Identification of a Maize Kernel Pathogenesis-Related Protein and Evidence for Its Involvement in Resistance to Aspergillus flavus Infection and Aflatoxin Production

Z.-Y. Chen, R. L. Brown, K. Rajasekaran, K. E. Damann, and T. E. Cleveland

ABSTRACT


Aflatoxins are carcinogens produced by Aspergillus flavus and A. parasiticus during infection of susceptible crops such as maize. Several aflatoxin-resistant maize genotypes have been identified and kernel proteins have been suggested to play an important role in resistance. In the present study, one protein (#717), which was expressed fivefold higher in resistant than susceptible maize kernels, was identified using proteomics. This protein was sequenced and identified as a pathogenesis-related protein (PR-10) based on its sequence homology. To assess the involvement of this PR-10 protein (ZmPR-10) in host resistance of maize against fungal infection and aflatoxin production, the corresponding cDNA (pr-10) was cloned. It encodes a protein of 160 amino acids wi...
based on peptide sequences obtained through electrospray ionization tandem mass spectrometry (ESI-MS/MS), examined its expression during kernel development with or without fungal infection using real-time reverse-transcriptase polymerase chain reaction (RT-PCR), and demonstrated RNase activity of the protein in vitro using the ZmPR-10 protein overexpressed in and purified from Escherichia coli. Further, we transformed tobacco plants with maize pr-10 and demonstrated increased antifungal activity in the leaf extracts of transgenic plants against A. flavus and Verticillium dahliae compared with the control plants. A preliminary report has been published (9).

MATERIALS AND METHODS

Chemicals and materials. Chemicals for two-dimensional (2-D) protein gels and kernels of resistant (Mp420, Mp313E, and GT-MAS:sk) and susceptible (B73, Mo17, and Va35) maize genotypes were obtained from the same sources as in a previous study (10). Resistance or susceptibility of these genotypes to aflatoxin accumulation was determined by breeders in field trials repeated over different environments and times using artificial and natural infection (6,39,46). Aflatoxin standards were purchased from Sigma-Aldrich (St. Louis) and prepared according to the manufacturer’s instructions.

Protein extraction and gel electrophoresis. Kernels (20 g) from each genotype were dissected into embryo and endosperm as described (10). Endosperm tissues were frozen in liquid nitrogen and ground to powder in an analytical mill (Tekmar A-10; Janke and Kunkel GmbH & Co., Staufen, Germany). Powdered endosperm was extracted with an extraction buffer (0.25 M NaCl, 50 mM Tris-HCl [pH 8.0], 14 mM β-mercaptoethanol) (2 ml/g) for 2 h at 4°C, and then supernatant was recovered and desalted as described (10). The extracted protein was resolubilized in lysis buffer (9.5 M urea, 2% CHAPS, 1% dithiothreitol, 0.8% [wt/vol] immobilized pH gradient buffer, pH 3 to 10, and 0.04% Pefabloc protease inhibitor) at a final concentration of 2 µg/µl for analytical gels and 20 µg/µl for preparative gels.

Endosperm protein (analytical, 50 µg; and preparative, 700 µg) was applied to rehydrated 17-cm Immobiline DryStrip gels (pH 3 to 10). First- and 2-D gel electrophoresis was performed as described (10). The extracted protein was resolubilized in lysis buffer (9.5 M urea, 2% CHAPS, 1% dithiothreitol, 0.8% [wt/vol] immobilized pH gradient buffer, pH 3 to 10, and 0.04% Pefabloc protease inhibitor) at a final concentration of 2 µg/µl for analytical gels and 20 µg/µl for preparative gels.

Protein extraction and gel electrophoresis. Kernels (20 g) from each genotype were dissected into embryo and endosperm as described (10). Endosperm tissues were frozen in liquid nitrogen and ground to powder in an analytical mill (Tekmar A-10; Janke and Kunkel GmbH & Co., Staufen, Germany). Powdered endosperm was extracted with an extraction buffer (0.25 M NaCl, 50 mM Tris-HCl [pH 8.0], 14 mM β-mercaptoethanol) (2 ml/g) for 2 h at 4°C, and then supernatant was recovered and desalted as described (10). The extracted protein was resolubilized in lysis buffer (9.5 M urea, 2% CHAPS, 1% dithiothreitol, 0.8% [wt/vol] immobilized pH gradient buffer, pH 3 to 10, and 0.04% Pefabloc protease inhibitor) at a final concentration of 2 µg/µl for analytical gels and 20 µg/µl for preparative gels.

Peptide sequencing and database sequence homology analysis. Protein #717 was recovered from five preparative 2-D gels and sequenced as previously described using PE SCIEX API 3000 (Applied Biosystems, Foster City, CA) ESI-MS/MS equipped with a Protana nanospray source (Odense, Denmark) (10). Peptide sequence homology searches were performed using BLAST (1) against known proteins or translated open reading frames of expressed sequence tags (ESTs) in databases at the National Center for Biotechnology Information (NCBI) and SWISS-Prot.

Cloning of full-length pr-10 cDNA. Two degenerate primers were made, 5′-CCIGT(G/C/T)GA(G/A)GGIGA(C/T)TG-3′ (PR-10-F1) and 5′-GG(G/A)TTTGCG(C/G/A)ACIAG(G/A)TAIGC(C/T)-TC-3′ (PR-10-R1), based on peptide sequences PVEGDG and EAYLVANP, respectively. The first round of PCR was performed in 50 µl using M13(-20) and PR-10-R1 primer pair and 1 µl of undiluted maize endosperm cDNA library made from Ohio 43 kernels 10 to 14 days after pollination (titer 1.1 × 10⁷ plaque forming units per ml) (provided by R. J. Schmidt, Division of Biology, University of California, San Diego). The second-round PCR was performed using the PR-10-F1 and PR-10-R1 pair and 1 µl of 1:20 diluted first-round PCR product as the template. The amplified 0.4-kb PCR product was cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). DNA sequences of five independent clones were identical and aligned to the rice pr-10 gene. The remaining sequences of pr-10 cDNA were cloned using 5′ and 3′ rapid amplification of cDNA ends (RACE) as previously described (11) with primers from the cDNA library vector and gene-specific primers (PR-10-F2: 5′-ACTTACAAGCTGCTGCC-3′ and PR-10-F3: 5′-AGGTGAAGAGGACGATCGGCC-3′ for cloning the 3′ end; PR-10-R2: TCCAGGAACTCGAGCTTCTC-3′ and PR-10-R3: GAACGGCATGACTGAGGTA-3′ for cloning the 5′ end) derived from the sequenced 0.4-kb PCR product.

Construction of vectors for bacterial and plant expression. The coding region of pr-10 cDNA was amplified using two primers (PR-10-Nco I: TGAAGATACGTCGCTCC, and PR-10-Sac I: AATGGAAGCTCATCCAGT). The nucleotide changes (lower-case) to incorporate an NcoI site at the 5′ end (by changing TA to CC) and a SacI site at the 3′ end (by changing T to C) did not alter any amino acid sequence of the ZmPR-10. The PCR product was digested with NcoI and SacI, gel purified, and then cloned into corresponding sites of the pET28c vector (Novagen, Madison, WI) and the pBS-d35S-D4E vector (provided by J. Cary, United States Department of Agriculture (USDA)–Agricultural Research Service, Southern Regional Research Center) to generate pET28c-PR-10 and pBS-d35S-PR-10, respectively. The latter then was digested with HindIII and SacI to excise the 1.4-kb d35S-PR-10 insert, which then was cloned into the HindIII and SacI double-digested and dephosphorylated pBl121 vector. The resulting construct was named pBl121-d35S-PR-10-nos (Fig. 1). Correct constructs were verified during each step of cloning through DNA sequencing.

Genomic DNA and total RNA isolation. Genomic DNA of resistant maize genotype Mp420 or transgenic tobacco was isolated from young leaf tissue according to the instructions of the Redextract-N-Amp Plant PCR kit (Sigma-Aldrich). The pr-10 genomic clone was obtained from Mp420 genomic DNA using primers corresponding to the 5′ and 3′ ends of pr-10 cDNA. Total RNA was isolated from immature maize kernels of different developmental stages or from tobacco leaf tissues using the RNeasy Plant mini kit (Qiagen, Valencia, CA) according to the manufac-
turer’s instructions. The optional DNase treatment was included to remove trace DNA contamination before proceeding to real-time RT-PCR.

Expression of the pr-10 gene during different stages of kernel development with or without A. flavus inoculation. The expression of the pr-10 gene during kernel development was examined in one resistant (GT-MAS-gk) and one susceptible (Mo17) genotype. One-third of the plants from each genotype was inoculated in the field with an A. flavus conidial suspension at 5 × 10⁶/ml using a syringe 7 days after pollination (DAP). Another one-third was wounded with a syringe needle without inoculum to serve as a wounded-only control. The remaining one-third was used as a noninfected (nonwounded) control. Kernels were collected at 0, 3, 8, and 15 days after inoculation (7, 10, 15, and 22 DAP, the time period with active gene expression, protein biosynthesis, and linear grain fill inside kernels). For wound-only and inoculated ears, only the healthy-looking kernels surrounding the wounded or infected kernels were harvested.

Total RNA was reverse transcribed into cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. The level of pr-10 expression was then quantified using ABI 5700 Thermal Cycler with SYBR Green dye chemistry (Applied Biosystems). Primers used in real-time PCR (PR-10-F, 5′-CACCTCAGTCA-AGCCGTCTCA; and PR-10-R, 5′-CCCCTCGATGCGGTGTTCTT) were designed using Primer Express 2.0 (Applied Biosystems). The amplicon size was 90 bp. PR-10-F was designed to span the intron of its genomic DNA sequence so that it only amplifies cDNA sequence. To check the specificity of annealing of the oligonucleotides, dissociation kinetics was performed by the machine at the end of the experiment. In addition, each amplified product was sequenced. The expressions of 18S and actin (forward: CT-CAGGCCAAGAGGACCAC, and reverse: TGCCATGCTCAATCGGGTA) in maize kernels were used as internal controls to normalize the expression of pr-10 gene (27). This experiment was repeated at least twice for cDNA prepared for two batches of isolated RNA samples. Using standardized conditions, deviations of threshold values were less than 1.0 cycle for independent cDNA preparations and less than 0.5 cycle for replicates of the same cDNA. The pattern of pr-10 expression was the same when normalized to either 18S or actin. Data are mean expression levels of pr-10 normalized to actin.

Overexpression of ZmPR-10 in E. coli and partial purification. The correct in-frame fusion of the pET28c-PR-10 construct was transformed into an E. coli BL21 (DE3) expression host. The overexpressed ZmPR-10 was predicted to be 16.9 kDa, containing the full-length mature ZmPR-10 sequence (160 amino acid residues) without extra sequence from the vector. Isopropyl-β-D-thiogalactopyranoside induction of pr-10 expression and E. coli cell disruption were performed as previously described (12). Cell disruption and fractionation were performed at 4°C. Inclusion bodies, which contained mainly the overexpressed ZmPR-10, then were recovered and resuspended in 100 ml of 50 mM Tris-HCl (pH 8.0) containing 6 M urea. Urea-soluble proteins were then separated from the urea-insoluble fraction by centrifugation (18,000 × g, 20 min) and used to refold into the active form as described previously (12). The purity of the protein then was assessed by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (29) before storage in aliquots at −70°C. The content of overexpressed ZmPR-10 in each fraction was quantified with a Bio-Rad GS-700 gel densitometer.

Nucleic acid degradation assay. The refolded 17-kDa ZmRP-10 protein (2 µg) isolated from E. coli was incubated with 15 µg (2 µg/µl in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) of total RNA from yeast (Sigma-Aldrich), A. flavus, and calf (Sigma) for 5 h at 37°C. At the end of incubation, the protein was removed by extraction with phenol-chloroform and the nucleic acids were separated on 1.2% agarose gels and stained with 0.003% ethidium bromide (2). The brighter the intensity of the stained RNA samples, the less RNA degradation and, therefore, the lower the RNase activity. Controls include RNA treated with sterile water, with proteins from noninduced E. coli cells, or with heat-inactivated purified ZmPR-10 protein from E. coli.

In vitro antifungal activity assay of ZmPR-10. The antifungal activity of overexpressed ZmPR-10 against the growth of phytopathogenic fungi, such as A. flavus and V. dahliae, was assayed in 10% potato dextrose broth (PDB) (Difco Laboratories, Detroit) using microtiter plates as described previously (13). Seven-day-old A. flavus conidia or V. dahliae microconidia were allowed to germinate and grow in the presence of 10% PDB supplemented with overexpressed ZmPR-10 at concentrations ranging from 5 to 400 µg/ml at 30°C (A. flavus) or 22°C (V. dahliae) for 24 h. The negative control was 10 mM phosphate buffer (pH 7.0) containing heat-inactivated proteins at a concentration of 100 µg/ml. The hyphal growth of control or treated fungi was examined after 8 and 24 h of incubation under a microscope as previously described (13), and at least 40 measurements of hyphal length were taken for each treatment. The data presented here were combined from two repeated experiments.

Tobacco transformation. The pBI121-d35S-PR-10 construct was transformed into electrocompetent Agrobacterium tumefaciens LBA4404 (Gibco-BRL, Bethesda, MD) via electroporation using a Cell-Porator (Bio-Rad, Hercules, CA) according to the manufacturer’s instruction. The transformation of tobacco (Nicotiana tabacum L. cv. SR-1) leaf disks and the selection and regeneration of transgenic tobacco plants expressing the maize pr-10 gene were done according to the procedures described by Horsch et al. (23).

Confirmation of pr-10 transformation and expression. PCR of the maize pr-10 gene from genomic DNA of transgenic tobacco leaves was performed with primers spanning from within the d35S promoter (5′-ATGACGGCAATCCACTATCCT-3′) (d35S-For) to the 3′ end of the pr-10 gene (PR-10-Sac I) using AmpliTaq Gold polymerase (Stratagene, La Jolla, CA). PCR products were analyzed by gel electrophoresis.

Real-time RT-PCR was used to examine the transcript level of pr-10. Total RNA was isolated from tobacco leaves of seven transgenic plants that were confirmed to have the gene, reverse-transcribed, and amplified as described above. In addition to 18S, the expression of actin also was determined and used as an internal control. The pattern of pr-10 expression was the same when normalized to either 18S or actin. This experiment was conducted twice, each time with two replicates. The level of pr-10 expression presented in this article is the mean value (combined from two repeated experiments) normalized to the level of actin.

Antifungal assays of transgenic tobacco plants. Leaves from selected transgenic (numbers 4, 2, and 5, which express maize pr-10 at high, medium, and low levels, respectively), and control (number 8) tobacco plants (transformed with pBI121) were ground with liquid N2 with no buffer added. Ground tissues were then centrifuged at 8,200 × g for 10 min at room temperature to collect leaf extract from each sample. The total protein concentration of the leaf extracts of different samples ranged from 0.27 to 0.34 mg/ml; each was adjusted to 0.27 mg/ml for the antifungal assay. Conidia (25 µl of 10⁵ conidia/ml) then were added to 225 µl of plant extracts containing ≈60 µg of total protein, mixed, and incubated for 12 h at 30°C (A. flavus) or 22°C (V. dahliae) in a 12-well macrotriter plate (7). At the end of incubation, fungal hyphal growth then was examined under a microscope as previously described (13) and measurements of at least 40 individual hyphae were taken for each treatment. The data presented here were combined from two repeated experiments.

Statistical analysis. All data were analyzed using the analysis of variance procedure of the Statistical Analysis System (SAS Institute, Cary, NC). Mean separations for pr-10 expression in
maize kernels during different developmental stages and in transgenic tobacco leaf tissues were performed using the method of Duncan’s multiple range test ($P \leq 0.05$).

**RESULTS**

Protein identification. Endosperm proteins from three resistant and three susceptible maize genotypes were compared using proteomics. Gel analysis of differential protein expression between resistant and susceptible genotypes identified one protein (#717), with expression fivefold higher in resistant genotypes GT-MAS:gk, Mp420 and Mp313E than in susceptible maize lines Mo17, B73, and Va35 (Fig. 2). This protein, with an estimated molecular weight (MW) of 15.86 kDa and a pI of 5.30, was recovered from Coomassie brilliant blue R-250-stained preparative 2-D gels and subjected to in-gel trypsin digestion. The digested peptides were sequenced using ESI-MS/MS (Fig. 3). The peptide sequences obtained from MS were shown in the inset of Figure 3. Peptide sequences of #717 matched portions of PR-10 from sorghum (T14718), and are highly homologous to a portion of intracellular pathogenesis-related proteins from asparagus (Asparagus officinalis, CAA10720), lily (Lilium longiflorum, AAF21625), and white lupin (Lupinus albus, AJ000108) (LaPR-10).

Cloning of pr-10 gene from maize and sequence homology analysis. A 0.4-kb PCR product was obtained from a maize endosperm cDNA library made from Ohio 43 immature kernels of 10 to 14 DAP after two rounds of PCR amplification using two degenerate primers. DNA sequencing of the cloned PCR product and homology of the deduced amino acid sequence to rice and sorghum PR-10 proteins confirmed that it is part of the pr-10 gene. The 5’ and 3’ ends of the pr-10 gene were cloned by RACE, revealing a complete cDNA of 620 bp with an open reading frame of 540 amino acids, with a calculated MW and pI of 16.9 kDa and 5.38, respectively (Fig. 4). These values are similar to those determined from 2-D gels. Cloning of the corresponding genomic DNA (713 bp) also revealed the presence of an intron of 93 bp (Fig. 4). The sequence has been deposited into GenBank under accession no. AY953127. The deduced ZmPR-10 sequence does not appear to contain a signal peptide sequence according to prediction by SignalP 3.0 Server, which agrees with previous reports that PR-10 is an intracellular protein (24). The protein is also quite hydrophilic, with only one possible membrane-spanning helix based on the hydrophobicity distribution analysis.

The homology search of the deduced amino acid sequence of ZmPR-10 showed that it shares 81.4 and 85.6% identity with PR-10 protein from sorghum and rice, respectively (Fig. 5), which confirmed the identity of the cloned gene. It also showed 51.9% identity to intracellular pathogenesis-related proteins from lily (AAF21625), and asparagus (CAA10720), and ≈30 to 35% identity to a pathogenesis-related protein from white lupin (AJ000108), a ribonuclease from ginseng (P80890) (33), and major food allergens of celery (S30055) (3) and apple (T17007) (22) (Fig. 5).

A BLAST homology search of maize ESTs identified two maize ESTs (A1812193 and BM073462) whose deduced amino acid sequences showed a complete match to the deduced peptide sequence of the cloned pr-10 cDNA, and three additional ESTs (AK367898, BI643519, and CF630679) with only one mismatch in the peptide sequence with Zm-PR-10. The pr-10 cDNA had only one nucleotide difference in the overlapping region with BM073462, its closest match.

Temporal expression of pr-10 in maize with or without fungal infection. The transcript level of pr-10 increases significantly during early kernel development in noninfected plants, with a fivefold increase in the 15-day period (7 to 22 DAP) in both resistant (GT-MAS:gk) and susceptible (Mo17) genotypes (Fig. 6). However, the pr-10 transcript level was higher in the noninfected Mo17 kernels than that in GT-MAS:gk (significant at day 3 and 15). Upon Aspergillus flavus infection, the expression of pr-10 was upregulated significantly at day 8 and 15 in GT-MAS:gk, whereas its expression remained the same or decreased at day 8 and 15 in the susceptible genotype compared with noninfected control kernels (Fig. 6). The expression of pr-10 did not change significantly in the wound-only controls compared with the nonwounded controls at the same stage (Fig. 6).

Expression of pr-10 in E. coli. After 3 h of induction, the level of ZmPR-10 was ≈35% of total E. coli cell proteins (Fig. 7, lane 3). However, the majority of ZmPR-10 protein was not soluble in

![Fig. 2. Sections of large-format two-dimensional gels containing endosperm proteins extracted from three resistant (top) and three susceptible (bottom) maize genotypes. One protein (#717), which was expressed fivefold higher in resistant lines than in susceptible ones, was indicated with arrows. R, resistant genotype; S, susceptible genotype.](image)
water or low salt buffers and remained in the pellet, although it became soluble in the presence of 6 M urea (Fig. 7). By taking advantage of this characteristic, ZmPR-10 protein was significantly enriched (accounting for 85% of urea-soluble protein) in urea-soluble protein fraction (Fig. 7, lane 5). Urea then was removed slowly through dialysis in the presence of cystamine to allow protein refolding. This refolded protein fraction contains mainly the 17-kDa ZmPR-10 and a few minor proteins (Fig. 7, lane 7). These minor proteins are possible breakdown products or multimers of ZmPR-10, as has been previously observed (12), because none of these protein bands were present in the control protein sample refolded from a urea-soluble fraction of non-induced E. coli cells (Fig. 7, lane 8).

**RNase activity of overexpressed ZmPR-10 protein.** The above overexpressed and refolded ZmPR-10 protein demonstrated RNase activity in vitro assays against yeast RNA. The RNase activity was abolished when the refolded ZmPR-10 was incubated at 80°C for 15 min (data not shown), confirming that the ribonucleolytic activity is of a protein origin. Less than 10% of total RNase specific activity was detected in the control sample prepared from noninduced E. coli cells or in the nonfolded preparations compared with the refolded ZmPR-10. The ZmPR-10 protein also was active in vitro against RNAs isolated from A. flavus and calf (data not shown).

**In vitro antifungal activities of ZmPR-10 from E. coli.** This partially purified and refolded ZmPR-10 protein also exhibited an inhibitory effect in vitro on conidia germination and hyphal growth of A. flavus. The A. flavus conidia treated with ZmPR-10 at 100 µg/ml showed delayed germination and slower hyphal growth once they germinated compared with the control treated with heat-inactivated ZmPR-10. The mean hyphal length of control culture and treated culture was 67.8 ± 12.6 µm (standard error) and 0 (not germinated yet) at 8 h, and 194.7 ± 31.1 and 76.9 ± 14.5 µm at 24 h, respectively (Fig. 8). The 50% inhibition of fungal growth compared with the control was achieved in the presence of ZmPR-10 at 80 µg/ml (4.73 µM). The inhibitory Fig. 3. Electrospray ionization peptide mass spectrum of a peptide mixture from in-gel tryptic digestion of protein #717. Peptide sequences corresponding to peaks a, b, and c are shown in the inset; “m/z” stands for mass to charge ratio.
effect on *A. flavus* was observed in the presence of as low as 25 µg of protein per milliliter (data not shown). It also was noticed that *A. flavus* is less sensitive to ZmPR-10 than *V. dahliae*, which requires approximately half as much protein to see the same growth inhibition effect (data not shown).

**Transgenic tobacco expressing maize pr-10.** Twenty tobacco leaf disks were infected with *Agrobacterium tumefaciens* containing the pBI121-d35S-PR-10-tnos construct (Fig. 1), and seven plants regenerated from seven independent transformation events were selected and tested for the presence of maize pr-10 gene using PCR. A 0.7-kb PCR product was amplified from genomic DNAs of all seven transformants, and DNA sequencing of the PCR product confirmed the presence of maize pr-10 gene (data not shown).

The expression level of maize pr-10 gene relative to actin in transgenic tobacco leaves varies among the seven transformants (Fig. 9). The highest level of pr-10 expression was detected in plant number 4 which was approximately five times the level of that in plant number 5, the lowest level of pr-10 expression among the seven transformants (Fig. 9). No pr-10 expression was detected in the control plant number 8 transformed with pBI121 vector (Fig. 9). It is interesting to note that leaf extracts from those plants that had high levels of pr-10 expression also demonstrated high level of RNase activities against yeast RNA in in vitro assays (data not shown).

**Antifungal properties of transgenic tobacco plants.** An increase in antifungal activity against *Aspergillus flavus* growth was observed in the leaf extracts of transgenic tobacco plants expressing maize pr-10 gene compared with the control leaf extract. The strongest inhibition on fungal hyphal growth was observed in conidia treated for 12 h with leaf extracts from plant number 4 with a mean hyphal length of 46.5 ± 3.8 µm (standard error), compared with that treated with leaf extract from the control plant (plant number 8) with a mean hyphal length of 135.6 ± 7.8 µm. Leaf extract from plant number 2, which showed an intermediate level of pr-10 expression, also demonstrated significant inhibition of fungal hyphal growth (66.8 ± 3.8 µm) when compared with the control. The leaf extract from plant number 5, which had the lowest level of pr-10 expression among the seven transformants, exhibited a level of inhibition on fungal growth (78.2 ± 4.7 µm) similar to that observed for plant number 2, and both were significantly different compared with the control based on the mean hyphal length of *A. flavus*.

![Fig. 6. Expression of pathogenesis-related (PR) protein 10 gene during kernel developmental stages with or without *Aspergillus flavus* infection in maize. Maize ears were inoculated 7 days after pollination. Transcript levels of pr-10 gene were measured at 0, 3, 8, and 15 days after inoculation using real-time reverse-transcriptase polymerase chain reaction and are expressed as relative to actin (internal normalizer) in both resistant (GT-MAS:gk, light bar) and susceptible (Mo17, dark bar) maize genotypes. N, nonwounded and noninoculated controls; W, wounded only; and I, inoculated. The bars labeled with the same letters are not statistically different within and across the genotypes based on Duncan’s multiple range test.](image-url)

![Fig. 5. Sequence homology comparisons of deduced amino acid sequences of pathogenesis-related protein PR-10 from *Zea mays* (this study, accession number AY953127, Maize) with a jasmonate inducible PR protein from *Oryza sativa* (AAL74406, Rice) (26), a PR protein from *Sorghum bicolor* (T14718, Sorghum) (30), an intracellular PR protein, plant MPR-10, from *Lilium longiflorum* (AA21625, Lily) (24), an intracellular PR protein, isoform 6, from *Asparagus officinalis* (CA1A0720, Asparagus.), a PR protein from *Lupinus albus* (AO000108, Lupin) (2), and a ginseng ribonuclease (P80890, Ginseng) (33), as well as major food allergens of celery (S30055) (3) and apple (T17007) (22). The underlined letters indicate the region where the peptide sequence was obtained from electrospray ionization tandem mass spectrometry of spot #717. Gaps (indicated with “-”) were introduced to achieve maximum sequence alignment. Uppercase letters in the consensus sequence indicate identical amino acid residues at the same position in most sequences, lowercase letters indicate highly conserved amino acid residues, and “.” indicates low or no amino acid consensus at that position.](image-url)
DISCUSSION

Comparative investigations of maize genotypes resistant or susceptible to *A. flavus* infection and aflatoxin production have demonstrated the existence of two levels of kernel resistance strategically distributed on the kernel surface (e.g., physical barrier, kernel wax and cutin layer) and inside kernels (e.g., proteins) (15). Recent work determined that both constitutive and induced kernel proteins are required for resistance to aflatoxin production (8), and that one distinct difference between resistant and susceptible lines is the higher level of constitutive expression of antifungal, stress-related, and highly hydrophilic storage proteins in the former, in both the endosperm and embryo (9,10). In the present study, one such protein (#717) was identified in the endosperm of resistant lines compared with susceptible ones as revealed through 2-D gel comparisons, was identified and further investigated. The peptide sequence homology analysis identified this protein as PR-10. Because ZmPR-10 is present in mature kernels, it should be called ZmPR-10-like protein according to the definition of PR proteins (43). However, in this study, it is referred to merely as ZmPR-10 protein for simplicity.

Currently, PR proteins are grouped into 17 independent families, PR-1 to PR-17, and antifungal activities have been described for some (16,44). PR-10s have been reported in a number of plant species, including asparagus (45), lily (24), and rice (32). They also share sequence similarities to major food allergens of celery (3) and apple (22), and to ginseng ribonuclease (33). For this reason, intracellular PR-10s also are classified as RNase-like proteins (43). In the present study, the corresponding cDNA encoding ZmPR-10 was cloned, and it showed significant sequence homology to proteins from the PR-10 family, including one from white lupin that has been shown to possess RNase activity (2).

Evidence linking PR-10 to host resistance has been reported. A barley *pr-10* gene was found to be specifically induced in the epidermis of resistant cultivars upon infection by *Rhynchosporium secalis*, but not in leaf tissues of near-isogenic susceptible plants (40). In cowpea, a *pr-10* homolog was specifically upregulated in resistant epidermal cells inoculated with the rust fungus *Uromyces vignae* Barclay (34). Also, in rice, a *pr-10* transcript was induced during infection with *Magnaporthe grisea* (31) or by salicylic or jasmonic acid applications to rice plants (25,31).

In the present study, the involvement of ZmPR-10 in host resistance against *A. flavus* infection and aflatoxin production was first supported by evidence obtained through proteome analysis of mature kernels demonstrating higher constitutive expression of PR-10 in resistant versus susceptible lines. Supporting evidence also includes developmental expression of its corresponding gene after fungal inoculation, and antifungal activity of both the protein overexpressed in *E. coli* and the transgenic tobacco leaf extracts. During development, the *pr-10* transcript level was observed to be higher in susceptible Mo17 kernels than in the resistant GT-MAS:gk uninoculated plants (at days 3 and 15). However, this higher transcript level during development in the susceptible genotype did not lead to a higher constitutive protein level (as was observed through proteomics) in mature kernels, the target of aflatoxin accumulation. It should be kept in mind that it is the proteins themselves, not the corresponding transcripts, that are involved in biological functions (e.g., antifungal activity).

Overexpressed ZmPR-10 was found to be inhibitory to both *A. flavus* hyphal growth and conidia germination. Based on the IC\textsubscript{50} value, ZmPR-10 is slightly less potent than the previously described 14-kDa TI protein (12). Similar inhibitory effects also were observed when the leaf extracts of transgenic tobacco plants expressing maize *pr-10* gene were used in in vitro bioassays. However, the level of *pr-10* expression in transgenic tobacco appears to be much lower than that observed in developing maize kernels based on data from real-time RT-PCR.

The partially purified and refolded ZmPR-10 from *E. coli* demonstrated, in vitro, a clear RNase activity against RNAs, not only from fungi (yeast and *A. flavus*) but also from an animal (calf

![Fig. 7. Overexpression of maize gene pr-10 in Escherichia coli and partial purification of its protein. Lane 1, MW marker; lane 2, total cell protein from noninduced *E. coli* cells; lane 3, total cell protein from isopropyl-β-D-thiogalactopyranoside-induced *E. coli* cells (the overexpressed protein represents ~30% of total cell proteins); lane 4, water- or salt-soluble protein fraction from induced cells; lane 5, urea-soluble protein fraction from induced cells; lane 6, urea-soluble protein from noninduced *E. coli* cells; lane 7, refolded ZmPR-10 protein from induced urea-soluble fraction; and lane 8, proteins refolded from urea-soluble fraction of noninduced *E. coli* cells.](image)

![Fig. 8. Antifungal bioassay of ZmPR-10 protein. Aspergillus flavus conidia were germinated in 10% potato dextrose broth medium containing refolded ZmPR-10 at 100 µg/ml (right) or heat-inactivated ZmPR-10 at 100 µg/ml to serve as controls (left) for 8 (top) and 24 h (bottom) at 30°C.](image)

![Fig. 9. Expression of the maize pr-10 gene in the leaves of transgenic tobacco plants. The pr-10 expression in transgenic (numbers 1 to 7) tobacco leaf tissues is reported as the ratio relative to the level of actin in the control plant transformed with pBH21 vector only (number 8). The bars labeled with the same letters are not statistically different based on Duncan’s multiple range test.](image)
tRNA). In addition, several transgenic tobacco plants expressing maize pr-10 also showed an increase in RNase activities compared with a vector-only transformed control plant. This is the first reported demonstration of RNase activity of a PR-10 protein from a crop. Furthermore, leaf extracts from plants expressing high levels of pr-10 also showed high levels of RNase activities against yeast RNA in vitro assays. In addition, the leaf extract from plant number 2, expressing the highest level of pr-10, demonstrated the strongest inhibition of A. flavus hylpah growth in vitro assays. However, the linkage between PR-10 RNase activity and its antifungal activity (inhibition on fungal growth) is not understood. The involvement of plant RNase activity in defense against pathogen infection and resistance has been reported (20). An increase in the extracellular RNase activity and enhanced resistance were observed after tobacco was challenged with Phytophthora parasitica var. nicotianae. This protection was found to be durable and effective against other unrelated pathogens.

One speculation concerning RNase activity in resistance is that it may function in determining the compatibility reactions during the interaction of the host and pathogen, similar to the role proposed for the PR-10 homologs as self-incompatibility determinants in pollen. It has been proposed in cases of pollen incompatibility reactions between plants that some PR-10 homologs play a role in the recognition of pollen grains by inducing an inhibition reaction on the stigma surface (5,38).

The relationship between ZmPR-10’s RNase activity and maize kernel aflatoxin resistance still needs much clarification. The findings of the present study do suggest, however, that ZmPR-10 protein is a component of the constitutive defense arsenal of the maize plant.

ACKNOWLEDGMENTS

This study was supported by USDA cooperative agreement 58-64352-2-130 and USDA National Research Initiative Competitive Grant 2002-35201-12541. We thank C. Ambrogio, D. Hadrick, and G. Windham for technical assistance; and H.-J. Kim, K. Ehrlich, and G. Windham for critical reviewing of the manuscript.

LITERATURE CITED


34. Mould, M. J., Xu, T., Barbara, M., Iscove, N. N., and Heath, M. C. 2003. cDNAs generated from individual epidermal cells reveal that differential gene expression predicting subsequent resistance or susceptibility to rust fungal infection occurs prior to the fungus entering the cell lumen. Mol. Plant-Microbe Interact. 16:835-845.


