A Corn Trypsin Inhibitor with Antifungal Activity Inhibits *Aspergillus flavus* α -Amylase

Z.-Y. Chen, R. L. Brown, J. S. Russin, A. R. Lax, and T. E. Cleveland

First and third authors: Department of Plant Pathology and Crop Physiology, Louisiana State University Agricultural Center, Baton Rouge 70803; and second, fourth, and fifth authors: Southern Regional Research Center, USDA-ARS, New Orleans, LA 70179.
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ABSTRACT

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In this study, we found that the inhibition of fungal growth in potato dextrose broth (PDB) medium by the 14-kDa corn trypsin inhibitor (TI) protein, previously found to be associated with host resistance to aflatoxin production and active against various fungi, was relieved when exogenous α -amylase was added along with TI. No inhibitory effect of TI on fungal growth was observed when *Aspergillus flavus* was grown on a medium containing either 5% glucose or 1% gelatin as a carbon source. Further investigation found that TI not only inhibited fungal production of extracellular α -amylase when *A. flavus* was grown in PDB medium containing TI at 100 $\mu\text{g ml}^{-1}$ but also reduced the enzymatic activity of *A. flavus* α -

amylase by 27%. At a higher concentration, however, TI stimulated the production of α -amylase. The effect of TI on the production of amylglucosidase, another enzyme involved in starch metabolism by the fungus, was quite different. It stimulated the production of this enzyme during the first 10 h at all concentrations studied. These studies suggest that the resistance of certain corn genotypes to *A. flavus* infection may be partially due to the ability of TI to reduce the production of extracellular fungal α -amylase and its activity, thereby limiting the availability of simple sugars for fungal growth. However, further investigation of the relationship between TI levels and fungal α -amylase expression in vivo is needed.

Additional keywords: antifungal protein, maize.

Plants do not contain an immune system and must rely on other mechanisms to protect themselves from fungal infection. These mechanisms include synthesis of inhibitory compounds such as phenols, melanins, tannins, or phytoalexins as well as accumulation of proteins that can directly inhibit fungal growth (20). A number of such antifungal proteins have been isolated from a variety of plant tissues (11,22,24,25,30–32). These include protease inhibitors, lectins, ribosomal inactivating proteins, zeamatin, chitinases, β -1,3-glucanases, and thionins. The most extensively studied protease inhibitors are the trypsin inhibitors (TI), proteins of different size and amino acid sequence that can inhibit the activity of trypsin. They have been isolated from various grains including corn kernels (29,35). Some TI have been reported to be inhibitors of both trypsin and α -amylase (9,28,29,33).

Gatehouse et al. (16) indicated that elevated levels of TI observed in cowpea (*Vigna unguiculata*) cv. Tvu2027 played a role in resistance to damage caused by the bruchid beetle *Callosobruchus maculatus*. Feeding studies using artificial diets found that ingestion of TI caused a significant elevation of the levels of trypsin in insect guts. Therefore, it was proposed that the true mode of action of the inhibitor was to cause pernicious hyperproduction of trypsin, which in turn leads to depletion of the sulfur-containing amino acids, this being detrimental to the insects (5). Direct evidence of involvement of TI in plant defense was demonstrated by expression of the cowpea TI gene in tobacco, which increased its resistance against herbivorous insects (18).

Peng and Black (26) showed that TI and chymotrypsin inhibitor levels in tomato plants increased following infection by the pathogenic fungus *Phytophthora infestans*, but this host response only occurred in those varieties of tomato that were resistant to the fungus and not in susceptible varieties. The possible involvement of TI in plant defense against fungal pathogens has also been implicated by their antifungal activities (20,36). However, the mode of action of many of these proteins against fungi has not been clearly demonstrated. A 14-kDa protein from corn kernels shown to be a specific inhibitor of bovine trypsin (35) also inhibited activated Hageman factor (factor XIIa) of the intrinsic blood-clotting process (19) as well as α -amylase from the red flour beetle (*Tribolium castaneum*) and the yellow mealworm (*Tenebrio molitor*) (9). However, this protein did not inhibit α -amylases from the fungi *Aspergillus niger*, *A. fumigatus*, or *A. oryzae*, the bacterium *Bacillus subtilis*, or human saliva (2). We recently demonstrated that this 14-kDa TI was associated with resistance to *A. flavus* infection and aflatoxin production in 11 corn genotypes (11). The purified TI inhibited both in vitro conidia germination and hyphal growth of *A. flavus*, the major aflatoxin-producing fungus in crops such as corn, cotton, peanut, and tree nuts. The reduced fungal growth of *A. flavus* has been shown to correlate with reduced aflatoxin production in laboratory studies (8,17). The ability of TI to inhibit the growth of *A. flavus* and some other fungal pathogens (10) suggests its potential importance in the control of aflatoxin contamination of corn and other crops as well as of some other fungal diseases.

Characterization of the mode of action of the 14-kDa TI could constitute a first step leading to the development of selectable marker traits for corn breeders as well as isolation of genes for use in developing resistant transgenic plants. Since certain TI have been shown to inhibit α -amylase activity, the current study sought to determine if the 14-kDa TI from corn resistant to *A. flavus* infection and aflatoxin production inhibited *A. flavus* α -amylase, thereby inhibiting fungal growth. Preliminary reports have been published (7,12).

Corresponding author: R. L. Brown; E-mail address: rbrown@nola.srrc.usda.gov

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MATERIALS AND METHODS

Chemicals. Soluble starch, β -nicotinamide adenine dinucleotide (NADP), amyloglucosidase (EC 3.2.1.3) from *A. niger*, and glucose dehydrogenase (NADP dependent) (EC 1.1.1.119) from *Cryptococcus unigutulatus* were purchased from Sigma Chemical Co. (St. Louis). α -Amylase (EC 3.2.1.1) from *B. subtilis* was purchased from United States Biochemical Corp. (Cleveland).

Fungal growth. The effect of TI on fungal growth in the presence or absence of exogenous α -amylase was examined using TI overexpressed from *Escherichia coli* (10). Conidia from 7-day-old *A. flavus* AF13 (13) cultures on V8 juice agar plates were suspended in deionized water and then added to each of the following four media in the wells of tissue culture plates to a final concentration of 10^5 conidia per ml: 10% potato dextrose broth (PDB) alone, PDB amended with *B. subtilis* α -amylase ($40 \mu\text{g ml}^{-1}$), PDB amended with TI ($200 \mu\text{g ml}^{-1}$), or PDB amended with both α -amylase and TI. The plates were incubated at 31°C in darkness. Hyphal length, a measure of fungal growth, was measured after 12 and 24 h. Similar experiments were conducted using A&M medium (1) containing 1% gelatin or 5% glucose as a carbon source in place of PDB for *A. flavus*. Amendments (α -amylase and TI) were added to these media as described above. For each treatment, hyphal length was measured from 40 randomly selected hyphae using a light microscope equipped with an ocular micrometer, and the mean hyphal length was used for comparison. This experiment was conducted twice.

TI effects on α -amylase activity and production. The effect of TI on α -amylase activity of *A. flavus* was evaluated based on its activity in a native gel being treated with or without TI. Concentrated filtrates of 1-day-old *A. flavus* cultures in 10% PDB and 3-day-old *A. flavus* cultures in 1% starch in A&M medium, which we found to have high levels of enzyme activity, were loaded equally on duplicated native polyacrylamide gels (8.0%) and re-

solved simultaneously at 4°C . The control gel was soaked in 10 ml of 0.1 M potassium phosphate buffer (pH 6.5) at 25°C for 30 min on a rotatory shaker, and the other gel was soaked in the same buffer containing TI ($100 \mu\text{g ml}^{-1}$). Both gels were then rinsed briefly with water and soaked in the same buffer containing 0.25% (wt/vol) soluble starch for 30 min. α -Amylase activity was visualized by staining the gels in 5 mM $\text{I}_2\text{-KI}$ for 3 min followed by rinsing with water. The intensity of clear bands resulting from α -amylase activity was quantified using a gel densitometer. This experiment was conducted three times.

Another experiment examined production of extracellular α -amylase by *A. flavus* in the presence of various concentrations of TI in growth medium. *A. flavus* conidia (final 10^5 ml^{-1}) were germinated in 4 ml of 10% PDB containing 0, 100, and $200 \mu\text{g}$ of TI per ml in the wells of tissue culture plates. Each treatment was replicated four times. After 10, 16, and 24 h of incubation at 31°C , fungal biomass was removed by centrifugation and the supernatant was concentrated fivefold at 4°C using Centricon-10 concentrators (Amicon, Beverly, MA). Concentrated filtrate ($15 \mu\text{l}$) from each treatment was resolved on an 8% native polyacrylamide gel. After electrophoresis, the gel was soaked in 0.25% soluble starch and stained as above. Since TI and α -amylase were dissociated during gel electrophoresis (data not shown), the bands representing α -amylase on the gel are a reflection of the inhibition of α -amylase production in the medium rather than the inhibition of its enzymatic activity. This experiment was conducted twice.

TI effects on amyloglucosidase activity and production. The effect of TI on the activity of amyloglucosidase, another enzyme involved in starch metabolism, was studied by comparing the rate of glucose liberation by the enzyme in the presence or absence of TI in the assay. Glucose liberation from starch was coupled to the reduction of NADP by glucose dehydrogenase (34). The production of reduced NADP (NADPH) was monitored spectrophotometrically at 340 nm for 15 min, the time point in which the reaction usually

Fig. 1. Growth of *Aspergillus flavus* conidia after 12 h at 31°C in **A**, 10% potato dextrose broth (PDB) alone; **B**, PDB amended with α -amylase from *Bacillus subtilis* ($40 \mu\text{g ml}^{-1}$); **C**, PDB amended with trypsin inhibitor ($200 \mu\text{g ml}^{-1}$); or **D**, PDB amended with both α -amylase ($40 \mu\text{g ml}^{-1}$) and trypsin inhibitor ($200 \mu\text{g ml}^{-1}$). Photos were taken under the same magnification.

reached its maximum velocity. The sources of amyloglucosidase evaluated were commercial amyloglucosidase from *A. niger*, concentrated 3-day-old 1% starch in A&M medium filtrate, and 1-day-old 10% PDB filtrate from *A. flavus*, which we found to have high levels of amyloglucosidase activity. The assay was conducted in 1-ml cuvettes with the following components added in order: 0.5 ml of 2× assay buffer (100 mM Na₂HPO₄ and 0.8 mM NADP, pH 7.0), 0.1 ml of amyloglucosidase (1 unit) or concentrated medium filtrate, and 0.2 ml of TI (0.5 mg ml⁻¹) or H₂O. The mixture was incubated at 25°C for 30 min before adding 0.1 ml of glucose dehydrogenase (2 units), and 0.1 ml of 5% (wt/vol) starch to start the reaction. For each treatment, the assay was repeated three times. This experiment was conducted twice. The protein concentration in the filtrates was assayed according to Bradford (4) using the Bio-Rad dye reagent (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin as a standard.

The effect of TI on amyloglucosidase production in 10% PDB medium was also studied. *A. flavus* conidia were germinated and grown in 4 ml of 10% PDB as described previously in the presence of TI at 0, 50, 100, and 200 µg ml⁻¹. After 10, 16, and 24 h of incubation at 31°C, fungal biomass was removed by centrifugation and the supernatant was concentrated fivefold at 4°C using Centricon-10 concentrators. Enzyme production was evaluated by measuring enzymatic activity of the concentrated filtrates in the same manner described above.

Statistical analysis. All data were analyzed using the analysis of variance procedure of the Statistical Analysis System (SAS Institute, Cary, NC). Means were separated by least significant difference ($P \leq 0.05$).

RESULTS

The effect of TI and α -amylase on fungal growth. It was found that TI inhibited hyphal growth of *A. flavus* when it was grown in 10% PDB medium, and hyphal growth was restored when exogenous α -amylase was added along with TI. After 12 h of incubation, there was no significant difference either visually (Fig. 1A and B) or quantitatively (Table 1) in fungal growth between 10% PDB media with exogenous α -amylase and without. However, in 10% PDB amended with TI at 200 µg ml⁻¹, hyphal length was reduced, on average, to 20 µm compared with 77 µm in 10% PDB medium (Fig. 1C, Table 1). Fungal growth in 10% PDB amended with TI at 200 µg ml⁻¹ was restored to about 82% of the control after 12 h of incubation when α -amylase from *B. subtilis* was added to the medium along with TI (Fig. 1D, Table 1). At the end of 24 h of incubation, the hyphal length in the PDB medium amended with TI at 200 µg ml⁻¹ remained nearly the same as that at 12 h, whereas the average hyphal length in the 10% PDB amended with both TI and α -amylase (230 µm) was even longer than that in 10% PDB medium alone (203 µm) (Table 1). However, an increase in fungal growth was observed after 30 h in the PDB medium amended with TI at 200 µg ml⁻¹ (data not shown).

Further, it was found that TI did not inhibit hyphal growth when *A. flavus* was grown on glucose or gelatin medium. When the fun-

gus was grown on simple sugar-enriched medium such as 5% glucose in A&M medium, there was no significant difference in the mean hyphal length between the medium with (176 µm) or without (162 µm) TI at 200 µg ml⁻¹ after 24 h of incubation (Table 1). No significant growth difference was observed in 5% glucose in A&M medium amended with TI only or amended with both TI and α -amylase. A similar pattern was also observed when the fungus was grown on protein-enriched medium such as 1% gelatin in A&M medium (Table 1).

The effect of TI on the activity and production of α -amylase. By visually comparing the band intensity in activity-stained native gels, a clear reduction of α -amylase enzymatic activity was observed in the TI-treated gel (Fig. 2). Quantitative analysis using gel densitometry showed that α -amylase activities in the gels treated with TI at 100 µg ml⁻¹ for 30 min were reduced by an average of 27% ($27.3 \pm 8.3\%$ [standard deviation]), using data pooled from three repeated experiments, when compared with those in the control gels. Also, different sources of α -amylase were found to have different isoforms, and all isoforms were inhibited equally by TI (Fig. 2). The 3-day-old starch medium filtrate of *A. flavus* had only one isoform of α -amylase (isoform A), which was also present in 3-day-old 10% PDB medium filtrate. However, in the 3-day-old PDB medium filtrate, there were two additional isoforms (B and C) of α -amylase present (Fig. 2).

Extracellular α -amylase production by *A. flavus* in 10% PDB medium filtrates was also affected by TI. In 1-day-old 10% PDB medium filtrate, only two isoforms (B and C) of α -amylase were observed (Fig. 3, inset). Extracellular α -amylase production increased in the control medium filtrate from 0 to 16 h and then decreased slightly at 24 h for both isoforms. In the fungal medium amended with TI at 100 µg ml⁻¹, the production of extracellular α -amylase was reduced to about one-fourth the level in the control medium. However, a different response in fungal α -amylase production was observed in fungal medium amended with TI at 200 µg ml⁻¹. α -Amylase production for both forms was undetectable at 10 h. The level of α -amylase was about one-third the level in the control at 16 h, but increased to twice the level in the control at 24 h (Fig. 3). Also, the two α -amylase isoforms were affected differently by

Fig. 2. α -Amylase activity-stained native polyacrylamide gel electrophoresis of concentrated filtrates from *Aspergillus flavus* cultures. Lanes 1 and 3, Three-day-old 1% starch in A&M filtrate (15 µl per lane); and lanes 2 and 4, 10% potato dextrose broth filtrate (15 µl per lane). Lanes 1 and 2 were soaked in 0.1 M phosphate buffer (pH 6.5) alone, and lanes 3 and 4 were soaked in 0.1 M phosphate buffer (pH 6.5) containing trypsin inhibitor at 100 µg ml⁻¹ for 30 min at 25°C prior to staining for α -amylase activity. The arrows indicate the three different isoforms of α -amylase (isoforms A, B, and C).

TABLE 1. Fungal hyphal growth (µm) in different media with different treatments

Treatment ^x	10% PDB ^y , 12 h	10% PDB, 24 h	1% Gelatin in A&M, 24 h	5% Glucose in A&M, 24 h
Control	77 a ^z	203 b	126 a	162 a
+ α -Amylase	83 a	221 ab	130 a	165 a
+ TI	20 c	23 c	119 a	176 a
+ Both	63 b	230 a	143 a	153 a

^x α -Amylase added at 40 µg ml⁻¹ and trypsin inhibitor (TI) added at 200 µg ml⁻¹.

^y PDB = potato dextrose broth. Hyphal length was measured in different media with indicated treatments after 12 or 24 h of incubation at 31°C. The values shown here are means of pooled data from two experiments.

^z Within columns, values followed by the same letter did not differ significantly by the least significant difference test.

TI; the induction of isoform B was slower than that of isoform C (lower band) (Fig. 3).

The effect of TI on amyloglucosidase activity and production. TI at a concentration of 100 µg ml⁻¹ did not inhibit the activity of amyloglucosidase from *A. niger* or of *A. flavus* grown in PDB (Table 2). However, an inhibition (10%) of the amyloglucosidase activity from *A. flavus* grown in starch medium was observed when the 3-day-old starch medium filtrate was preincubated with TI at 100 µg ml⁻¹ for 30 min (Table 2).

The production of extracellular amyloglucosidase in the presence of various concentrations of TI in the medium was affected differently when compared with that of α-amylase. The level of extracellular amyloglucosidase was very similar between 10% PDB medium alone or amended with TI at 50 µg ml⁻¹ at all sample times (Fig. 4). When the medium was amended with TI at 100 or 200 µg ml⁻¹, enzyme production increased three- to fourfold during the first 10 h as compared with that in 10% PDB medium alone, and then it decreased with time. By 24 h, the level of amyloglucosidase in the medium containing TI at 100 µg ml⁻¹ decreased to the level of 10% PDB medium alone. In the medium containing TI at 200 µg ml⁻¹, however, the amount of enzyme was still about twice that in the 10% PDB medium alone (Fig. 4).

DISCUSSION

It was previously demonstrated that constitutive levels of a 14-kDa TI in corn kernels are associated with resistance to *A. flavus* infection and aflatoxin production and that TI purified from corn kernels ruptured conidia and inhibited both conidia germination and hyphal growth of *A. flavus* (11). It was also shown that this TI over-expressed in *E. coli* inhibited conidia germination and hyphal growth of eight other fungal pathogens (10). Despite these discoveries, little is known about the mode(s) of action of this protein as a fungal growth inhibitor. Since some TI have been reported to inhibit both trypsin and α-amylase (9,28,29,33), we hypothesized that one of the ways TI affected fungal growth of *A. flavus* was by inhibiting fungal α-amylase.

Fig. 3. Time course production of *Aspergillus flavus* α-amylase in 10% potato dextrose broth in the presence of different trypsin inhibitor (TI) concentrations. Total α-amylase production (mean ± standard error) in filtrates (per ml) amended with TI at 0 (●), 100 (■), or 200 (▲) µg ml⁻¹ was calculated from the total enzyme activity of isoforms B and C quantified from native gels after separation from TI. The inset is a photograph of a representative native gel at three different time points.

In the current study, it was found that *A. flavus* grew similarly on 10% PDB medium with or without external α-amylase. This indicated that 10% PDB medium provided sufficient simple sugars for growth. It also was found that TI, used at concentrations comparable to that found in resistant corn kernels (about 234 µg g⁻¹ based on the data reported by Brown et al. [6]), inhibited fungal growth. However, when *B. subtilis* α-amylase was added along with TI, fungal growth was restored within 24 h. These findings suggest that inhibition of fungal growth by TI may result from inhibition of fungal α-amylase. Limiting this hydrolase would limit the availability of simple sugars for growth. This hypothesis was further supported by the failure of TI to inhibit the growth of *A. flavus* on growth medium containing either simple sugars such as glucose or protein such as gelatin.

Further investigation revealed that TI inhibited α-amylase production and, to a lesser extent, its activity. The reduction of enzymatic activity by only 27% agrees with earlier findings that many known amylase inhibitors demonstrate little or no inhibition of fungal α-amylase enzymatic activity (3,23). The effect of TI on *A. flavus* α-amylase production depended on TI concentration. The level of α-amylase in 10% PDB medium amended with TI at 100 µg ml⁻¹ was about one-fourth that in the nontreated control during the time period studied. This corresponded to earlier work,

TABLE 2. The effect of trypsin inhibitor (TI) on amyloglucosidase activity^v in different media

Treatment ^w	Amyloglucosidase from <i>Aspergillus niger</i>	10% PDB ^x filtrate	Starch medium filtrate
Control	38.9 a ^y	32.5 a	75.3 a
+ TI	39.3 a	32.3 a	67.0 b
+ Autoclaved TI	38.6 a	ND ^z	75.8 a

^v Amyloglucosidase activity (µmol of glucose per min per mg of protein). 1 OD₃₄₀ = 9.603 µmol of NADPH = 9.603 µmol of glucose.

^w TI added at 100 µg ml⁻¹ and autoclaved TI added at 100 µg ml⁻¹.

^x PDB = potato dextrose broth. The values shown here are means of pooled data from two experiments.

^y Within columns, values followed by the same letter did not differ significantly by the least significant difference test.

^z ND = not determined.

Fig. 4. Time course production of *Aspergillus flavus* amyloglucosidase in 10% potato dextrose broth in the presence of different trypsin inhibitor (TI) concentrations. The production of amyloglucosidase (mean ± standard error) in filtrates (per ml) amended with TI at 0 (●), 50 (■), 100 (▲), or 200 (▼) µg ml⁻¹ was calculated from the total amyloglucosidase enzymatic activity.

demonstrating that fungal growth is inhibited at this same TI concentration (10). However, when the medium was amended with TI at 200 $\mu\text{g ml}^{-1}$, an initial reduction of fungal α -amylase production in the first 16 h followed by a dramatic increase in production was observed. This observation is not well understood, since this same concentration of TI is comparable to that found in kernels resistant to *A. flavus* infection (6). Overproduction of proteins as a means of adaptation to unfavorable conditions has been widely reported in other organisms. Cells of the plastid-free mutant line of *Euglena gracilis* var. *bacillaris* can be adapted to tolerate 6 mM glyphosate in the culture medium through selective approximately 10-fold overproduction of the multifunctional *arom* protein (27). The overproduction of ATP-binding cassette transporters was found to cause multidrug resistance in laboratory-generated mutants of *A. nidulans* (14,15). Here, the overproduction of α -amylase by *A. flavus* in the presence of TI at 200 $\mu\text{g ml}^{-1}$ in growth medium may further suggest a relationship between TI effects on fungal growth and its effect on α -amylase.

Overproduction of fungal α -amylase was observed after 16 h, while fungal growth was still inhibited even at 24 h. However, these data do not contradict each other, but merely indicate the presence of an expected time lag between increased enzyme production and its resultant fungal growth. The effect of TI on the production of fungal α -amylase inside resistant corn kernels (in vivo) can be significantly different from in vitro observations and needs to be documented. The overproduction of α -amylase in the presence of a high concentration of TI may, however, discount the possibility that TI lowers α -amylase levels by blocking its secretion into the medium rather than by inhibiting its actual production.

Several other observations in the current study are worth noting. First, an inverse correlation was observed between the level of α -amylase and the level of amyloglucosidase in the fungal growth medium. This correlation suggests that amyloglucosidase may play an important role in providing the fungus with glucose as a carbon source for growth when the α -amylase level is low. It also suggests that complete inhibition of fungal growth and, therefore, aflatoxin production may not be feasible by merely inhibiting α -amylase. Second, a slight reduction of amyloglucosidase activity on a per-milligram-of-protein basis by TI at 100 $\mu\text{g ml}^{-1}$ was observed for the enzyme in starch medium filtrate. This could be the result of more α -amylase being present in the starch medium filtrate than in the PDB medium filtrate on a per-milligram-of-protein basis (*A. flavus* produces many additional hydrolytic enzymes when grown in PDB medium) (Z.-Y. Chen, R. L. Brown, K. E. Damann, and T. E. Cleveland, unpublished data) and a reduced amount of maltose available for conversion to glucose.

Three isoforms of α -amylase were observed in filtrates of *A. flavus* from 3-day-old PDB cultures in activity-stained native gels, even though only one α -amylase (21,37) and one gene (C. P. Woloshuk, personal communication) coding for α -amylase have been found in *A. flavus*. It is possible that these isoforms were the products of the same gene, but underwent different posttranslational modifications under different growth conditions. It is also possible that these isoforms were the products of different, but homologous genes, that were expressed under different growth conditions. Further research is needed to clarify this issue.

In summary, our results suggest that inhibition of fungal growth by TI may be partially due to its inhibition of fungal α -amylase production and, to a lesser extent, its activity, thereby limiting the amount of carbon source for fungal growth. However, the ability of *A. flavus* to adapt to TI inhibition is also evident in the transient up-regulation of amyloglucosidase levels during initial incubation with TI at a concentration of 100 $\mu\text{g ml}^{-1}$ or higher and the overproduction of α -amylase in the presence of TI at 200 $\mu\text{g ml}^{-1}$. Reduced α -amylase activity and production by TI may also be directly related to lower levels of aflatoxin production in resistant kernels. In a recent study, Woloshuk et al. (37) found that inducers of aflatoxin biosynthesis were generated by an amylase activity from *A.*

flavus. Further investigation is needed to clarify the relationship between TI levels and fungal α -amylase production in vivo.

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