense mechanism.

Relative Expression level of Chitinase mRNA Gene

All resistant lines in this study showed that the rates of chitinase mRNA expression were constantly expressed without stimulation by FAW (Figure 8), and they were almost the same as the susceptible line, Tx601, when attacked by FAW. After infested by FAW, all mRNA of chitinase genes were increased, especially in Mp708. Compare to Figures 7, the 3’-end of the chitinase leading peptide shortage does not affect the transcription or translation of chitinase genes, which means the length mRNA cannot lead to pre-mature RNA or stop early during protein synthesis. This implies that the leading peptide only play the role of helping transport chitinase protein to vacuoles.
Figure 7  Comparison of 3’-end Sequences of Selected Inbred Lines.
Figure 8  Relative Expression of Chitinase mRNA Transcripts.

F: Plant fed by FAW. C: Control.
Chitinase Protein in Vacuoles

The protein amount in Mp708 was larger than Mp716 and Mp496. Apparently, chitinase protein was barely detected in Mp496 as susceptible line Tx601 (Figure 9). It was indicated that the 3’-end sequence of chitinase mRNA were essential and it played the key role to accumulate chitinase protein in vacuoles. Lacking few base pairs does not mean that chitinase has no ability to be accumulated in vacuoles but these sequences did affect the efficiency of protein transportation. In a surprise, activities of chitinase protein were not affected by the sequences of leading sequences, no matter it has completed sequence or few bases shortage (Figure 10).
Figure 9   Western Blot of Chitinase Protein in Vacuoles.

Each lane contained 10 ug of protein. Chitinase protein with deletion of 8 to 10 bases in the 3’-end mRNA (Mp496 and Tx601) accumulated less protein than others with complete sequences (Mp708 and Mp716)
Figure 10  Graphical Representation of the Ration of Vacuolar Chitinase Activities (μg) Protein.

C: Control, F: Attacked by FAW.
CHAPTER V  
SUMMARY AND DISCUSSION

_A. flavus_ Fingerprinting

*Aspergillus* species are widely distributed fungi that release large amounts of airborne conidia that are dispersed in the environment. Several *Aspergillus* specie have been described as human pathogens. *Aspergillus flavus* is the species most frequently found in crops. To investigate the epidemiological relationship in crops and soils molecular typing techniques for fungal isolates have been developed. Fingerprinting techniques for _A. flavus_ required a high discriminatory power and need to be highly reproducible, since a high extent of genetic variability has been demonstrated. The RFLP method in combination with the hybridization probe is highly discriminatory. However, this method is time-consuming and labor intensive performance and the RFLP banding pattern are difficult to interpret. This makes the RFLP method far from ideal for typing a large number of isolates. In contrast, typing techniques which yield sequence data, such as MLST, is 100% reproducible and the data can be readily exchanged between laboratories. Unfortunately these methods lack sufficient discriminatory power for large provide a high level of large scale studies. Microsatellites or STRs, however, have shown to provide a high level of discrimination between wide varieties of micro-organisms. STR analysis yielded highly reproducible and exact typing results, which allow the easy exchange of data, but using repeat sequences in a single PCR amplification may lead to
the formation of fragments of the same length. Successful classification of *Aspergilli* based on Tm profile was first reported in efforts to distinguish *A. flavus*, *A. niger*, *A. terreus* and *A. fumigatus* (Faber et al. 2009). However, in biocontrol studies there is a need to distinguish strains within the same species, and this is beyond the capabilities of most classification methods. Tm profiling will provide the accuracy needed for classification, even among highly related fungal samples. However, as the number of strains/species to be identified increases, the number of PCR reactions required to distinguish them also increases.

The temperature at which a DNA strand separates or melts when heated can vary over a wide range, depending on the sequence, the length of the strand and the GC content of the strand. Furthermore, melting temperature can vary for products of the same length but different GC/AT ratio, or for products with the same length and GC contents, but with a different GC distribution. Also, base pair mismatches between two DNA molecules lead to a decrease in melting temperature. This effect is more pronounced for short DNA hybrids. The measurement of fluorescence emitted by DNA denaturation was used to determine the melting temperature of each sample. The melting temperature of a sample is defined as the point at which half of the DNA has melted. In this study of the SYBR Green I dye, this is due to the separation of DNA strands and consequently the release, of SYBR Green I molecules. As SYBR Green I only fluoresces at 530 nm if bound to double-strand DNA, melting dramatically decreases fluorescence at this wavelength. The analysis depended on displaying a melting peak(s) chart that plots fluorescence versus temperature (Figure 3). Display the melting temperatures as peaks were set limitations to two peaks and make it easier to distinguish a sample’s
characteristic melting profiles. Thus, non-specific amplification may occur and present more then two repeats at the melting curve display chart. Using SYBR Green I to acquire and analyze obtained DNA melting temperature is very sensitive; any possible double-stranded DNA can be monitored and acquired its reprehensive melting temperature. The peak limitation parameters can avoid biases such as primer dimer formation and non-specific amplification.

In this study, a novel assay for fingerprinting *Aspergillus spp.* is described and evaluated. Although a total 18 primers were used to establish the database of Tm values for known fungal VCGs (Figure 5), after generation of the decision tree it was found that only 9 primers were necessary to identify all Georgian VCGs down to the level of the strain. The primers that were not included by the decision tree learning algorithm provide less information on melting temperature variations than the primers that were included and are not required to uniquely identify each strain. As additional strains and fungal species are examined, these primers or new ones may be needed when the decision tree is reconstructed to include the additional species/strains. For the known species and strains in this study, the decision tree requires a minimum of two and a maximum of six primers to uniquely identify all strains, and the average number of primers required to do so is 3.4. Classical fingerprinting approaches require all primers to be run on all samples and compare the banding patterns on electrophoresis gel. Thus, Tm based fungal fingerprinting coupled with the decision tree analysis is more efficient for distinguishing samples than other methods especially under a large amount of fungal sample needed to be identified such as discrimination of soil fungal isolations. This assay demonstrated several characteristics important for a good typing assay. Major importances are the
abilities to discriminate between *Aspergillus* spp. and *Aspergillus flavus* populations. Typing results from epidemiological isolates showed identical to AV test. Table 4 shows that the Tm classification and the AV test results were consistent and the samples shown to produce aflatoxin via the AV tests were identified via Tm as strains known to produce aflatoxin with the exception of the four that could not be identified. Although our Tm database contains known fungal strains including *A. flavus* VCGs and some other *Aspergillus* spp., all selected SSR primers were designed based on genomic sequences from identified strains of *Aspergillus* spp. At any decision point in the tree, there is an implied choice of “unknown” if the observed Tm is not one of the choices given in the flowchart for that primer (Figure 6B). If a Tm value cannot be found in the flowchart at one decision point, we assume the sample was not in the known Tm database or it is a fungal strain other than an *Aspergillus* spp. This type of mismatch can potentially occur anywhere in the tree, although we only encountered this situation at the first decision point for primer AFPM2. Of the selected samples run, 96% could be placed into a strain group by the Tm identification method. The database can be improved over time as new strains are correctly identified via traditional taxonomic methods and their Tm values for the SSR loci presented here (or, if necessary, new markers) are added to the database. We anticipate the percentage of strains that can be identified will increase as additional VCG strains are added to the database. Other advantages include the tolerances of DNA quantification error and DNA manipulation error (pipeting error). In research settings, quantitative real-time PCR is mainly used to provide quantitative measurements of gene abundances. Therefore, real-time PCR is very sensitive to template DNA and primers (probes). The goal of developing this method is to establish a rapid identification method
having a potential to exchange the Tm database between different laboratories. Along with the improving of molecular techniques, DNA extraction kits are varies. Different researchers use DNA extraction kits depending on their own personal preferences. For DNA quantification, instrument variation and researcher’s pipeting error in DNA dilution are still the concern in developing this method. According to the operation manual of LightCycler® 480 (Roche), the ideal template concentration for real-time PCR is 50 ng. According to the Table 4, increasing template DNA concentration from 50 ng to 100 ng did not influence results in acquiring Tm. For the typing procedures, the most efficient primers were selected and discriminated the PCR results without comparing the banding pattern. Furthermore, transferring banding pattern to digital numbers (Tm values) makes it easy to interpret the data. Together with the use of computer-based sorting program, it is even possible to exchange fingerprinting typing data between laboratories. The high throughput of samples, the low cost, the robustness and the inter-laboratory reproducibility together with the easiest manipulated Tm values makes this method suitable for fingerprinting large numbers of isolates and exchange of results between laboratories.

**Chitinase**

In mature plant cells, the vacuoles are the largest of the membrane-bound organelles. Plant vacuoles often serve as both a lytic compartment for the degradation of materials and as a storage area for proteins. Protein concentrations in the vacuoles determine the efficiency of functions that are essential for the regulation and maintenance of plant growth and development. Soluble plant vacuolar proteins are sorted away from
secreted proteins into small vesicles at the Golgi network. This process requires the presence of sorting signals on the vacuolar proteins. Some protein contains N-terminal peptide that functions as a sorting signal and others contain COOH-terminal peptides such as chitinase. In this study, maize defense reaction was triggered by FAW infestation so that the abundance of chitinase mRNA increased, and the deletion of 3’-end seemed has no effect on the transcription and translation level. Activities of vacuoles chitinase protein were nearly the same in resistant and susceptible inbred maize lines. Deletion of 8 to 10 bases in the COOH-terminal sequences in chitinase affects its accumulation in vacuoles. This result gave us a conjecture that there is a potential vacuolar sorting receptor at the membrane of the vacuoles. This receptor may combine with the domain of chitinase leading peptide. The region of 8 to 10 bases may project the combination domain to the sorting receptor after translation. The future work is to obtain the complete chitinase mRNA sequence so that the amino acid sequence can be translated. It can help us to realize the chitinase hypothetical binding domain by protein structure/prediction program. Moreover, cloning the 3’end chitinase gene and membrane receptor gene of maize vacuole are necessary and utilize the yeast two hybrid system to verify the efficiency of combination of these two proteins.
REFERENCES


