A Maize Trypsin Inhibitor (ZmTIp) with Limited Activity against Aspergillus flavus

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ABSTRACT

Infection of maize both pre- and postharvest by Aspergillus flavus is a severe agricultural problem in the southern United States. Aflatoxins are secondary metabolites produced by A. flavus and are carcinogenic to humans and animals upon ingestion. Extensive research has been conducted to identify sources of resistance to A. flavus in maize. Some maize genotypes exhibit greater resistance to A. flavus than others. Many research groups have validated the role of plant trypsin inhibitors (TIs) as a means of plant defense against fungal infection. Research consisting of the cloning, expression, and partial characterization of one previously uncharacterized TI protein has been conducted. The overexpressed protein displayed TI activity, as expected, and some ability to alter germination of conidia (8%) from several fungal pathogens and to inhibit hyphal growth (30%). This effect on fungal growth, although less than that of previously investigated TIs, marks this protein as a potential source of resistance to aflatoxin accumulation in maize.

Aflatoxins are secondary metabolites produced by Aspergillus flavus and are potent carcinogens. Their presence in food and feed products reduces the marketability of these goods and poses serious health risks to humans and domestic animals (3). Contamination of maize by A. flavus is a recurrent problem in the southern United States, and post-harvest elimination strategies are not sufficient to prevent contamination. Consequently, research efforts have focused on prevention of preharvest contamination (7). Agricultural losses associated with mycotoxins average $630 million to $2.5 billion annually (14). Yield losses, restricted markets, increased transportation costs, increased costs to sell, and large amounts of nonmarketable product all result from aflatoxin contamination (8). Widespread aflatoxin infection impacts farmers directly in terms of monetary losses and consumers as a whole when contaminated agricultural products reach the market.

Contamination of a crop with aflatoxins can occur both preharvest and during storage. One approach to reducing crop contamination by aflatoxin is through the utilization of host resistance to A. flavus colonization (9). Trypsin inhibitors (TIs) from several plant species such as wheat and maize have been associated with antifungal activity (3). TIs are protease inhibitors, of various sizes and amino acid sequences, that inhibit the activity of trypsin (2). The ability of these proteins to provide protection to a plant by inhibiting fungal infection may warrant their use in the breeding and screening of maize genotypes resistant to A. flavus infection. A 14-kDa maize TI has been linked to resistance to A. flavus (3). Seven maize genotypes resistant to aflatoxin accumulation exhibited a higher level of TI accumulation than did more susceptible genotypes (3). On closer examination, the researchers determined that the TI protein also deterred fungal growth and spore germination, often causing the rupture of conidia (3). Reduced fungal growth is correlated with reduced aflatoxin production (2). Although overall antifungal activity may be largely dependent upon a series of processes that include proteins working in concert and the environment within the plant, this 14-kDa TI plays an important role in the resistance of maize to A. flavus infection.

In the present study, a previously uncharacterized TI from maize was cloned and expressed, and its potential fungicidal properties were investigated. Characterization of the mode of action of such TI proteins could aid in the future development of selectable markers to be used by programs breeding for resistance to A. flavus infection.

MATERIALS AND METHODS

Amplification and cloning of the ZmTI gene. The primers 5’ ATGCGGTGTCAGCCGAC 3’ and 5’ CTAATCGTCGTTGTCTGTCGTCGAC 3’ were used to amplify maize cDNA for ZmTI (NCBI accession no. AY111599) from a maize cDNA library (Dr. Robert Schmidt, University of California, Berkeley).

Protein expression and purification. The Champion pET Directional TOPO Expression Kit with Lumio Technology (Invitrogen, Carlsbad, CA) was used to express ZmTI. Primers used were 5’ CACCATGCGGTGTCAGCCGAC 3’ (forward) and 5’ CTAATCGTCGTTGTCTGTCGTCGAC 3’ (reverse). A CACC overhang was added to the forward primer to ensure directional insertion during cloning. The PCR was conducted with GoTaq
Flexi DNA polymerase (Promega, Madison, WI) under the following conditions: one cycle at 95°C for 2 min; 35 cycles of 1 min at 95°C, 30 s at 57°C, and 1 min at 72°C; with a final extension of 15 min at 72°C. PCR products amplified from the cDNA were cloned into the vector pEAT160/GW/D-TOPO (Invitrogen) according to the protocol provided. The Escherichia coli strain BL21-CodonPlus (DE3)-RILP (Stratagene, Cedar Creek, CA) was used to express the protein with the addition of 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG). Expression of the ZmTI was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis on a 12.5% (wt/vol) 29:1 bis-acrylamide:acrylamide gel and was further verified through utilization of Lumio technology (Invitrogen). The 6xHis-tagged expressed protein was purified with an Ni-NTA agarose column (Invitrogen). The purified protein was dialyzed against a 1 M phosphate buffer (pH 7.0). The final concentrations of the proteins were determined using a Bradford assay kit (Bio-Rad, Hercules, CA) with bovine albumin as a standard.

BAPNA-based inhibition assays. Activity of the protein, ZmTIp, was assayed using N-benzoyl-DL-arginine-p-nitroanilide (BAPNA; Acros, Morris Plains, NJ); 0.1 ml of a 100 μg/ml solution of trypsin from porcine pancreas (Sigma Aldrich, St. Louis, MO) was mixed with 0.06 ml of 1 mM BAPNA in 0.1 M Tris-HCl buffer (pH 8.2), for a final volume of 0.2 ml per cuvette. Trypsin activity was monitored at 405 nm with a Spectronic Genesys 5 spectrophotometer for 30 min. To test for inhibitory activity, the inhibitor protein was incubated with the trypsin at 25°C for 10 min before BAPNA was added. A comparable amount of three control TIs from Glycine max (T9003, 93618, and 93620; Sigma Aldrich) were used as positive controls.

Fungal strains and culturing. A. flavus, Aspergillus parasiticus, Fusarium graminearum, F. verticilloides, and Penicillium aurantiogriseum were cultured on potato dextrose agar plates (Becton Dickinson, Sparks, MD) and incubated at room temperature for 2 weeks. To prepare the conidia for assays, plates were scraped with an excess of phosphate-buffered saline (PBS) buffer (pH 7.4), cells were counted, and dilutions of the suspension were made as needed.

RESULTS

Expression and purification of ZmTIp. The primers 5’ CACCATGGCGTCTCAGAC 3’ (forward) and 5’ CTATCCGTCGTTGTCACGC 3’ (reverse) were used to amplify AY111559 from a maize endosperm cDNA library prior to cloning in the expression vector. IPTG-induced expression of BL21-CodonPlus E. coli and subse-
sequent purification by affinity chromatography with an Ni-NTA column resulted in the isolation of a protein that separated as a single band, close to 10 kDa in size, with SDS PAGE analysis (Fig. 1).

**BAPNA-based inhibition assays.** The ability of ZmTIp to inhibit trypsin activity was determined via a BAPNA-based assay, using three trypsin inhibitors from soybean as controls. ZmTIp inhibition of a trypsin from porcine pancreas was comparable to that exhibited by the control inhibitors. ZmTIp total overall inhibition was approximately 84% after 30 min of incubation; control inhibitors from soybean also produced roughly 84% inhibition under the same conditions.

**Antifungal activity of ZmTIp.** Conidia from maize ear–infecting and mycotoxin-producing fungi *A. flavus*, *A. parasiticus*, *F. graminearum*, *F. verticillioides*, and *P. aurantiogriseum* were exposed to various concentrations of ZmTIp to assess the effect of the protein on the germination of conidia and the development of hyphae. After 1 week of incubation with the conidia, 600 μg/ml ZmTIp caused an average of 8% decrease in germination in all the fungal species tested (Figs. 2 and 3). At this high concentration, ZmTIp also seemed to alter the shape of the germination tubes (Fig. 2); the tubes were thicker and more segmented than those produced by conidia suspended in a PBS buffer control. The micrograph in Figure 2 shows hyphae from *A. flavus*. Similar alterations in the hyphae were observed in the other fungal species (not shown). The effect of ZmTIp on hyphal growth was assessed after 24 h of incubation with the protein. At 600 μg/ml, ZmTIp reduced hyphal length by approximately 30% (Fig. 3).

In a previous study, a 14-kDa TI from maize delayed fungal germination, shortened hyphal length, and ruptured walls of *A. flavus* and *F. verticillioides* and affected the conidia of eight other fungal plant pathogens that often co-exist in vivo (2, 3). The 14-kDa protein inhibited germination and hyphal growth in *A. flavus* by 90% when conidia were exposed to the trypsin inhibitor at 100 μg/ml.

**Temperature and pH Stability of ZmTIp.** ZmTIp remained stable and continued to demonstrate strong inhibitory activity after incubation at temperatures ranging from 30 to 90°C; inhibition remained at 83% or higher. ZmTIp activity also remained stable between pH 4.0 and pH 12.0, with inhibition ranging from 73 to 83%.

**DISCUSSION**

TIs function by limiting the bioavailability of trypsin. They prevent trypsin from binding chymotrypsin, an en-
zyme capable of performing proteolysis. TIs are often constitutively expressed within a plant, but induced expression has been noted in plant defense responses. Although not noted to be highly toxic to humans or animals, plants with high concentrations of TIs have lower digestibility. Treatments such as boiling to denature TI proteins can increase the nutritional quality of seeds with a TI high content (10). Several TI proteins, including a 14-kDa TI and a 22-kDa TI from maize, a wheat seed TI, and a barley TI, have been linked to resistance to A. flavus infection and aflatoxin accumulation (3, 4, 13). Opaque-2, a maize line noted to contain high concentrations of expressed TIs, is less likely to be contaminated with A. flavus, F. verticillioides, Alternaria tenuis, and Periconia circinata (3).

ZmTlp has a relatively small size compared with previously documented TIs, which range from 14 to more than 20 kDa. The antifungal activity of ZmTlp, although not as significant as that reported for other TIs, is still potentially detrimental to the fungus and thus makes ZmTlp important for its ability to provide resistance to fungal infection.

Several TIs have been characterized that possess stability similar to that of ZmTlp in terms of temperature and pH. PpTI, a TI from Poecilanthe parviflora seeds, remained stable after exposure to various temperatures and pH conditions; protein activity remained after incubation for 30 min at temperatures ranging from 37 to 100°C and from pH 2.0 to pH 10.0 (6). Similar stability was reported for TIs from Schizolobium parahyba and Inga laurina seeds (8, 11). The inhibitor from S. parahyba, SpTCl, maintained a high level of activity from pH 2.0 to pH 12.0 (12) as did an inhibitor from Enterolobium contortisiliquum seeds (1) and a TI from onion bulbs (5).

We investigated a previously uncharacterized TI protein from maize with some antifungal activity against A. flavus and several other plant pathogens. Although the antifungal activity in our assays was less than that reported for other TIs, we cannot rule out the possibility that the activity of ZmTlp in the plant may be enhanced by the presence of other antifungal compounds, rendering ZmTlp an important source of resistance to fungal pathogens in maize.

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REFERENCES


