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Proteomics to identify resistance factors in corn - a review

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Abstract

The host resistance strategy for eliminating aflatoxins from corn has been advanced by the discovery of natural resistance traits such as proteins. This progress was aided by the development of a rapid laboratory-based kernel screening assay (KSA) used to separate resistant from susceptible seed, and for investigating kernel resistance. *A. flavus* GUS transformants have also been used, in conjunction with the KSA, to assess the amount of fungal growth in kernels and compare it with aflatoxin accumulation. Several proteins associated with resistance (RAPs) have been identified using 1 D PAGE. However, proteomics is now being used to further the discovery of RAPs. This methodology has led to the identification of stress-related RAPs as well as other antifungals. Characterization studies being conducted, including RNAi gene silencing experiments, may confirm roles for RAPs in host resistance.

Keywords: biochemical markers, maize resistance, mycotoxigenic fungi, review

Introduction

A number of approaches to eliminating aflatoxins from susceptible crops such as corn, cottonseed, peanut, and treenuts have been proposed, however, the most widely explored strategy is the development of preharvest host resistance. This is because *Aspergillus flavus* infects affected crops prior to harvest (1). In corn production, the host resistance strategy has also gained prominence because of advances in the discovery of natural resistance traits such as proteins (2). The primary objective of this review is to highlight these discoveries and their contributions to the development of host resistance against aflatoxigenic fungi.

Kernel screening assay

A laboratory kernel screening assay (KSA) was developed and used to study resistance to aflatoxin production in kernels of resistant population, GT-MAS:gk (3). This rapid assay employs a very simple and inexpensive procedure (4). Kernels screened by the KSA are usually incubated for seven days in 100% humidity and at 31 °C, a temperature favorable to both the growth of *A. flavus* and aflatoxin production. Aflatoxin data from KSA experiments can be obtained two to three weeks after experiments are initiated. KSA experiments confirmed GT-MAS:gk resistance to aflatoxin production and demonstrated that the resistance in otherwise viable kernels is maintained, when the pericarp barrier is breached (3). The results of this study indicated the presence of two levels of resistance: at the pericarp and at the subpericarp level. The former was supported by KSA studies which demonstrated a role for pericarp waxes in kernel resistance (5, 6, 7) and highlighted quantitative and qualitative differences in pericarp wax between GT-MAS:gk and susceptible genotypes (8). The KSA also confirmed sources of resistance among 31 inbreds tested in Illinois field trials (4, 9).

The KSA has several advantages as compared to traditional breeding techniques (4): 1) it can be performed and repeated several times throughout the year and outside of the growing season; 2) it requires few kernels; 3) it can

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detect/identify different kernel resistance mechanisms expressed; 4) it can dispute or confirm field evaluations (*e.g.* identify escapes); and, 5) relationships between laboratory findings and inoculations in the field have been demonstrated. The KSA can, therefore, be a valuable complement to standard breeding practices for preliminary evaluation of germplasm. Field trials, however, are irreplaceable for confirmation of resistance.

A. flavus *GUS transformants*

Three resistant inbreds (MI82, CI2, and T115) among the 31 tested in Illinois field trials, were examined by a modified KSA, which included an *A. flavus* strain genetically engineered with a gene construct consisting of a β-glucuronidase (GUS) reporter gene linked to an *A. flavus* β-tubulin gene promoter for monitoring fungal growth. Results demonstrated, both visually and quantitatively, kernel resistance to fungal infection in nonwounded and wounded kernels, and a statistically significant positive relationship between the degree of fungal infection and aflatoxin levels was established (4, 10). Thus, it is now possible to accurately assess fungal infection levels and to predict the corresponding aflatoxin levels, as a result of fungal infection, in the same kernels. *A. flavus* GUS transformants with the reporter gene linked to an aflatoxin biosynthetic pathway gene may also provide a quick and economical way to indirectly measure aflatoxin levels (11, 12, 13), based on the extent of the expression of the pathway gene.

The KSA was also used to screen corn inbred lines for aflatoxin resistance that had been selected for ear rot resistance in West and Central Africa (14). The large number of promising lines observed in these experiments provides the basis for a current collaborative effort between the International Institute of Tropical Agriculture (IITA) and USDA-ARS, New Orleans. The best African lines (as determined by the KSA) were crossed with U.S. aflatoxin-resistant lines at IITA, while markers linked to resistance to aflatoxin production in progeny derived from these crosses are being identified and characterized in the USDA-ARS laboratory (15). The goal is to develop aflatoxin-resistant, agronomically-superior germplasm for use in both the U.S. and West Africa.

Resistance-associated proteins (RAPs) identified in corn

Developing resistance to fungal infection in wounded as well as intact kernels would go a long way toward solving the aflatoxin problem (16). Studies demonstrating subpericarp (wounded-kernel) resistance in corn kernels have led to research to identify subpericarp resistance mechanisms. Previous observations (17) suggest that even susceptible kernels contain proteins capable of being induced during imbibition that can inhibit growth and/or fungal elaboration of aflatoxins. A recent investigation into corn kernel resistance (18), however, determined that both constitutive and induced proteins are required for resistance to aflatoxin production. In fact, one major difference demonstrated between resistant and susceptible genotypes is the relatively high level of constitutively expressed antifungal proteins in the former compared to the latter. A function of these high levels of constitutive proteins may be to delay fungal invasion, and subsequent aflatoxin formation, until infection-induced antifungal proteins can be synthesized. Examinations of kernel proteins of several corn genotypes revealed differences between resistant and susceptible lines (19). Susceptible kernels, imbibed prior to fungal inoculation and showing reduced aflatoxin levels, contained germination-induced ribosome inactivating protein (RIP) and zeamatin (20). Both zeamatin and RIP have been demonstrated to exhibit *in vitro* inhibition of *A. flavus* growth (20). These studies implicate corn proteins as potential targets for imparting kernel resistance to *A. flavus* infection and aflatoxin production.

In another study, two kernel proteins were identified from resistant corn inbred Tex6, which may contribute to resistance to aflatoxin production (21). One of the proteins, 28 kDa in size, inhibited *A. flavus* growth, while a second larger protein inhibited toxin formation with little effect on fungal growth. Recently, the antifungal protein was identified as an endo-chitinase and was shown to be a major contributor to Tex6 resistance (22). In a study where a commercial corn hybrid was inoculated with aflatoxin and nonaflatoxin-producing strains of *A. flavus* at milk stage, one induced chitinase and one ß-1,3-glucanase isoform was detected in maturing infected kernels, while another isoform was detected in maturing uninfected kernels (23).

An examination of kernel protein profiles of 13 corn genotypes revealed that a 14 kDa trypsin inhibitor protein (TI) is present at relatively high concentrations in seven resistant corn lines, but is present only in low concentrations in six susceptible ones (24). The mode of action of TI against fungal growth may be partially due to its inhibition of fungal α-amylase, limiting *A. flavus* access to potential simple sugars (25) required for toxin production (26). TI was shown to inhibit conidial germination and hyphal growth of eight other phytopathogenic fungi including mycotoxin producing taxa, *A. parasiticus, F. graminearum,* and *F. moniliforme* (27). Using a subtractive approach emanating from comparisons of susceptible with resistant kernel protein profiles may shorten the time it takes to identify RAPs.

Enhancement of RAP identification through proteomics

Two-dimensional (2-D) gel electrophoresis, which sorts proteins according to two independent properties, isoelectric points and then molecular weights, has been recognized for a number of years as a powerful biochemical separation technique. Improvements in map resolution and reproducibility (28, 29), rapid analysis of proteins, analytical software and computers, and the acquisition of genomic data for a number of organisms has given rise to another application of 2-D electrophoresis: proteome analysis. Proteome analysis or "proteomics" is the analysis of the protein complement of a genome (30, 31). This involves the systematic separation, identification, and quantification of many proteins simultaneously. 2-D electrophoresis is also unique in its ability to detect post- and cotranslational modifications, which cannot be predicted from the genome sequence.

Through proteome analysis and the above-mentioned subtractive approach, it may be possible to identify important RAPs, as well as genes encoding these proteins. This could facilitate marker-assisted breeding and/or genetic engineering efforts. Endosperm and embryo proteins from several resistant and susceptible genotypes have been compared using large format 2-D gel electrophoresis, and over a dozen such protein spots, either unique or 5-fold upregulated in resistant lines (Mp420 and Mp313E), have been identified, isolated from preparative 2-D gels and analyzed using ESI-MS/MS after in-gel digestion with trypsin (32, 33). These proteins, all constitutively expressed, can be grouped into three categories based on their peptide sequence homology: 1) storage proteins, such as globulins and late embryogenesis abundant proteins; 2) stress-responsive proteins, such as aldose reductase, a glyoxalase I protein and a 16.9 kDa heat shock protein, and 3) antifungal proteins, including the above-described TI. Another antifungal RAP, a pathogenesis-related protein, PR 10, was identified through proteome analysis, and characterized as possessing both antifungal and RNase activities against *A. flavus* (34).

During the screening of progeny developed through the IITA–USDA/ARS collaborative project, near-isogenic lines from the same backcross differing significantly in aflatoxin accumulation were identified, and proteome analysis of these lines has been conducted (15). Investigating corn lines from the same cross with contrasting reaction to *A. flavus* should enhance the identification of RAPs clearly without the confounding effect of differences in the genetic backgrounds of the lines. Results of this investigation demonstrate constitutive expression of the same three above-mentioned categories of proteins (Z. Chen *et al.*, submitted).

Heretofore, most RAPs identified have had antifungal activities. However, increased temperatures and drought, which often occur together, are major factors associated with aflatoxin contamination of corn kernels (35). It has also been found that drought stress imposed during grain filling reduces dry matter accumulation in kernels (35). This often leads to cracks in the seed and provides an easy entry site to fungi and insects. Possession of unique or of higher levels of hydrophilic storage or stress-related proteins, such as the aforementioned, might put resistant lines in an advantageous position over susceptible genotypes in the ability to synthesize proteins and defend against pathogens while under stress conditions. Therefore, the necessary requirements for developing commercially-useful, aflatoxin-resistant corn lines may include, aside from antifungal proteins, a high level of expression of stress-related proteins.

The direct involvement of a purported stress-related protein, glyoxalase I (36), in resistance to aflatoxin accumulation was recently investigated. The substrate for glyoxalase I, methylglyoxal, is a potent cytotoxic compound

produced spontaneously in all organisms under physiological conditions from glycolysis and photosynthesis intermediates, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Methylglyoxal is an aflatoxin inducer even at low concentrations; experimental evidence indicates that induction may be through upregulation of aflatoxin biosynthetic pathway transcripts including the AFLR regulatory gene. Therefore, glyoxalase I may be directly affecting resistance by removing its aflatoxin-inducing substrate, methylglyoxal.

Further studies including physiological and biochemical characterization, genetic mapping, plant transformation using RAP genes, and marker-assisted breeding should clarify the roles of stress-related RAPs in kernel resistance. RNAi gene silencing experiments involving RAPs, currently being conducted, may also contribute valuable information (15). Favorable outcomes of these investigations may yield commercially-useful, aflatoxin-resistant corn lines. Since certain RAPs, that may exhibit enhanced expression in the new resistant lines, have been recognized as potential allergens (*e.g.* glyoxalase; PR 10), further investigation may be needed to determine the risk of higher allergenicity of these lines.

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