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Association of a 33-Kilodalton Cysteine Proteinase Found in Corn Callus with the Inhibition of Fall Armyworm Larval Growth[1]

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Protein patterns of callus from corn (*Zea mays* L.) inbreds that are either resistant or susceptible to fall armyworm (*Spodoptera frugiperda* [J.E. Smith]) were analyzed by two-dimensional electrophoresis. Fall armyworm larvae reared on callus initiated from resistant inbreds were significantly smaller than those reared on callus of susceptible inbreds. A 33-kD protein found in callus from the resistant inbreds Mp704 and Mp708 was absent in callus from the susceptible inbreds Tx601 and Ab24E. However, a 36-kD protein found in Ab24E callus immunoreacted with polyclonal antibody raised against the 33-kD protein. When Mp704 nonfriable callus changed to friable, larval growth was not inhibited and the 33-kD protein was absent. There was a significant negative correlation between the concentration of the 33-kD protein in the callus and the weight of the larvae feeding on the callus in the F_2 progeny of Mp704 × Tx601. The N-terminal amino acid sequence of the 33-kD protein suggested that it was cysteine proteinase. Purification of the 33- (Mp708) and 36-kD (Ab24E) proteins indicated that they were both cysteine proteinases. The 33-kD cysteine proteinase had 7-fold higher specific activity than the 36-kD enzyme.

Fall armyworm (*Spodoptera frugiperda* [J.E. Smith]) and southwestern corn borer (*Diatraea grandiosella* Dyar) are serious insect pests of corn (*Zea mays* L.) in the southern United States. Larvae of both species damage plants by feeding on leaves within the whorls. Several germplasm lines with resistance to these two insects have been developed and released (Williams and Davis, 1982, 1984; Williams et al., 1990b). These lines also show resistance to several additional lepidopterous insects, including the sugarcane borer (*Diatraea saccharalis* [Fab]), the corn earworm (*Helicoverpa zea* [Boddie]), and the European corn borer (*Ostrinia nubilalis* [Huber]) (Davis et al., 1988).

Field experiments indicated that fall armyworm and southwestern corn borer larvae feed more extensively on whorls of susceptible than resistant plants, and that larvae recovered from resistant plants are smaller than those recovered from susceptible plants (Williams et al., 1989). When larvae of both species were reared in vitro on vegetative tissues (leaves and whorls), those reared on tissue of resistant plants weighed less than those reared on susceptible tissue (Wiseman et al., 1981; Ng et al., 1985; Williams et al., 1990a). Both antibiosis and nonpreference appear to be operating as mechanisms of resistance (Wiseman et al., 1981, 1983).

Similar results have been obtained using callus initiated from mature embryos of resistant and susceptible genotypes (Williams et al., 1983). Larvae of fall armyworm (Williams et al., 1985) or southwestern corn borer (Williams and Davis, 1985; Williams et al., 1987) reared for 7 d on callus from resistant hybrids weighed significantly less than those reared on callus initiated from susceptible genotypes. Since the growth of larvae reared on resistant callus was inhibited, there may be some biochemical difference between callus from the resistant and susceptible genotypes that contributes to the differential feeding response. Attempts to identify specific secondary metabolites that may be involved in resistance have been inconclusive (Hedin et al., 1984).

Recently, genes encoding the toxin from *Bacillus thuringensis* have been transferred to several crop species for the control of lepidopteran pests (Shade et al., 1994). The *B. thuringensis* toxin is highly toxic to these insects, and it is believed that they will rapidly develop resistance to the toxin when the transgenic crops are grown in the field (Shade et al., 1994). Several endogenous plant proteins may confer resistance to insect pests. These include the Ser proteinase inhibitors, lectins, and α-amylase inhibitors

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Abbreviations: CLN, *p*-nitrophenyl-N[a]-benzylcarbonyl-L-lysinate; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guandino)-butane; 2-D, two-dimensional.

(Shade et al., 1994). Proteins such as these may not be highly toxic, but they can control the rate of development of pest insect populations (Shade et al., 1994). These moderately toxic proteins exert a less stringent selection pressure than highly toxic insecticides and slow the development of resistance (Tabashnik, 1994).

This study was conducted to determine if proteins associated with reduced larval growth could be identified in callus initiated from corn genotypes exhibiting resistance to fall armyworm and southwestern corn borer larvae. Callus was used as an experimental material because the phenotype, retardation of larval growth, was expressed in callus. In addition, previous studies with whorl tissue, the site of insect feeding, indicated that a single whorl contained many developmentally distinct regions with unique protein patterns. Differences in sampling within the same whorl from a single genotype resulted in inconsistent protein patterns (Callahan et al., 1992). The use of callus also eliminated the interference of photosynthetic proteins in 2-D gel analysis. The results of this study indicate that a unique 33-kD Cys proteinase is present in callus initiated from resistant genotypes.

MATERIALS AND METHODS

Plant Materials

Corn (*Zea mays* L.) genotypes used in this study were the resistant inbreds Mp704 and Mp708 (Williams and Davis, 1982; Williams et al., 1990b) and the susceptible inbreds Tx601 and Ab24E (Williams et al., 1989). F_1 and F_2 progeny of the resistant × susceptible hybrid Mp704 × Tx601 were also used. F_2 seeds were obtained by self-pollinating Mp704 × Tx601 plants.

Callus was initiated and maintained using modifications of the method of Williams et al. (1987). Mature kernels were surface sterilized in 5.25% sodium hypochlorite (Clorox, 100%, v/v) containing a few drops of Tween 20 for 10 min. The kernels were transferred to 70% (v/v) ethanol for 10 min and then rinsed four times with sterile, deionized, distilled water. Kernels were wrapped in three layers of paper towels moistened with sterile water containing 0.1 mg/mL gentamicin sulfate and 1.2 mg/mL sorbic acid and incubated at 28°C for approximately 2 d. Embryos were excised from the germinated kernels and placed on initiation medium (Murashige and Skoog, 1962) containing 1.0 mg/L thiamine-HCl, 0.5 mg/L pyridoxine-HCl, 0.5 mg/L nicotinic acid, 100 mg/L *myo*-inositol, 20 g/L Suc, 6 g/L agar, 15 mg/L 2,4-D, and 1.5×10^{-4} mg/L zeatin. Callus was produced by incubating at 28°C with a 5-h-light and 19-h-dark photoperiod in a growth chamber. Approximately 1 month later, the developing callus was transferred to maintenance medium, which was the same as the initiation medium except that the concentrations of 2,4-D and zeatin were reduced to 5 mg/L and 1.0×10^{-4} mg/L, respectively. Callus was transferred monthly to fresh maintenance medium. Callus in culture for at least 2 months was used for all analyses. For enzyme purification, callus was frozen in liquid nitrogen and stored at −70°C.

Preparation and Collection of Embryo Samples

To determine if the synthesis of the 33-kD protein was dependent on the length of culture time or the presence of plant growth regulators, kernels of Mp704 and Tx601 were germinated at 28°C for 2 d. Several germinated embryos from each genotype were excised and stored at −70°C for controls. On d 0, embryos excised from 2-d germinated kernels of Mp704 or Tx601 were placed on initiation medium and on initiation medium without plant growth regulators (basal medium). Six embryos or calli from each treatment were collected at 3-d intervals for 35 d (d 0–35) and stored at −70°C. After 35 d, all embryos cultured on basal medium were transferred to fresh basal medium, and all calli cultured on initiation medium were transferred to maintenance medium. Six embryos or calli from each treatment were collected at 3-d intervals for 26 additional d (d 38–61) and stored at −70°C. Samples were used for the ELISA analysis as described below.

2-D PAGE

Total proteins were extracted from callus samples (4 g each) using the phenol extraction method (Hurkman and Tanaka, 1986). The protein pellet was dissolved in lysis buffer (O'Farrell, 1975) that contained 1% (v/v) 3-[(3-chloamidopropyl)-dimethylammonia]-2-hydroxy-1-propanesulfonate) in addition to the 1% (v/v) Nonidet P-40. Aliquots of 2-mercaptoethanol were added to the protein samples to give a final concentration of 5% (v/v). The protein concentrations of the samples were determined using the Pierce bicinchoninic acid protein assay reagent following the directions of the manufacturer. BSA was used as a standard.

2-D PAGE was conducted as described by O'Farrell (1975) and O'Farrell et al. (1977), except that the IEF gels contained 9 M urea, 4.2% (w/v) acrylamide (acrylamide: bisacrylamide, 15.7:1), 1% (v/v) Nonidet P-40, 1% (v/v) 3-[(3-chloramidopropyl)-dimethylammonia]-2-hydroxy-1-propanesulfonate), 5.5% (v/v) ampholines (4.4% pH 5–8 and 1.1% pH 3.5–10), 0.1% (w/v) of 10% (w/v) ammonium persulfate, and 0.07% (v/v) N,N,N′,N′-tetramethylethylene diamine. After pre-electrophoresis (O'Farrell, 1975), samples containing 400 or 500 μg of total protein were layered onto each tube and electrophoresed at 400 V for 12 h and at 800 V for 1 h. Gels were removed from the tubes and equilibrated in 5 mL of SDS-PAGE sample buffer (Laemmli, 1970). Proteins were separated in the second dimension by SDS-PAGE (Laemmli, 1970) on gels containing a 10 to 15% (w/v) gradient of polyacrylamide. After electrophoresis in the second dimension, gels were stained overnight with staining solution containing 1 volume of methanol and 4 volumes of the mixture of 0.1% (w/v) Coomassie brilliant blue R-250 (United States Biochemical) and 10% (w/v) ammonium sulfate in 2% (v/v) phosphoric acid. Gels were destained with a mixture of methanol: glacial acetic acid:water (50:75:875) until the background was clear. The gels were dried by placing each gel between two pieces of Gel Drying Film (Promega) for 3 d at room temperature.

Antiserum Production

The procedure of Meyer et al. (1988) was used to obtain sufficient quantities of the 33-kD protein for polyclonal antibody production. Approximately six protein samples (500 μg each) were separated on IEF tube gels as described above. The acidic regions of each gel (the area where the 33-kD protein migrated) were excised and aligned in tandem in the well for SDS-PAGE. Electrophoresis in the second dimension and staining were conducted as described above. Several of these gels were run, and each gel had a row of spots containing the 33-kD protein. Approximately 20 to 25 of these spots were excised, pooled, and ground in liquid nitrogen using a mortar and pestle in 200 μL of PBS buffer (8 g of NaCl, 0.2 g of KH_2PO_4, 1.13 g of Na_2HPO_4, 0.2 g of KCl, and 0.2 g of NaN_3/L, pH 7.4). An equal volume of Freund's complete adjuvant was added to form an emulsion. Antibody was raised in New Zealand White rabbits by the staff at the College of Veterinary Medicine at Mississippi State University using standard immunological techniques. Two booster injections were given, each containing 20 to 25 spots of the 33-kD proteins. Antiserum was prepared as previously described (Luthe, 1986).

Immunoblot Analysis

Proteins (100 μg) extracted from calli of several genotypes were separated by SDS-PAGE and blotted onto nitrocellulose membrane using the ABN Polyblot system (American Bionetics, Inc., Hayward, CA) following the instructions of the manufacturer. Immunoreacting proteins were detected using the alkaline phosphatase reaction as previously described (Luthe, 1991).

ELISA Procedure

Protein for ELISA was extracted by homogenizing approximately 0.3 g of callus frozen in liquid N_2 in 1.0 mL of PBS buffer containing 0.35 mg/mL PMSF using a mortar and pestle. The homogenate was centrifuged at 13,000*g* for 7 min. The supernatant was removed and samples were stored at 4°C. The protein concentration was determined using the Pierce bicinchoninic acid protein assay as described above. Protein (15 μg) extracted from each callus sample was diluted to 200 μL in ELISA coating buffer (Hampton et al., 1990). Aliquots of each sample (200 μL) were placed in wells of a polystyrene (Immulon I) microtiter plate (Dynatech Laboratories, Inc., Chantilly, VA). The ELISA was conducted using the method of Hampton et al. (1990), and reaction products were quantified using a Bio-Tek Microplate Reader model EL309 (Burlington, VT) at 405 nm.

Insect Feeding Bioassays

Insect feeding bioassays were conducted using methods similar to those described by Williams et al. (1985, 1987). For all bioassays, one fall armyworm neonate larva was placed in a 30-mL plastic insect diet cup containing approximately 500 mg of callus. Cups with callus of the appropriate genotype were arranged in a randomized complete block design with 10 to 40 replications per experiment. Larvae were weighed after 7 d. Data were analyzed using the General Linear Model procedure of the SAS Institute (Cary, NC). Means were separated using Fisher's protected LSD.

N-Terminal Sequencing of the 33-kD Protein

The N-terminal amino acid sequence of a spot containing the 33-kD protein was determined by microsequencing in the Department of Biochemistry at the University of Mississippi, Mississippi Medical Center (Jackson). The N-terminal sequence was compared to others in the GenBank data base using the Hibio protein analysis program (Hitachi Software Engineering Co., Brisbane, CA).

Preparation and Purification of Cys Proteinases

The enzyme purification procedure was based on that reported by Koehler and Ho (1988). Ten to 20 g of callus, frozen in liquid nitrogen, was pulverized with a mortar and pestle. The ground material was mixed with 2 to 3 volumes of extraction buffer (100 mM Hepes-KOH [pH 8.3], 2 mM DTT, 2 mM EGTA, 1 mM $MnCl_2$, 2 M NaCl, and 0.5% PVP) filtered through Miracloth (Calbiochem) and centrifuged at 10,000*g* for 15 min. The supernatant was fractionated by $(NH_4)_2SO_4$ in two steps. The first step was a 0 to 30% fractionation, the precipitate of which was discarded. The second step was a 30 to 80% fractionation, and the resulting precipitate was pelleted by centrifugation at 10,000*g* for 15 min at 4°C. The 30 to 80% pellet was resuspended in a minimal volume of extraction buffer without 2 M NaCl and dialyzed overnight in dialysis buffer containing 20 mM Mes (pH 6.0), 2 mM DTT, 2 mM EGTA, 1 mM $MgCl_2$, and 1 mM $MnCl_2$. The dialysate was applied to a Toyapearl DEAE-650M (Tosohaas, Montgomeryville, PA) column (1 × 8 cm) equilibrated in equilibration buffer, which was identical to dialysis buffer except that the Mes concentration was reduced to 15 mM. The column was washed with equilibration buffer at a flow rate of 1 mL min^{-1} and then washed again with equilibration buffer containing 150 mM NaCl until the baseline was re-established. The proteinase activity was eluted with a linear gradient of 200 to 300 mM NaCl. Sample purified by DEAE-exchange chromatography was loaded onto a Superdex 200 HR 10/30 fast protein liquid chromatography column (Pharmacia) equilibrated with 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl. The flow rate of the column was 0.5 mL min^{-1}. The column was calibrated with Bio-Rad gel filtration standards.

Proteinase Assays

Esterolytic activity was monitored spectrophotometrically by measuring the increase in A_{340} in 1.0 mL of assay solution containing 10 mM Na-acetate (pH 4.6), 0.1 M KCl, 1 mM L-Cys, and 0.3 mM CLN (Heinrikson and Kezdy, 1976). Activity was calculated based on the molar extinction coefficient for the chromogenic substrate CLN at 6320 M^{-1} cm^{-1}. Inhibitors or activators were added to the above

assay solution as detailed in the text except that SDS activation could be monitored only if the KCl was removed to prevent precipitation of the SDS. Removal of KCl did not affect the activity of the enzyme. The production of TCA-insoluble peptides from a 0.2% azocasein solution dissolved in 10 mM Na-acetate (pH 4.5), Mes (pH 6.0), 2-([2-amino-2-oxoethyl]-amino)ethansulfonic acid (pH 7.0), or Tris (pH 8.0), and 10 mM 2-mercaptoethanol was used to determine the pH optimum (Koehler and Ho, 1988). Proteinase (1.2 μg) was incubated in a 0.8-mL solution of 0.2% (w/v) azocasein at the indicated values for 3 h, 30°C. The reaction was terminated by the inclusion of 0.2 mL of 50% (w/v) TCA. Insoluble material was removed after 10 min by centrifugation and the supernatant was read against an appropriate blank at 280 nm. One unit is defined as a ΔA_{280} $(3\ h)^{-1}$ of 0.018 (Kunimitsu and Yasunobu, 1970). Protein concentrations were determined using the dye-binding method of Bradford (1976).

Electrophoresis

Gel electrophoresis for activity staining was conducted on mildly denaturing 10% (w/v) polyacrylamide gels containing 0.1% (w/v) gelatin (Michaud et al., 1993) using the Bio-Rad Mini-Protean System. Electrophoresis and activity staining were conducted using the procedure of Michaud et al. (1993). For molecular weight determination, samples were analyzed by SDS-PAGE (Laemmli, 1970) on gels containing a 10 to 15% polyacrylamide gradient. Callus extracts were prepared by homogenizing callus in sample buffer (Laemmli, 1970) containing 1 mM PMSF (tissue: buffer, 1:3) using a mortar and pestle. Samples were boiled for 5 min and centrifuged at 13,000g for 5 min at room temperature. Supernatants were used for SDS-PAGE and subsequent immunoblot analysis.

RESULTS

2-D Gel Analyses

Total proteins isolated from callus of several resistant and susceptible corn genotypes were separated by 2-D gel electrophoresis. Comparison of the callus protein patterns of the resistant inbred Mp704 and the susceptible inbred Tx601 indicated that they were similar (Fig. 1) except for the presence of a prominent 33-kD protein in Mp704. This protein was absent in callus from the inbreds Tx601 and Ab24E, which are susceptible to fall armyworm feeding (Williams et al., 1989). The 33-kD protein was also present in callus from the resistant inbred Mp708 (Fig. 1) and four other resistant inbreds (Jiang, 1994). Computer-aided image analysis indicated that the 33-kD protein was approximately 1% of the total protein on the gel. The staining intensity of the 33-kD protein was less in the hybrid Mp704

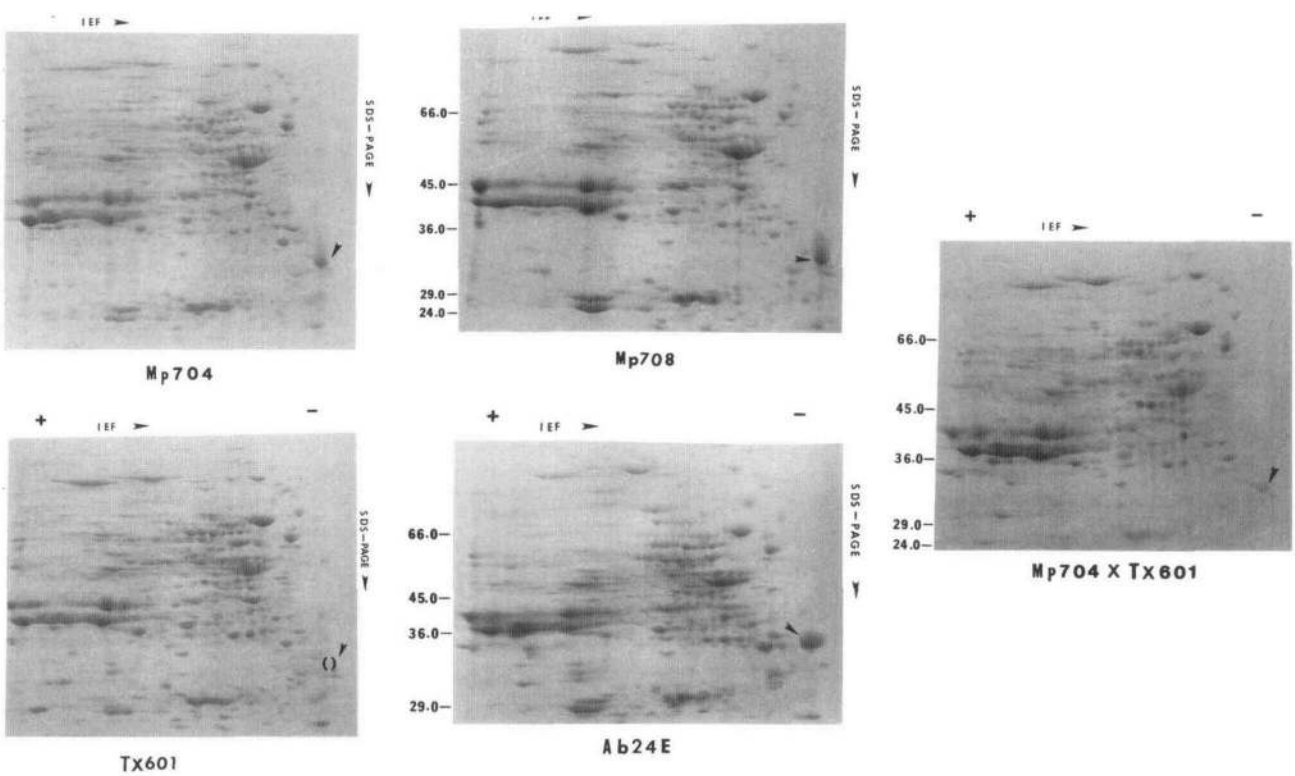

Figure 1. 2-D PAGE analysis of protein patterns of several corn genotypes: the resistant inbreds (Mp704, Mp708), susceptible inbreds (Tx601, Ab24E), and hybrid (Mp704 × Tx601). Arrows indicate the position of the 33-kD protein in Mp704, Mp708, and Tx601 × Mp704 and the position of the 36-kD protein in Ab24E. Brackets indicate the absence of the 33-kD protein in Tx601. Numerals in the left margins indicate the molecular mass (kD) of the protein standards. Direction of IEF is indicated at the top of the gels. Five hundred micrograms of protein were loaded on each gel.

× Tx601 (Fig. 1). Because the 33-kD protein was relatively abundant in the callus of all resistant genotypes tested, further studies focused on identifying this protein and determining if it was correlated with the inhibition of fall armyworm larval growth.

Immunological Detection of the 33-kD Protein

Immunoblot analysis of callus proteins separated on denaturing gels indicated that the antibody raised against the 33-kD protein from Mp708 recognized a 33-kD protein in all of the resistant genotypes tested (Fig. 2). The presence of bands appearing to be doublets was due to compression of the gel during the immunoblotting procedure. No proteins from the susceptible line Tx601 were detected on the immunoblot (Fig. 2). The inbred Va35 had a less intense 33-kD band on the immunoblot (Fig. 2). However, field experiments indicated that Va35 was less susceptible to leaf-feeding damage than several other susceptible genotypes (Williams et al., 1989). This "partially" resistant genotype appears to have a lower concentration of the 33-kD protein in the callus. Alternatively, the polyclonal antibody may have reduced specificity for the 33-kD protein found in Va35.

In the susceptible genotype Ab24E, a 36-kD protein cross-reacted with the antibody (Fig. 2). Although they differ in size, these two proteins appear to have similar antigenicity. Inspection of the 2-D gel in Figure 1 indicated that Ab24E contains a prominent 36-kD protein that appears to have a pI similar to that of the 33-kD protein. Immunoblot analysis of callus proteins separated on nondenaturing gels indicated that the polyclonal antibody cross-reacted with a native protein in Mp708 but not in Tx601 (data not shown).

Nonfriable versus Friable Callus

Typically, the morphology of the callus used in this study was classified as nonfriable. On one occasion, when callus from Mp704 had been in culture for several months, a portion of the callus changed from a nonfriable to a friable morphology. In preliminary larval feeding bioassays using Mp704 callus, we observed that larvae reared on friable callus were larger than those reared on nonfriable callus (data not shown). A comparison of larval growth on friable and nonfriable callus is shown in Table I. Fall armyworm larvae reared on friable callus from Mp704 or Mp704 × Tx601 were significantly larger than those reared on nonfriable callus from the same genotypes. In fact, they were significantly larger than those reared on nonfriable callus from the susceptible genotype Tx601 (Table I). When total protein isolated from Mp704 friable callus was analyzed by immunoblotting, no proteins cross-reacted with the antibody raised against the 33-kD protein (Fig. 2, lane 1). Friable callus from Tx601 was not available for testing. Because the 33-kD proteinase was not present in Tx601 nonfriable callus, it seems unlikely that it would be present in the friable callus from that inbred. These results suggest that the 33-kD protein, or a linked metabolite, is required to retard the growth of larvae feeding on the callus.

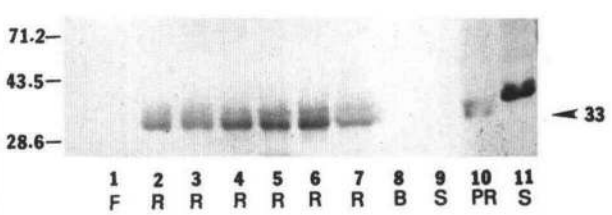

Figure 2. Immunoblot analysis of proteins isolated from callus of several resistant (R) and susceptible (S) corn genotypes. Lane 1, Friable (F) callus from the resistant inbred Mp704; lanes 2 through 7, nonfriable callus from the resistant inbreds Mp704, Mp708, Mp88:5186, Mp88:5253, Mp89:5464, and Mp704 × Mp707, respectively; lane 8, blank; lanes 9 and 11, nonfriable callus from susceptible inbreds Tx601 and Ab24E, respectively; lane 10, nonfriable callus from the partially resistant inbred Va35. Numerals in the left margin indicate the molecular mass (kD) of protein standards. The arrowhead in the right margin indicates the position of the 33-kD protein. PR, Partially resistant.

Table I. *Mean weights of fall armyworm larvae raised on the nonfriable and friable calli of Mp704 and Mp704 × Tx601 and on nonfriable callus of Tx601 for 7 d*

Callus	Larval Mean Weight
	mg
Mp704 nonfriable callus	59.4
Mp704 friable callus	129.6
Mp704 × Tx601 nonfriable callus	93.2
Mp704 × Tx601 friable callus	131.3
Tx601 nonfriable callus	100.0
LSD (0.05)	15.7

Correlation of the Concentration of the 33-kD Protein and the Weight of Larvae Feeding on Calli of F_2 Progeny

The concentration of the 33-kD protein was measured in callus tissues initiated from approximately 200 F_2 progeny of Mp704 × Tx601 using the ELISA. Twenty-six callus lines containing the highest, lowest, and intermediate levels of the protein were used for insect feeding bioassays and immunoblot analysis (Table II). An ELISA reading of 0.4 was required for the 33-kD protein to be visible on an immunoblot. The data in Table II indicate that there was a significant negative correlation ($r = -0.59$, $P < 0.01$) between the concentration of the 33-kD protein as measured by ELISA and larval weight. The weights of larvae reared on callus from lines 57 and 40, which had the highest concentration of the 33-kD protein as determined by ELISA, were among the lowest in the study. Insects reared on callus from lines 145 and 23, which had undetectable levels of the 33-kD protein on western blots and very low concentrations of the 33-kD protein, had two of the highest larval weights (Table II). For several callus lines, the concentration of the 33-kD protein and larval weight did not follow this trend. For example, calli 75 and 134 had a low concentration of the 33-kD protein and low larval weight. This discrepancy could be due to variability among larvae or mis-measurement of the concentration of the 33-kD protein.

Table II. *Relationship between concentration of the 33-kD protein measured by immunoblot and ELISA and the weight of larvae raised for 7 d on calli initiated from F_2 progeny of Mp704 × Tx601 and the parent lines*

F_2 Callus Code	Immunoblot[a]	ELISA[b]	Larvae Weight
		A_{405}	mg
57	++	1.627	26.40
40	++	1.370	31.90
47	++	1.258	47.20
115	++	1.033	36.00
169	++	0.948	30.82
175	++	0.828	79.82
167	++	0.819	43.36
155	++	0.665	75.08
183	++	0.650	10.60
84	++	0.622	87.50
129	++	0.559	27.62
83	+	0.452	39.00
8	+	0.442	108.25
106	+	0.424	76.21
14	+	0.400	87.08
127	+	0.384	47.25
156	−	0.240	73.73
159	−	0.230	100.92
17	−	0.201	138.33
75	−	0.191	62.20
190	−	0.157	133.33
145	−	0.139	178.75
23	−	0.137	160.83
96	−	0.111	84.50
38	−	0.084	158.67
134	−	0.029	34.70
Mp704	++	0.741	66.20
Tx601	−	0.051	131.82

[a] ++ indicates a high concentration of the 33-kD protein, + indicates an intermediate concentration of the 33-kD protein, and − indicates that the 33-kD protein was not detected by immunoblot analysis. [b] The ELISA reading (A_{405}) was directly proportional to the concentration of the 33-kD protein.

Presence of the 33-kD Protein in Other Tissues

Because the presence of the 33-kD protein in callus appeared to retard fall armyworm growth, immunoblot analysis was conducted to determine if the protein could be found in vegetative tissues, especially the whorl, the site of insect feeding. The 33-kD protein was not detected in the leaves or whorls of resistant inbreds (data not shown). To determine if callus formation was required for expression of the 33-kD protein, embryos from both Tx601 and Mp708 were cultured in the presence and absence of 2,4-D and zeatin. Using the ELISA, the 33-kD protein was not detected in the following tissues: mature (not allowed to imbibe) embryos of Mp704 and Tx601, embryos of Mp704 and Tx601 that had been allowed to imbibe, embryos of Mp704 and Tx601 cultured in medium without 2,4-D and zeatin, and calli initiated from the embryos of Tx601 cultured on medium with 2,4-D and zeatin (data not shown).

When Mp704 embryos were cultured in the presence of plant growth regulators, low levels of the 33-kD protein could be detected after 3 d in culture using the ELISA (Fig. 3). At this stage the embryos were swollen, but there was no visible callus formation. During the 2 months of culture, callus proliferated and the relative amount of the 33-kD protein gradually increased. The low ELISA reading on d 27 may be due to experimental error, since the 33-kD protein was apparent on the immunoblot at this day (data not shown). The largest increase in concentration occurred during the 2nd month of culture after the callus was transferred to fresh medium. It appears that the accumulation of the 33-kD protein in Mp704 is dependent on the formation of nonfriable callus.

N-Terminal Sequence of the 33-kD Protein

The N-terminal sequence of the first 23 amino acids in the 33-kD protein is shown in Table III. A data base search indicated that this portion of the protein had approximately 50% homology with several plant Cys proteinases or cDNA clones encoding Cys proteinases. This value takes into consideration identical amino acids and those that have R-groups with similar properties. The N-terminal sequence indicated that the 33-kD protein may be a Cys proteinase that is related to the papain family of Cys proteinases (Ritonja et al., 1989). The cDNA clone pZmcp1 (Table III) encodes a possible Cys proteinase that was isolated from a cDNA library prepared from Mp708 callus (Jiang, 1994), but it does not encode the 33-kD protein. The sequences from corn (pMCP10A), pea, rice, and barley are those of Cys proteinase cDNAs expressed during seed germination. The sequences from Arabidopsis are Cys proteinase cDNAs induced by drought. Major differences between the N-terminal sequence and those of other proteinases occur at positions 7, 10, 17, 20, and 22. The absence of a Cys at position 22 in the 33-kD protein is unexpected, because this Cys is involved in disulfide bonding (Koehler and Ho, 1988). The reason for these differences is unknown. Confirmation of the sequence awaits isolation and sequencing of the appropriate cDNA clones.

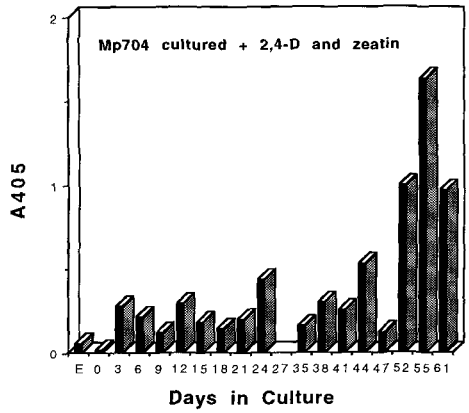

Figure 3. ELISA quantification of the 33-kD protein in embryos or nonfriable callus from the resistant inbred Mp704 cultured in the presence of 2,4-D and zeatin. A_{405} is directly proportional to the concentration of the 33-kD protein. E, Ungerminated Mp704 embryos.

Table III. *Comparison of N-terminal amino acid sequence of the 33-kD protein with those of several Cys proteinases*

A hyphen (-) indicates amino acids conserved in all proteins listed. References: corn pZmcp1, Jiang (1994); corn pMCP10A, deBarros and Larkins (1990); rice oryzain α, β, Watanabe et al. (1991); barley EPA, Koehler and Ho (1988); barley EPB, Koehler and Ho (1990); Arabidopsis RD19 and RD21, Koizumi et al. (1993); pea, Granell et al. (1992).

Source	Amino Acid Sequence
Corn 33-kD protein	L P D A I D R R Q I G A V T E V D D Q A A D G
Corn pZmcp1	- - E S V - W - A K - - - A E V K D - G S C -
Corn pMcP10A	- - R S V - W - Q K - - - T A V K N - G R C -
Rice oryzain α	- - E S V - W - T K - - - A - I K - - G G C -
Rice oryzain β	- - E S V - W - E K - - - A P V K N - G Q C -
Barley EPA	- - P A V - W - Q K - - - T A I K ? -
Barley EPB	- - P S V - W - Q K - - - T G V J D -
Arabidopsis RD19	- - E D F - W - D H - - - T P V K N - G S C -
Arabidopsis RD21	- - E S V - W - K K - - - A E V J D - G G C -
Pea	- - E S V - W - K E - - - V G V K D - G S C -

Purification of the 33- and 36-kD Proteins

The 33-kD protein was purified from callus of the resistant inbred Mp708 and analyzed for Cys proteinase activity. The 30 to 80% ammonium sulfate fraction of the callus extract had proteinase activity (0.095 units mg^{-1}) when it was assayed using CLN as a substrate. When anion-exchange chromatography was performed on this fraction, a sharp peak of activity eluted between 220 and 250 mM NaCl. This sample had a specific activity of 6.2 units mg^{-1}, which was approximately a 65-fold purification of the 30 to 80% ammonium sulfate fraction. Material collected on the leading edge of eluted activity was enriched for the 33-kD protein (Fig. 4A). Fast protein liquid chromatography gel filtration confirmed this molecular mass (data not shown) and did not appreciably alter the specific activity of the enzyme. Molecular weight determinations under both native and denaturing conditions demonstrated that the proteinase was monomeric in nature. Although proteinase activity eluted at other NaCl concentrations, these fractions were not studied further. No proteinase activity eluted in the range of 220 to 250 mM NaCl when an extract from the susceptible genotype Tx601 was applied to the column.

The kinetic properties of the proteinase are shown in Table IV and Figure 5. The specific activity was 6.18 μmol min^{-1} mg^{-1}, the K_m for CLN was 3.85 μM, the k_{cat} was 3.4 s^{-1}, and the k_{cat} K_m^{-1} was 8.83×10^5. The k_{cat} K_m^{-1} value indicated that the enzyme had a relatively high catalytic efficiency (Matthews and Van Holde, 1990). When malate dehydrogenase (1 mg mL^{-1}) was incubated with 0.1 mg mL^{-1} of the protease, 77% of the malate dehydrogenase activity was lost within 24 min, suggesting that the enzyme had proteinase activity against a native protein. Incubation of the purified enzyme with iodoacetate and E-64, known inhibitors of Cys proteinase activity (Barrett et al., 1982), inhibited proteinase activity by 79.7 and 100%, respectively (Table V). These data demonstrated that the 33-kD protein purified from Mp708 callus had Cys proteinase activity. The activity of the 33-kD Cys proteinase was the highest at the acidic pH values of 4.5 and 6.0 (5.18 and 5.51 units mg^{-1}, respectively), decreased approximately 70% at pH

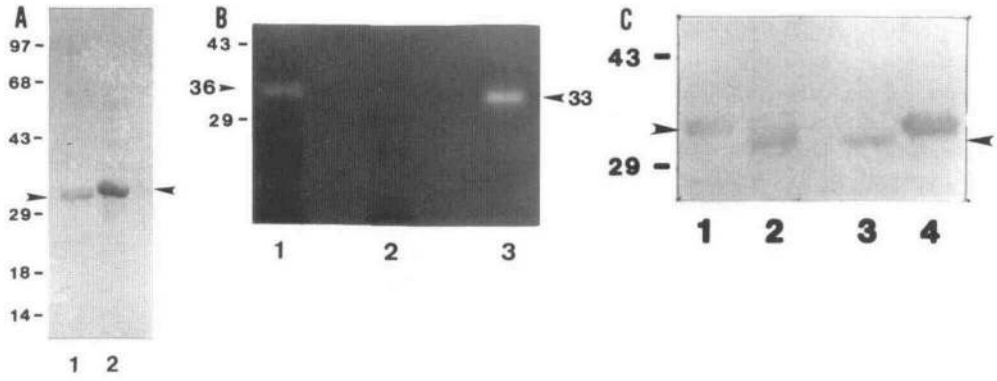

Figure 4. A, SDS-PAGE analysis of proteinases purified from callus of Mp708 (lane 1) and Ab24E (lane 2). Arrowheads on the left and right indicate the 33- and 36-kD proteinase, respectively. B, Gelatinolytic activity of the proteinases purified from callus of Mp708 (lane 3) and Ab24E (lane 1), and a crude extract from Mp708 (lane 2). Arrowheads on the left and right indicate the 36- and 33-kD proteinase, respectively. C, Immunoblot analysis of callus extracts from Ab24E (lane 1) and Mp708 (lane 2) and purified proteinases from Mp708 (lane 3) and Ab24E (lane 4). Arrowheads on the left and right indicate the positions of the 36- and 33-kD proteinase, respectively. Numerals in the left margins indicate the molecular mass (kD) of the protein standards.

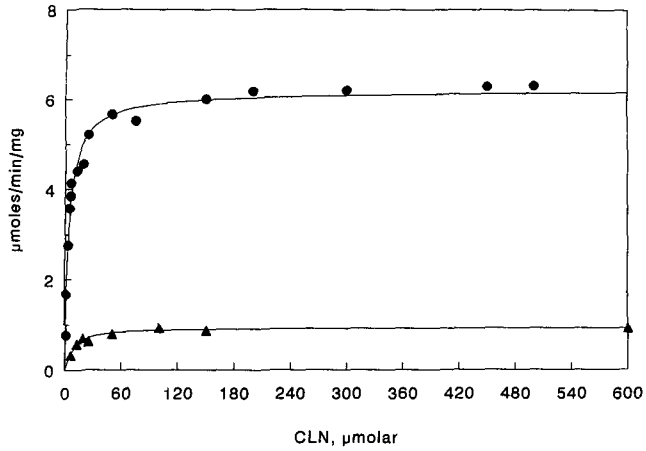

Figure 5. Substrate saturation analysis for the 33- (●) and 36-kD (▲) Cys proteinases isolated from callus of resistant (Mp708) and susceptible (Ab24E) corn inbreds, respectively.

7.0, and was undetectable at pH 8.0. The 33-kD Cys proteinase remains active after incubation at 60°C for 1 h but loses activity at 70°C. It can be stored at 27°C for 3 d without loss of activity.

To determine if the proteinase could be purified by preparative SDS-PAGE or assayed on activity gels containing SDS, activity was measured in the presence of SDS using CLN as substrate. The activity was stimulated approximately 60% in the presence of 0.1% (w/v) SDS, and there was no inhibition of activity at 0.2% (w/v) SDS. Therefore, the purified protein was analyzed on an activity-stained gel using gelatin as a substrate. Figure 4B (lane 3) shows a single, clear band of gelatinolytic activity at approximately 33 kD. This band was not present when the sample was incubated with E-64 or iodoacetate prior to electrophoresis (data not shown).

Immunoblot analysis indicated that purified 33-kD proteinase immunoreacted with the polyclonal antibody raised against the 33-kD protein and that the band was in the same position as the 33-kD immunoreactive band in the callus extract from Mp708 (Fig. 4C). Preimmune serum did not cross-react with the purified 33-kD proteinase or proteins in the callus extract. These results indicated that the purified 33-kD proteinase was immunologically identical to the 33-kD protein found in Mp708 callus.

The 36-kD protein found in Ab24E callus that immunoreacted with the polyclonal antibody raised against the 33-kD protein was also purified. The inbred Ab24E is susceptible to fall armyworm feeding, and larvae reared on callus of Ab24E hybrids are significantly larger than those reared on Mp704 hybrids (Williams et al., 1985). The peak eluting between 220 and 250 mM NaCl contained a proteinase with specific activity of 0.92 μmol mg^{-1} (Table IV). Analysis by SDS-PAGE indicated that the molecular mass of the proteinase was 36 kD (Fig. 4A). In addition, the Ab24E proteinase exhibited slightly lower mobility than the 33-kD proteinase on the activity-stained gel (Fig. 4B). When the Ab24E proteinase was analyzed on an immunoblot, a 36-kD protein cross-reacted with the polyclonal antibody raised against the 33-kD protein (Fig. 4C). This protein had the same mobility as the 36-kD protein in the Ab24E callus extract. This confirms that the 36-kD protein in Ab24E is a proteinase that is antigenically similar to the 33-kD proteinase.

The kinetic properties of the proteinases from Mp708 and Ab24E are compared in Figure 5 and Table IV. The specific activity of the 36-kD Ab24E proteinase was approximately 7-fold lower than that of the Mp708 proteinase. The Ab24E proteinase had a 2.5-fold higher K_m (9.57 μM) and lower k_{cat} (0.55 s^{-1}) and k_{cat} K_m^{-1} (0.57 × 10^5) than the 33-kD proteinase from Mp708. Not only did the 36-kD proteinase have lower specific activity and higher K_m, it also had a catalytic efficiency that was 15-fold less than the 33-kD proteinase. The 36-kD proteinase appeared to be less stable than the 33-kD enzyme. Cys proteinase activity was confirmed by incubation with iodoacetate and E-64 (Table V), which inhibited enzyme activity 82.9 and 100%, respectively.

DISCUSSION

We have shown that a 33-kD protein with Cys proteinase activity is present in nonfriable callus initiated from corn genotypes resistant to fall armyworm feeding. Several pieces of evidence suggest that the presence of the 33-kD proteinase, or one of its metabolic products, retards the growth of larvae reared on the callus. First, analysis of callus lines from F_2 progeny of Mp704 × Tx601 indicated that the concentration of the 33-kD proteinase and the weight of the fall armyworm larvae reared on the callus were negatively correlated. The correlation coefficient (r = −0.59) was statistically significant ($P < 0.01$). The coefficient of determination (r^2 = 0.35) indicated that 35% of the

Table IV. *Kinetic properties of the proteinase purified from callus of resistant (Mp708) and susceptible (Ab24E) corn inbreds*

Property	Mp708	Ab24E
Specific activity (μmol^{-1} min^{-1} mg^{-1})	6.18	0.92
$K_{m_{CLN}}$ (μM)	3.85	9.57
k_{cat} (s^{-1})	3.40	0.55
k_{cat} K^{-1} × 10^5	8.83	0.57

Table V. *Effects of Cys proteinase inhibitors on proteinase activity purified from callus of resistant (Mp708) and susceptible (Ab24E) corn inbreds*

Enzyme preparations were incubated with iodoacetate and E-64 at the indicated final concentration for 30 min, 25°C prior to assay.

Inbred and Treatment	Rate	Percent Inhibition
	µmol min^{-1}	
Mp708		
Control	12.3	0
Iodoacetate, 5 mM	2.5	79.7
E-64, 0.1 mM	0	100.0
Ab24E		
Control	15.2	0
Iodoacetate, 5 mM	2.6	82.9
E-64, 0.1 mM	0	100.0

variation in larval weight was accounted for by concentration of the 33-kD protein. This suggests that other factors, such as the heterogeneity of the larval population, or other biochemical factors contribute to the variability in larval weight.

The second observation implicating the 33-kD proteinase was the apparent loss of this protein from the resistant inbred Mp704 during the transition from nonfriable to friable callus. Insect larvae reared on the friable callus were larger than those reared on nonfriable callus from Mp704. Originally, we suspected that the increased size of the larvae was due to the friability of the callus, which resulted in greater feeding. However, immunoblot analysis indicated that the 33-kD proteinase was not present in the friable callus. An alteration in gene expression during the morphological change from nonfriable to friable callus may account for the loss of the 33-kD protein. The gene encoding the 33-kD protein could have been eliminated by a somatic mutation, or its expression could have been blocked at the transcriptional, translational, or posttranslational level. However, if the last case were true, a precursor should have been detected on the immunoblot.

The third piece of circumstantial evidence is the difference in Cys proteinase activity between the 33- and 36-kD Cys proteinases isolated from callus of the resistant (Mp708) and susceptible (Ab24E) inbreds, respectively. Although both proteinases immunoreacted with polyclonal antibody raised against the 33-kD protein, there was a remarkable difference in the activities of the two enzymes. The activity of the 33-kD proteinase was 7-fold greater than that of the 36-kD enzyme, and its efficiency was 15-fold higher. The reason for this difference in catalytic activity is currently unknown. There may have been an insertion in the Ab24E gene encoding the proteinase that resulted in a larger but less active enzyme. An insertion of approximately 80 bp into the coding region would result in a 3-kD increase in molecular mass. Since similar Cys proteinases are synthesized as preproproteins (Mitsuhashi and Minamikawa, 1989), the enzyme must be posttranslationally modified. A defect in this step might result in a larger protein with reduced proteolytic activity. Therefore, it appears that the presence of a 33-kD protein with a relatively high Cys proteinase activity may be required to inhibit larval growth in callus. Ingestion of the Cys proteinase could directly harm the insect digestive system by destroying gut proteins. Alternatively, the proteinase could catalyze a reaction leading to a substance that is toxic to the larvae.

Although there is little specific information about the role of proteinases in insect resistance, it is generally believed that hydrolytic enzymes are involved in the plant defense response (Boller, 1986). Typically, hydrolytic enzymes are sequestered in the vacuole and are released when it is broken. The released hydrolases may be the first line of defense against pathogen or herbivore attack (Boller, 1986). Some proteinases such as the one found in *Phaseolus vulgaris* leaves (Van der Wilden et al., 1983) are present in the cell wall and thus provide another site of defense. Feeding by *Spodoptera littoralis* induces Leu aminopeptidase in tomato (Pautot et al., 1993). This proteinase may be involved in systemic response to wounding, which leads to the expression of plant defense proteins (Pearce et al., 1991).

The 33-kD proteinase has not yet been detected in vegetative tissues of resistant genotypes. The immunoblot analysis linked with alkaline phosphatase may not have been sufficiently sensitive to detect the protein in the whorl. Alternatively, wounding or insect feeding may be required for the 33-kD proteinase to accumulate. Since callus often emits large amounts of ethylene, it could be mimicking "super-wounded" tissue, enhancing expression of 33-kD proteinase. Incision wounding of tobacco (Linthorst et al., 1993) and ethylene treatment of chickpea cotyledons (Cervantes et al., 1994) have been shown to induce Cys proteinase mRNAs. Finally, it is possible that the 33-kD proteinase is not expressed in vegetative tissues, and that other resistance mechanisms are functioning in the plant.

Nevertheless, the finding that the 33-kD Cys proteinase is correlated with the inhibition of larval growth on callus is significant because of its potential practical applications. If it can be demonstrated that the 33-kD proteinase is a direct retardant of fall armyworm growth, it could be used to develop plants with increased resistance to a number lepidopteran insect pests.

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