Comparison of Constitutive and Inducible Maize Kernel Proteins of Genotypes Resistant or Susceptible to Aflatoxin Production

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ABSTRACT

Maize genotypes resistant or susceptible to aflatoxin production or contamination were compared for differences in both constitutive and inducible proteins. Five additional constitutive proteins were found to be associated with resistance in over 8 of the 10 genotypes examined. Among these, the 58- and 46-kDa proteins were identified as globulin-1 and globulin-2, respectively. Differences in the ability to induce specific antifungal proteins, such as the higher synthesis of the 22-kDa zeamatin in resistant genotypes, were also observed between resistant and susceptible kernels incubated under germinating conditions (31°C, 100% humidity). Both constitutive and inducible proteins appear to be necessary for kernel resistance. Embryo-killed kernels (unable to synthesize new proteins) supported the highest level of aflatoxins, whereas imbibed kernels (to hasten protein induction) supported the lowest among all treatments. This suggests that the synthesis of new proteins by the embryo plays an important role in conferring resistance. However, significantly lower levels of aflatoxin production in embryo-killed resistant kernels than in susceptible ones suggest that, in reality, high levels of constitutive antifungal proteins are indispensable to kernel resistance.

Plants do not contain an immune system and must rely on other mechanisms to protect themselves from fungal infection (26). These include both passive and active defense mechanisms (25). The passive or preexisting defense mechanisms involve structural barriers, such as waxy cuticle, or strategically positioned reservoirs of antimicrobial compounds that function to prevent colonization of plant tissue (27, 37). In addition, plants have inducible defense mechanisms such as newly synthesized proteins (pathogenesis-related proteins) to prevent further colonization of plant tissue once the structural barriers of the host have been breached (2, 15, 32, 34, 39, 47). These induced defenses are described as active defense mechanisms because they are a response to an invading pathogen and require an active host metabolism to function (25, 28).

Infection of maize (Zea mays L.) by Aspergillus flavus, and the subsequent production of the toxic, highly carcinogenic secondary metabolites called aflatoxins (44), is a recurrent problem (19). Aflatoxin contamination not only reduces the value of grain as an animal feed and as an export commodity (35), but it also poses health hazards to humans (23, 38) and domestic animals (35, 43). In the past decade, studies have identified maize genotypes as resistant to aflatoxin production (4, 5, 7, 42, 46). Recent investigations have indicated that mature maize kernel resistance traits against aflatoxin contamination may include physical barriers (cutin and layers) on the kernel surface (21, 41) and kernel proteins (6, 11, 24), as well as metabolic activities of living embryos (6). Genetic studies also have identified multiple chromosome regions associated with resistance to A. flavus infection or inhibition of aflatoxin production by restriction fragment length polymorphism analysis (8, 18), suggesting that the resistant trait is quantitatively inherited.

A comparison of constitutively expressed kernel proteins from 13 resistant and susceptible genotypes identified a 14-kDa trypsin inhibitor protein that is associated with resistance (11). This protein was shown to be bioactive against A. flavus as well as eight other fungal pathogens (10). The trypsin inhibitor protein also was shown to inhibit A. flavus growth through inhibition of fungal α-amylase (13). A study (24) testing the inhibitory activity of kernel extracts from resistant genotype Tex6 and susceptible genotype B73 found two proteinaceous fractions inhibitory to aflatoxin formation from Tex6. The above studies indicate that certain constitutive kernel proteins may serve as resistance factors against fungal infection. Further investigation may, however, identify additional constitutive kernel proteins involved in resistance.

Cordero et al. (16) and Wu et al. (47) found that in addition to the expression during some normal developmental stages, the expression of β-1,3-glucanases and chitinases, the most studied pathogenesis-related proteins (2, 15, 26, 32, 34, 39), increased upon A. flavus infection of maize kernels (32, 47). Also, both β-1,3-glucanases and chitinases have demonstrated in vitro antifungal activities (2, 26, 33). When increased resistance to aflatoxin production was observed in susceptible kernels allowed to imbibe

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moisture under germinating conditions before inoculation with A. flavus, it was speculated that the synthesis of new proteins during imbibition might be responsible for this phenomenon (20, 22). However, the synthesis of new kernel proteins other than chitinases and β-1,3-glucanases has not been closely studied.

While constitutive differences in protein expression may account for part of the ability of resistant kernels to accumulate less aflatoxin than susceptible ones, induced expression of other proteins also may play a role. To examine this hypothesis, the present study sought to (i) identify additional constitutive proteins whose expression may associate with resistance; and (ii) identify changes in protein synthesis in imbibed kernels under germinating conditions with or without A. flavus inoculation. Representative resistant and susceptible genotypes were used in this study for comparison of differences in constitutive and inducible proteins. The contributions of constitutive and induced proteins to kernel resistance were also evaluated, and the relative significance of each is discussed. A preliminary report has been made (12).

MATERIALS AND METHODS

Materials. Kernels of commercial maize hybrids Pioneer 3154 (P3154), Deltapine G4666 (G4666), ORO200, and Dekalb 689 (DK689) were obtained from their respective companies; their susceptibility to A. flavus infection has been established (22). Kernels of resistant breeding population GT-MAS:gk were obtained from the U.S. Department of Agriculture (USDA)-Agricultural Research Service, Insect Biology and Population Management Research Laboratory, Tifton, Ga. Kernels of resistant inbreds T115, C12, and Tex6, as well as susceptible inbred B73 (6, 7), were obtained from the Department of Plant Pathology, University of Illinois, Urbana. Kernels of resistant inbred MP420 (42) were obtained from the USDA-Agricultural Research Service, Corn Host Plant Resistance Research Unit, Mississippi State, Miss. A. flavus (SRRC strain AF13) was used in all inoculations. This strain produces large quantities of α/bullet6 atoxins in culture, developing cotton-seed, and maize kernels (17). The fungus was grown on V8 juice agar plates (5% V8 juice, wt/vol, pH 5.2; 2% agar, wt/vol) at 30°C. Conidia from 7-day-old cultures suspended in deionized water were used as inocula. The rabbit polyclonal antiserum to maize ribosomal-inactivating protein (RIP) was supplied by Dr. R. S. Boston (North Carolina State University, Raleigh); the antibody against maize zeamatin was provided by Dr. C. P. Selitrennikoff (University of Colorado, Denver), and the antibody against bean chitinase was provided by Dr. J. W. Cary (SRRC, USDA-Agricultural Research Service, New Orleans, La.).

Expression and identification of constitutive kernel proteins. Five resistant (GT-MAS:gk, C12, MP420, T115, and Tex6) and five susceptible (B73, G4666, P3154, DK689, and ORO200) maize genotypes (5, 22) were used in this study. To increase protein resolution on sodium dodecyl sulfate (SDS)-polyacrylamide gels, embryo and endosperm were hand separated using a scalpel after soaking the kernels in water overnight at 0°C. These conditions were employed to facilitate the separation without protein induction (confirmed using Western blot analysis). Embryo proteins were extracted with the extraction buffer (0.25 M NaCl, 50 mM Tris-HCl pH 8.0, 14 mM β-mercaptoethanol) (4 ml/g) for 10 min on ice with a mortar and pestle. Endosperm proteins were extracted using the same buffer in a flask with continuous shaking at 4°C for 1 h after endosperm tissues were first frozen in liquid nitrogen and ground in an analytical mill (Tekmar A-10, Janke and Kunkel GmbH & Co., Staufen, Germany). Supernatants containing proteins were recovered by centrifugation (17,000 × g, 20 min) and stored at −70°C in 1.5-ml aliquots until further use. Protein concentrations were assayed according to Bradford (3). This experiment was conducted three times.

Protein samples were resolved using SDS-polyacrylamide gel electrophoresis (PAGE) according to Laemmli (31). Five micrograms of protein from each sample was loaded per lane onto gels. The gels were electrophoresed, stained, and destained as described (11). The relative content of particular proteins was quantified using Bio-Rad’s GS-700 Gel Densitometer with the associated “Molecular Analyst” software.

Expression of induced proteins in noninoculated kernels over time. To determine the changes in protein synthesis that occur during the germination process, proteins of kernels kept under germinating conditions during a 7-day period were examined. Kernels of representative resistant (GT-MAS:gk, C12) and susceptible (B73, G4666) maize genotypes (50 g per genotype) were surface sterilized with 70% ethanol and incubated at 31°C under 100% humidity for 7 days (standard Kernel Screening Assay conditions) (5, 6). Ten grams of kernels from each genotype was taken out of the incubator at 0, 1, 3, 5, and 7 days and extracted with an extraction buffer (0.25 M NaCl, 50 mM Tris-HCl pH 8.0, 14 mM β-mercaptoethanol) as previously described (11). The protein samples were stored at −70°C until further use.

For Western blot analysis, 2.5 µg of protein was loaded per lane, and gels were blotted immediately after electrophoresis as described earlier (14). The antiserum used in Western blot analysis were against bean chitinase (1:1,000 dilution), maize 14-kDa trypsin inhibitor (1:2,000 dilution), maize inactive RIP (1:1,000 dilution), and maize 22-kDa zeamatin protein (1:1,500 dilution). This experiment was conducted twice.

Expression of germination- and infection-induced proteins in kernels and determination of the relative contribution of constitutive and inducible proteins to resistance. Surface-sterilized GT-MAS:gk, CI2, B73, and G4666 kernels (60 g per genotype) were first soaked at 0°C for 3 days to imbibe water with minimum protein synthesis. One-third of these kernels were then inoculated with A. flavus, and one-third were subjected to two rounds of freeze-thaw treatment (1 h at −70°C, 45 min at 25°C; to kill the embryo and abolish kernel ability to synthesize new proteins) prior to inoculation with A. flavus. The remaining one third were left uninoculated to serve as a control. Kernels were subjected to a freeze-thaw treatment to assess whether the level of aflatoxins accumulated were associated with the presence of high levels of constitutive antifungal proteins, antifungal proteins induced after fungal infection, or a complementary effect of both. After 7 days of incubation at 31°C and under 100% humidity, half of the number of kernels from each of the above treatments were used for protein analysis (only proteins from embryo tissue were examined, since it is where most proteins are made). The other half were assayed for aflatoxin accumulation (6). Surface-sterilized and inoculated dry kernels also served as controls. This experiment was conducted twice.

Partial characterization of putative marker proteins. Five (22-, 34-, 46-, 58-, and 72-kDa proteins) of the putative resistance-associated proteins identified from the above studies were selected for N-terminal amino acid sequence analysis. The 7-day GT-MAS: gk total protein extract, dry GT-MAS:gk embryo extract, CI2 endosperm extract, and B73 endosperm extract were used to obtain
amino acid sequences of the 22-, 58- and 46-, 34-, and 72-kDa proteins, respectively. The protein extracts (200 μg) were first resolved using SDS-PAGE. Thioglycolic acid was added to the upper buffer to 0.1% (vol/vol) to prevent chemical blockage of the N-terminus. The proteins were then transferred to a polyvinylidene difluoride membrane, stained, and destained as described by Chen et al. (14). Fifty picomoles of each protein were used for N-terminal amino acid microsequencing at Baylor Medical School (Houston, Tex.).

Statistical analysis. All relative content of specific proteins and aflatoxin data were analyzed using the analysis of variance procedure of the Statistical Analysis System (SAS Institute, Cary, N.C.). Means were separated by least significant difference (P ≤ 0.05). Aflatoxin data were log transformed prior to analyses to equalize variances.

RESULTS

Expression and identification of constitutive kernel proteins. Differences in the level of constitutive embryo and endosperm proteins extracted with a pH 8.0 buffer were compared between five resistant and five susceptible genotypes. A representative SDS-PAGE is shown in Figure 1. Resistant genotypes generally contained high levels of a 46-kDa embryo protein and low levels of a 72-kDa endosperm protein (Fig. 1 and Table 1). A 58-kDa embryo protein and a 34-kDa endosperm protein were also found at high levels in all five resistant genotypes and at low levels in three (G4666, P3154, and ORO200) and four (B73, G4666, P3154, and ORO200), respectively, out of five susceptible genotypes studied (Table 1). Also, three of the five resistant genotypes contained relatively high levels of a 46-kDa endosperm protein (2.26 to 5.44%). The level of this protein was much lower in the other two resistant genotypes and in all susceptible genotypes (0 to 0.73%) (Table 1).

Western blot analysis using antibodies against specific

<table>
<thead>
<tr>
<th>Genotype</th>
<th>58 kDa</th>
<th>46 kDa</th>
<th>72 kDa</th>
<th>46 kDa</th>
<th>34 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>GT-MAS:gk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BT3</td>
<td>12.12</td>
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<td>1.41</td>
<td>2.26</td>
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</tr>
<tr>
<td>CI2</td>
<td>16.69</td>
<td>9.94</td>
<td>1.51</td>
<td>0.20</td>
<td>8.73</td>
</tr>
<tr>
<td>T115</td>
<td>12.93</td>
<td>12.04</td>
<td>1.32</td>
<td>0.73</td>
<td>6.39</td>
</tr>
<tr>
<td>MP420</td>
<td>11.48</td>
<td>9.61</td>
<td>2.10</td>
<td>4.96</td>
<td>5.76</td>
</tr>
<tr>
<td>Tex6</td>
<td>10.04</td>
<td>14.38</td>
<td>1.30</td>
<td>5.44</td>
<td>7.11</td>
</tr>
<tr>
<td>B73</td>
<td>12.58</td>
<td>3.66</td>
<td>3.49</td>
<td>0.31</td>
<td>3.97</td>
</tr>
<tr>
<td>G4666</td>
<td>1.67</td>
<td>6.25</td>
<td>3.67</td>
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</tr>
<tr>
<td>P3154</td>
<td>1.29</td>
<td>4.00</td>
<td>2.05</td>
<td>0.31</td>
<td>3.36</td>
</tr>
<tr>
<td>DK689</td>
<td>11.23</td>
<td>3.82</td>
<td>3.43</td>
<td>0.31</td>
<td>6.46</td>
</tr>
<tr>
<td>ORO200</td>
<td>7.37</td>
<td>3.90</td>
<td>2.96</td>
<td>0.31</td>
<td>4.80</td>
</tr>
</tbody>
</table>

a Resistant genotypes are GT-MAS:gk, CI2, T115, MP420, and Tex6; susceptible genotypes are B73, G4666, P3154, DK689, and ORO200.

b Within columns, values followed by the same letter did not differ significantly by the least significant difference test (or Duncan’s test at P = 0.05).
antifungal proteins also revealed some qualitative and quantitive differences in protein induction between resistant and susceptible genotypes under germinating conditions (Fig. 3). In GT-MAS:ɡk and CI2, zeamatin levels increased during the 7-day period and were about threefold higher than in G4666, which remained unchanged. In B73, the zeamatin level increased during the incubation period, however, to a lesser extent, compared to GT-MAS:ɡk and CI2 (Fig. 3a). In resistant genotypes, the inactive 34-kDa RIP decreased during the incubation. It decreased dramatically in GT-MAS:ɡk and became undetectable after 3 days. A similar decrease was also observed in CI2, although at a slower rate. The level of RIP in B73 and G4666 remained constant over the 7-day period (Fig. 3b).

The major difference in antifungal proteins during the 7-day experiment was the 28-kDa chitinase, which was present in GT-MAS:ɡk and CI2 mature dry kernels and decreased during the incubation period. This protein was undetectable or present at low levels in B73 and G4666 dry mature kernels but increased during incubation, becoming detectable after 3 days in both genotypes (Fig. 3c). The expression of the 14-kDa trypsin inhibitor during the 7-day period also differed between resistant and susceptible genotypes. The level increased over twofold in the resistant genotypes and peaked around 5 days, whereas it remained unchanged in B73 and increased slightly in G4666 during the incubation (Fig. 3d).

Expression of germination- and infection-induced proteins in kernels. Freezing kernels at -70°C followed by slow defrosting at room temperature was shown to effectively kill the embryo without noticeable physical damage to kernels, and the embryo-killed kernels showed no signs of germination or protein induction when compared to dry kernels in a preliminary study (data not shown). Significant changes in embryo protein profiles were observed between imbibed and embryo-killed kernels of all genotypes 7 days after inoculation (Fig. 4). In embryo-killed inoculated kernels, the embryos contained significantly fewer proteins than those of imbibed and inoculated kernels. One of the major remaining proteins in the embryos of inoculated kernels has been confirmed to be a protease of fungal origin (9). However, the protein profiles of imbibed kernels of both inoculated and noninoculated controls were similar with two exceptions (Fig. 4). One was the increase of a 70-kDa protein (from 3.03 to 10.96%) in imbibed GT-MAS:ɡk embryos after inoculation. This protein also slightly increased (from 6.08 to 7.73%) in CI2 after inoculation but remained unchanged in the embryos of the two susceptible genotypes. Another difference was the pres-
ence of two proteins with a molecular mass of about 46 and 47 kDa in the imbibed noninoculated GT-MAS:gk and CI2 embryos; the 46-kDa protein (in G4666) or both (in B73) was missing in susceptible genotypes (Fig. 4).

Relative contribution of constitutive and inducible proteins to kernel resistance. Seven days after inoculation, the imbibed kernels, which retained most of their proteins, accumulated low levels of aflatoxins (Table 2), whereas the embryo-killed kernels, in which the embryo lost most of its constitutive proteins, supported the highest levels of aflatoxin production among all three treatments (Table 2). Aflatoxin levels in embryo-killed GT-MAS:gk and CI2 kernels were 5- to 12-fold higher, respectively, when compared to levels supported by dry kernel controls. The aflatoxin levels increased nine- to twofold in embryo-killed B73 and G4666, respectively, when compared to dry kernel controls. However, the levels of aflatoxin in embryo-killed GT-MAS:gk and CI2 were still two- to fourfold lower than those in susceptible lines, B73 and G4666. The imbibition of maize kernels at 0°C before inoculation did not significantly change the level of aflatoxins supported in GT-MAS:gk or CI2 (Table 2). However, this treatment dramatically reduced the level (five- to sixfold) of aflatoxins supported in susceptible genotypes compared to dry kernels (Table 2).

Identification and partial characterization of putative marker proteins. Five proteins associated with kernel resistance in the above studies were selected for further characterization through N-terminal amino acid sequence analysis. The N-terminal amino acid sequence of a 22-kDa protein (Table 3), whose level of expression increased during a 7-day incubation, showed 100% homology to the previously identified 22-kDa antifungal protein zeamatin (20, 40). The 34- and 72-kDa endosperm protein had blocked N-termini; therefore, no sequence data were obtained. N-terminal amino acid sequences were obtained for the 46- and 58-kDa embryo proteins (Table 3). Sequence comparison revealed that the N-terminal sequence of the 46-kDa protein shared 100% homology with the previously reported deduced amino acid sequence (between residue 24 and 43) of the maize globulin-2 gene (45) and that the 58-kDa protein showed 100% homology with the maize globulin-1 protein between residue 78 and 88 (1). Based on amino acid sequences, this 58-kDa mature protein is nine amino acid residues longer than the mature GLB1-S, a globulin-1 encoded by a Glb1-s allele (1).

**DISCUSSION**

In a previous study (11), a 14-kDa trypsin inhibitor protein associated with resistance was identified through comparison of total kernel proteins extracted from 13 maize genotypes. In the current study, five more proteins putatively associated with resistance were identified also by employing a comparative analysis of embryo and endosperm proteins of resistant and susceptible maize genotypes. Based on N-terminal amino acid sequences, two of the putative resistance markers were identified as globulin-1 and globulin-2, two of the most abundant embryo storage proteins (30). Maize embryo globulins appear to be unique among seed storage globulins in that each of the major components is encoded by only a single gene, whereas other storage proteins are usually encoded by multigene families (29), and they are highly hydrophilic and contain high levels of glycine (~9%), suggesting that they may have

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**TABLE 2.** Aflatoxin B1 levels (ppb) in maize kernels underwent different treatments before inoculation; data presented here are means of two independent experiments, each with 10 replications.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Dry</th>
<th>Imbibed</th>
<th>Embryo-killed</th>
</tr>
</thead>
<tbody>
<tr>
<td>GT-MAS:gk</td>
<td>6,083 A</td>
<td>4,434 A</td>
<td>34,668 A</td>
</tr>
<tr>
<td>CI2</td>
<td>5,229 A</td>
<td>5,749 A</td>
<td>66,037 A</td>
</tr>
<tr>
<td>B73</td>
<td>15,124 B</td>
<td>2,972 A</td>
<td>141,550 B</td>
</tr>
<tr>
<td>G4666</td>
<td>56,008 C</td>
<td>9,020 AB</td>
<td>115,950 B</td>
</tr>
</tbody>
</table>

*a* Within columns, values followed by the same letter did not differ significantly by the least significant difference test (or Duncan’s test at *P* = 0.05).
TABLE 3. Summary of partially characterized potential proteins associated with kernel resistance against Aspergillus flavus

<table>
<thead>
<tr>
<th>Size of protein</th>
<th>N-terminal amino acid sequence</th>
<th>Identity of the protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>22-kDa protein</td>
<td>AVFTVNVQCPF</td>
<td>Zeamatin (39)*</td>
</tr>
<tr>
<td>34-kDa protein</td>
<td>Blocked</td>
<td>RIP (suspected)</td>
</tr>
<tr>
<td>46-kDa protein</td>
<td>TESGSRPYYGHEEFSRHW</td>
<td>Globulin-2 (29, 44)</td>
</tr>
<tr>
<td>58-kDa protein</td>
<td>RSGEGSSELER</td>
<td>Globulin-1 (1)</td>
</tr>
<tr>
<td>72-kDa protein</td>
<td>Blocked</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

* Reference numbers.

other functions besides serving as storage proteins. Additionally, maize globulin-1 and globulin-2 were found to be present at higher levels in dry kernels of resistant genotypes than in those of susceptible ones. Further, the 46-kDa globulin-2 protein disappeared in susceptible genotypes after fungal infection, whereas it remained in resistant genotypes. In a previous investigation, an oat (Avena sativa) globulin gene was found to be linked to the Pg13 locus, a gene conferring resistance to pathogen Puccinia graminis f.sp. Avenae, causing stem rust disease (36). The above evidence indicates that the expression of globulins may be related to kernel resistance. However, further studies are necessary to establish the involvement of globulins in kernel resistance.

The presence of high levels of constitutive antifungal proteins, such as chitinase, zeatin, and trypsin inhibitor in the dry resistant kernels, possibly serving as a passive defense mechanism, may account for the difference in aflatoxin accumulation between resistant and susceptible genotypes. The significantly lower aflatoxin production in embryo-killed resistant kernels than in similarly treated susceptible kernels observed in the current study also may be due to the presence of high levels of these proteins.

When kernels were imbibed prior to inoculation, susceptible kernels supported levels of aflatoxins equivalent to resistant lines (Table 2), which is not surprising. A similar induction of kernel resistance in susceptible genotypes in pre-imbibed kernels was reported by Guo et al. (20). Previous studies (20, 22) support a role for kernel proteins induced under germinating conditions in aflatoxin resistance. It was found that imbibition under germinating conditions before inoculation increased kernel resistance in susceptible genotypes and that germination induces accumulation of specific antifungal proteins (such as the 22-kDa zeatin and the 18-kDa active RIP). In the present study, we demonstrated that imbibition at 0°C prior to inoculation also significantly induced kernel resistance in susceptible

FIGURE 5. Summary of factors contributing to resistance or susceptibility of maize kernels. Mature maize kernels with high levels of constitutive antifungal proteins and viable embryos exhibit resistance to fungal infection and aflatoxin production. Kernels containing no or low levels of constitutive antifungal proteins exhibit susceptibility. Kernels with viable embryos, however, have the ability to synthesize new proteins upon fungal infection and, when imbibed prior to infection, accumulate aflatoxins at low levels comparable to “resistant” kernels. Without viable embryos, kernels are susceptible to fungal infection and aflatoxin production.
genotypes, along with the induction of an antifungal chitinase. We also demonstrated that embryo-killed kernels, which were not able to synthesize new proteins, lost their resistance and supported the highest level of aflatoxin of any treatment. These data suggest that inducible proteins (including some of those proteins, if not all, produced during germination) are also required for the kernel resistance.

The above evidence highlights the importance of an inducible defense mechanism in maize kernel resistance. However, in reality, without high levels of constitutive antifungal proteins as a passive, preexisting defense mechanism, kernels of susceptible genotypes are likely colonized by A. flavus and contaminated with a/bullet6 atoxins before inducible mechanisms can be activated. The real function, therefore, of the high levels of constitutive antifungal proteins may be to delay fungal invasion, and subsequent aflatoxin formation, until other antifungal proteins can be synthesized to form an active defense system. The factors contributing to resistance or susceptibility of maize kernels discussed above are summarized in Figure 5. The current study also suggests that moisture level may be the primary determinant as to whether a kernel can immediately respond to fungal infection; reductions of aflatoxin levels were obtained in kernels of susceptible genotypes imbibed at 0°C in the present study similar to those obtained in a previous study (22), where kernels were imbibed for 3 days at 31°C to facilitate protein induction prior to fungal inoculation.

In summary, the present study identifies additional constitutive proteins that may associate with resistance, which include the globulin-1 and globulin-2 proteins. The level of inducible antifungal proteins, such as chitinase and zymatin, also appears to be associated with kernel resistance. This study also demonstrates that both constitutive and inducible proteins are required for kernel resistance to A. flavus infection and aflatoxin contamination. These findings may contribute not only to a clearer understanding of the factors required for kernel resistance, but also to the development of resistant commercial inbreds through the incorporation of newly identified traits by marker-assisted breeding or genetic engineering. Further investigation might benefit from using large-format 2-D gels (proteomics), which, because of increased resolution, may enhance the ability to identify more potential resistance markers.

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