Isoform Patterns of Chitinase and *β*-1,3-Glucanase in Maturing Corn Kernels (*Zea mays* L.) Associated with *Aspergillus flavus* Milk Stage Infection

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Isoform patterns of chitinase and *β*-1,3-glucanase of maturing kernels of yellow dent corn (Pioneer 3394) infected with *Aspergillus flavus* at the milk stage were investigated through polyacrylamide gel electrophoresis (PAGE). Proteins on the sodium dodecyl sulfate (SDS) gel with an apparent molecular mass range of 23–46 kDa were differentially present in the kernels infected with both aflatoxin-producing and non-aflatoxin-producing strains of *A. flavus*. From in-gel (native PAGE) enzyme activity assays, three bands corresponding to chitinase isoforms and two bands corresponding to *β*-1,3-glucanase isoforms were detected in the infected kernels. One chitinase isoform of 29 kDa was present only in the infected kernels, and another one of 28 kDa was present in both infected and noninfected kernels. They were judged to be acidic on the basis of their migration on an acrylamide isoelectric focusing (IEF) gel. For the *β*-1,3-glucanase, one isoform of 35 kDa was present in both infected and noninfected kernels, but another one, a 33 kDa isoform, was present only in the infected kernels. Both acidic and basic *β*-1,3-glucanase isoforms were detected in the IEF gel. The results of this study are the first to demonstrate patterns of enhanced or inducible proteins in maturing corn kernels in response to *A. flavus* infection at the milk stage. The results also indicate that only particular isoforms of the two hydrolytic enzymes are involved in the maturing corn kernels infected at the milk stage with *A. flavus*.

INTRODUCTION

Sporadic outbreaks of aflatoxin contamination of preharvest corn (*Zea mays* L.) can be a serious problem in the Midwestern corn belt (Hurburgh, 1991). Aflatoxins, produced by the fungus *Aspergillus flavus*, are toxic and carcinogenic to humans and other animals (Smith and Moss, 1985). Current attempts to prevent *A. flavus* infection and aflatoxin contamination include isolation and formulation of competitive microbes for use in biocontrol; characterization of structural, chemical, and biochemical factors associated with the resistance of corn varieties to *A. flavus* infection, aflatoxin accumulation, and/or insect damage to the ear; and identification of genes through marker-assisted gene mapping responsible for *A. flavus* resistance (Brown et al., 1999). Antimicrobial factors of protein or peptide nature have been extensively investigated because such factors could be rapidly introduced into corn through genetic engineering. Several families of pathogenesis-related (PR) proteins are recognized as biochemical factors possessing antimicrobial activity in vitro or the ability to enhance disease resistance when overexpressed in transgenic plants (Bushnell et al., 1998; Ryals et al., 1996). Some of these proteins are hydrolytic, including chitinase and *β*-1,3-glucanase (Boller, 1985; Nasser et al., 1988, 1990). Studies with various plant systems have indicated that several isoforms of the two hydro-lytic enzymes tend to appear in response to pathogen infection or other stress conditions (Darnetty et al., 1993; Ryals et al., 1996; Yi and Hwang, 1996), and only certain isoforms have defensive roles against particular fungal pathogens (Sela-Buurlage et al., 1993; Sticher et al., 1997). Activities of chitinase and *β*-1,3-glucanase have recently been reported to exist and inhibit the growth of *A. flavus* in mature corn kernels (Neucere et al., 1991, 1995; Lozovaya et al., 1998). However, little information has been published on what isoforms of the two hydrolytic enzymes in the corn kernels are induced by *A. flavus* infection.

In this paper, we provide evidence that proteins and particularly isoforms of chitinase and *β*-1,3-glucanase were differentially induced in maturing corn kernels infected with *A. flavus*. By utilizing in-gel (native polyacrylamide gel) enzyme activity assays (Trudel and Asselin, 1989), we further observed that certain isoforms of both chitinase and *β*-1,3-glucanase were associated with infection of *A. flavus* but not with infection of another fungus, *Fusarium moniliforme*, or with drought stress.

MATERIALS AND METHODS

Corn Inoculations. A commercial corn hybrid (Pioneer 3394) was grown in 1997 to maturity in experimental irrigation control plots at the 40-acre Illinois River Valley Sand Field (IRVSF), Kilbourne, IL. Within each plot, up to 20 corn ears in the late-milk to early-dough stage of kernel maturity (21 days after silking; August 7, 1997) were inoculated at three points separated by 4 cm, in two vertical rows on opposite sides

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of the ear by inserting a sterile wooden toothpick through the husk to wound underlying kernels. A second toothpick contaminated with *A. flavus* NRRL A-27837 (10^5 spores/mL), an aflatoxin-producing strain isolated from corn grown at IRVSF, was then inserted into each wound site and left until the naturally dried ears (15.5% moisture content) were hand harvested and shelled on October 6, 1997. Grain samples were selected from two irrigation control treatments: A, full-season irrigation; H, full-season nonirrigation. A single row of 20 corn plants under continuous irrigation was wound-inoculated with a non-aflatoxin-producing strain of *A. flavus*, NRRL A-27668 (10^5 spores/mL), to contrast the enzymatic response of infected kernels. The grain samples were examined for bright greenish yellow fluorescent (BGYF) kernels (Shotwell et al., 1972) using a black light at 365 nm and then grouped into three categories: category 1, intact non-BGYF kernels that were considered to be symptom-free kernels; category 2, intact kernels with limited fluorescence to the germ and endosperm (8000–17000 ppb of aflatoxin); category 3, kernels with full BGYF/discolored and shriveled appearance (28000–30000 ppb of aflatoxin). All kernel samples were stored at −20 °C. In selecting BGYF kernels for analyses we know that the fungus has gained entry to the seed proper and infected the germ or endosperm, sites of aflatoxin production. Infected kernels (Pioneer 3394) from ears inoculated with *Fusarium moniliforme* (strain M-3125) and having visible mold growth were provided by R. D. Plattner at the USDA–ARS, National Center for Agricultural Utilization Research, Peoria, IL (Desjardins and Plattner, 1998).

Fungal Growth and Harvest. *A. flavus* strains that produced aflatoxin (NRRL A-27837) or did not produce aflatoxin (NRRL A-27668) were grown separately for 7 days (25 °C) on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI). Portions of the colony were removed using a spatula for protein extraction. Each fungal strain was also inoculated onto soaked (12 h) and autoclaved corn kernels (25 g of dw; 50% moisture) and incubated for 7 days (25 °C). Following incubation, the molded kernels were extracted for proteins using the procedure described below.

Aflatoxin Analyses. All samples were analyzed for aflatoxin by the Aflatest (Vicam Inc., Waterown, MA) procedure according to the manufacturer's instructions, but adjusted to accommodate 10-g samples of ground corn. The standard procedure could detect aflatoxin as low as 1 ppb. Aflatoxin-containing wastes were immersed in Clorox bleach (regular) for 1 h to destroy the aflatoxin and then autoclaved.

Protein Extraction. The whole kernels or the lower thirds of the kernels were milled in a mini-miller (model IDS-50, Mr. Coffee, Inc.), and 1 g of the resulting powder was homogenized with a blender (Polytron) by adding 10 mL of an acetate buffer (pH 5.1), which contained 0.05% (w/v) sodium acetate, 4 mM of ascorbic acid, 2 mM PMSF, and 2% (w/v) PVPP. Proteins were extracted with gentle shaking at 4 °C overnight. The homogenates were then centrifuged at 15000g for 60 min at 4 °C. The isolated supernatants were concentrated ~10 times by filtering through ultrafiltration membranes (NMWL5,000, Millipore). The protein filtrates were then dialyzed against the same buffer for 8 h at 4 °C. The final protein extracts were stored at −20 °C and analyzed within 2 weeks. The same procedure was used to extract proteins of *A. flavus* or *F. moniliforme*, but the extraction was started by adding 10 mL of the acetate buffer to 5 g (fresh weight) of fungi. Protein concentration was determined using the Bio-Rad protein assay kit using bovine γ-globulin as standard (Bradford, 1976).

Electrophoretic Separation of Proteins. SDS–polyacrylamide gel electrophoresis (PAGE) and PAGE under native conditions were performed according to previously described methods (Trudel and Asselin, 1989; Ji and Kuc, 1995, 1996). The SDS–PAGE contained 12.5% (w/v) polyacrylamide and 0.1% SDS. Stacking gels were made of 4.5% (w/v) polyacrylamide containing 0.1% (w/v) SDS. The native PAGE contained 15% (w/v) polyacrylamide in the resolving gel and 5% polyacylamide in the stacking gel.
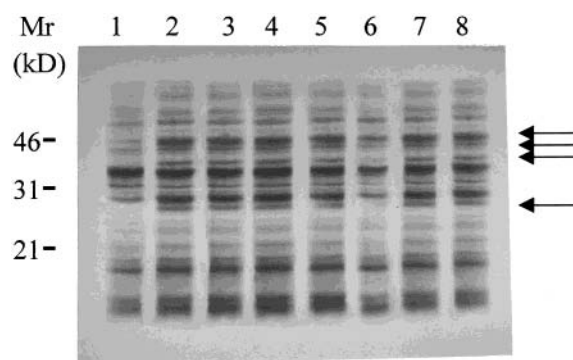

Figure 1. Proteins of maturing corn kernels associated with *A. flavus* infection. Proteins were separated by 12.5% SDS–PAGE and stained with GelCode Blue stain reagent (Pierce). Each lane was loaded with 100 μg of proteins from the lower third of the kernels. Samples were from symptom-free kernels (lanes 1 and 6), intact kernels with limited BGYF distribution (lanes 2, 4, and 7), kernels with full BGYF distribution (lanes 3, 5, and 8), kernels from irrigation treatment (lanes 1–5), kernels of nonirrigation treatment (lanes 6–8), and kernels infected with *A. flavus* strain NRRL A-27837 (lanes 2, 3, 7, and 8) and strain NRRL A-27668 (lanes 4 and 5). Mr, molecular mass markers.

Isoelectric focusing (IEF) was performed using precast wide range gels (pH 3.5–9.5, Pharmacia-LKB), as previously described (Dowd, 1994).

In-Gel Chitinase and β-1,3-Glucanase Isoform Assays. Detection of chitinase isoforms after native PAGE and IEF was based on the use of an overlay gel containing 0.01% (w/v) glycol chitin as substrate (Trudel and Asselin, 1989). After 2 h of incubation at 37 °C, the overlay gel was stained with fluorescent brightener 28 (Sigma) for 5 min. Lytic zones corresponding to chitinase activity on the gels were visualized by placing the gels on a Chromato-Vue C-62 transilluminator (UV Products). The fluorescent background could be reduced by washing the gel several times with distilled water. The bands on the gels were then photographed with Polaroid film (type 107) with UV-haze and 02 orange filters. Detection of β-1,3-glucanase isoforms after native PAGE and IEF was performed as described by Pan (1989) and Ji and Kuc (1995). Gels were incubated in a solution containing 1% of laminarin for 90 min at 40 °C. β-1,3-Glucanase activity in the gels was then visualized by staining the gels for 5–10 min at 100 °C in a 1 M NaOH solution containing 0.3% (w/v) 2,3,5-triphenyltetrazolium chloride (Sigma).

Partial Purification of Chitinase and β-1,3-Glucanase. After a single electrophoretic separation of proteins in the protein extracts by the native PAGE or IEF, all bands that corresponded to kernel chitinase or β-1,3-glucanase were cut out of the native PAGE. The proteins in the bands were eluted with an Electro-Eluter (model 422, Bio-Rad) and concentrated according to the manufacturer's instructions. The enzymatic nature of the partially purified proteins was checked by performing enzyme activity assays (Ji and Kuc, 1995).

RESULTS

Proteins from maturing corn kernels that are associated with *A. flavus* infection were analyzed on an SDS–polyacrylamide gel (PAGE) as shown in Figure 1. There were at least four corn kernel proteins enhanced or induced in response to *A. flavus* infection (Figure 1, lanes 2–5, 7, and 8) in contrast to the proteins from symptom-free kernels without infection (Figure 1, lanes 1 and 6). The four proteins, indicated by arrows, had apparent molecular masses of 23–46 kDa. The inducible proteins were similar among kernels infected with the aflatoxin-producing strain (Figure 1, lanes 2 and 3) or the non-aflatoxin-producing strain (Figure 1, lanes 4

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Figure 2. Isoform pattern of chitinase activity on a native polyacrylamide gel: (lanes 1−8) same as those described in Figure 1; (lane 9) protein extracts of *A. flavus* NRRL A-27668; (lane 10) protein extracts of *F. moniliforme*; (lane 11) symptom-free corn kernels; (lane 12) corn kernels infected with *F. moniliforme*. Each lane was loaded with 150 μg of proteins.

and 5) and among kernels from irrigated (Figure 1, lanes 2 and 3) or nonirrigated (Figure 1, lanes 7 and 8) treatments.

Results in Figure 2 show chitinase activity in a native PAGE after a single electrophoretic separation of the proteins. Three bands indicating different chitinase isoforms were detected in the kernels infected with *A. flavus* (Figure 2, lanes 2−8) or *F. moniliforme* (Figure 2, lane 12). On the basis of the intensity of each band, the two upper bands appeared to be inducible or enhancible, whereas the lowest band exhibited little difference among samples from both symptom-free (Figure 2, lanes 1 and 11) and infected kernels. The second inducible band (Figure 2, lane 12) by *F. moniliforme* was slightly different in migration rate from the one induced by *A. flavus*. Because proteins of fungi in the infected kernels might also contribute to the detected chitinase activity, protein extracts from *A. flavus* and *F. moniliforme* were used as controls. One band from either *A. flavus* (Figure 2, lane 9) or *F. moniliforme* (Figure 2, lane 10) was observed, and it appeared to be different from the kernel inducible chitinase isoforms.

Three bands corresponding to β-1,3-glucanase isoforms were also observed in the protein extracts of kernels infected with *A. flavus* (Figure 3, lanes 2, 3, 5, and 6). One band of β-1,3-glucanase at the lowest position was not associated with *A. flavus* infection because this band could also be detected in the symptom-free corn kernels (Figure 3, lanes 1 and 9). Another band of β-1,3-glucanase at the middle position might not be of corn kernel origin becuase this band was present in the protein extracts of *A. flavus* (Figure 3, lane 7). The third band at the highest position of the gel seemed specifically associated with *A. flavus* infection. This was supported by the observation that the third band was different from the one at a similar migration position detected in the corn kernels infected with *F. moniliforme* (Figure 3, lane 10).

To determine the isoelectric points of corn kernel chitinase and β-1,3-glucanase, IEF was conducted using precast wide range (pH 3.5−9.5). Bands indicative of acidic chitinase activity were detected (Figure 4A), and the pI values for the acidic chitinases were ∼5.0. Interestingly, bands indicating basic chitinase activity were not detected. Both acidic (pI ∼ 4.3) and basic (pI ∼8.7) β-1,3-glucanases were observed (Figure 4B).
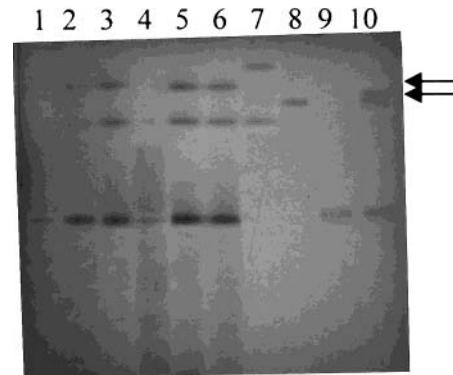

Figure 3. Isoform pattern of β-1,3-glucanase activity on a native polyacrylamide gel: (lanes 1−6) same as those described in Figure 1; (lane 7) protein extracts of *A. flavus* NRRL A-27668; (lane 8) protein extracts of *F. moniliforme*; (lane 9) symptom-free corn kernels; (lane 10) corn kernels infected with *F. moniliforme*. Each lane was loaded with 150 μg of proteins.
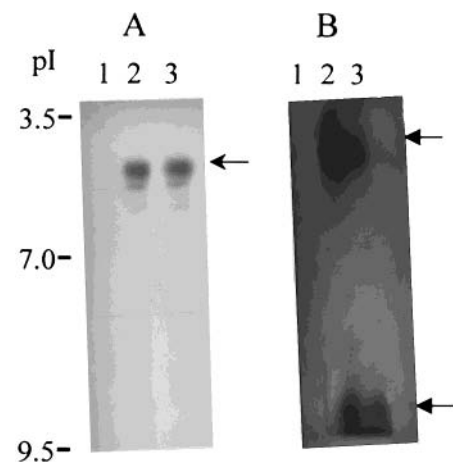

Figure 4. Isoforms of chitinase and β-1,3-glucanase on isoelectric focusing gels: (lane 1) symptom-free corn kernels with an irrigation treatment; (lane 2) corn kernels with limited BGYF distribution; (lane 3) corn kernels with full BGYF distribution; (A) chitinase activity; (B) β-1,3-glucanase activity. Each lane was loaded with 50 μg of proteins.

To identify individual proteins contained in the bands of the native PAGE that had either chitinase or β-1,3-glucanase activity (Figures 2 and 3), partial purification of the proteins was conducted with a direct electroelution (see Materials and Methods). The yield for eluted proteins was ∼40%. Figure 5 demonstrates the profiles of eluted proteins that represent possible isoforms of chitinase or β-1,3-glucanase. Four proteins of chitinase in the kernels infected with *A. flavus* (Figure 5A, lane 2) and three proteins of the kernels infected with *F. moniliforme* were visible (Figure 5A, lane 4). One of the four chitinase isoforms, with a molecular mass of 28 kDa, was present in both symptom-free and infected corn kernels. An additional 34 kDa protein was present in corn kernels infected with either *A. flavus* or *F. moniliforme*. Interestingly, a chitinase isoform of 29 kDa was present specifically in the corn kernels infected with *A. flavus* (Figure 5A, lane 2). Isoform proteins particularly associated with fungal infection were also observed in the case of β-1,3-glucanase. One protein, with a molecular mass of 33 kDa, was detected only in corn kernels infected with *A. flavus* (Figure 5B, lane 2), and another one of 30 kDa was present only in the kernels infected with *F. moniliforme* (Figure 5B, lane 4). A 35 kDa isoform protein of β-1,3-glucanase existed in both symptom-free and infected kernels.
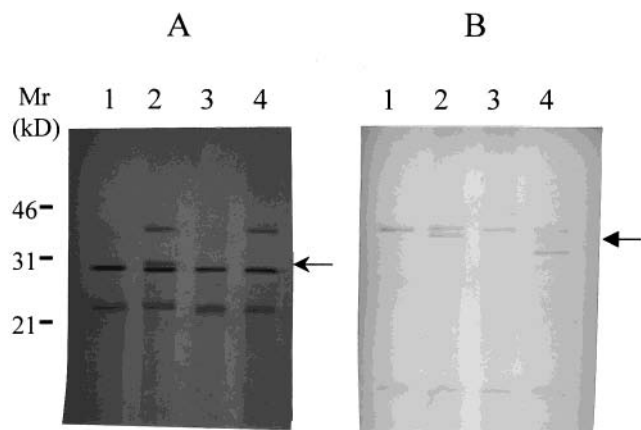
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Figure 5. Partially purified chitinase and β-1,3-glucanase proteins revealed on an SDS–polyacrylamide gel: (A) chitinase proteins; (B) β-1,3-glucanase proteins; (lanes 1) symptom-free corn kernels from an irrigation treatment; (lanes 2) corn kernels infected with *A. flavus* NRRL A-27668; (lanes 3) symptom-free corn kernels from a nonirrigation treatment; (lanes 4) corn kernels infected with *F. moniliforme.* The proteins were eluted from bands on native gels corresponding to either chitinase or β-1,3-glucanase activity.

DISCUSSION

In this study, four proteins were observed to be differentially present in corn kernels as a result of interaction between the kernels and *A. flavus.* The number of the enhanced or inducible proteins in corn kernels in this hybrid appeared to be less than the number of 10 pathogenesis-related proteins in corn leaves reported by Nasser et al. (1988, 1990). This probably is due to relatively low metabolic activities in corn kernels. To our knowledge, the results of this study are the first to demonstrate detailed patterns of enhanced or inducible proteins in maturing corn kernels in response to *A. flavus* infection at the milk stage, although induction of peroxidase isozymes has been reported in other studies (Dowd, 1994; Dowd et al., 1995). The induction of pathogenesis-related proteins in maturing corn kernels is of significance because it provides opportunities for us to identify resistance-related proteins or inducible and specific gene promoters even in kernels that are of reduced physiological activities and are the vulnerable targets of *A. flavus* (Payne, 1992). It holds promise in the genetic engineering of corn with resistance factors to prevent *A. flavus* infection or aflatoxin accumulation in a more efficient and tissue specific way.

There were different isoforms of chitinase and β-1,3-glucanase in the kernels. Some of the isoforms were present in both symptom-free and infected kernels, whereas others were enhanced or induced by *A. flavus* infection and were not observed in sterilized kernels molded with *A. flavus* (data not shown). They, like the chitinase and β-1,3-glucanase isoforms constitutively expressed in corn leaves and other plant systems, may be regulated developmentally (Carpita, 1996) and may not be involved in the interaction between the kernels and *A. flavus* or be responsible for defense against other fungi or bacteria that do not cause disease. Quite interestingly, the 29 kDa chitinase and 33 kDa β-1,3-glucanase isoforms were particularly associated with *A. flavus* infection but not with infection of another fungus, *F. moniliforme,* or with drought stress. Since, in corn kernels, correlations of the two enzyme activities with antifungal properties and to disease resistance of a few

corn varieties have been demonstrated (Lozovaya et al., 1998; Neucere et al., 1995), it is reasonable to assume that the two particular isoforms of chitinase and β-1,3-glucanase are involved in combating *A. flavus* infection. Rigorous studies are necessary to characterize the individual proteins, to find out how the particular isoforms of chitinase and β-1,3-glucanase relate structurally to other published isoforms and how the isoform patterns vary in kernels of other corn varieties in response to *A. flavus* infection. These studies might be useful when the two fungal degradative enzymes are applied in corn through genetic engineering to prevent *A. flavus* infection and aflatoxin contamination.

However, the question must be asked: why were the corn kernels infected by *A. flavus* in the presence of a considerable level of chitinase and β-1,3-glucanase? The reason is complex. The phenomenon has often been observed in other plant–pathogen systems. One possible explanation is that it is the speed and intensity of the defense reaction that determines resistance to the pathogen (Ryals et al., 1996). During the course of interaction of the kernels and *A. flavus,* effective chitinase and β-1,3-glucanase isoforms may not be produced in sufficient amounts or at the right time or cellular location. Synergistic interactions of the two hydrolases may not be established in kernels due to grain maturation. Other growth-enhancing factors, such as sugars and other nutrients, may allow *A. flavus* to overcome the rapid expression and function of chitinase and β-1,3-glucanase (Neucere et al., 1991). There is also the possibility that *A. flavus* has developed resistance to these enzymes. Environmental conditions may also affect the outcome of the pathogen–microbe interactions (Bhardwaj, 1995). We have shown that drought stress does not induce additional isoforms of chitinase or β-1,3-glucanase among the intact, non-BGYF kernel fraction. At the same time, although aflatoxin contamination of discolored and shriveled BGYF grains harvested from nonirrigation plots (30000 ppb) was equivalent to that of grain from irrigated plots (28000 ppb), the intact BGYF kernels harvested from nonirrigated corn were contaminated with substantially more aflatoxin (17000 ppb) than the intact BGYF kernel fraction from irrigated corn (9000 ppb). Application of corn resistance factors through genetic engineering may be integrated with other strategies and enhance the control of *A. flavus* infection or aflatoxin contamination (Brown et al., 1999).

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