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Association of a Specific Cationic Peroxidase Isozyme with Maize Stress and Disease Resistance Responses, Genetic Identification, and Identification of a cDNA Coding for the Isozyme

PATRICK F. DOWD* AND ERIC T. JOHNSON

Crop Bioprotection Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, 1815 North University Street, Peoria, Illinois 61604

The presence of a p*I* 9.0 cationic peroxidase isozyme from milk stage pericarp of six susceptible and five resistant inbreds was correlated significantly with previously reported field data on percentage infection by *Aspergillus flavus* in the inbreds and their hybrids. The isozyme was constitutively expressed in some additional maize tissues and lines examined, and frequently induced by mechanical damage, heat shock, *Fusarium proliferatum*, and/or *Bacillus subtilis* in other lines tested. Native/IEF two-dimensional electrophoresis identified the isozyme as the previously genetically identified *px5*. A cDNA clone expressed in black Mexican sweet (BMS) maize cell cultures produced the p*I* 9.0 isozyme. In addition to potential use in marker-assisted breeding, enhanced expression of this cationic peroxidase through breeding or genetic engineering may lead to enhanced disease or insect resistance.

KEYWORDS: Maize; peroxidase; *Fusarium*; *Aspergillus*; disease resistance

INTRODUCTION

Peroxidases appear to be associated with plant disease resistance based on physiological responses at the cellular level, induction during disease challenge, and the presence or absence of isozymes in resistant versus susceptible varieties. Despite these associations, there are few examples of peroxidases consistently linked to a disease resistance. This problem has been attributed to not looking in depth at all associated disease resistance factors that could be associated with peroxidases (*1*).

In some cases, there is no evidence for peroxidase involvement in disease resistance in maize (*Zea mays* L.) (*2*, *3*). However, in other cases, peroxidase disease resistance associations have been reported for maize and have been demonstrated by histochemical evidence (*4*), induction (*5*−*9*), and different constitutive isozyme profiles in susceptible versus resistant varieties (*8*, *10*). Further complicating the interpretation of peroxidase roles in disease resistance is the fact that the isozymes have different substrate "preferences" (*11*, *12*). Reports of these isozyme preference differences range from a 1 (low) to 4 (high) preference rate for various peroxidase substrates, including those not found in maize (*13*), to absolute absence of activity for some isozymes versus others when maize allelochemicals are used as substrates (*10*, *11*). Peroxidases are also known to convert maize allelochemicals to products more toxic to maize pathogens (*14*) and insects (*15*). Expression of a tobacco anionic peroxidase in maize also led to enhanced insect resistance (*16*).

Mycotoxins can be produced by fungi that colonize maize ears, such as *Aspergillus flavus*, *Fusarium verticillioides* (*F. moniliforme*), and *Fusarium proliferatum* (*17*). Examination of milk stage kernel pericarps from two inbreds that were resistant (contained characteristics that inhibited kernel colonization) and susceptible to *A. flavus* (*18*, *19*) and insects (*10*, *20*) indicated the susceptible inbred pericarps constitutively expressed several fewer isozymes than the resistant inbred, including a cationic peroxidase (*10*). A cationic peroxidase from barley also has been associated with disease resistance response (*21*). Challenging maize kernels with potential pathogens at milk stage also demonstrated induction of a cationic peroxidase isozyme (*9*). Conditions that contribute to *A. flavus* and aflatoxin severity, heat and drought, are also reported to reduce peroxidase activity in maize (*22*, *23*). This background information prompted further examination of the cationic peroxidase isozyme profile in kernels of several inbreds previously rated for relative susceptibility to *A. flavus*, which included a wide range of percent infection (*18*, *19*). Cationic peroxidase profiles were also examined in additional maize tissues at constitutive levels and after challenge with potential inducing agents. Additionally, this cationic isozyme was matched with a known, genetically determined peroxidase gene by relating the corresponding peroxidase isozyme band from the native gel to its isoelectric point and comparing expression profiles of several inbreds that had

* Author to whom correspondence should be addressed [telephone (309) 681-6242; fax (309) 681-6686; e-mail dowdpf@ncaur.usda.gov].

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previously been genotyped as to expression profile. Finally, a cDNA clone coding for this cationic peroxidase isozyme was identified.

MATERIALS AND METHODS

Plants. Eleven inbreds previously rated for resistance to *A. flavus* (Gt106, Mo18W, Mp313E, Mp339, Mp428, Mp420, Mp68:616, Sc212M, Sc54, Tx601, Tx805) were grown in Mississippi as described previously (*18*, *19*). At least two milk stage ears were shipped on dry ice to Peoria, IL. Seedlings used in experiments were grown in G-10 growth chambers (EGC, Chagrin Falls, OH) with 14:10 h light/ dark photoperiods in previously described soils (*24*) supplemented with 5 vol % Osmocote. Plants were also grown in the greenhouse using the same photoperiod with supplemental lighting and 18.3 °C night, 29.4 °C day target temperatures. Plants were grown in outside plots from transplants as described previously (*10*) except supplemental nitrogen was added using Miracle-Gro Lawn Food 36−6−6 NPK (Stern's Miracle Gro Products, Port Washington, NY) at a weekly interval. Heat shock was induced by holding plants in growth chambers at 38 °C for 16 h, followed by a 24 h recovery period. For induction assays, husks were surface sterilized using 70% ethanol, a push drill with a 2 mm bit was used to make a hole in the husk, and 20 μL of either inoculum of *Bacillus subtilis* Kodiak formulation (Gustafson Corp., Plano, TX) at 10 mg/mL [$\sim 10^8$ cfu/mL (*25*)] or a microconidial spore suspension of 10^6 cfu/mL of *F. proliferatum* strain M5991 (which was isolated from maize and produces fumonisin, provided by G. Bennett, USDA) was added to the hole. Kernels were harvested 48 h after inoculation and frozen at −20 °C until analysis.

Assays. Frozen tissues were homogenized in ice-cold 0.1 M, pH 7.4, phosphate buffer as described previously (*10*, *24*). Typically, 1 cm^2 sections of leaf tissue or one split pericarp was homogenized per milliliter of buffer. Homogenates were centrifuged at 10000*g* for 15 min, and the supernatant was used as an enzyme source. Prior examination of these preparations had indicated extraction of all peroxidases corresponding to known peroxidase isozymes (*10*, *24*). Material from at least two different plants receiving the same treatments was analyzed.

Peroxidase isozymes in supernatants were separated by isoelectric focusing using wide-range pH 3.5−9.5 precast gels (Pharmacia Biotechnology, Piscataway, NJ) as described previously (*10*). Gels were stained for peroxidase activity using 1-chloronaphthol and dimethylphenylene diamine (Aldrich Chemical Co., Milwaukee, WI) as substrate and coupling agent, respectively (*10*). In some cases, native gel electrophoresis was performed using 7.5% polyacrylamide gels as described previously (*10*). To identify which previously described genetically identified isozyme corresponded to the cationic p*I* 9.0 isozyme, cationic bands were cut from the 7.5% native gel (*10*) and laid on top of the isoelectric focusing gel, followed by isoelectric focusing and staining as just described.

Analysis. For assays with pericarps from inbreds previously tested for *A. flavus* resistance, bands were scored by eye as either readily visible/positive or faint/negative. In some cases, alleles of the same isozyme gene appeared to be present on the basis of similar isoelectric points, but only if bands appeared to have the same isoelectric point as in resistant inbreds were they scored as positive. Genetic scores for inbreds and hybrids made from scored inbreds were subjected to linear regression analysis using the Proc Reg, Max-R method (*26*), with inbred and hybrid percent infection ratings previously published (*18*, *20*) as the dependent variable. Previously reported values for hybrids from different years and different inoculation methods (silk channel versus puncture) were used separately.

Other isozyme results are reported on the basis of scoring by relative intensity compared to "control" type material. Gels were "developed" until at least one isozyme became readily visible. Gels were stained for up to 1 h and examined at frequent intervals to discriminate between fine degrees of band intensity.

Preparation of Constructs To Identify a cDNA Clone Coding for the p*I* 9.0 Isozyme. A series of full-length cDNA clones derived from EST libraries and tentatively identified as peroxidases on the basis of sequence homology were obtained from Pioneer Hi-Bred International and were grown as colonies on 0.01% ampicillin or kanamycin (depending on the clone) plates. Colonies were individually picked and inoculated into terrific broth with ampicillin or kanamycin (Sigma Chemical Co., St. Louis, MO) and grown at 37 °C overnight. Plasmid DNA was isolated from the cultures using standard methods (Qiagen, Inc., Valencia, CA). A *Sma*I 5′ restriction site and a *Sst*I 3′ restriction site were added to the peroxidase coding sequence by PCR using the forward primer 5′GCGCCCGGGCCATGNNNNNNNNNNNNNNN-NNNNNNNN3′ and the reverse complement primer 5′GCGCGC-GAGCTCTTANNNNNNNNNNNNNNNNN3′ (with N dependent upon the sequence of the respective clones). The PCR mix was composed of 10 μL of Pfu polymerase and 5 μL of 10× buffer (Stratagene USA, La Jolla, CA), 5 μL of each dNTP as supplied (Stratagene), 5 μL of each primer (100 ng/μL), 5 μL of the plasmid preparation (200 ng/μL), and 200 μL of water, subdivided into five equal volume 50 μL aliquots, and overlaid individually with 50 μL of mineral oil (Applied Biosystems, Inc., Foster City, CA). PCR was performed using an MJ Minicycler (MJ Research, Waltham, MA) with cycle conditions of initial denaturation at 96 °C for 45 s; followed by 30 cycles of 96 °C for 45 s, 68 °C for 45 s and 70 °C for 4 min; and a final extension of 72 °C for 10 min. The PCR products were separated in 0.8% agarose gels and purified from the gels using a matrix binding method (Q-Biogene, Carlsbad, CA). The purified PCR products were sequentially digested with *Sma*I and *Sst*I according to the manufacturer's instructions (InVitrogen Life Technologies, Carlsbad, CA), then separated on 0.8% gels, and purified from the gel as just described. The plasmid pAHC25, which contains a GUS gene driven by a ubiquitin promoter, and a BAR selectable marker gene (*27*), was used as a host plasmid for the peroxidase clones by removing the GUS gene and substituting the peroxidase gene. The GUS gene was removed using *Sma*I and *Sst*I, separating the products in 0.8% agarose gel, purifying the host plasmid band, and then ligating in the *Sma*I and *Sst*I ended cDNA peroxidase clones using a Rapid DNA Ligase kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. The ligation mix was transformed into *Escherichia coli* DH10B cells according to the manufacturer's instructions (Invitrogen Life Technologies), and colonies were selected on 50 μg/mL ampicillin or 50 μg/mL kanamycin containing plates. Colonies that grew were subcultured in liquid terrific broth, and plasmid DNA was prepared as just described. The presence of the correct-sized insert was verified by digesting with *Sma*I and *Sst*I, separating the products with 0.8% agarose gels, and comparing with molecular weight markers (Gene Choice, PGC Scientific Corp., Frederick, MD) to determine which inserts were of the correct size. The peroxidase sequence of the new plasmids containing the correct-sized inserts was verified using sequencing reactions following the manufacturer's instructions (Applied Biosystems Inc.) and an ABI 3100 sequencer (Applied Biosystems, Inc.).

Stable Transformation with cDNA Clones Potentially Coding for the p*I* 9.0 Isozyme. The peroxidase cDNA clones under the control of the maize ubiquitin promoter described above were introduced individually into maize BMS cells (*28*) using biolistic technology. The plasmid pAHC25 was also introduced into these cells as a transformant control. Maize BMS cells were maintained in a shaking incubator at 28 °C and 130 rpm in BMS medium (Murashige and Skoog plant salts plus Gamborg's vitamins, 20 g/L sucrose, 0.169 g/L asparagine, 2 mg/L 2,4-D, pH 5.8) in the dark. One day before bombardment, a thin layer of BMS cells was placed on a sterile filter paper disk that was resting in a sterile Büchner funnel. Any excess BMS liquid was removed by applying a vacuum. The disk with the BMS cells was placed on BMS medium plates containing 3 g/L Gelrite and kept at 28 °C in the dark overnight. The next day, each plasmid was separately bound to 1.0 μm gold particles (Bio-Rad Laboratories, Hercules, CA) as follows. A 40 mg/mL ethanol suspension of the gold particles was vortex mixed for 45 s, and then 37 μL was added to a 1.5 mL microfuge tube. The tube was centrifuged at high speed for 30 s, the ethanol was removed, and 1 mL of sterile distilled water was added. The tube was vortex mixed and then centrifuged at high speed for 5 min. The water was removed, 25 μL of sterile water was mixed with the gold particles, and 10 μL of water containing 4 μg of plasmid DNA was added. The suspension was vortex mixed for 20 s, the tube was laid on its side, and 20 μL of filter-sterilized 0.1 M spermidine plus 50 μL of filter-

sterilized 2.5 M $CaCl_2$ were added as separate droplets. The tube was slowly tilted so that the three solutions made contact simultaneously and then vortex mixed for 2 min. The suspension was centrifuged for 3 s at high speed, and the supernatant was discarded. The gold particles were suspended in 200 μL of ethanol, and the tube was sonicated. The tube was centrifuged for 3 s at high speed, the supernatant was discarded, and 100 μL of ethanol was added. Suspensions were held on ice until added to each macrocarrier. The suspension was sonicated, and 10 μL was added to each sterile macrocarrier. One macrocarrier, coated with gold particles and DNA, was bombarded into one plate of BMS cells using the PDS-1000 biolistic device (Bio-Rad Laboratories) according to the manufacturer's instructions. The macrocarrier was placed ~2 cm below the 1100 psi rupture disk assembly. The plate of BMS cells was placed on a shelf two tiers (~5 cm) below the macrocarrier assembly, and the fire key was depressed once the vacuum reached 28−29 mmHg. BMS medium (0.5−1 mL) was added to each plate of cells after bombardment, and then the plates were placed in a 28 °C incubator overnight in the dark. The next day, cells were removed from the filters and placed on BMS medium with 3 g/L Gelrite and 1 mg/L Bialaphos (the selection agent, PhytoTechnology Laboratories, Shawnee Mission, KS). Cells were kept on this selective medium for ~3 weeks at 28 °C in the dark. Growing clumps of cells were then transferred to fresh selective media. Putative transformants were maintained by transferring to new selective medium on a weekly basis. Clumps of cells 1 week old and ~1 cm wide were homogenized in 250 μL of phosphate buffer and then prepared, electrophoresed, and stained as described above for peroxidase isozyme detection.

Evaluation of Stable Transformants Producing the p*I* 9.0 Isozyme for the Presence of the cDNA. The presence of the peroxidase gene construct in BMS transformants that produced the p*I* 9.0 isozyme was verified using PCR analysis. Nucleic acids were prepared by taking a small amount of transformed cells and placing them in a 1.5 mL microfuge tube and holding at −20 °C for at least 30 min. Four hundred microliters of urea extraction buffer [168 g of urea (6.9 M final), 25 mL of 5 M NaCl (0.31 M final), 20 mL of pH 7.9 2M Tris-HCl (0.1 M final), 16 mL of 0.5 M EDTA (0.02 M final), and 20 mL of 20% (saturated) *N*-lauryl sarcosine (0.037 M final) in a total volume of 400 mL] was added to the tube of thawing cells. The mixture was ground with a small plastic pestle, and 500 μL of phenol/chloroform/isoamyl alcohol 25:24:1 was added. The tubes were shaken at room temperature for at least 15 min and then centrifuged for 10 min at high speed. The aqueous layer was removed, reextracted with 400 μL of the phenol/chloroform/isoamyl alcohol and then centrifuged for 5 min at high speed. The aqueous extract was removed and measured to volume, and $^1/_{10}$ volume of 3 M, pH 5.2, sodium acetate was added and mixed. Two volumes of ethanol was added to the tube, and the tube was mixed and held at −20 °C for at least 30 min. The extract was centrifuged at high speed for at least 10 min, and the pellet was washed with 70% ethanol and recentrifuged for 5 min. The pellet was resuspended in TE or water.

At least 240 ng of total nucleic acid was analyzed by PCR using the following two primers: the forward primer located within the ubiquitin promoter (5′GATGATGGCATATGCAGCAGCTATATGTGG3′) and a reverse complement primer located within the peroxidase gene (5′GACGGGCAAATGCTCTTGAGGAGGAACG3′) (MaizeSeq Database 54759C.1). The PCR mix totaled 50 μL and consisted of 1× PCR buffer, 200 μM of each dNTP, 2 mM $MgCl_2$, 0.5 μM of each primer, 1.25 units of Amplitaq DNA polymerase (Applied Biosystems), and 5% DMSO. The PCR program involved an initial denaturation step at 94 °C for 3 min; followed by 40 cycles of 94 °C for 1 min, 57.1 °C for 1 min, and 72 °C for 1 min; then a final extension cycle at 72 °C for 10 min, using a PTC-200 thermal cycler (MJ Research). Products were separated in a 1.2% agarose gel containing SYBRSafe DNA stain (Molecular Probes, Inc., Eugene, OR) and compared with standard molecular weight markers as just described using an ImageMaster VDS-CL imaging system (Amersham Biosciences). The plasmid containing the peroxidase gene served as a positive control, and transformants containing the GUS gene served as negative controls.

Table 1. Associations of Milk Stage Maize Pericarp p*I* 9.0 Peroxidase Isozyme Presence or Absence with Field Percentage Infection by *A. flavus*[a]

year	*n*	*y* intercept	*x* peroxidase	R^2	*P*
inbreds					
1984 PB	8	15.5	–10.0	.82	0.0021
1985 PB	8	30.5	–20.3	.62	0.0203
1986 PB	7	77.5	–58.9	.83	0.0045
1986 NC	7	58.3	–50.0	.92	0.0006
1986 SN	7	66.2	–55.7	.94	0.0003
hybrids					
1987/88 PB	14	35.0	–9.6	.74	0.0001
1987/88 PB	13	34.8	–9.2	.76	0.0001

[a] PB, pinbar; NC, needle in channel; SN, side needle. The 1984–1986 field data for *A. flavus* of inbreds scored in the present study for production of the p*I* 9.0 peroxidase isozyme are from Scott and Zummo (*18*), and the 1987–1988 field data are from Scott and Zummo (*19*). R^2 and *P* values were obtained using the SAS program Proc Reg with MaxR selection. A second series from 1987/1988 ($n = 13$) was run due to the presence of a statistical outlier for $N = 14$ analysis, which was removed for the $n = 13$ analysis.

RESULTS

The p*I* 9.0 peroxidase isozyme extracted from milk stage pericarp of the six susceptible and five resistant inbreds to *A. flavus* appeared to be important in explaining the resistance to *A. flavus* from 1984 to 1988 for both inbreds and hybrids, with R^2 values ranging from 0.62 to 0.94 depending on the year of field data and inoculation method used for the hybrids (**Table 1**). Percentage infection by *A. flavus* was inversely related to the presence of the cationic peroxidase isozyme. However, the value of the *y*-intercept indicates additional factor(s) present in all inbreds that contribute to resistance, which varied in degree from year to year.

The p*I* 9.0 peroxidase isozyme was often present at constitutively higher levels in commercial inbreds resistant to *F. verticillioides* (pericarps) compared to near isogenic lines or lines with known susceptibility to pathogens, such as B73, A632, and commercial susceptible inbreds (**Table 2**). If not already constitutively expressed in tissues and lines, this isozyme was also often induced by mechanical damage, heat shock, *F. proliferatum*, and/or *B. subtilis* (**Table 2**). Of the three possible cationic isozyme bands corresponding to *px1*, *px4*, and *px5* (*13*, *29*) removed from the native gel and separated by isoelectric focusing, only the excised band corresponding to *px5* produced a band at p*I* 9.0. Leaf tissue profiles of the *px5* isozyme for the characteristic inbreds were consistent with previously reported allele types. The bands for A295, B37, and M14 all were of the same type (p*I* 9.0). The *px5* allele from L289 had a null scoring previously based on ref *13*, but in our study, it appeared as a band with a slightly higher isoelectric point than the other allele forms.

The commercial clone Px15 putatively coding for a peroxidase produced a p*I* 9.0 peroxidase isozyme band in all transformants of BMS cells assayed, with varying activity (**Figure 1**). The isozyme had the same isoelectric point as the peroxidase isozyme produced in B73 leaf tissue. This isozyme was not present in the sets of BMS cells transformed with the GUS construct. PCR analysis of these peroxidase transformants using a primer located within the ubiquitin promoter sequence and a complementary reverse sequence primer located in the peroxidase gene amplified a band of expected size of ~850 base pairs (**Figure 2**).

Table 2. Constitutive or Induced Expression of Peroxidase Isozymes Related to Disease Resistance or Induction by Mechanical Damage, Induction, or Heat Shock

source/treatment	p*I* 9.0 response
BMS	
7-day-old seedling leaf	+
callus, normal	–
callus, apoptotic	+
GH70	
7-day-old seedling leaf	+
7-day-old seedling leaf, heat shocked	+
21 days after pollination, pericarp	f
21 days after pollination, pericarp, wounded	f
21 days after pollination, pericarp plus *B. subtilis*	+
A632	
7-day-old seedling leaf	+
7-day-old seedling leaf, heat shocked	++
21 days after pollination, pericarp	f
21 days after pollination, pericarp, wounded	+
21 days after pollination, pericarp and *F. proliferatum*	+
CM45	
7-day-old seedling leaf	+
21 days after pollination, pericarp	+
21 days after pollination, pericarp, wounded	f
21 days after pollination, pericarp and *B. subtilis*	f
PHN46	
7-day-old seedling leaf	+
21 days after pollination, pericarp	–
21 days after pollination, pericarp, wounded	–
21 days after pollination ,pericarp and *F. proliferatum*	+
21 days after pollination, pericarp, heat shocked	++
PHG61 (ear rot resistance)	
21 days after pollination, pericarp	+
21 days after pollination, pericarp, wounded	+
21 days after pollination, pericarp and *F. proliferatum*	+
B73	
7-day-old seedling leaf	+
7-day-old seedling leaf, heat shocked	++
21 days after pollination, pericarp	f
peroxidase allele variants	
A295	
7-day-old seedling leaf	++
21 days after pollination, pericarp	++
B37	
7-day-old seedling leaf	f
21 days after pollination, pericarp	+
L289	
7-day-old V8 leaf	+2
M14	
7-day-old seedling leaf	f

[a] Ratings: –, not or barely visible; f, faint; +, solid black, readily visible; ++, very intense or enhanced over control material (as for induction studies); 2, different allele type. To help discriminate between induced band ratings, gels were examined at frequent intervals from placement in the staining solution until 1 h after being placed in the staining solutions.

DISCUSSION

Peroxidase involvement in maize resistance to pathogens has been previously suggested using a variety of methods. One source of evidence is based on papilla formation associated with peroxidase activity when challenged with fungi (*4*). Induction of peroxidase is sustained in varieties resistant to *H. maydis* race T (*6*). Activity levels of general peroxidase activity have been reported to be up to 3-fold higher in pericarps of the *A. flavus* resistant inbred Mp313E compared to the susceptible SC212M (*10*).

Our identification of a maize cationic peroxidase isozyme associated with disease resistance, stress, or wounding response is consistent with past reports of peroxidase associations with disease resistance response in maize and other monocots.

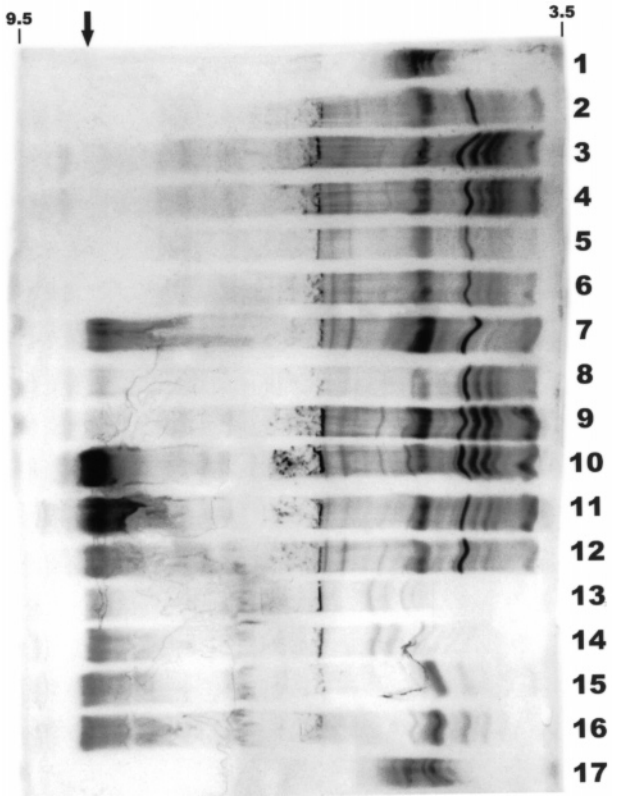

Figure 1. Isoelectric focusing gel showing expression of p*I* 9.0 peroxidase isozyme by "PX15" cDNA clone expressed in BMS cells: (lanes 1 and 17) Evans's blue isoelectric focusing standard; (lanes 2–6) GUS transformants; (lanes 7–12) peroxidase transformants; (lanes 13 and 14) B73 leaf extracts; (lanes 15 and 16) L289 leaf extracts.

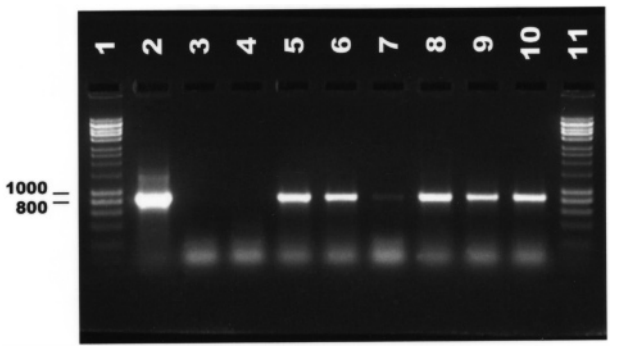

Figure 2. PCR product from primers based in ubiquitin promoter and p*I* 9.0 peroxidase gene from transformed BMS cells: (lanes 1 and 11) molecular weight standards (DNA ladder); (lane 2) positive control peroxidase plasmid; (lanes 3 and 4) GUS transformants; (lanes 5–10) peroxidase transformants.

However, most prior studies on peroxidase and disease resistance in maize have involved anionic peroxidases. Isozyme analysis has previously indicated the induction of anionic peroxidases when maize tissues are challenged with disease organisms such as *F. moniliforme* (*5*) or *Exserohilum turcicum* (*8*, *30*). Constitutive expression of anionic peroxidases is also reported for maize lines resistant to *E. turcicum* (*8*).

Information on cationic peroxidases in maize disease resistance is more limited. Part of the problem in interpreting past studies is that calculated p*I* values based on cloned DNA sequence may not correspond to the actual determined p*I* of the native isozyme. For example, a barley peroxidase induced by disease has an actual expressed p*I* of 8.5 (*31*, *32*), but the p*I* calculated from the corresponding clone is 6.4 (*21*). Thus, the

following discussion is primarily based on information for which actual isozyme data are available, rather than data where p*I* values are calculated on the basis of DNA sequence only. The *px5* peroxidase isozyme (p*I* 9.0) was also mentioned as potentially important in resistance to *A. flavus* when isozyme patterns of Mp313E versus SC212M were initially examined (*10*). The p*I* 9.0 peroxidase isozyme was only weakly expressed in maize pericarp of B73, an inbred with reported susceptibility to *A. flavus* and aflatoxin production (e.g. ref *33*) and *Fusarium* ear rot and fumonisin accumulation (e.g., ref *34*). A cationic peroxidase was also induced in maize, along with other disease response proteins, when an avirulence protein was expressed in maize (*35*). High p*I* isozymes induced/involved in disease resistance have also been reported for other monocots such as barley (p*I* 8.5; *31*, *36*), rice (p*I* 8.6; *37*), and wheat (p*I* 8.1; *38*, *39*). However, in one case, transient expression of a cDNA of a high p*I* barley peroxidase that was induced when leaves were challenged by powdery mildew increased the number of colonies colonized by powdery mildew compared to control cells (*40*). In the present study, constitutive expression of the p*I* 9.0 isozyme was more common in *A. flavus* and *F. verticillioides* disease resistant material than was induction from null expression when challenged by potential pathogens in disease susceptible material. Thus, constitutive expression of this isozyme may be necessary to promote significant disease resistance. Constitutive expression of other proteins potentially involved in maize resistance to *A. flavus* was also thought to be important (*41*). Proteins other than peroxidases have also been implicated in contributing to disease resistance of different inbreds to *A. flavus* (*41*, *42*).

The molecular weight of the p*I* 9.0 isozyme determined by gel filtration chromatography is higher (Dowd, unpublished data) than the predicted molecular weight of the clone producing the p*I* 9.0 peroxidase or another peroxidase clone described from maize (*43*). The p*I* 9.0 maize peroxidase is also retained by a concanavalin A column (Dowd, unpublished data), suggesting the isozyme is glycosylated. There are nine predicted sites of glycosylation in the sequence of the PX15 cDNA clone of the putative peroxidase expressed in maize cells in the present study. Glycosylation is a property that is associated with proper folding, thermal stability, and catalytic ability of peanut peroxidase, depending on the position of the glycosyl group (*44*).

The maize p*I* 9.0 peroxidase isozyme is capable of oxidizing a broad range of maize-derived substrates (*10*), several of which become more toxic to both fungi (*14*) and insects (*11*, *15*) when oxidized by peroxidases. Mapping of disease resistance genes indicates the involvement of multiple chromosome BIN sites, some of which also occur at previously reported insect resistance QTLs (*45*). Association of both fungal and insect resistance QTLs at the same BIN sites would be consistent with a single gene peroxidase involvement with both fungal and insect resistance.

In conclusion, the results of this study indicate a specific peroxidase isozyme of p*I* 9.0 is associated with disease resistance to *A. flavus*. It is also often constitutively present in other disease resistant material and/or is induced by stress conditions such as mechanical damage, inoculation with pathogens, or heat shock. This isozyme appears to potentially be a good marker for following disease and perhaps insect resistance in breeding programs. As breeding programs directed toward yield have reported reductions in null peroxidase scoring (i.e., expression of more isozymes) (*46*), expression of "missing" peroxidases may not only confer insect or disease resistance but also may result in increases in yield. The present study suggests that when peroxidases are contributed by both parents (hybrid scoring versus infection by *A. flavus*), better disease resistance can result. Because peroxidase activity is substrate dependent as well, increases in disease resistance may be limited by substrate content, as has been shown for insect resistance in tomato expressing high levels of tobacco anionic peroxidase (*47*). However, because the peroxidase substrate complex is also important in determining the ability of the plant to defend itself against insects and fungi (*14*, *15*), studies of the involvement of the p*I* 9.0 peroxidase are likely to be needed in maize lines of different backgrounds to more fully determine its role in resistance. There is some information to support studying the association of this isozyme and insect resistance, as kernel resistance to insect feeding has been reported for an inbred that produces the isozyme in the pericarp compared to one that does not (*10*, *20*). In addition, regression analysis of six inbreds for which both quantitative values for leaf resistance to the European corn borer (*48*) and p*I* isozyme scoring (present study) are available has indicated a correlation coefficient of 0.79 and *P* value of 0.066 (Dowd, unpublished data). If a role in resistance by this isozyme is demonstrated by expression or knockout studies, mapping of the responsible gene would also allow for marker-assisted breeding, as has been described for other resistance traits in inbreds resistant to *A. flavus* (*49*).

ABBREVIATIONS USED

BMS, black Mexican sweet; IEF, isoelectric focusing; NPK, nitrogen−phosphorus−potassium; PCR, Polymerase Chain Reaction; p*I*, isoelectric point.

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