Host genes involved in the interaction between Aspergillus flavus and maize

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Host genes involved in the interaction between *Aspergillus flavus* and maize

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Abstract

Aflatoxin contamination caused by *Aspergillus flavus* is a major concern in maize production. Understanding the complex interrelationships of genes during the *A. flavus*–maize interaction may be the key to developing strategies to interrupt the aflatoxin contamination process. The *A. flavus* Genome Sequencing and Expressed Sequence Tags projects are providing opportunities to identify a number of candidate genes involved in plant invasion as well as aflatoxin biosynthesis. Many *A. flavus* hydrolase genes have been presumed to function in polymer degradation and nutrient capture. With the application of proteomics and genomics technology, many resistance-associated proteins and genes have also been identified by comparing resistant with susceptible maize lines. This research facilitates an understanding of defense mechanisms in maize kernels at physical and biochemical levels. Although environmental stress, such as drought and high temperature, has been observed to greatly enhance aflatoxin contamination of maize kernels, and the related genomics research has been initiated, more work needs to be done to determine the environmental impacts on gene expression in maize kernels.

Keywords: *Aspergillus flavus*, aflatoxin, maize kernel, host resistance, gene interaction

Introduction

Since the occurrence of *Aspergillus flavus* in a maize field was first reported in 1920 (Taubenhaus, 1920), *A. flavus* as well as *A. parasiticus* have been found to colonize many crops such as peanuts, cottonseed, tree nuts, rice, etc., and be the predominant species responsible for aflatoxin contamination of these crops prior to harvest or during storage (Brown et al., 1999; Cotty et al., 1994; Diener et al., 1987). Aflatoxins are secondary metabolites that can be highly toxic and carcinogenic when animals or humans consume the contaminated feeds or food (Brown et al., 1999; Diener et al., 1987; Payne and Brown, 1998). In maize production, aflatoxin contamination is a major concern (Brown et al., 1998; Cleveland et al., 2003; Payne, 1992). Understanding the complex interrelationships of genes during the host plant–*A. flavus* interaction may be the key to developing strategies to interrupt the aflatoxin contamination process (Cleveland et al., 2004).

*A. flavus* is a saprophytic fungus that survives on decaying organic and plant materials, but it is also an opportunistic pathogen that invades susceptible hosts. The fungus has a broad range of nonspecific hosts, including crops as well as insects (Cotty et al., 1994; Geiser et al., 1998; Leger et al., 2000; Scheidegger and Payne, 2003). Recently, the identification and annotation of *A. flavus* genes has made significant progress through Genome Sequencing and Expressed Sequence Tags (EST) projects (Yu et al., 2004). The genes involved in the host invasion process and aflatoxin biosynthesis have been further disclosed through functional characterization.

In the past 20 years, several resistant maize lines have been identified and developed against *A. flavus*...
infection and aflatoxin contamination (Busboom and White, 2004; Menkir et al., 2006; Tubajika and Damann, 2001; Williams, 2006; Windham and Williams, 2002). These provide an opportunity for researchers to study host-resistance mechanisms. With the application of proteomics and genomics technology to high-throughput gene identification, many resistance-associated proteins (RAPs) and genes have been identified by comparing resistant and susceptible maize lines (Brown et al., 2006; Chen et al., 2002, 2007). During the interaction between maize and A. flavus, environmental stress such as drought and high temperature has been observed to greatly enhance A. flavus invasion and aflatoxin biosynthesis in kernels (Munkvold, 2003; Paul et al., 2003; Scheidegger and Payne, 2003; Williams, 2006), yet very little has been reported about their impact on gene expression of the host. Because defense mechanisms in maize kernels are not fully understood, no efficient biomarkers are available for breeding resistant lines, and therefore, no resistant commercial hybrids have been developed through marker-assisted selection (MAS) (Munkvold, 2003).

This review aims to discuss genes indentified in the A. flavus–maize interaction, and to explore the mechanisms of A. flavus aggression and maize defense from the viewpoint of gene expression. Investigation of the genes will accelerate the efficient selection of target genes, which can be used in MAS breeding or in genetic engineering to develop maize lines with good agronomically important traits and that support low aflatoxin accumulation.

**Invasion of maize kernels by A. flavus**

* A. flavus appears to grow predominantly as a saprophyte in the soil, but the fungus can also directly invade kernels as a pathogen and colonize living tissue (Diener et al., 1987; Payne and Brown, 1998). Preharvest contamination by aflatoxin has been observed on many crops, but most research on the infection process was conducted on maize with ear rot disease caused by *A. flavus* (Scheidegger and Payne, 2003). The epidemiology of *A. flavus* involves sclerotia (a compact mass of hyphae) and conidia (asexual spores; *A. flavus* has no known sexual stage) produced by the fungus growing on crop debris and in the soil, and serving as primary inoculum for young host plants in the spring. Later in the growing season, conidia dispersed in the soil and air on infected kernels provide high levels of secondary inoculum when environmental conditions are conducive for disease development (Horn et al., 1996; Payne and Brown, 1998; Scheidegger and Payne, 2003). Insects or wind play an important role in spreading the fungus to maize where infection follows (Diener et al., 1987).

Early research found that invasion of kernels by *A. flavus* was associated with wounding by insects. Therefore, insect injury to the maturing ear was thought to be necessary for infection (Taubenhaus, 1920). Insects were implicated in transporting inoculum to the developing ear, moving inoculum from silks into the kernel region, and providing wounds for establishment of the fungus in damaged kernels (Widstrom, 1979). However, later observations revealed a high incidence of *A. flavus* infection in ears and kernels free of obvious insect damage, therefore showing insect feeding as not necessary for establishment of the fungus or aflatoxin biosynthesis (Jones et al., 1980; Marsh and Payne, 1984; Widstrom, 1996). However, the exact process of fungal invasion of ears is still not clear. The fungus may invade thin-walled cells at the junction between the bracts and their rachillas or it may grow through the air space leading from the cob into the spikelet (Payne and Brown, 1998; Smart et al., 1990). *A. flavus* also can colonize silk tissue that is in the yellow-brown stage of senescence and grow down silks to the kernels where it can infect developing kernels (Jones et al., 1980; Marsh and Payne, 1984). Here, germination of conidia occurs first near the pollen grains, and the hyphae spread rapidly across the silk, producing extensive growth and lateral branching. Preharvest invasion of maize kernels was also thought to occur via the tip cap (Marsh and Payne, 1984).

When conidia reach the surface of maize kernels, they germinate under favorable conditions and can produce extracellular enzymes to hydrolyze the insoluble pericarp comprising the outermost barrier of kernels (Kolattukudy, 1980, 1985). Smart et al. (1990) thought that fungal growth occurred in the layer of thin-walled tissue between the outer pericarp and the aleurone/testa layer, which contains steryl esters, however, this probably becomes a factor only when drought or stress conditions result in cracks in the pericarp.

Once *A. flavus* accesses plant cells or is present in plant tissues, it can continue to grow and produce aflatoxins; toxin levels in infected plant tissues can continue to increase long after harvest (Scheidegger and Payne, 2003). As for the trigger for aflatoxin biosynthesis in kernels, research suggests that the process is induced by the degradation products of complex storage components, with fungal α-amylase playing an important role (Brown et al., 2001; Chen et al., 1999b; Woloshuk et al., 1997).
The ubiquity of *A. flavus* in nature and its role as a saprophyte/facultative pathogen suggests that this fungus is biochemically equipped for a lifestyle involving less host specialization (Leger et al., 2000). Therefore, it would be expected that the fungus would have the capacity to produce a large array of extracellular hydrolases to degrade complex substrates in nutrient sources for growth, and also to macerate the structural barriers in the host to invade living tissues. Until the mid-1990s, few studies had been conducted to identify the extracellular proteins. In 1999, Chen et al. (1999a) identified a dominant protein observed in *A. flavus*-infected maize kernels as a 33 kDa fungal alkaline protease. Out of over 1,600 entries of *Aspergillus* protein sequences in the SWISS-PROT/TrEMBL database, only about 70 entries are of secreted proteins, seven of which are from *A. flavus* (Hemming, 1995). However, the recent technology of genomics and proteomics is providing opportunities to identify a number of candidate genes governing the processes involved in plant invasion as well as aflatoxin biosynthesis (Bhatnagar et al., 2008; Cleveland et al., 2004; Medina et al., 2004; Mellon et al., 2007; Yu et al., 2004).

Genomic research indicates that the *A. flavus* genome is 36.3 Mb in size and consists of eight chromosomes and 13,071 predicted genes (Payne et al., 2006; Yu et al., 2005). To date, there are a total of 12,604 gene sequences (http://www.broad.mit.edu/), and 7,218 contigs or singleton EST sequences (http://compbio.dfci.harvard.edu/) provided in public databases. The sequences from genome and EST sequencing projects have revealed the encoding and expressed genes in the genome of this fungus, and the gene information also provides an excellent resource for researchers to gain insight into fungal processes necessary for plant invasion and aflatoxin biosynthesis. This includes cell wall degradation, fungal development, and aflatoxin biosynthesis and regulation. Thus far, however, from the functional annotation of the genes, a large number of genes are still unknown, and a great deal more functional characterization needs to be performed.

By searching the gene index of the *A. flavus* genome, many genes characterized as hydrolases were found, such as proteinases, pectinases, and amylases. Many hydrolases have been presumed to function in polymer degradation and nutrient capture (De Vries and Visser, 2001; Mellon et al., 2007). From the gene survey of the *A. flavus* genome, a wide range of glycosylhydrolase encoding genes were found, especially the enzymes related to plant cell wall degradation in the infection process (Table 1). For instance, the extracellular pectinases, which degrade cellulose and hemicellulose components of plant cell walls, are crucial for fungal invasion and pathogenesis. These enzymes include endoglucanases, exoglucanases, xylanases, and mannanases, which are involved in the degradation of cellulose, hemicellulose, and pectin, respectively. The presence of these enzymes in the *A. flavus* genome highlights the fungus's ability to efficiently infect and degrade plant tissues, thereby gaining access to nutrients necessary for its growth and survival.

### Table 1. Predicted cell wall hydrolases in the *A. flavus* genome sequences.

<table>
<thead>
<tr>
<th>Components</th>
<th>Enzymes</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>Glucanase</td>
<td>endoglucanase (1, 3, EG-1), endo-β-1,3 (4)-glucanase, exo-β-1,3-glucanase, α-1,3-glucanase</td>
</tr>
<tr>
<td></td>
<td>Glucosidase</td>
<td>α-glucosidase (I, II), β-glucosidase (I, III)</td>
</tr>
<tr>
<td>Hemicellulose</td>
<td>Xylanase</td>
<td>endo-1,4-β-xylanase (A, B, C, F1)</td>
</tr>
<tr>
<td></td>
<td>Xylosidase</td>
<td>(α, β)-xylosidase</td>
</tr>
<tr>
<td></td>
<td>Mannosidase</td>
<td>α-1,2-mannosidase (1A), β-mannosidase, endo-1,4-β-mannosidase</td>
</tr>
<tr>
<td></td>
<td>Mannanase</td>
<td>β-mannanase</td>
</tr>
<tr>
<td></td>
<td>Glucuronidase</td>
<td>β-glucuronidase, α-glucuronidase</td>
</tr>
<tr>
<td></td>
<td>Feruloyl esterase</td>
<td>feruloyl esterase (A, B)</td>
</tr>
<tr>
<td></td>
<td>Acetylxyylan esterase</td>
<td>acetyl xylan esterase</td>
</tr>
<tr>
<td>Pectin</td>
<td>Polygalacturonase</td>
<td>endo-polygalacturonase, exo-polygalacturonase, polygalacturonase I</td>
</tr>
<tr>
<td></td>
<td>Rhamnogalacturonase</td>
<td>rhamnogalacturonase A</td>
</tr>
<tr>
<td></td>
<td>Pectin lyase</td>
<td>pectin lyase (A, B, D, F)</td>
</tr>
<tr>
<td></td>
<td>Pectate lyase</td>
<td>pectate lyase (A)</td>
</tr>
<tr>
<td></td>
<td>Pectin methylesterase</td>
<td>pectin methylesterase</td>
</tr>
<tr>
<td></td>
<td>Arabinase</td>
<td>endo-arabinase, endo-1,5-α-L-arabinosidase A</td>
</tr>
<tr>
<td></td>
<td>Galactosidase</td>
<td>β-galactosidase, arabinogalactan endo-1,4-β-galactosidase, α-galactosidase (A, C)</td>
</tr>
<tr>
<td></td>
<td>Rhamnosidase</td>
<td>α-L-rhamnosidase (A), rhamnosidase B</td>
</tr>
<tr>
<td></td>
<td>Methyl esterase</td>
<td>methyl esterase</td>
</tr>
<tr>
<td>Lignin</td>
<td>Arabinofuranosidase</td>
<td>α-N-arabinofuranosidase (A), α-L-arabinofuranosidase (A)</td>
</tr>
<tr>
<td>Cutin</td>
<td>Cutinase</td>
<td>cutinase (1)</td>
</tr>
<tr>
<td>Tannin</td>
<td>Tannase</td>
<td>tannase</td>
</tr>
<tr>
<td>Others</td>
<td>Laccase</td>
<td>laccase</td>
</tr>
</tbody>
</table>
plant cell walls, have been shown to have important roles in *A. flavus* invasion of developing cotton bolls (Brown et al., 1992; Cleveland and Cotty, 1991; Cotty et al., 1991). Although only several hydrolases in *A. flavus* were reported as extracellular enzymes, such as polygalacturonase (Cleveland and Cotty, 1991; Cotty et al., 1991), pectin lyase (Brown et al., 1992), cutinase (Guo et al., 1996), and alkaline protease (Chen et al., 1999a), most of them have been found in other *Aspergillus* strains (De Vries and Visser, 2001; Leger et al., 2000). In addition, gene coding sequences for proteinase, lipase, and amylase are found in the *A. flavus* genome; these enzymes are necessary to break down plant storage proteins, lipids, and starch to acquire nutrition for fungal growth and aflatoxin biosynthesis (Brown et al., 2001; Leger et al., 2000; Mellon et al., 2005; Yu et al., 2003). The wide array of hydrolase genes in the genome may explain the ability of the fungus to degrade a broad range of substances in a saprophytic lifestyle, as well as the ability to infect and adapt to a wide variety of hosts as an opportunistic pathogen.

**Maize defense mechanisms against *A. flavus***

From the investigation of hydrolases, it was determined that *A. flavus* possesses the necessary enzymes to degrade cutin, cellulose, hemicellulose, pectin, etc., the main components in the physical structure of plants. Therefore, it appears that it should not be difficult for the fungus to invade maize kernels. However, plants are also equipped with complex defense systems to defend pathogen invasion. In maize kernels, the pericarp and aleurone layers constitute the first line of defense against pathogens. These layers were thought to play a crucial part in the resistance against *A. flavus* invasion into kernels after gaining entry through the silk or through breaks in the pericarp (Diener et al., 1987). In addition to the physical barrier, biochemical components are considered important parts of the defense system. Some of the defensive proteins can be either constitutively produced or induced as a result of pathogen invasion or wounding. The constitutive resistance from storage components comprises the second defense line, and induced proteins, the third line of defense (Chen et al., 2004b).

Gembeh et al. (2001) compared the pericarp wax components of the *A. flavus*-resistant line GT-MAS:gk with a group of susceptible lines, and found that GT-MAS:gk wax, alone, was bioactive against *A. flavus*, and that a higher percentage of phenol-like compounds was present in GT-MAS:gk pericarp wax. In fact, the amount of a specific phenolic compound, alkylresorcinol, was dramatically higher. When a fungal inhibition assay was conducted, an alkylresorcinol, 5-methylerosorcinol, inhibited the growth of *A. flavus* (Gembeh et al., 2001). Besides the constitutive components in the first defense line, enzyme analysis also indicated that a cationic peroxidase isozyme (PX15) is significantly induced in the pericarp of *A. flavus*-resistant lines compared with susceptible lines (Dowd and Johnson, 2005). The above research suggests that the pericarp is not only a physical barrier, but may also chemically inhibit *A. flavus* growth.

It has been observed that *A. flavus* growth and aflatoxin biosynthesis occur primarily in the embryo and aleurone layer rather than the endosperm during infection (Brown et al., 1993; Keller et al., 1994; Norton and Dowd, 1996; Smart et al., 1990). This raises the question: what factor or chemical signal stimulates fungal growth after breaking down the first line of defense? Since the embryo and aleurone layer are known to store the majority of seed lipids (Watson, 1987), lipids and related metabolic products become candidates. Lipases seem to be important among related enzymes, since detailed histological work has shown that *A. flavus* first destroys the lipid bodies but not the starch granules of the maize scutellum (Smart et al., 1990). Lipid peroxidation is an important feature of plant development and defense responses to a wide range of stresses including fungal invasion (Gardner, 1991, 1995). In plants, lipoxygenase (LOX) catalyzes the incorporation of molecular oxygen into free fatty acids, primarily linoleic (C18:2) and linolenic (C18:3) acids, either at position 9 or 13 of their carbon chains. The 9(S)-hydroperoxide derivative of linoleic acid promotes transcription of aflatoxin genes, whereas the 13(S)-hydroperoxide derivative from the 13S-LOX pathway inhibits aflatoxin biosynthesis (Gao et al., 2007; Wilson et al., 2001). The 9(S)-hydroperoxylinoleic acid has been implicated as an aflatoxin-signaling molecule, and gene *cssap 92* in the pathway clearly responds to *A. flavus* infection with significant expression during the infection of susceptible maize lines (Wilson et al., 2001).

**The genetics of maize host resistance**

Since the mid-1980s, the identification of maize germplasm resources against *A. flavus* infection and aflatoxin contamination has been extensively sought through field trials and laboratory studies (Brown et al., 2003; Munkvold, 2003; Williams, 2006). A number of resistant lines with low aflatoxin contamination were reported, such as the inbred lines: C12, LB31, M182, Mo18W, Mp313E, Mp420, Mp715, Mp717, Oh516, Tex6, Tx601, etc., and the population,
GT-MAS:Gk (Busboom and White, 2004; Campbell and White, 1995; Maupin et al., 2003; McMillian et al., 1993; Scott and Zummo, 1990, 1992; Williams and Windham, 2001, 2006). Although many native resistant lines were identified and developed, none of them have useful agronomic features and, therefore, none are used directly in commercial hybrids (Betran et al., 2002; Munkvold, 2003; Williams and Windham, 2006). Currently, maize hybrids with improved resistance to A. flavus infection and aflatoxin biosynthesis are being used, but the level of resistance is not yet adequate to prevent unacceptable aflatoxin concentrations in some fields (Munkvold, 2003).

Recently, sixbredlines were developed and released to the public as sources of resistance to aflatoxin contamination (Menkir et al., 2008). These lines were developed through a collaborative project between the International Institute of Tropical Agriculture (IITA) and the Southern Regional Research Center (SRRC) of the USDA-ARS (Menkir et al., 2006). Selection was also based on good agronomic characteristics, and these lines have good levels of resistance to southern corn leaf blight and southern corn rust. These features may facilitate their rapid incorporation into breeding programs for development of commercially useful lines with competitive aflatoxin resistance.

In order to analyze the genetic resistance of maize in the A. flavus–host interaction, inheritance of resistance to A. flavus infection and aflatoxin accumulation have been conducted at several locations (Williams et al., 2008). Results indicate that aflatoxin resistance is quantitatively inherited. The additive and dominant genetic effects are important in the inheritance of resistance to aflatoxin accumulation in grain from crosses in diallel analysis, and additive gene effects generally are more important (Busboom and White, 2004; Campbell and White, 1995; Campbell et al., 1997; Hamblin and White, 2000; Zhang et al., 1997). The associated traits with high genotypic correlation to aflatoxin accumulation included endosperm texture, silking date, kernel integrity, husk coverage, and pericarp thickness (Betran et al., 2002; Gardner et al., 2006; Rector et al., 2002; Russin et al., 1997). The environmental effects and genotypes multiplied with environment interactions contribute significantly to the variation of aflatoxin accumulation in maize. Drought, high temperature, and insect damage are among factors linked to highly variable responses of genetic resistance to environmental factors (Williams et al., 2008).

Mapping populations were developed to characterize the genetic factors involved in resistance by analyzing significant quantitative trait loci (QTL) (Brooks et al., 2005; Gardner et al., 2006; Paul et al., 2003; Williams et al., 2008). Results from a Tex6 x B73 population using simple sequence repeat (SSR) and restriction fragment length polymorphism (RFLP) markers suggested that environment strongly influenced the detection of QTLs for lower aflatoxin production. In different years of the investigation, there were very few consistent chromosome regions associated with QTLs. The resistance-associated QTLs were located on chromosomes 3, 4, and 10 in population BC1S1, and chromosome 5 in population F2:3 from the single year results (Paul et al., 2003). The variation of resistance-associated QTLs was observed in the chromosomal regions from different mapping populations of Mp313E x Va35 and Mp313E x B73 using SSR as analysis markers, in which the results indicated that QTLs varied with environments, but the locus af3 on chromosome 2 and af5 on chromosome 4 were identified in different environments and in different years (Brooks et al., 2005).

Identification of RAPs and genes in maize kernels

Generally, the majority of seed storage proteins don’t have known biological activity or function except as nutrition for seed germination. But there are storage proteins associated with defense against the action of pests and pathogens. They are proteinase inhibitors, lectins, lectin-like proteins, amylase inhibitors, ribosome-inactivating proteins, lipid transfer proteins, glucanases, and chitinases (Sales et al., 2000). One-dimensional SDS-PAGE electrophoresis, immunoblot, activity assays, protein N-terminal sequencing, and cDNA cloning were used to identify RAPs in kernels (Table 2). With the development of high-throughput analysis methods, proteomics technology has greatly facilitated the process of protein identification, in which 2-D gel electrophoresis, mass spectrometry (MS), and homology comparison based on protein databases are the main components of this approach (Chen et al., 2002; Domon and Aebersold, 2006; Vercauteren et al., 2007).

In maize kernels, RAPs may comprise important parts of the host defense system against A. flavus growth and aflatoxin biosynthesis after the fungus penetrates the pericarp and aleurone layers. The investigation of RAPs is not only to explore the mechanism of host–pathogen interaction, but also to develop biomarkers that would greatly facilitate the incorporation of resistance into desirable genotypes. Several RAPs have been associated with resistance to A. flavus infection based on comparative protein analysis (Table 2), including storage proteins such as globulin 1, globulin 2, late embryogenesis abundant proteins (LEA3, LEA14), etc.; antifungal proteins such
## Table 2. RAPs reported in resistance against the *A. flavus* infection or aflatoxin production.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Molecular weights</th>
<th>Resistant genotypes</th>
<th>Protein resource</th>
<th>Identification methods</th>
<th>Biological activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>aldose reductase (ALD)</td>
<td>39.2 kDa,</td>
<td>GT-MAS:gi, Mp420, Mp313E, T115</td>
<td>embryo (constitutive or induced)</td>
<td>2-D, ESI-MS/MS, sequence homology</td>
<td>stress tolerance</td>
<td>Chen et al., 2002</td>
</tr>
<tr>
<td>catalase (CAT3)</td>
<td>54.7 kDa,</td>
<td>Mp313E, Mp420,Tx601</td>
<td>activity assay, cDNA sequencing</td>
<td>response to stress, maintain reactive oxygen</td>
<td>Magbanua et al., 2007</td>
<td></td>
</tr>
<tr>
<td>cationic peroxidase (PX5)</td>
<td>38 kDa</td>
<td>Mo18W, Mp313E, Mp420, Sc54, Tx601</td>
<td>pericarp (induced)</td>
<td>activity assay, cDNA sequencing</td>
<td>disease resistance</td>
<td>Dowd and Johnson, 2005</td>
</tr>
<tr>
<td>chitinase 1 (CHT1)</td>
<td>29 kDa</td>
<td>Tex6, Pioneer 3394</td>
<td>kernel (constitutive or induced)</td>
<td>activity assay</td>
<td>degrade polysaccharides of the fungal cell wall</td>
<td>Ji et al., 2000; Moore et al., 2004</td>
</tr>
<tr>
<td>chitinase</td>
<td>28 kDa</td>
<td>Pioneer 3394</td>
<td>kernel (induced)</td>
<td>activity assay</td>
<td>degrade polysaccharides of the fungal cell wall</td>
<td>Ji et al., 2000</td>
</tr>
<tr>
<td>cold-related protein (COR18)</td>
<td>20.1 kDa</td>
<td>GT-MAS:gi, CI2, MI82, Mp420, Mp313E</td>
<td>endosperm (constitutive)</td>
<td>2-D, ESI-MS/MS, sequence homology</td>
<td>stress tolerance</td>
<td>Chen et al., 2007</td>
</tr>
<tr>
<td>globulin 1 (GLB1)</td>
<td>58 kDa, 66.2 kDa</td>
<td>GT-MAS:gi, CI2, Mp420, Mp313E, T115, Tex6</td>
<td>embryo (constitutive)</td>
<td>1-D, 2-D, ESI-MS/MS, sequence homology</td>
<td>antifungal</td>
<td>Chen et al., 2001, 2002</td>
</tr>
<tr>
<td>globulin 2 (GLB2)</td>
<td>24.9 kDa, 25.6 kDa, 46 kDa</td>
<td>GT-MAS:gi, CI2, Mp420, Mp313E, T115, Tex6</td>
<td>embryo, endosperm (constitutive)</td>
<td>1-D, 2-D, ESI-MS/MS, sequence homology</td>
<td>antifungal</td>
<td>Chen et al., 2001, 2002, 2007</td>
</tr>
<tr>
<td>β-1,3-glucanase</td>
<td>33 kDa, 35 kDa</td>
<td>Pioneer 3394, Tex6</td>
<td>kernel (constitutive or induced)</td>
<td>activity assay</td>
<td>degrade polysaccharides of the fungal cell wall</td>
<td>Ji et al., 2000; Lozovaya et al., 1998</td>
</tr>
<tr>
<td>glyoxalase (GLX-I)</td>
<td>32 kDa</td>
<td>GT-MAS:gi, Mp420, T115</td>
<td>embryo (constitutive or induced)</td>
<td>2-D, ESI-MS/MS, sequence homology</td>
<td>stress tolerance</td>
<td>Chen et al., 2007</td>
</tr>
<tr>
<td>heat-shock protein (HSP16.9)</td>
<td>16.9 kDa</td>
<td>GT-MAS:gi, Mp420, Mp313E, T115</td>
<td>embryo (constitutive)</td>
<td>2-D, ESI-MS/MS, sequence homology</td>
<td>stress tolerance</td>
<td>Chen et al., 2002</td>
</tr>
<tr>
<td>heat-shock protein (HSP17.2)</td>
<td>17.2 kDa</td>
<td>GT-MAS:gi, CI2, MI82, Mp420, Mp313E</td>
<td>endosperm, embryo (constitutive)</td>
<td>2-D, ESI-MS/MS, sequence homology</td>
<td>stress tolerance</td>
<td>Chen et al., 2002, 2007</td>
</tr>
<tr>
<td>late embryogenesis abundant protein (LEA3)</td>
<td>24.7 kDa</td>
<td>GT-MAS:gi, CI2, MI82, Mp420, Mp313E</td>
<td>endosperm, embryo (constitutive)</td>
<td>2-D, ESI-MS/MS, sequence homology</td>
<td>stress tolerance</td>
<td>Chen et al., 2002, 2007</td>
</tr>
<tr>
<td>late embryogenesis abundant protein (LEA14)</td>
<td>14.2 kDa</td>
<td>GT-MAS:gi, CI2, MI82, Mp420, Mp313E</td>
<td>endosperm (constitutive)</td>
<td>2-D, ESI-MS/MS, sequence homology</td>
<td>stress tolerance</td>
<td>Chen et al., 2007</td>
</tr>
<tr>
<td>peroxiredoxin antioxidant (PER1)</td>
<td>24.9 kDa</td>
<td>GT-MAS:gi, CI2, MI82, Mp420, Mp313E</td>
<td>endosperm (constitutive)</td>
<td>2-D, ESI-MS/MS, sequence homology</td>
<td>response to stress, maintain reactive oxygen</td>
<td>Chen et al., 2007</td>
</tr>
<tr>
<td>ribosome-inactivating proteins (RIP)</td>
<td>9 kDa, 18 kDa, 32 kDa</td>
<td>GT-MAS:gi, MP420, opaque-2, W64A,</td>
<td>kernel, endosperm (constitutive)</td>
<td>2-D, ESI-MS/MS, sequence homology</td>
<td>block protein synthesis by modifying the large ribosomal RNA</td>
<td>Bass et al., 1992; Nielsen et al., 2001</td>
</tr>
<tr>
<td>trypsin inhibitor (TI)</td>
<td>14 kDa</td>
<td>GT-MAS:gi, CI2, MI82, Yellow Creole</td>
<td>kernel, endosperm (constitutive)</td>
<td>activity assay, 2-D, ESI-MS/MS, sequence homology</td>
<td>Inhibits fungal trypsin, amylase</td>
<td>Chen et al., 1998, 2007</td>
</tr>
<tr>
<td>water-stress inducible protein (WSI18)</td>
<td>24.9 kDa</td>
<td>GT-MAS:gi, Mp420, Mp313E, T115</td>
<td>embryo (constitutive)</td>
<td>2-D, ESI-MS/MS, sequence homology</td>
<td>stress tolerance</td>
<td>Chen et al., 2002</td>
</tr>
<tr>
<td>zeamatin</td>
<td>22 kDa</td>
<td>GT-MAS:gi, CI2, Mp420, T115, Tex6</td>
<td>kernel (constitutive)</td>
<td>activity assay, 1-D, protein N-terminal sequencing</td>
<td>Changes fungal membrane permeability</td>
<td>Chen et al., 2001; Roberts and Selitrennikoff, 1990</td>
</tr>
<tr>
<td>pathogenesis-related (ZmPR-10)</td>
<td>16.9 kDa</td>
<td>Mp420, Mp313E, GT-MAS:gi</td>
<td>endosperm (constitutive or induced)</td>
<td>2-D, ESI-MS/MS, sequence homology, cDNA sequencing</td>
<td>RNase activity, antifungal</td>
<td>Chen et al., 2006</td>
</tr>
</tbody>
</table>
as trypsin inhibitor, ribosomal inactivating proteins, pathogenesis-related proteins (β,1,3-glucanase, chitinase, cationic peroxidase, PR10), and stress-related proteins such as aldose reductase, cold regulated protein, water-stress inducible protein, glyoxalase I, heat shock proteins (HSP14.9, HSP17.2), etc. For the majority of proteins, their molecular weights are between 14-35 kDa. The RAPs were extracted from embryo, endosperm, or whole seed, and very few were reported from the aleurone layer in the maize–A. flavus interaction. However, several antifungal proteins were reported in specific areas of the kernel in other studies, such as BAP2 of BAPs located in the placentochalazal cells of the pedicel, adjacent to the basal endosperm transfer layer (BETL) cells (Serna et al., 2001). The mature BAP2 peptide exhibits potent broad-range activity against a range of filamentous fungi, including several plant pathogens (Serna et al., 2001).

Luo et al. (2008) used a maize microarray to investigate the constitutive RAP gene profiles in kernels of resistant line Tex6 during the developmental phases from 25 days after pollination (DAP) to 45 DAP. A total of 8,497 spots with unique gene IDs were detected, in which 4,247 genes expressed in all stages. Of the total expressed genes, 823 were classified as RAP-related, including pathogenesis-related protein genes, phenylalanine ammonia lyase pathway genes, etc.; stress response genes such as lipoxygenase, low molecular weight heat shock proteins, dehydrin, lipid transfer protein, absciscic acid-inducible gene, antifreeze proteins, etc.; and reactive oxygen species scavengers such as ascorbate peroxidase, glutathione S-transferases, superoxide dismutase (Cu-Zn), etc. Compared with storage protein genes, whose expression decreased as kernels matured, over 83% of RAP genes were expressed as up-regulated or unchanged with high expression levels even after 40 DAP. The advantage of microarray technology is the ability for high efficiency in the selection of RAP candidate genes, and in the identification of gene family members with different RAP isoforms. In the comparison of resistant line Tex6 with susceptible line B73 using qRT-PCR, it was found that most of RAP genes between these two lines differed in quantitative expression. This is similar to results obtained in proteomics studies, where differences in the production of certain proteins between resistant and susceptible lines were determined to be quantitative, such as with aldose reductase (Chen et al., 2002), peroxidase (Dowd and Johnson, 2005), glyoxalase I (Chen et al., 2004a), and peroxidredoxin antioxidant (PER1) (Chen et al., 2007). Qualitative differences between specific isoforms have been observed with peroxidase (Dowd and Johnson, 2005), catalase (Magbanua et al., 2007), chitinase, and β,1,3-glucanase (Ji et al., 2000).

As evidence of induced resistance existing in the A. flavus–maize interaction, several RAPs were found induced in pre- or post-harvest kernels inoculated by A. flavus, such as aldose reductase, anionic peroxidase, catalase, chitinase, β,1,3-glucanase etc. (Table 2). However, little is known about this type of resistance in plant seeds, nor about the basic knowledge of systemic acquired resistance (SAR), which has been investigated in plant vegetative parts (Durrant and Dong, 2004). Microarray was used to compare gene expression profiles in 16 DAP kernels of resistant inbred line Mp313E with those of susceptible inbred line Va35 (Kelley et al., 2006; Wilkinson et al., 2007), in which the ears were inoculated with A. flavus in the field and collected at 48 hours after inoculation. A clear expression profile of induced genes was identified. A total of 123 of these genes were up-regulated in the susceptible line Va35 and 95 in the resistant line Mp313E. The genes in Va35 showed increased response in electron transport, signal transduction, generation of precursor metabolites, response to abiotic and endogenous stimulus, protein metabolism, photosynthesis, carbohydrate metabolism, etc. However, the genes in Mp313E showed marked increases only in amino acid and derivative metabolism, and lipid metabolism.

In the A. flavus–maize interaction, research on resistance mechanisms has been focused on the identification of resistant proteins and genes. However, little has been reported on small molecular components in the interaction, which are found to be involved in antifungal resistance in other crop seeds (Jwa et al., 2006; Wink, 2003). With the development of metabolomics, techniques such as LC-MS and HPLC have been widely used in the identification of small molecular components, such as plant secondary metabolites (Fernie, 2007; Oldiges et al., 2007). Applying these techniques could facilitate the discovery of small molecular components with resistance in the A. flavus–maize interaction, and enhance our understanding of host resistance mechanisms.

**Effect of abiotic factors on host–A. flavus interaction**

Environmental conditions, including high soil or air temperature, drought stress, nitrogen stress, plant density, and conditions that aid dispersal of conidia during silking play important roles in the host–A. flavus interaction (Council for Agricultural Science and Technology, 1989; Diener et al., 1987). A. flavus had been isolated at all latitudes, but has been found at higher frequencies in desert climates and at latitudes ranging from 16 to 35 degrees. This range of latitudes
Host genes involved in the interaction between Aspergillus flavus and maize

includes tropical, subtropical, and warm temperate climates. Unlike most fungi, A. flavus is favored by hot and dry conditions. Its optimal range for growth is at 28°C to 37°C, and humidity > 85%. But the fungus can grow in a wide range of temperatures from 12°C to 48°C and at low water potentials (~35 MPa). The ability of A. flavus to survive in harsh conditions allows it to out-compete other organisms for substrates in the soil or in the plant (Bhatnagar et al., 2000; Diener et al., 1987; Jones et al., 1980; Payne and Brown, 1998).

The infection by A. flavus and subsequent production of aflatoxins in maize kernels before harvest varies year to year depending upon the environmental conditions. Cultural practices that tend to expose plants to high temperature and greater drought stress will lead to higher levels of aflatoxins (Munkvold, 2003; Payne and Brown, 1998; Scheidegger and Payne, 2003). Maize breeding for low levels of aflatoxin production can also be severely hindered by environmental effects on the phenotype (Paul et al., 2003). Jones et al. (1980) found that irrigating corn fields to reduce drought stress also reduced fungal infection and aflatoxin contamination. Irrigation not only relieved drought stress, but also reduced soil temperature. The reason A. flavus can infect maize kernels under drought stress and high temperatures in natur, might be that stress compromises physiological defense systems and lead to cracks in the seed (Bhatnagar et al., 2000; Marsh and Payne, 1984; Payne and Brown, 1998; Smart et al., 1990).

Although the significant effects of environmental factors have been well documented, little is known about direct impacts of environmental factors on gene expression in the maize kernels during the host–A. flavus interaction. Investigations of abiotic stress indicate that stress can influence the expression of numerous genes in plants, such as dehydrins, oxidant protectants, heat shock proteins (HSPs), compatible solute synthetic pathways, and senescence-related proteins (Mittler, 2006; Zhu, 2002). In maize, investigations of the effect of drought stress on kernel development have mainly focused on the early stage after pollination. The results suggest that the early phase is particularly sensitive to water deficit, whereas later phases of kernel development, when starch and zein synthesis are at their maximum, are usually less affected (Grant et al., 1989; Mambelli and Setter, 1998; Setter et al., 2001). Yu and Setter (2003) used a cDNA microarray to compare maize gene profiles in the endosperm (9 DAP) under drought stress, and found that 82% of the affected genes were down-regulated, including cell wall degradation gene β-1,3-glucanase, β-D-glucan exohydrolase, β-galactosidase, and endoxylanase. Luo et al. (2006) used a similar approach to compare the gene expression profiles in the drought-stressed kernels at 35 DAP, and found 341 genes were down-regulated. Of these, 153 have known functions, including RAPs, such as chitinase, trypsin inhibitor, pathogenesis related protein (PR1, PR10), glutathione S-transferase, phenylalanine ammonia-lyase, etc. The down-regulation of RAP genes and metabolic genes during drought stress may indicate a mechanism for increased host susceptibility to aflatoxin contamination.

Conclusion

In recent years, identification of RAPs in the A. flavus–host interaction has made great progress using high-throughput technology. The identified RAPs came primarily from postharvest kernels through comparing protein profiles of resistant with susceptible lines. Results clearly indicate that constitutive resistance is involved in kernel defense against A. flavus infection. While research on gene expression profiles in preharvest kernels in response to A. flavus infection has been initiated, much needs to be done to clarify induced resistance. Unlike postharvest kernels, in which the protein profile is the final accumulation of the gene products with less variation, the gene profile in preharvest kernels, will be dynamically affected by many factors, such as kernel developmental stage, environmental stress, fungal inoculation method, sampling date, etc. A reliable and standardized experimental system for preharvest maize kernels is still needed.

Thus far, many RAPs in kernels have been identified in the A. flavus–maize interaction. From QTL investigations, it was also established that maize resistance to A. flavus infection and aflatoxin contamination is controlled by multiple genes. The challenge is to identify the key RAPs contributing to resistance, and develop them as biomarkers for breeding.

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References


Host genes involved in the interaction between Aspergillus flavus and maize


