Identification of Maize Kernel Endosperm Proteins Associated with Resistance to Aflatoxin Contamination by *Aspergillus flavus*

Z.-Y. Chen, R. L. Brown, K. E. Damann, 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 and fourth authors: Southern Regional Research Center, United States Department of Agriculture–Agricultural Research Service, New Orleans 70179.

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ABSTRACT

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Aflatoxins are carcinogens produced mainly by *Aspergillus flavus* during infection of susceptible crops such as maize (*Zea mays*). Previously, embryo proteins from maize genotypes resistant or susceptible to *A. flavus* infection were compared using proteomics, and resistance-associated proteins were identified. Here, we report the comparison of maize endosperm proteins from five resistant and five susceptible genotypes, and the identification of additional resistance-associated proteins using the same approach. Ten protein spots were upregulated twofold or higher in resistant lines compared with susceptible ones. Peptide sequencing of these proteins identified them as a globulin-2 protein, late

embryogenesis abundant proteins (LEA3 and LEA14), a stress-related peroxiredoxin antioxidant (PER1), heat-shock proteins (HSP17.2), a cold-regulated protein (COR), and an antifungal trypsin-inhibitor protein (TI). The gene encoding one such upregulated protein, PER1, was cloned and overexpressed in *Escherichia coli*. The overexpressed PER1 protein demonstrated peroxidase activity in vitro. In addition, *per1* expression was significantly higher in the resistant genotype Mp420 than in the susceptible genotype B73 during the late stage of kernel development, and was significantly induced upon *A. flavus* infection, suggesting that it may play an important role in enhancing kernel stress tolerance and aflatoxin resistance. The significance of other identified proteins to host resistance and stress tolerance also is discussed.

Additional keywords: corn, oxidative stress.

Infection of maize (*Zea mays* L.) kernels in the field (preharvest) and during storage (postharvest) by *Aspergillus flavus* Link:Fries and subsequent contamination with aflatoxins is a recurring problem in the southern United States, especially in dry and hot weather conditions (40). The dominant aflatoxin produced during the infection is aflatoxin B₁, which is the most potent carcinogenic substance produced in nature (46). Aflatoxin contamination not only reduces the value of grain as an animal feed and as an export commodity (38) but also has been linked to increased mortality in farm animals (45) and increased incidence of liver cancer in humans (28).

Currently, measures for controlling aflatoxin contamination in susceptible crops, such as maize, include cultural practices (early planting, crop rotation, irrigation, and rapid drying after harvest), biocontrols, and chemical controls (insecticide) (40). In addition, the genetic engineering approach to enhance maize plant insect resistance (such as Bt corn) has been shown to reduce aflatoxin contamination in areas where high southwestern corn borer infestations occur (58). Enhancing host plant resistance to *A. flavus* infection and aflatoxin contamination in maize, however, is deemed the most cost-effective control measure (3). Several maize genotypes, such as Mp420, Mp313E, MI82, and GT-MAS:sk, have demonstrated resistance to aflatoxin accumulation in both field and laboratory studies (4,5,22,32,43,57). However, progress in incorporating resistant traits from these lines into genotypes with desirable agronomical traits has been slow, mainly due to the lack of markers known to be consistently associated with resistance

(4). Recent genetic studies further indicated that resistance of maize kernels to aflatoxin is a multigene-controlled quantitative trait, and is under strong environmental control (17,39).

Studies to understand host resistance mechanisms in maize against *A. flavus* infection and aflatoxin contamination indicate that proteins are a major factor contributing to kernel resistance (12,15,24,29). The expression of a 14-kDa trypsin-inhibitor protein (TI) was shown to be associated with resistance in vivo (12). In addition, TI demonstrated strong antifungal activity in vitro against *A. flavus* and other fungal pathogens through inhibiting the production and activity of *A. flavus* α -amylase (11). Reduced α -amylase production and activity can limit the available carbon source, which has been shown to reduce fungal growth and aflatoxin biosynthesis (60). Further investigation determined that both constitutive and induced proteins are required for maize kernel resistance to aflatoxin production (8).

Recently, a comparative proteomic approach was employed to identify maize kernel proteins that may serve as markers for breeders to use in marker-assisted breeding strategies. In these investigations, the expression of over a dozen constitutive kernel embryo proteins were found to be associated with resistance (either unique or fivefold upregulated in resistant lines) (9). One of the identified proteins is a stress-related protein, called glyoxalase I, which had been shown to enhance stress tolerance (54). Further examination of glyoxalase I revealed a potentially important role for it in resistance through controlling the level of its substrate, methylglyoxal, which is an aflatoxin inducer (10). In addition to glyoxalase I, other stress-related resistance-associated proteins (RAPs) identified in the comparison include a small heat-shock protein (17.9 HSP), a water-stress-inducible protein (WSI 18), and an aldose reductase (9). In light of the positive relationship between drought and aflatoxin contamination in the field

Corresponding author: Z.-Y. Chen; E-mail address: zchen@agcenter.lsu.edu

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(40), the identification of these stress-related proteins may indicate a correlation between a kernel's resistance to *A. flavus* infection or aflatoxin production and its ability to tolerate stress (9).

Among the maize RAPs identified in the above investigations was a pathogenesis-related protein (PR-10) found in the kernel endosperm (13). Leaf extracts of transgenic tobacco plants over-expressing this PR-10 showed increased RNase activity and antifungal activity toward *A. flavus*, indicating the potential for a direct involvement in host resistance (13). However, PR-10 was the only differentially expressed endosperm protein characterized in the earlier study. Therefore, the objectives of this study were to identify other proteins differentially expressed in the endosperm tissue between resistant and susceptible maize lines, and further characterize one of such proteins, a peroxidoredoxin antioxidant (PER1) protein. This protein was expressed at higher levels in resistant lines than in susceptible ones. In addition, the over-expressed PER1 demonstrated peroxidase activity in vitro, and the expression of its corresponding gene was induced in the resistant line upon fungal infection, suggesting that PER1 may play an important role in enhancing kernel stress tolerance and aflatoxin resistance.

MATERIALS AND METHODS

Chemicals and materials. Immobilized pH gradient (IPG) buffer (pH 3.0 to 10.0, linear), Immobiline DryStrip (pH 3.0 to 10.0, 18 cm), dithiothreitol (DTT), IPGphor, and a Hoefer-Dalt 2-D electrophoresis system were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). 3-([3-Cholamidopropyl]-dimethylammonio)-1-propane-sulfonate (CHAPS), ammonium persulfate, and N,N,N',N'-tetramethyl-ethylenediamine (TEMED) were purchased from Bio-Rad (Hercules, CA). Kernels of five aflatoxin-resistant (GT-MAS:sk, CI2, MI82, Mp420, and Mp313E) and five aflatoxin-susceptible (P3154, G4666, B73, Mo17, and Va35) maize genotypes were obtained from the same sources as described in a previous report (9). Their resistance or susceptibility to *A. flavus* infection or aflatoxin production has been established in earlier studies (5,25,43).

Protein extraction. Kernels (20 g) from each genotype were separated into embryo and endosperm as previously described (9). Endosperm tissues were frozen in liquid nitrogen, ground, and extracted at 4°C as previously described (9) with an extraction buffer (0.25 M NaCl, 50 mM Tris-HCl pH 8.0, 14 mM β -mercaptoethanol, and 0.04% Pefabloc proteinase inhibitor) (2 ml/g). Protein concentration in each extract was assayed according to Bradford (2) using the Bio-Rad dye reagent and bovine serum albumin as a standard. Then, an appropriate amount of proteins from each genotype was precipitated twice with five volumes of cold acetone to remove salt (9) and dried under vacuum before resolubilizing in lysis buffer (9.5 M urea; 2% CHAPS; 1% DTT; 0.8% wt/vol IPG buffer, pH 3 to 10; and 0.04% Pefabloc proteinase inhibitor) at a final concentration of 2 μ g/ μ l. The resultant mixture was centrifuged for 30 min at 35,000 $\times$ g (15°C) to prepare the supernatant for loading onto a rehydrated IPG gel strip. Two protein extractions were performed for each genotype used in this study, and each protein sample was resolved in triplicate gels.

Two-dimensional gel electrophoresis. First-dimensional isoelectric focusing was performed at 20°C using rehydrated IPG gel strips (pH 3 to 10) as previously described (9). Of the above supernatant, 50 μ g (analytical) to 700 μ g (preparative) was loaded per gel strip. At the end of isoelectric focusing, gel strips were equilibrated immediately for 15 min in 10 ml of sodium dodecyl sulfate (SDS) equilibration buffer (50 mM Tris-HCl buffer, pH 8.8; 6 M urea; 30% wt/vol glycerol; 2% wt/vol SDS; and 1% wt/vol DTT) (23). After equilibration, IPG strips were embedded in 1% agarose solution at the top of the second-dimensional (2-D) gel. SDS-polyacrylamide gel electrophoresis (PAGE) was per-

formed in 13.5% polyacrylamide gels (235 by 190 by 1.5 mm, width by length by height) at 10°C at a constant voltage of 120 V for 2,000 Vh as described (9).

Staining and gel analysis. Protein spots in analytical or preparative gels were stained automatically with Silver Stain Kits (Genomic Solutions, Chelmsford, MA), or Coomassie Brilliant Blue R 250, respectively, in an Investigator Gel Processor (Genomic Solutions). All stained gels were scanned using a UMAX PowerLook II scanner (UMAX data systems, Taiwan), and analyzed using Progenesis Discovery 2003 software package (Non-linear Dynamic, Durham, NC). Protein spots between replicated gels of the same extraction first were matched automatically after warping each gel to a reference gel, with some post-matching manual editing to correct mismatches. After spot matching, gels from resistant and susceptible genotypes were organized into two subgroups. Composite gels were constructed from each subgroup, and were used to compare for protein differences to homogenize genetic differences between the genotypes. The normalized volume, which had adjusted for background, loading, and staining variations between gels, was used as a criterion to compare protein expression between genotypes. The matched spots that showed significant differences in their expression between two subgroups based on least significant differences analysis then were highlighted by the software as possible candidates. Protein spots with mean expression levels from two extractions that were at least twofold higher in the resistant subgroup compared with the susceptible subgroup then were selected for peptide sequencing as described below.

Peptide sequencing and database sequence homology analysis. Protein spots were excised from the Coomassie Brilliant Blue R 250-stained 2-D gels with a combined amount of 10 to 20 pmol. These protein spots then were subjected to in-gel trypsin digestion and sequenced as previously described (9) using an electrospray ionization tandem mass spectrometry (ESI-MS/MS) equipped with a Protana nanospray source (Odense, Denmark). Peptide sequence homology searches were performed using BLAST (1) against known proteins and translated open reading frames of expressed sequence tags (ESTs) in databases at the National Center for Biotechnology Information (NCBI) and SWISS-Prot.

Cloning of the full-length 1-cys peroxidoredoxin antioxidant gene (*per1*) from a maize endosperm cDNA library. Two degenerate primers were synthesized, 5'-GAGCT(G/C/T)GA-(C/T)TCIACICA(C/T)GGIAA-3' (PER1-F0) and 5'-GGT(C/T)-TC(G/A)AAICC(C/T)TGIGG(G/A)AACAT-3' (PER1-R0), based on peptide sequences ELDSTHGK and MFPQGFET, respectively. The first-round polymerase chain reaction (PCR) was performed in a 50- μ l volume using a cDNA library-specific primer J19 (5'-ATCGAATTAGGATCCTCTGCT-3') and the PER1-R0 primer. The template was 1 μ l of an undiluted endosperm cDNA library (in pAD-GAL4-2.1 vector) made from Ohio 43 kernels that were 10 to 14 days after pollination (titer 1.1×10^9 pfu/ml) (kindly provided to us by Dr. Robert J. Schmidt, Division of Biology, University of California, San Diego). The second-round PCR was performed using the PER1-F0 and PER1-R0 pair and 1 μ l of the 1:20 diluted first-round PCR product as a template. The amplified 0.6-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 *per1* gene from barley. The remaining sequences of *per1* cDNA were cloned using 5' and 3' rapid amplification of cDNA ends (RACE) with primers from the cDNA library vector (J19 and J20: 5'-CTC-GAGATGCTAGCGAGTATCCTTTTTTTT-3') and gene-specific primers (PER1-F1: 5'-GACGACGAAGGTGACGTT-3' and PER1-F2: 5'-TCCGGCAGCTGAACATGGT-3' for cloning the 3' end; PER1-R1: 5'-TCAGCTTGACGGCCTTGTC-3' and PER1-R2: 5'-ATCGGGAACGTCACCTTCG-3' for cloning the 5' end) derived from the sequenced 0.6-kb PCR product. The resulting PCR products then were cloned as above and sequenced using a

dye termination reaction on an ABI 377 DNA sequencer (Applied Biosystems Inc., Warrington, UK).

Overexpression of PER1 in *Escherichia coli*. The coding region of *per1* cDNA was amplified using two primers (PER1-*NcoI*: 5'-AACCATGgCGGGGCTCAC-3' and PER1-*SacI*: 5'-AGAgcTCCGTGCCGTACGTC-3'). The nucleotide substitutions (lowercase) to incorporate a *NcoI* site at the 5' end (by changing C to G) and a *SacI* site at the 3' end (by changing CA to GC) changed only the second amino acid residue from P to A at the N-terminus of the maize PER1. The PCR product was digested with *NcoI* and *SacI*, gel purified, and cloned into the corresponding sites of the pET28c vector (Novagen, Madison, WI) to generate the pET28c-PER1. Correct construct was verified through DNA sequencing.

The construct then was transformed into an *E. coli* expression host BL21 (DE3). The molecular mass of the overexpressed maize PER1 was predicted to be 24.9 kDa, containing only the complete mature PER1 sequence (229 amino acid residues). The induction of PER1 expression and the fractionation were performed according to Chen et al. (11). Each fraction then was resolved using SDS-PAGE according to Laemmli (33). The content of overexpressed PER1 in the total cell extract and in the soluble fraction was quantified with a Bio-Rad GS-700 gel densitometer.

Gel staining of peroxidase activity. Various fractions of *E. coli* protein extracts first were resolved using native polyacrylamide gels (13.5%), and then the peroxidase activity was visualized according to Shimoni et al. (44). Gels first were incubated in 150 ml of sodium acetate buffer solution (0.05 M, pH 5.0) containing 10 mM H₂O₂. After 1 to 2 min, 3 ml of N,N-dimethylformamide containing 30 mg of 3-amino-9-ethylcarbazol was added with slow shaking. Brown bands were visible in 5 to 10 min.

Expression of *per1* during maize kernel development with and without *A. flavus* inoculation. The effect of fungal colonization on *per1* expression during maize kernel development was studied in a resistant (Mp420) and a susceptible (B73) maize genotype in spring 2004. Seven days after pollination, one-third (≈ 100) of the plants from each genotype were inoculated in the field with 5 ml of *A. flavus* suspension (5×10^6 conidia/ml) per ear at multiple locations in the mid-ear using a syringe. Another one-third of the plants were wounded with a syringe needle without inoculum to serve as a wounded-only control. The remaining one-third of the plants were used as a noninfected (nonwounded) control. Six wounded-only or inoculated ears were harvested at the following time intervals: 0, 1, 2, 3, 5, 8, 12, 16, and 24 days after inoculation (DAI) and divided into three replicates, each with two ears. Six noninfected control ears also were harvested on the same days. Kernels harvested from two randomly selected ears of the same genotype and treatment were combined as one replicate and frozen in liquid nitrogen before storing at -80°C .

For inoculated and wounded ears, only healthy-looking, intact kernels surrounding the inoculation or wounding sites were collected. Ears were harvested at a fixed time during the day to avoid any circadian effect on gene expression. For each sampling date, kernels were removed from the cob as soon as ears were harvested. This experiment was repeated in a different field in the same growing season.

Total RNA was isolated from immature maize kernels of different developmental stages using the RNeasy Plant mini kit (Qiagen, Valencia, CA) and reverse transcribed into cDNA as previously described (13) according to the manufacturer's instructions. The level of *per1* expression then was quantified using an ABI 5700 Thermal Cycler with SYBR Green dye chemistry (Applied Biosystems). Primers used in real-time reverse-transcriptase PCR (PER1-F, 5'-CAAGATCCGCATCCACGACTA-3' and PER1-R, 5'-TCGGCTGGGTGGGAGAA-3') were designed using Primer Express 2.0 (Applied Biosystems). The amplicon size was 90 bp. Dissociation kinetics was performed by the machine at the end of the experiment to examine the annealing specificity of primers. In addition, the amplified product was sequenced to confirm the specific amplification. The expression of 18S rRNA in maize kernels was used as an internal control to normalize the expression of the *per1* gene (13). Using standardized conditions, deviations of threshold values usually were <1.0 cycle for independent cDNA preparations from replicated samples of the same time point and treatment, and <0.5 cycle for replicates of the same cDNA. The replication variation of data between the repeated experiments was determined by comparing the means using least significant difference and was not statistically different ($P = 0.05$). Therefore, the data were combined and reported here.

Statistical analysis. All statistical analyses were conducted using the SAS software package (version 8.2; SAS Institute, Cary, NC). Means for protein expression were separated using a least significant difference test at $P = 0.05$. Mean separations for *per1* expression in maize kernels during different developmental stages were performed using the method of Duncan's multiple range test ($P = 0.05$).

RESULTS

Identification of differentially expressed endosperm proteins. Endosperm proteins first were detected, quantified, and matched for each gel using Progenesis Software. The number of protein spots detected in the endosperm was usually between 800 and 1,200 when stained with silver, less than that observed in the embryo. Of the matched proteins, 109 spots were identified as present in all resistant genotypes and 114 spots were present in all susceptible genotypes. Of the 109 spots present in all resistant genotypes, 42 were missing in one or more susceptible genotypes, and 47 of those present in all susceptible genotypes were missing in one or more of the resistant genotypes. However, none of these

TABLE 1. Summary of twofold or more upregulated protein spots identified in endosperm of five aflatoxin-resistant maize genotypes compared with five susceptible genotypes

Spot number	Normalized volume in R ^a	Isoelectric point ^b	Molecular weight ^b	Fold of upregulation
529	0.625 $\pm$ 0.091	9.56 $\pm$ 0.01	24,740.8 $\pm$ 248.3	2.16
533	0.282 $\pm$ 0.063	5.27 $\pm$ 0.02	27,518.0 $\pm$ 1,227.6	2.74
537	0.116 $\pm$ 0.039	6.21 $\pm$ 0.03	27,304.4 $\pm$ 1,327.5	2.08
546	0.180 $\pm$ 0.071	6.80 $\pm$ 0.04	26,785.0 $\pm$ 1,194.2	2.17
646	0.216 $\pm$ 0.106	5.44 $\pm$ 0.03	20,101.9 $\pm$ 429.1	2.31
678	0.280 $\pm$ 0.133	5.48 $\pm$ 0.02	19,084.5 $\pm$ 659.1	2.52
717	0.176 $\pm$ 0.083	5.19 $\pm$ 0.03	14,934.4 $\pm$ 474.5	3.10
798	1.977 $\pm$ 0.708	6.07 $\pm$ 0.05	11,682.7 $\pm$ 226.1	2.09
852 ^c	0.166 $\pm$ 0.089	6.03 $\pm$ 0.03	9,124.7 $\pm$ 564.5	2.04
1433	0.127 $\pm$ 0.014	6.20 $\pm$ 0.02	11,320.5 $\pm$ 341.3	2.45

^a Normalized volume: the values presented here are means $\pm$ standard error in all resistant genotypes.

^b The values for isoelectric point and molecular weight are the means of the same spot obtained from replicated gels in two experiments.

^c The spot was not visible in prep gel.

spots were completely missing in the other group. The remaining 67 spots were present in all genotypes. Of these, 10 were expressed at a level twofold or higher in resistant genotypes than in susceptible genotypes (Table 1), and 6 of them were down-regulated between four- to twofold in the resistant genotypes compared with susceptible ones. The protein that showed the highest upregulation (3.1-fold) in resistant lines was PR-10. Examples of one upregulated protein spot (533) and one downregulated protein spot (1022) are shown in Figures 1 and 2, respectively.

Peptide sequencing and homology analysis of resistance-associated proteins. After gel analysis, the upregulated protein spots in resistant lines were recovered, digested, and sequenced using ESI-MS/MS. Two to four peptides were sequenced from each spot for positive protein identification. The peptide sequences obtained from each spot and their homology identified through database searches are summarized in Table 2. Spot 529 is identified as a maize group 3 late embryogenesis abundant protein (LEA3) based on its peptide sequence homology. The peptide sequence of spot 533 is highly homologous (98.2% identity) to a maize globulin 2 protein (GLB2). However, the molecular mass of spot 533 is only 25.6 kDa based on gel estimation, and the peptide sequence is aligned to the middle region of GLB2. This suggests that spot 533 is derived from GLB2 through a partial proteolytic processing.

For spots 537 and 546, four and three peptide sequences were obtained, respectively; and two of them were identical between the two spots (Table 2). All of these sequenced peptides showed high homology (94.8 to 82.8% identity) to a peroxiredoxin

antioxidant protein from barley (X96551), a 1-cys peroxiredoxin protein (PER1) from wheat (AF327046), and an ABA-responsive 24-kDa protein with probable peroxiredoxin (thioredoxin peroxidase) activity from rice (RAB24, P52573). PER1 is a subgroup of the most recently discovered types of enzymatic antioxidants with one conserved cysteine residue at the N terminus and possessing peroxidase activities. The two protein spots have similar molecular masses, but different isoelectric points (pIs), suggesting that the two proteins are encoded by two highly homologous genes or by one gene with different post-translational modifications.

Three peptide sequences were obtained for spot 646 (Table 2). They are highly homologous to cold-regulated proteins from wheat (*cor18*, accession no. AB097412), barley (accession no. AJ291295, 81.8% identity), and the deduced amino acid sequences of ESTs from maize (CO456082) and rice (XM_475410). The deduced amino acid sequences of the maize EST showed the highest sequence similarity (93.2% identity) to the sequenced peptides. Peptide sequences of spot 678 (Table 2) showed high homology to a group 4 LEA (LEA14) from cotton (accession no. P46518, 60.9% identity), a desiccation-related protein from *Arabidopsis thaliana* (accession no. AAC62908, 56.5% identity), a desiccation protectant protein from soybean (accession no. P46519, 56.5% identity), and a putative LEA from rice (accession no. BAB19059, 86.9% identity) (Fig. 3). These peptides also are highly homologous to the deduced amino acid sequences of several maize ESTs, such as AW331170 (73.9%) and AW566229 (78.3%) (Fig. 3). These data suggest the presence of multiple genes encoding group 4 LEA proteins in maize.

Fig. 1. Subsections of representative two-dimensional gels showing an up-regulated endosperm protein spot 533 (indicated with filled arrows) in the proteomic comparison between five aflatoxin-resistant and five susceptible maize genotypes. The gel images on the left side are from resistant maize genotypes GT-MAS:gk, CI2, Mp313E, MI82, and Mp420 (from top to bottom) and the gels on the right side are from susceptible genotypes G4666, Mo17, P3154, B73, and Va35 (from top to bottom).

Fig. 2. Subsections of representative two-dimensional gels showing a twofold downregulated endosperm protein spot 1022 (indicated with open arrows) in the proteomic comparison between five aflatoxin-resistant and five susceptible maize genotypes. The gel images on the left side are from resistant maize genotypes GT-MAS:gk, CI2, Mp313E, MI82, and Mp420 (from top to bottom) and the gels on the right side are from susceptible genotypes G4666, Mo17, P3154, B73, and Va35 (from top to bottom).

Based on the obtained peptide sequences, both spots 798 and 1433 are identified as the maize 14-kDa TI (Fig. 4). The peptide sequences also showed high sequence identity to TIs from barley (55.2%) and finger millet (62.1%) (Fig. 4). The mismatch (L/M) between one peptide sequence from spot 1433 and the maize 14-kDa TI1 (CCA37998) at amino acid residue position 68 appears to be real because it completely matched to another maize 14-kDa

TI2 (ABA34134) (Fig. 4). This indicates that the two discrete TI spots identified on 2-D gels may have arisen from two homologous genes rather than from a single gene with different post-translational modifications.

The peptides of spot 1113 are highly homologous (88.1% identity) to small HSPs from maize (accession no. S23212), pearl millet (S72545), and rice (P27777) (Fig. 5). Three of the peptide

TABLE 2. Summary of peptide sequences obtained from 10 protein spots that were upregulated in the endosperm of aflatoxin-resistant maize genotypes through electrospray ionization tandem mass spectrometry

Spot no.	Peptide sequences ^a	Accession ^b	Identity or homology	E value ^c	References
529	ASDTGSYLGK DAVMSTLGMGGDDK	Z29512, U05226	LEA3 from maize	4e-05	6,56
533	EGEGVIVLLSGGK GAVVYSANTHQSEWFR VVMLLSPVSTSGR GEITTASEEQIR	X53715	Globulin 2 from maize	8e-09	50,55
537	VTFPI(L/I)ADPAR LSFLYPATTGR AVDSLTLAAK MFPQGFETADLPSK	X96551	1-Cys peroxiredoxin antioxidant (PER1) from barley	8e-04	37,47
546	VPNLELDSTHGK VTFPI(I/L)ADPAR MFPQGFETADLPSK	P52572	1-Cys peroxiredoxin antioxidant (PER1) from barley	0.11	37,47
646	SPPLEWYGVPGGAAAR GLPEGFSGAAGAGAGAGR LGGLQEGVNDWK	AB097412, AJ291295	Close to cold-related protein from wheat	0.011	16
678	VPYDF(I/L)VSLAK VG(I/L)TVDLPPVVGK	P46518	Close to putative LEA14 from cotton	4.2	21
717	VEPAAGGGSVVK, EAYLVANP VVASAQPVGDDGGVGSVR	AY751553, AY953127	Pathogenesis-related protein 10 from sorghum and maize	0.28	13
798	ELADIPAYCR SILMDGAIPPGPDAQLEGR	DQ898140, X54064	Bifunctional trypsin or amylase inhibitor	6e-07	12
1113	TSSETAAFAGAR, ETPEAHVFK VEVEDGNVLQISGER, RFRLPENAK	X94193	Close to heat-shock protein 17.9 from pearl millet	0.073	53
1433	LPWPPEMK ELADIPAYCR PPGPDAQLEGR	DQ147267, DQ898140, X54064	Bifunctional trypsin/ amylase inhibitor from maize	0.010	12

^a The amino acid L and I shown in parentheses in the peptide sequences cannot be distinguished by mass spectrometry.

^b The accession numbers of sequences that show highest homology to the query peptide sequences in the BLAST search.

^c Expected (E) value is a parameter that describes the number of expected hits by chance when searching a database of a particular size. It takes into account the length of the query sequence. A relatively high E value is possible even though a significant homology exists between the query and the database sequence when a short query sequence is used for database search, as in this study, due to the fact that shorter sequences have a high probability of occurring in the database purely by chance.

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BAB19059      SGTTNLELPVKVPYDFIVSLIKDVS GDWDIDYVLEVGITIDLPVVG SFTIPLATEGEMKL 60
SPOT 678      -----VPYDFIVSLAK-----VGITVDLPVVGK----- 23
AW331170      SGSTKLDLPVKVPYDFIVSLMKDLGGDWDIDYMLEVGLTVDLPAIGAFTIPLTTEGEIKL 60
SPOT 1868      -----VPYDFIVSLAK-----DDIDYEMER--TVDLPPVVGK----- 29
AW566229      SGSTALEIPAKVPYDFIVSLVLRDVG RDWDIDYELQVGLTVDLPIVGNFTIPLSTSGEFKL 60
LEA14-GH      SDTTMLDVPVKVPYNI LVS LKDIGADWDIDYELGLTIDLPVGNFTIPLSQKGEIKL 60
LEA14-GM      SDTTMLDVPVKVPHS ILLSLAKDIGADWDIDYQLDLGLVIDLPVGNFTIPLSQKGEIKL 60
AAC62908      SGTTVLDVPVKVAYS IAVSLMKDMCTDWDIDYQLDIGLTFDIPVVG DITIPVSTQGEIKL 60
               *      **      :
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Fig. 3. Partial amino acid sequence alignments of plant group 4 late embryogenesis abundant proteins with the peptide sequences of spot 678 and a previously identified putative late embryogenesis abundant protein (LEA) (spot 1868) (9). LEA14-GH, an lea14-A from cotton (*Gossypium hirsutum*, P46518); LEA14-GM, a desiccation protectant protein lea14 homolog from soybean (*Glycine max*, P46519); AAC62908, a putative desiccation-related protein from *Arabidopsis thaliana*; AW331170 and AW566229, two maize expressed-sequence tags; and BAB19059, a putative late embryogenesis abundant protein from rice (*Oryza sativa*). The conserved amino acid residues are indicated with “*” and the one amino acid mismatch between spots 678 and 1868 is indicated with “:”.

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TI1-ZM      PRLPWPELKRRCRELADIPAYCRCTALSILMDGAIPPGPDAQLEGRLEDLPG-CPREVQ 119
SPOT 798      -----ELADIPAYCR-----SILMDGAIPPGPDAQLEGR----- 29
SPOT 1433      --LPWPPEMK-----ELADIPAYCR-----PPGPDAQLEGR----- 28
TI2-ZM      PRLPWPEMKRRCRELADIPAYCRCTALSILMDGAIPPGPDAQLEGRLEDLPG-CPREVQ 118
0903195A      PRLATQEMKARCCRQLEAIPAYCRCEAVRILMDGVVTPS--GQHEGRLLQDLPGCPRQVQ 90
CAA35188      PRLTSDMKRRCCDELSAIPAYCRCEALRIIMQGVVTWQ--GAFEGAYFKDSPNCPREVRQ 113
               :      *      *****      **
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Fig. 4. Sequence homology comparison of peptide sequences from spots 798, 1433, and the 14-kDa trypsin inhibitor proteins from maize (CCA37998 for maize TI1-ZM and ABA34134 for maize TI2-ZM), barley (CAA35188), and finger millet (0903195A). The conserved amino acid residues are indicated with “*” and the one amino acid mismatch between TI1-ZM and spot 1433 is indicated with “:”.

sequences also were identical to a previously sequenced putative embryo HSP (spot 1724) (9). The peptide sequences also completely match the deduced amino acid sequences of two maize ESTs (BE123268 and AW258080) and are highly homologous (97.5 to 92.9% identity) to that of three other ESTs (BE056217, AW067565, and BE639130).

Cloning of full-length cDNA encoding 1-cys peroxiredoxin antioxidant protein. To further investigate the role of RAPs identified through proteomics in host resistance, the PER1 protein was selected for further characterization. The *perl* gene was cloned as described in Materials and Methods. The full-length cDNA (1,037 bp), under accession no. DQ378060 in GenBank, has an open reading frame for 229 amino acids with a putative poly A site (CTAGTATATA) located at nucleotide position 861 (Fig. 6). This putative maize PER1 protein has a calculated molecular mass and pI of 24,912 Da and 6.71, respectively. BLAST search of the cDNA sequence found several maize ESTs (AW258068, AW261289, and AW255107) with identical DNA sequence to the cloned *perl* cDNA except at the very 5' end. These ESTs, along with the cloned *perl* cDNA, appear to have several single-nucleotide polymorphisms compared with other maize *perl* homologous ETs, such as CF000383, CO459331, CO455640, and CD443434 (data not shown).

The deduced amino acid sequence of *perl* shares high homology to 1-cys peroxiredoxin antioxidant from other plants, such as barley (X96551, 82%), wheat (AF327064, 81%), and rice (AY336994, 76%) (Fig. 7), and contains a conserved active site (PVCTTE) at the N-terminus (Fig. 7). The *perl* deduced amino acid sequence was 98 and 94.8% identical to the deduced amino acid sequences of a maize clone contig519 mRNA sequence (BT016686) (Fig. 7) and a maize EST (AW225107) (data not shown), respectively. The deduced amino acid sequence also possesses a conserved domain, similar to those observed in the alkyl hydroperoxide reductase (AhpC) family, which contains proteins related to alkyl hydroperoxide reductase; and the thiol specific

antioxidant (TSA) family, which contains peroxiredoxin proteins involved in posttranslational modification, protein turnover, and chaperones (35).

Peroxidase activity of maize PER1 overexpressed in *E. coli*.

The maize PER1 was successfully overexpressed in *E. coli* cells and consisted of $\approx 6\%$ of total proteins of *E. coli* cells when induced by IPTG (Fig. 8A). The apparent molecular mass of the overexpressed protein was ≈ 25.0 kDa based on SDS-PAGE, which was close to the predicted value of 24.9 kDa. The majority of the overexpressed protein remained in the water- or salt-soluble fraction, and some was present in the urea-soluble fraction (data not shown). The peroxidase activity (brown band) was detected in the water- or salt-soluble protein fraction of the induced *E. coli* cells, but not from that of the noninduced control cells when the proteins were resolved using native PAGE gels (Fig. 8B). The areas corresponding to the peroxidase activity from an unstained duplicated native gel were recovered from both the control and induced cells. The proteins recovered from the excised gels were resolved on a new SDS-PAGE. It was found that the major difference in protein profiles between the proteins recovered from the native gel of induced cell extract and those from noninduced control cell extract was the presence of the 25-kDa protein in the induced cell extract (Fig. 8C). This protein was missing in the noninduced cell extracts, indicating that the observed peroxidase activity in the native gel was contributed by the overexpressed PER1 protein.

Expression of *perl* during kernel development with or without fungal infection. Under normal kernel developmental conditions without fungal inoculation, *perl* expression was very low in the early stage (up to 3 DAI) in both resistant and susceptible genotypes (Fig. 9). It increased slightly between 3 to 8 DAI and rapidly thereafter to ≈ 5 to 10-fold the level of 8 DAI at 24 DAI (Fig. 9), coinciding with kernel maturation and dehydration. The transcript level in the resistant genotype Mp420 under either inoculated or noninoculated conditions was sevenfold higher than that in the susceptible B73 during the late stage

HSP16.9-PG	MSLVRRGNVDFPFSMDLWDPF-----NMFRSIVP--SSSSS-DTAAFANARIDWKETP	51
HSP16.9-OS	MSLVRRSNVDFPFSLDLWDPF-----SVFRSVVP--ATSDN-DTAAFANARIDWKETP	51
AW067565	-----ETAAFASARIDWKETP	16
BE639130	MSLVRRSSVDFPFSVDLDFDPF-----SMFRSIVPSSSSAAASETAAFASARIDWKETP	54
HSP17.2-ZM	MSLVRRSNVDFPFSMDLWDPF-----TMFRSIVPSATSTNS-ETAAFASARIDWKETP	53
AW258080	MSLIRRSNVDFPFSLDLWDPFEGFPFGSGSSSLFPSFPRTSS-ETAAFAGARIDWKETP	59
SPOT 1113	-----TSS-ETAAFAGAR-----ETP	15
BE056217	-----APFAGARIDWKETP	14
	* * * *	
HSP16.9-PG	EVHVFKADLPGVKKEEVKVEVEDGNVLVISGQRSK-EKEDKNDRWHRVERSSGQ-FVRRF	109
HSP16.9-OS	ESHVFKADLPGVKKEEVKVEVEEGNVLVISGQRSK-EKEDKNDKWHRVERSSGQ-FMRRF	109
AW067565	EAHVFKADLPGVKKEEVKVEVEDGNVLLISGQRSR-EKEDKGDKWHRVERSSGQ-FVRRF	74
BE639130	EAHVFKADLPGVKKEEVKVEVEDGNVLLISGQRSRXXRRRTXGDKWHRVERSSGQ-FVRRF	113
HSP17.2-ZM	EAHVFKADLPGVKKEEVKVEVEDGNVLVISGQRSR-EKEDKDDKWHRVERSSGQ-FIRRF	111
AW258080	EAHVFKADVPLKKKEEVKVEVEDGNVLQISGERNK-EQEEKTDTWHRVERSSGR-FLRRF	117
SPOT 1113	EAHVFK-----VEVEDGNVLQISGER-----RF	38
BE056217	EAHVFKADVPLKKKEEVKVEVEDGNVLQISGERNK-EQEEKTDTWHRVERSSGR-FLRRF	72
	* * * * *	
HSP16.9-PG	RLPEDAKTDQVNAGLENGVLTVTVPKAEKKPEVKAIEISG	150
HSP16.9-OS	RLPENAKVDQVKAGLENGVLTVTVPKAEVKKPEVKAIEISG	150
AW067565	RLPENAKTEEVRAALENGVLTVTVPKAEVKKPEVKSIIQISG	115
BE639130	RLPENAKTEEVRAALENGVLTVTVPKAEVKKPEVKSIIQIS-	153
HSP17.2-ZM	RLPDDAKVDQVKAGLENGVLTVTVPKAEKKPEVKAIEISG	152
AW258080	RLPENAKTEQIRAAMENGVLTVTVPKEDVKKPEVK-----	152
SPOT 1113	RLPENAK-----	45
BE056217	RLPENAKTEQITAAMENGVLTVTVPKEDAKKPEVKSIIQISG	113
	* * *	

Fig. 5. Alignment of amino acid sequences of spot 1113 with low molecular weight heat-shock proteins (HSPs) from *Pennisetum glaucum* (HSP16.9-PG, S72545), *Oryza sativa* (HSP16.9-OS, P27777), *Zea mays* (HSP17.2-ZM, S23212), and the deduced amino acid sequences of several maize expressed sequence tags (AW258080, AW067565, BE056217, and BE639130). The conserved amino acid residues are indicated with “*”.

of kernel development. Upon fungal inoculation, a threefold induction of *per1* expression was observed in both resistant and susceptible kernels between 8 and 16 DAI (Fig. 9). However, the transcript level was still fivefold higher in Mp420 than in B73 at 24 DAI. The *per1* expression in the wounded-only control samples collected between 1 and 5 DAI did not show a clear induction by wounding, and the level was not significantly different from the corresponding nonwounded controls (Fig. 9). Therefore, the remaining wounded-only control samples collected at a later time were not examined.

DISCUSSION

Comparisons of kernel proteins between resistant and susceptible maize genotypes through proteomics have identified that the expression of three categories of proteins was higher in resistant than in susceptible lines and, therefore, associated with aflatoxin resistance (RAPs). These are storage proteins, stress-related proteins, and antifungal proteins. In a previous report comparing

embryo proteins, two globulins (GLBs) and a couple of LEAs were identified as RAPs (9). The identification of a third GLB2 (spot 533) and another LEA3 (spot 529) in the endosperm as a RAP further suggests a potential importance of storage proteins in kernel resistance. The similar molecular masses but different pIs among the three GLB2 proteins suggest that these proteins may arise from different posttranslational modifications (50). The accumulation of LEA3 proteins was shown to occur late in embryogenesis to enhance stress tolerance (61), coinciding with the onset of dehydration (52) and the outbreak of aflatoxin production in infected kernels (40).

The expression of two antifungal proteins has been described as associated with resistance in the maize endosperm. One is a PR-10 shown to be an RNase and antifungal (13). The other is the 14-kDa TI, which also is a potent antifungal protein (12). Two of the RAPs sequenced in the present study also are TIs, supporting the potential importance of TI to host resistance.

The stress-related RAPs identified in the endosperm include small HSPs, a cold-regulated protein, and a peroxiredoxin anti-

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1      GAATTCTTCCGATGAGGGTAATGCGTAACCATAGCCAGCAAGCCAGCGCACGTCGGTAGG
61     CACGCCACCATGCGGGGGCTCACCATCGGCGACACCGTCCCCAACCTGGAGCTGGACTCC
1      M P G L T I G D T V P N L E L D S
121    ACCCACGGCAAGATCCGCATCCACGACTACGTCGGCGACGGCTACGCCATCATCTTCTCC
18     T H G K I R I H D Y V G D G Y A I I F S
181    CACCCAGCCGACTTCACGCCCCGTGTGCACAACGGAGATGGCGGCGATGGCGGGGTACGCC
38     H P A D F T P V C T T E M A A M A G Y A
241    AAGGAGTTCGAGAAGCGTGGCGTGAAGCTGCTGGGCATCTCGTGCGACGACGTGGAGTCG
58     K E F E K R G V K L L G I S C D D V E S
301    CACAGGCAGTGGACCAAGGACGTGGAGGCGTACGGCGGGAAGCAGCAGCAGCAGCAGGCG
78     H R Q W T K D V E A Y G G K Q Q Q Q Q A
361    ACGACGACGAAGGTGACGTTCCCGATCCTGGCGGACCCGGCGCGGGACGCCATCCGGCAG
98     T T T K V T F P I L A D P A R D A I R Q
421    CTGAACATGGTGGACCCCGACGAGAAGGACGCGGCGGGGCGGAGCATGCCGTCCCGCGCG
118    L N M V D P D E K D A A G R S M P S R A
481    CTCCACGTCGTCGGGCCCCACAAGGCGTCAAGCTGAGCTTCCTGTACCCGGCCACCACG
138    L H V V G P D K A V K L S F L Y P A T T
541    GGGCGGAACATGGACGAGGTGCTGCGCGCTGTGCGACTCGCTGCTCACCGCCGCCAAGCAT
158    G R N M D E V L R A V D S L L T A A K H
601    GGCGGGAAGGTGGCCACGCCCGCCAAGTGAAGCCCGGGGAGTGCGCCGTCATCGCGCCC
178    G G K V A T P A N W K P G E C A V I A P
661    GGCGTCTCCGACGAGGAGGCCAGGAAGATGTTCCCGCAGGGGTTGAGACCGCCGACCTG
198    G V S D E E A R K M F P Q G F E T A D L
721    CCCTCCAAGAAGGGCTACCTCCGTTTACCAAGGTTTAGCTAGCCGACGCACGGCACGGA
218    P S K K G Y L R F T K V
781    CATCTAGCTCGTCTGTCTAGTCCGCGTCGACCGGGGTGCGCGGATCTTTTGTGCTGCTGG
841    TGGTTTCTCTGACTAGTACTAGTATATATATAGGCTCTATGTATGTGCGTACTATGCCGT
901    GCCGTGTTGTCTCGTGTAGTGGTGGCAACCGGTTTCGTGGCACTACCGCGCTAGTATTGTC
961    CACCCATATCAATGTCCTCTTCTGAAGTTAGCGGCGCACATGTTTGTGCGTGTGTGTAGC
1021   ATCTGCTAAAAAA

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Fig. 6. Cloning of the full-length *per1* cDNA encoding a maize peroxiredoxin antioxidant protein and the deduced amino acid sequence. The putative poly A site is shown in bold.

oxidant protein. Their high level of expression in the resistant maize genotypes supports a connection between a kernel's resistance to *A. flavus* infection or aflatoxin production and its stress tolerance. High temperature and drought, which often occur together in nature, are two major factors associated with increased aflatoxin contamination of maize kernels in the field (40). Reducing drought stress through irrigation has been shown to reduce aflatoxin contamination under field conditions (30,41). Possession of unique or high levels of the hydrophilic storage proteins (which are a major part of kernel dry mass) or stress-related proteins inside maize kernels, such as those identified in the present study, may put resistant lines in an advantageous position over susceptible genotypes in the ability to maintain biological activities and to defend against pathogens, especially while under stress.

A high-level expression of stress-related proteins may not only confer stress tolerance but also enhance disease resistance. The role of a glyoxalase I protein from *Brassica juncea* in enhancing tolerance to salt, water, and heavy metal stresses was demonstrated in an earlier study (54). The expression of this protein was found to associate with resistance in maize against aflatoxin accumulation (10). Further investigations suggest an important role for glyoxalase I in aflatoxin resistance through the removal of its aflatoxin-inducing substrate, methylglyoxal (10). Sugar beet overexpressing a stress-related superoxide dismutase from tomato exhibited increased tolerance to oxidative stress as well as to leaf infection by *Cercospora beticola* (51). Overexpression of another stress-related protein, peroxiredoxin Q, from *Gentiana triflora* in transgenic tobacco plants improved not only host stress tolerance

but also disease resistance against *Alternaria alternata* and *Botrytis cinerea* (31).

To continue investigating the roles of stress-related RAPs in host resistance, the maize 1-cys peroxiredoxin antioxidant protein (PER1) identified in the present study was further characterized. Peroxiredoxins are one of the most recently discovered types of enzymatic antioxidants with peroxidase activities on substrates such as hydroperoxides and alkyl hydroperoxides (6,19,37). It has been speculated that the function of peroxiredoxins is to protect plants from oxidative damages to nucleic acids, lipids, and proteins caused by rapid production of reactive oxygen species during stress or fungal infection (27,34,59). Other studies also suggest a role for peroxiredoxins in pathogen defense. Peroxiredoxins were upregulated in the highly resistant *B. carinata* compared with the highly susceptible *B. napus* (canola) when challenged by the fungal pathogen *Leptosphaeria maculans* (49). The expression of a peroxiredoxin Q from poplar exhibited differential responses during compatible and incompatible interactions with two races of *Melampsora larici-populina*, the causal agent of the poplar rust (42).

The maize PER1 identified in the present study represents a subgroup of the peroxiredoxin family of thiol-requiring antioxidants with one conserved cysteine residue (1-cys) (18,48). The high expression at protein level in the resistant maize genotypes, the constant higher transcript level of *perl* during kernel development in the resistant line, the induction of *perl* expression after inoculation, and its demonstrated peroxidase activity indicate a possible role for PER1 in host defense in response to *Aspergillus flavus* infection. Rapid production of reactive oxygen

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AF327046      MPGLTIGDTPVPLELDSTHGKIRIH DYVNGYVILF SHPGDFTPVCTTELAAMANYAKEF 60
X96551        MPGLTIGDTPVPLELDSTHGKIRIH DYVNGYVILF SHPGDFTPVCTTELAAMANYAKEF 60
P52573        MPGLTIGDTPVPLELDSTHGKIRIH DFVGD TYVILF SHPGDFTPVCTTELAAMAAYAKEF 60
AY336994      MPGLTIGDTPVPLELDSTHGKIRIH DFVGD TYVILF SHPGDFTPVCTTELAAMAGYAKEF 60
ZmPER1        MPGLTIGDTPVPLELDSTHGKIRIH DYVGDGYAII FSHPADFTPVCTTEMAAMAGYAKEF 60
BT016686      MPGLTIGDTPVPLELDSTHGKIRIH DYVGDGYAII FSHPADFTPVCTTEMAAMAGYAKEF 60
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****:*****

AF327046      EKRGVKLLGISCD DVQSHKEWTKDIEAYKPGS-----KV TYPIMADPDRSAIKQLNM 112
X96551        EKRGVKLLGISCD DVQSHKEWTKDIEAYKPGS-----KV TYPIMADPDRSAIKQLNM 112
P52573        DKRGVKLLGISCD DVQSHKDWFKDIEAYKPGN-----RV TYPIMADPSREAIAIKQLNM 112
AY336994      DKRGVKLLGISCD DVQSHKDWIKDIEAYKPGN-----RV TYPIMADPSREAIAIKQLNM 112
ZmPER1        EKRGVKLLGISCD DVESHQWTKDVEAYGGKQQQQATTTKVTFPILADPARDAIRQLNM 120
BT016686      EKRGVKLLGISCD DVESHQWTKDVEAYGGKQ---QT TTKVTFPILADPARDAIRQLNM 117
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****

AF327046      VDPDEKDAEG-QLPSRTLHIVGPDKKVKLSFLYP SCTGRNMDEVVRVAVDSL LTAAKH--K 169
X96551        VDPDEKDAQG-QLPSRTLHIVGPDKKVKLSFLYP SCTGRNMDEVVRVAVDSL LTAAKH--K 169
P52573        VDPDEKDSNGGHLPSRALHIVGPDKKVKLSFLYP PACVGRNMDEVVRVAVDALQTA AKT--R 170
AY336994      VDPDEKDSNGGHLPSRALHIVGPDKKVKLSFLYP PACVGRNMDEVVRVAVDALQTA AKHA-- 170
ZmPER1        VDPDEKDAAGRSMPSRALHVVGPDKAVKLSFLYP ATTGRNMDEVLRVAVDSL LTAAKHGGK 180
BT016686      VDPDEKDAAGRSMPSRALHVVGPDKAVKLSFLYP ATTGRNMDEVLRVAVDSL LTAAKHGGK 177
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****

AF327046      VATPANWNPGE CVVIAPGVSDDGAKKMFPQGFETADLP SKKGYLRF TKV- 218
X96551        VATPANWKPGE CVVIAPGVSDDEEAKKMFPQGFETADLP SKKGYLRF TKV- 218
P52573        LATPVNWKPGEP FVIPPGVSDDEAKEKFPQGFDTADLP SGKGYLRF TKVG 220
AY336994      VATPVNWKPGER VVIPPGVSDDEAKEKFPQGFDTADLP SGKGYLRF TKVG 220
ZmPER1        VATPANWKPGE CAVIAPGVSDDEEARKMFQGFETADLP SKKGYLRF TKV- 229
BT016686      VATPANWKPGE CAVIAPGVSDDEEARKMFQGFETADLP SKKGYLRF TKV- 226
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****

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Fig. 7. Sequence homology comparisons between PER1 from maize and other plants. PER1-HV, a peroxiredoxin antioxidant protein encoded by a dormancy-related gene from barley (*Hordeum vulgare*, X96551) (47); PER1-TT, a 1-cys peroxiredoxin protein from wheat (*Triticum turgidum* subsp. *durum*, AF327046) that is induced under salt stress; PER1-OS, a rice (*japonica* cultivar group) peroxiredoxin gene (AF336994); P52573, a RAB24 protein from rice; PER1-ZM, the deduced amino acid sequences of the cloned maize *perl* cDNA (accession no. DQ378060, this study); and BT016686, the deduced amino acid sequences of a maize clone contig519 mRNA. The underlines indicate the peptide sequences of spot 537 obtained from electrospray ionization tandem mass spectrometry. The conserved amino acid residues are indicated with "*" and the putative conserved active site sequence is highlighted.

Fig. 8. Overexpression of maize PER1 and demonstration of peroxidase activity in vitro. **A**, Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showing overexpressed PER1 protein (arrow) in induced total *Escherichia coli* cell extract (lane 2) compared with the control (lane 1). M = protein markers (from top, albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; and trypsin inhibitor, 20 kDa). **B**, The same two samples were resolved in a native PAGE gel and stained for peroxidase activity. **C**, The gel areas corresponding to the activity band in lanes 1 and 2 were recovered from B, eluted, and reloaded on another SDS-PAGE on lanes 3 and 4, respectively, confirming the observed activity was from the overexpressed maize PER1 protein.

species in host plants during a pathogen attack or stress has been documented (59). However, the possibility of PER1 involved in a secondary stress-related response cannot be ruled out. It has been demonstrated that biotic stresses could induce the expression of genes involved in abiotic stress responses and, vice versa, through some common regulatory factors (7,14,20,26,36). High-level expression of PER1, other stress-related proteins, storage proteins, and antifungal proteins identified in proteomic comparisons may act in concert either directly through inhibiting fungal infection or indirectly through enhancing host stress tolerance to reduce aflatoxin contamination in resistant maize genotypes. However, uncovering the exact role of PER1 in host resistance will require further investigation. RNA silencing of PER1 expression may provide direct evidence of this protein's importance to maize aflatoxin-resistance.

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Fig. 9. Expression of peroxiredoxin antioxidant gene during kernel developmental stages with or without *Aspergillus flavus* inoculation in maize. Maize ears were inoculated 7 days after pollination. Transcript levels (mean with standard error bars) of the *per1* gene were measured at 0, 1, 2, 3, 5, 8, 12, 16, and 24 days after inoculation using real-time reverse-transcription polymerase chain reaction and are expressed as relative to 18S (internal normalizer) in the resistant (Mp420) and susceptible (B73) maize genotypes; ◆, *per1* expression in nonwounded control kernels; ■, *per1* expression in wound inoculated kernels; and ▲, *per1* expression in wounded only control kernels.

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