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The *Fusarium solani*-Induced Expression of a Pea Gene Family Encoding High Cysteine Content Proteins

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Two pea genes, pI39 and pI230, which are specifically induced by two forma speciales of *Fusarium solani*, encode closely related proteins with predicted molecular masses (M_r) of 8.2 and 8 kDa, respectively. Both proteins contain a signal sequence and are cleaved to mature proteins of M_r 5 kDa as indicated by an *in vitro* translation system. The mature proteins contain about 17% cysteine residues and have the potential to form four disulfide bonds. The two proteins share extensive homology in their signal sequences but much less homology as mature proteins. The cysteine residues of the mature proteins are highly conserved, suggesting functional importance. Southern hybridization suggests these genes belong to a multigene family. The relative accumulations of mRNA levels indicate that the two genes are expressed somewhat differentially. In both the compatible (susceptible) and incompatible reactions between *F. solani* and pea tissue, pI39 mRNA accumulates more slowly than pI230 mRNA and accumulates to relatively high levels after 24 hr of inoculation. The increase in accumulation of pI230 mRNA occurs within 6 hr and thus correlates with an initial suppression of the growth of both the compatible and incompatible pathogen, which is cytologically observable at 6 hr. pI39 and pI230 belong to a distinct class of pathogenesis-related proteins characterized previously, which are associated with and thus may contribute to nonhost resistance in plants.

Additional keywords: disease resistance response genes, plant-fungal interaction, induced genes, protein processing.

The response of plants to pathogenic fungi is multifaceted (Bailey 1983) but is often consistent for a given plant pathogen interaction. In pea-*Fusarium* interactions, the host tissue initially suppresses both the compatible (*Fusarium solani* (Mart.) Sacc. f. sp. *pisi* (F.R. Jones) W.C. Snyder)) and incompatible (*F. s.* f. sp. *phaseoli* (Burkholder) W.C. Snyder & H.N. Hans) pathogens, but eventually the interaction develops susceptible and resistance reactions, respectively. The initial suppression breaks down after 12 hr in the compatible interaction (Loschke and Hadwiger 1981). A hypersensitive response (yellow-green discoloration) can be observed 16 hr after inoculation in the incompatible response and after 18 hr in the susceptible host. Necrotic symptoms are detectable in the susceptible host tissue 20 hr after fungal inoculation (Kendra and Hadwiger 1987). The molecular basis of this differential response is complex. Molecular analyses of pea-fungal interactions reveal that at least 20 different proteins are synthesized in response to fungal challenge (Loschke *et al.* 1983; Riggleman *et al.* 1985; Wagoner *et al.* 1982). Some of the induced genes encode proteins of known functions such as phenylalanine ammonia lyase (Loschke *et al.* 1981; Loschke *et al.* 1983), chitinases, and β-1,3-glucanases (Mauch *et al.* 1984; Mauch *et al.* 1988a; Mauch *et al.* 1988b; Nichols *et al.* 1980). Also induced are the genes with unknown functions called disease resistance response genes (DRRG) (Chiang and Hadwiger 1990; Fristensky *et al.* 1988). Genes homologous to pea DRRG49 have been reported in parsley (Somssich *et al.* 1988), potato (Matton and Brisson 1989), and soybean (Chiang and Hadwiger 1990), indicating the homology extends to widely diverse species. Therefore, the functions of such induced genes appear, by circumstances, to be of some importance during host-pathogen interactions.

Previously, we have reported the isolation of induced pea genes in response to *Fusarium* inoculations (Riggleman *et al.* 1985). In this paper, we have sequenced and characterized two cDNAs whose mRNA accumulate somewhat differently upon fungal challenge. The genes appear to encode low molecular weight proteins with a high content of cysteine residues. Computer searches have revealed only low homology between their sequence predicted proteins and other known proteins; however, they share some similarities with the fungal-toxic thionins. Thionins are low molecular weight cysteine-rich proteins that accumulate in barley after challenge by pathogenic fungi (Bohlmann *et al.* 1988). The properties of these pea proteins and other cysteine-rich proteins will be discussed.

MATERIALS AND METHODS

DNA sequencing. Plasmids carrying either pI39 or pI230 (Riggleman *et al.* 1985) were digested with different restriction enzymes and the fragments subcloned into Bluescript vector pKS+ (Stratagene, Inc., La Jolla, CA). The recombinant plasmids were isolated and sequenced as previously described (Del Sal *et al.* 1989).

DNA and RNA isolation. Total pea DNA was isolated from *Pisum sativum* L. 'Alaska' endocarp tissue according to Polans *et al.* 1985. Total pea RNA was isolated from immature pea pods after treatment with water, *F. s.* f. sp.

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phaseoli or *F. s.* f. sp. *pisi* macroconidia (3×10^6 spores per milliliter) as described previously (Wagoner *et al.* 1982).

Northern probe construction. The pI39 probe was constructed by inserting the *Hin*dIII/*Spe*I fragment (Fig. 1) into the Bluescript vector under a T7 promoter control. The resulting plasmid was restricted with *Nhe*I, and an antisense RNA probe was generated using T7 RNA polymerase in the presence of [^{32}P]rUTP. Similarly, a 143-bp *Mn*II/*Eco*RI fragment of pI230 (Fig. 1) was subcloned into the Bluescript vector to generate an antisense RNA probe.

Nucleic acid hybridizations. Total pea DNA was digested with restriction enzymes and separated on a 0.8% agarose gel. Southern transfer and hybridization were performed as previously described (Chiang and Hadwiger 1990). The *Hin*dIII/*Sph*I fragment of pI39 cDNA devoid of the poly(A)$^+$ tail was ^{32}P-labeled by random oligonucleotide priming (Boehringer Mannheim Biochemicals, Indianapolis, IN). The blots were washed in 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7) and 0.1% sodium dodecyl sulfate (SDS) at 65° C.

For northern hybridization, 15 μg of total RNA extract from pea endocarp tissue after different treatments was separated in a 1.5% agarose gel containing 10 mM NaH_2PO_4 (pH 7.5) and 2.2 M formaldehyde. The RNAs were transferred to GeneScreen Plus membranes (Du Pont) in a 10× SSC solution. The membranes were hybridized to ^{32}P-labeled antisense RNA of either pI39 or pI230 specific regions (Fig. 1, thick lines). The membranes were washed in 0.1× SSC and 0.1% SDS at 68° C.

***In vitro* transcription and translation.** A Bluescript plasmid containing the *Hin*dIII/*Eco*RI fragment of pI230 under the control of a T3 promoter was restricted with *Nco*I/*Hin*cII, filled in with Klenow enzyme and religated with T4 DNA ligase. The resulting plasmid, pCC67, has neither the 5′ end of the GC tail nor the poly(A)$^+$ region of pI230, which were used during the cDNA synthesis. Removal of these regions is required for the efficient transcription and translation of pI230 *in vitro*. The pCC67 plasmid was linearized with *Eco*RI and transcribed with T3 RNA polymerase according to manufacturer's protocols (Stratagene). The plasmid pCC68 was constructed by inserting the *Nco*I/*Eco*RI restriction fragment of pI230 into the pCITE-1 vector (Novagen Inc., Madison, WI). The pCC68 plasmid was linearized with *Eco*RI and transcribed with T7 RNA polymerase.

In vitro translation of the synthetic transcripts of pI230 was performed using a rabbit reticulocyte lysate (Promega Biotech, Madison, WI) containing [^{35}S]cysteine (Du Pont) in the presence or absence of canine pancreatic microsomal membranes (Promega). Proteinase K was added to some reactions to a final concentration of 500 μg/ml and incubated on ice for 30 min in the presence or absence of 0.1% Triton X-100. All reactions were stopped with 10 mM PMSF (phenyl methylsulfonylfluoride) and separated through 10–20% SDS-polyacrylamide gradient gels. After electrophoresis, the gels were treated with 5 volumes of enhancer (Du Pont) fluorography reagent before being dried and exposed to preflashed X-ray film at −70° C.

Computer analysis. All computer manipulation was done using the University of Wisconsin Genetics Computer Group (UWGCG), Madison, package provided by the Visualization, Analysis and Design in the Molecular Science (VADMS) Laboratory at Washington State University.

RESULTS

Structural analysis of cDNAs. Nucleotide sequence analysis of pI39 and pI230 cDNA revealed open reading

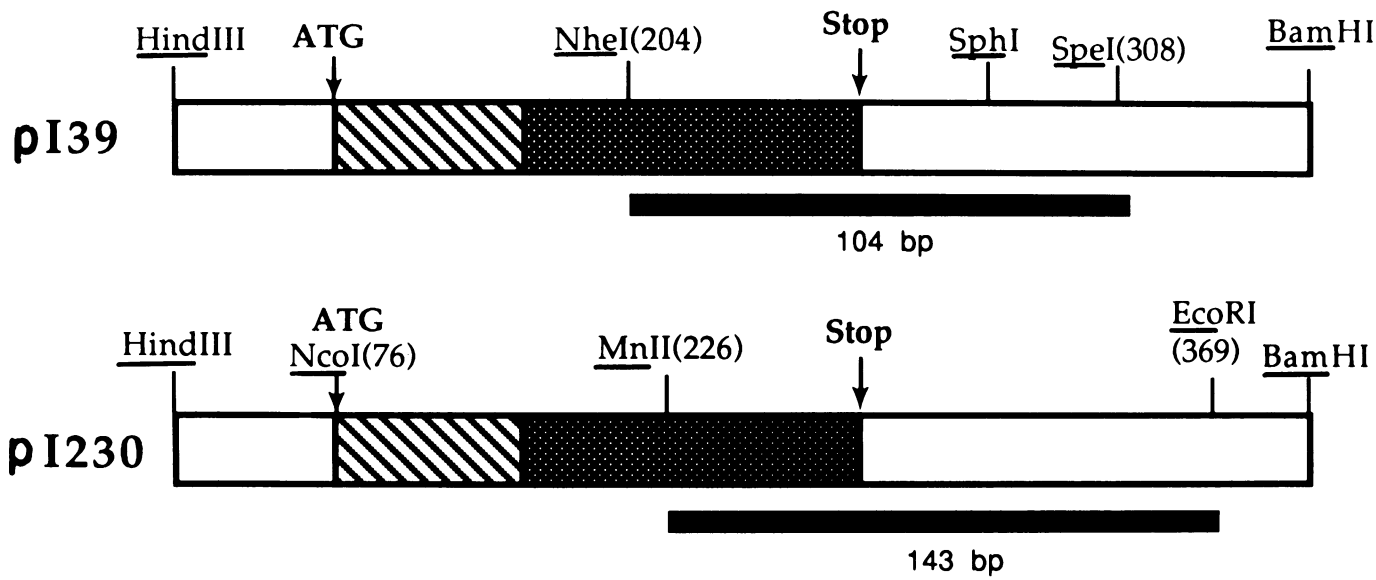

Fig. 1. Structure of the two cDNAs. The two cDNAs are indicated by the boxes. The open blocks correspond to the 5′ and 3′ untranslated regions. The hatched blocks represent the signal sequence (domain 1) and the stippled blocks indicate the cysteine-rich domain (domain 2). The initiation (ATG) and stop (TAA) codons are represented by arrowheads. The location of the restriction enzymes is shown. The numbers in parentheses correspond to the nucleotide sequence numbers shown in Figure 1. There are multiple MnII sites within the pI230 cDNA, and only one relevant site is shown. pI39 and pI230 gene probes used for northern hybridizations are indicated by the thick lines.

A

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                    20                  40                  60
AAACACACAACATATTAAGTGAAGTGAATCATATATATATTAAATCTATATTCATCATCA

                    80                 100                 120
CTAAAGTTATGGAGAAGAAATCACTAGCTGCCTTGTCCTTCCTCCTCCTCCTCGTTCTCT
          MetGluLysLysSerLeuAlaAlaLeuSerPheLeuLeuLeuLeuValLeuP

                   140                 160                 180
TTGTTGCACAAGAAATTGTGGTGACAGAGGCAAACACTTGTGAGCATTTGGCTGATACAT
heValAlaGlnGluIleValValThrGluAlaAsnThrCysGluHisLeuAlaAspThrT

                   200                 220                 240
ACAGGGGAGTATGCTTCACGAATGCTAGCTGTGATGATCACTGCAAGAACAAAGCGCACT
yrArgGlyValCysPheThrAsnAlaSerCysAspAspHisCysLysAsnLysAlaHisL

                   260                 280                 300
TAATCAGTGGCACGTGCCATGACTGGAAATGTTTCTGCACTCAAAACTGTTAAAGACGTT
euIleSerGlyThrCysHisAspTrpLysCysPheCysThrGlnAsnCysEnd

                   320                 340                 360
ATAAGTATAATAATATAAATAAATAAAATGCATGCAGTTATAGCAACTACCGCTGTATCT

                   380                 400                 420
GTGTATGTATTTGAATAAGTTATGTGTGTACTCATCGTGTATAACCTACTAGTTATGCAC

                   440
TTTTATCTTATGGAATAAAACTTTAATCAATAAAAA
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B

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                    20                  40                  60
GATCACACAAACACACATAACACATTAAGTGAAGTGAGTCATATTAAGTTTTTATATTCA

                    80                 100                 120
TCACTACTTAAGAAGCCATGGAGAAGAAATCACTAGCTTGCTTGTCCTTCCTCCTCCTCG
                  MetGluLysLysSerLeuAlaCysLeuSerPheLeuLeuLeuV

                   140                 160                 180
TTCTCTTTGTTGCACAAGAAATAGTGGTGAGTGAAGCAAACACATGTGAGAATTTGGCTG
alLeuPheValAlaGlnGluIleValValSerGluAlaAsnThrCysGluAsnLeuAlaG

                   200                 220                 240
GTTCATATAAGGGAGTATGCTTCGGTGGATGTGACCGTCACTGTAGAACACAAGAGGGCG
lySerTyrLysGlyValCysPheGlyGlyCysAspArgHisCysArgThrGlnGluGlyA

                   260                 280                 300
CAATTAGCGGCAGATGCAGGGATGACTTTCGCTGTTGGTGCACTAAAAACTGTTAAATCC
IaIleSerGlyArgCysArgAspAspPheArgCysTrpCysThrLysAsnCysEnd

                   320                 340                 360
CTTTTCTCCAACACCAACAACACCCATATATAATACTATAATATAAATAAATAAACAAGT

                   380                 400                 420
GTTGTTTCGAATTCTATGTGTGTACTCAATATCGTGTATAACGTGTTTGTTATGCACTTT

                   440                 460
TATCATATCATATGGAATAAAAAGTAATCAATCATTTCCTTTCCAAAA
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Fig. 2. DNA sequence and deduced amino acid sequence of genes **A**, pI39 and, **B**, pI230. The putative signal sequence cleavage site is obtained from Von Heijne (1986) and indicated by an arrowhead. The 3′ AATAAA processing signals are underlined.

frames (ORFs) of 222 and 219 bp, respectively (Fig. 2). Both cDNAs contain multiple AATAAA sequences typical of polyadenylation signals. The ORF of pI39 encodes 74 amino acids and the ORF of pI230 encodes 73 amino acids. These translates are 70.4% identical. The N-terminal portions of the two ORFs are highly hydrophobic (Fig. 3A), whereas the C-terminal portions are generally hydrophilic and rich in basic amino acids; therefore, each of the ORFs can be divided into two domains. The first domain is composed of a long stretch of hydrophobic residues and the second domain contains all of the cysteines (Fig. 1). The two cDNAs share extensive sequence homology within the hydrophobic domain with characteristics prominent in protein export or targeting. The cysteine-rich domains of the two cDNAs share much less homology; however, the position and number of cysteine residues are very conserved (Fig. 3B). In addition, the similarity of the hydrophobicity plots shown in Figure 3 indicates that the two proteins may also be functionally conserved. The predicted molecular masses of the preproteins for pI39 and pI230 are 8.2 and 8 kDa, respectively. The mature proteins, as indicated by *in vitro* translation, are 5 kDa.

Southern hybridization where pI39 cDNA insert was used as a probe indicates the multigenic nature of this cDNA (Fig. 4). Sequence homology searches have failed to reveal close homologies with any other proteins recorded in the Genbank or NBRF.

***In vitro* protein processing.** The presence of long stretches of hydrophobic residues at the N-terminus of the cDNAs prompted us to further characterize the processing of pI230 gene product *in vitro*. Two plasmids carrying the pI230 cDNA were constructed under the control of either the T3 or T7 promoters (Fig. 5A). The pCC68 plasmid carries 586 bp of the Cap-independent translational enhancer

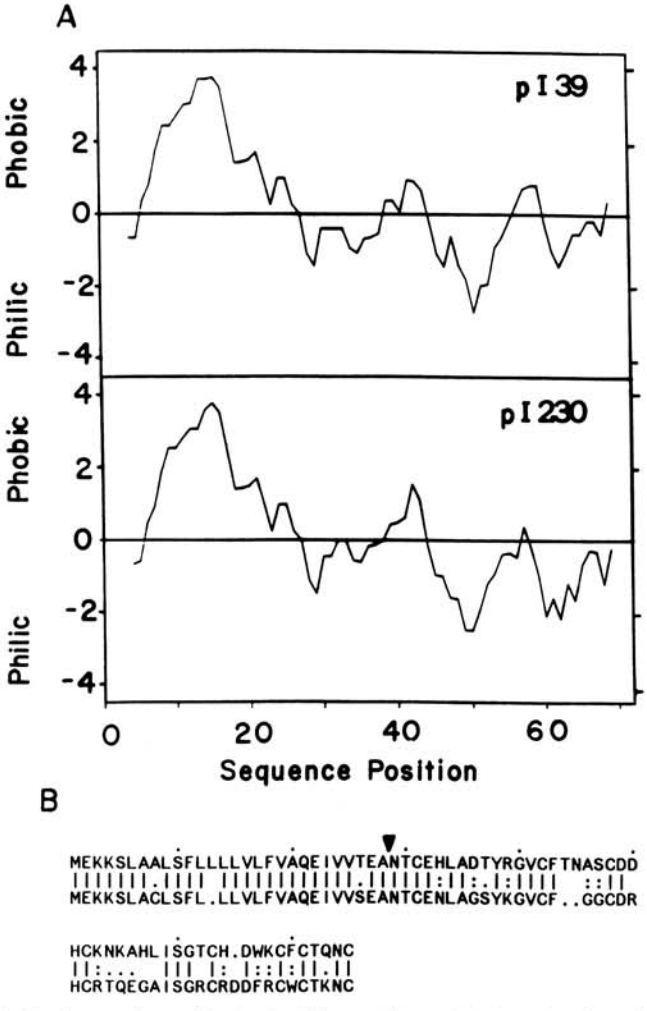

Fig. 3. Comparison of hydrophobicity profiles and deduced amino acid sequence of the two cDNAs. **A,** Plots were derived by using the algorithm of Kyte and Doolittle (1982) with a window of nine amino acids. **B,** The putative signal sequence cleavage site is indicated by the arrowhead. The lines and dots between two residues represent identical and conserved amino acids.

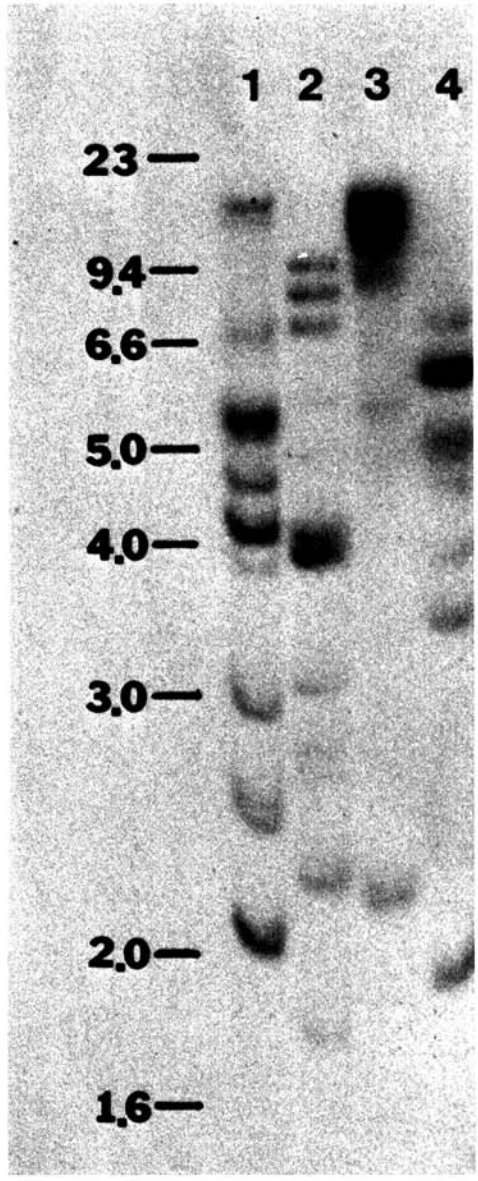

Fig. 4. Southern analysis of pea genomic DNA. Total DNA was isolated from immature pea pods. Lanes 1–4 indicate pea genomic DNA digested with *Eco*RI, *Hin*dIII, *Bam*HI, and *Xba*I, respectively. The size marker is shown on the left. The blot was hybridized to pI39 cDNA.

(CITE), which was derived from a 5′ untranslated region of encephalomyocaditis virus (Parks *et al.* 1986). It has been shown that the presence of CITE can increase the reporter gene expression severalfold in the translation of synthetic RNA *in vitro* by rabbit reticulocyte lysates (Elroy-Stein *et al.* 1989). The pCC68 plasmid also contains 12 additional base pairs between CITE and the start of the pI230 gene. This results in the translation of four amino acids (met-ala-thr-thr) in addition to the ATG codon of pI230. As expected, the translation product of pI230 is slightly larger from the construct pCC68 than from pCC67 (Fig. 5B, lanes 1 and 4). When *in vitro* translation was carried out in the presence of microsomal membranes, the product is visible as a band with molecular mass of 5 kDa. No difference in the size of this band was observed between the two constructs (Fig. 5B, lanes 2 and 4). The translation of pI230 in the absence of RNA and in the presence of membranes yielded no visible bands (lane 5). Therefore, the hydrophobic domain of pI230 appears to be cleaved *in vitro* in the presence of microsomal vesicles that cause a mobility shift in the protein from about 8 to 5 kDa.

To ascertain if the mature protein is targeted into the endoplasmic reticulum (ER) *in vitro*, the translated protein was incubated with proteinase K in the presence or absence of Triton X-100. As shown in Figure 6 (lane 3), the translation products synthesized in the absence of membranes were degraded but were protected from proteolysis in the presence of membranes (lane 4). Proteinase K treatment in the presence of 1% (v/v) Triton X-100 resulted in the degradation of the protected 5-kDa protein band (lane 5). Therefore, the preprotein appears to be processed to the mature protein form and translocated into the ER *in vitro*.

mRNA induction kinetics. The accumulation of RNA in compatible and incompatible reactions was followed with probes constructed to encompass part of the nonhomologous cysteine-rich domain and a portion of the 3′ untranslated region (Fig. 1). When these probes were hybridized with the RNA from the compatible and incompatible reactions a clear difference in the steady state mRNA levels was observed between the two genes (Fig. 7). After inoculation with the compatible pathogen, the mRNA levels of pI39 initially accumulate slowly and subsequently accumulate to a maximum at about 32 hr (Fig. 7A). After inoculation with the incompatible pathogen, the mRNA levels of pI39 also accumulate slowly and reach about 50% of the compatible mRNA levels. In contrast, the accumulation of pI230 mRNA occurs earlier and at a faster rate (Fig. 7B). A higher mRNA accumulation of pI230 in the incompatible than the compatible reaction is apparent throughout

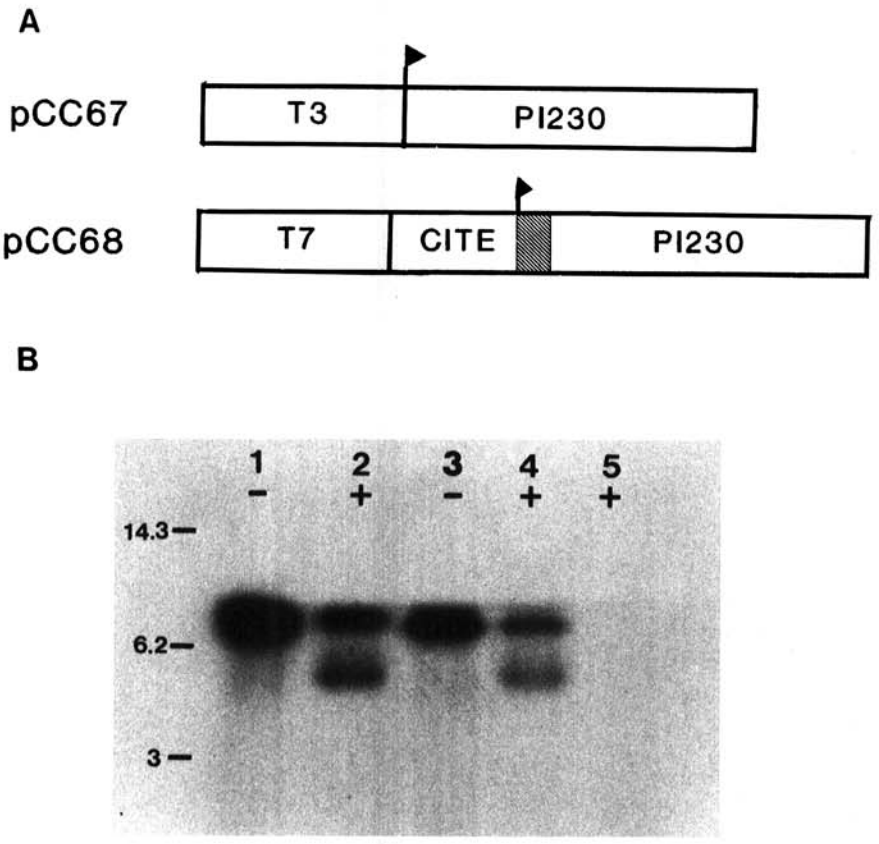

Fig. 5. Cell-free translation products of the pI230. **A,** The constructs used for *in vitro* transcription of pI230 cDNA. T3 and T7 are the phage promoters used to transcribe the pI30 cDNA. Arrows indicate the translation start sites. The hatched block indicates a 12-bp segment generated from the vector sequence. The synthetic RNAs from these constructs were used for cell-free translation. **B,** *In vitro* translations were performed in the presence (+) or absence (−) of the microsomal membranes. Lanes 1 and 2, translation product of pCC68; lanes 3 and 4, product of pCC67; lane 5, absence of synthetic RNA. The molecular weight marker in kilodaltons is shown on the left lane.

the initial 20-hr period of fungal challenge. No transcripts were detectable with either probe in the accumulating RNA from water-treated tissue at zero hour, but low levels developed as the incubation time proceeded, which may relate to the trauma of excising and splitting the pods.

DISCUSSION

The characterization of the two cDNA species increased in the presence of the *Fusarium* pathogens reveals several interesting features. The deduced amino acid sequences of protein products from the cDNAs can be divided into two domains (Fig. 1). The hydrophobic domain is highly conserved and the cysteine-rich domain is much less conserved in each gene product. The number and position of cysteine residues with respect to the proteins are almost identical, suggesting that the two proteins share similar conformations. The nearly identical hydrophobic patterns (Fig. 3A) of these cDNAs provide an argument for their functional similarities. The cysteine-rich domains contain eight cysteine residues, which have the potential to form four disulfide bonds. The presence of these disulfides may provide extra stability to the native proteins. Proteins that are functional in extracellular spaces very often contain disulfide bridges (Schulz and Schirmer 1979), for example, low molecular weight proteins such as snake venoms, spider toxins, and peptide hormones.

In vitro transcription and translation systems have allowed us to further characterize the gene product of pI230. Previously, such *in vitro* systems have been used to study individual subunits of glutenin (Bulleid and Freedman 1988) and protein targeting into the ER (Iturriaga *et al.* 1989). Both of the constructs developed for *in vitro* translation gave identical results, suggesting that these proteins are also processed and targeted into the ER *in vivo*. However, the construct containing the CITE sequences fused to the pI230 gene was more efficiently translated than the construct without the CITE sequence. Interestingly, an additional four amino acids at the 5′ terminus of pI230 did not alter the processing of the signal peptide.

The steady state mRNA induction study revealed differential regulation between pI39 and pI230. The differential regulation of the multigenic genes in response to the pathogens or elicitors has also been observed in phenylalanine ammonia lyase genes (Lois *et al.* 1989; Loschke *et al.* 1983) and chalcone synthase genes (Ryder *et al.* 1987). The differences in the rates of accumulation of the two mRNA species pI39 and pI230 are not surprising because the sequence heterogeneity within their cysteine-rich domains indicate they are indeed coded by different genes. The induction of pI39 mRNA occurs at a much slower rate than pI230 but remains at relatively high levels in the compatible interaction after 20 hr of inoculation when necrosis is occurring. The early induction of pI230 mRNA appears to correlate with the suppression of the fungal growth and resistance of pea tissue. The expression of pI39 appears to be correlated more with the extent of hypersensitivity and necrosis observed in the pea tissue. Although these genes may contribute actively to the host response, they do not appear to be determinants of the reaction type, initially. The expression of these genes is more likely to be important in the general host defense response and eventually in the cytologically observable hypersensitive response. Alternately, some precedent exists for cysteine-rich proteins to cause necrosis in plant tissue and, therefore, breakdown of the host defense responses. A necrosis-inducing peptide has been isolated from the apoplastic fluids of tomato infected with *Cladosporium fulvum* Cooke (Schottens-Toma and DeWitt 1988). The secreted peptide has a low molecular mass (3 kDa) and also has a high percentage of cysteine residues, which is also characteristic of the pI39 and pI230 gene products.

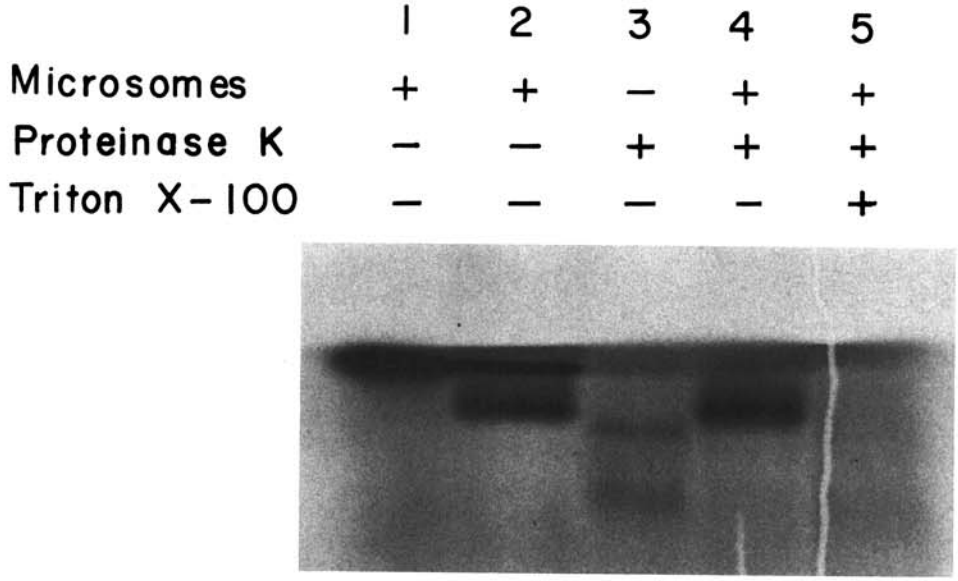

Fig. 6. Analysis of protein translocation using *in vitro* translation products. All the analyses were done using *in vitro* transcribed pCC67 RNA. Lanes 2, 4, and 5, translation in the presence of microsomal membranes; lanes 3 and 4, translation products were treated with proteinase K; lane 5, translation products were treated with 1% (v/v) Triton X-100 and proteinase K.

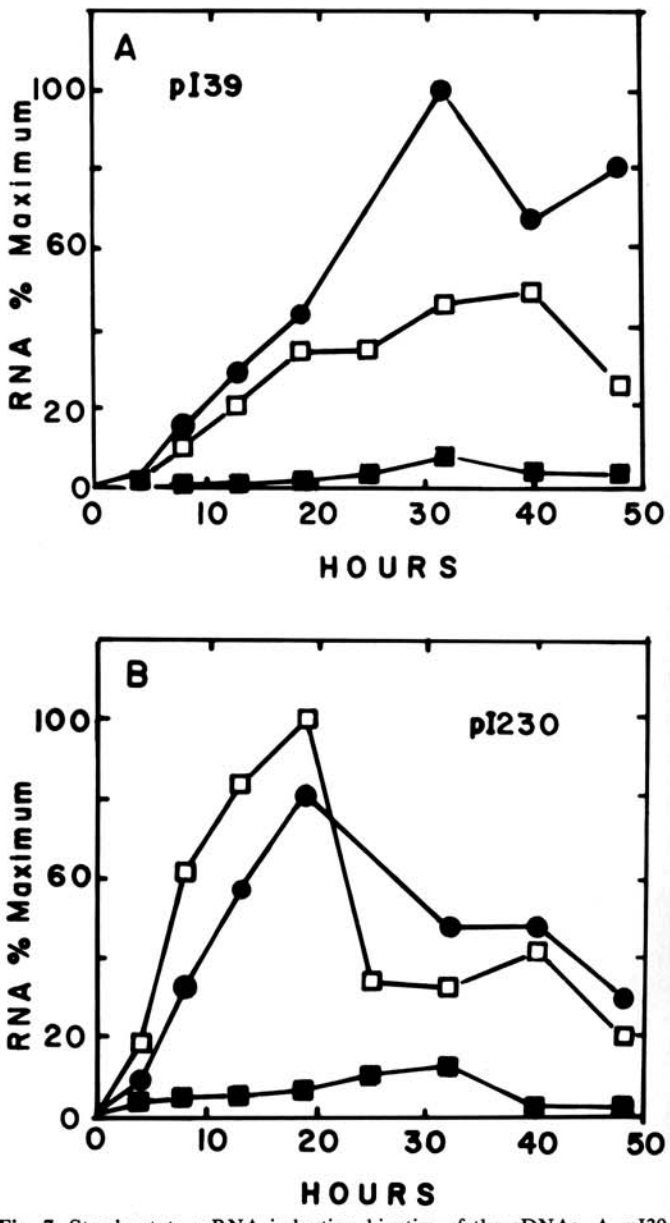

Fig. 7. Steady state mRNA induction kinetics of the cDNAs, **A,** pI39 and, **B,** pI230. Total RNA was isolated from pea pods treated with the incompatible pathogen *Fusarium solani* f. sp. *phaseoli* (open squares), the compatible pathogen *F. s.* f. sp. *pisi* (closed circles), or water (closed squares) for different postinoculation periods. The levels of mRNA were quantified using AMBIS Radioanalytic Imaging System (AMBIS Systems Inc., San Diego, CA).

Additionally, the fungal toxic thionin (Bohlmann *et al.* 1988) isolated from barley and other monocots (Ramshaw 1982) shares similar features with the pea genes. Thionin is also encoded by a multigenic family and has a molecular mass of 5 kDa. The thionin protein has eight cysteine residues, which form four disulfide bonds, and has many basic residues. The similarities of the predicted mature protein of gene 39 to human defensin HNP-1 an (endogenous antibiotic peptide [Lehrer *et al.* 1991]) include six cysteine residues, four arginine/lysine residues, and low molecular weight (46 and 30 amino acids, respectively). However, there is no conserved amino acid sequence homology. Lastly, the predicted mature protein product of gene 39 is 33% identical to the potato gene p322 (Stiekema *et al.* 1988), and p322 is 35% identical to the Bowman-Birk C11 proteinase inhibitor from soybean (Odani and Ikenada 1977). The gene 39 product is only 15% identical and 21% similar to C11 (Fig. 8); however, the match of cysteine residues certainly warrants future investigation of a possible proteinase inhibitor function for this pea gene product. The potential functions and differential responses of the pea genes reiterate the complexity of disease resistance and reemphasize the need to genetically evaluate all components of the response to derive a final definition of disease resistance. Further, inducible disease resistance response genes with DNA sequences capable of traversing products through the ER may be valuable in targeting other cloned genes controlling major functions to the host parasite interaction as a part of the future engineering of disease resistance in plants. We are currently synthesizing the segment of the gene 230 product, which constitutes the mature processed protein to enable a direct test of its biological functions toward both the plant and the fungus.

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Fig. 8. Comparison of the amino acid sequences of two putative mature proteinase inhibitors against the predicted Pi39 polypeptide. The upper sequence is pea gene pI39, the middle sequence is the potato gene p322, and the lower sequence is the soybean proteinase inhibitor C11. Identical amino acids are indicated by vertical match bars, similar substitutions by dots, and more conserved substitutions by colons, with the most conserved substitutions using capitalized symbols. The putative active site of the Bowman-Birk soybean C11 inhibitor is indicated at Arg-37 by an asterisk. Pi39 is 51% similar to and 33% identical to p322, and soybean is 51% similar and 35% identical to C11, whereas pI39 is only 21% similar and 15% identical to C11. Similarly, values and match symbol threshholds were determined by the Genetics Computer Group's GAP and PRETTY programs using default settings.

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