JOURNAL OF BACTERIOLOGY, Dec. 1995, p. 7070–7077
0021-9193/95/$04.00+0

Vol. 177, No. 24

# Cloning of a Novel Constitutively Expressed Pectate Lyase Gene *pelB* from *Fusarium solani* f. sp. *pisi* (*Nectria haematococca*, Mating Type VI) and Characterization of the Gene Product Expressed in *Pichia pastoris*

WENJIN GUO, LUÍS GONZÁLEZ-CANDELAS,† AND P. E. KOLATTUKUDY*

*Neurobiotechnology Center, The Ohio State University, Columbus, Ohio 43210*

Received 13 July 1995/Accepted 2 October 1995

**Since plant-pathogenic fungi must penetrate through pectinaceous layers of the host cell wall, pectin-degrading enzymes are thought to be important for pathogenesis. Antibodies prepared against a pectin-inducible pectate lyase (pectate lyase A [PLA]) produced by a phytopathogenic fungus, *Fusarium solani* f. sp. *pisi* (*Nectria haematococca*, mating type VI), was previously found to protect the host from infection. The gene (*pelA*) and its cDNA were cloned and sequenced. Here we report the isolation of a new pectate lyase gene, *pelB*, from a genomic library of *F. solani* f. sp. *pisi* with the *pelA* cDNA as the probe. A 2.6-kb DNA fragment containing *pelB* and its flanking regions was sequenced. The coding region of *pelB* was amplified by reverse transcription-mediated PCR, using total RNA isolated from *F. solani pisi* culture grown in the presence of glucose as the sole carbon source. The predicted open reading frame of *pelB* would encode a 25.6-kDa protein of 244 amino acids which has 65% amino acid sequence identity with PLA from *F. solani* f. sp. *pisi* but no significant homology with other pectinolytic enzymes. The first 16 amino acid residues at the N terminus appeared to be a signal peptide. The *pelB* cDNA was expressed in *Pichia pastoris*, yielding a pectate lyase B (PLB) which was found to be a glycoprotein of 29 kDa. PLB was purified to homogeneity by using a two-step procedure involving ammonium sulfate precipitation followed by Superdex G75 gel filtration chromatography. Purified PLB showed optimal lyase activity at pH 10.0. A rapid drop in the viscosity of the substrate and Mono Q anion-exchange chromatography of the products generated by the lyase showed that PLB cleaved polygalacturonate chains in an endo fashion. Western blotting (immunoblotting) with antibodies raised against PLA showed that PLB and PLA are immunologically related to each other. The 5′ flanking regions of both *pelA* and *pelB* were translationally fused to the β-glucuronidase gene and introduced into *F. solani* f. sp. *pisi*, and β-glucuronidase activities of the transformants were measured. Expression of the marker gene by the transformants showed that *pelA* expression is induced by pectin and repressed by glucose, whereas expression of *pelB* is constitutive and is not subject to glucose repression. Reverse transcription-mediated PCR showed that both *pelA* and *pelB* are expressed when *F. solani* f. sp. *pisi* infects pea epicotyl.**

Plant cell walls provide plants shape and support, help to regulate physiological processes including defense responses, and act as physical barriers to pathogen invasion (13). Many plant pathogens produce an array of enzymes capable of degrading plant cell wall components. Among the different cell wall-degrading enzymes known to be produced by pathogens, most attention has been focused on those that depolymerize pectin, a major component of the primary plant cell wall and middle lamella (29). Pectin-degrading enzymes are frequently the first cell wall-degrading enzymes produced by plant fungal pathogens in infected tissues (6, 21, 28). Depolymerization of pectin would not only provide a carbon source for fungal growth and development (13) but also expose other cell wall components to degradation, causing further cell wall breakdown (3, 23). Purified pectinases can cause tissue maceration and host cell death (6), a major symptom of some plant diseases. While the role of pectin-degrading enzymes in bacterial pathogenesis has been extensively studied (2, 6), the function of such enzymes in fungal pathogenesis remains less clear.

Many plant-pathogenic fungi, when grown with pectin as the sole carbon source, produce different forms of pectinolytic enzymes (7, 8, 39). Several genes encoding pectinolytic enzymes have been cloned from phytopathogenic fungi as well as nonpathogenic fungi (4, 9, 10, 15, 31, 33, 35). It is known that expression of genes encoding these enzymes is induced by substrates such as polygalacturonic acid (PGA) and is repressed by the presence of readily metabolized carbon sources such as glucose (9, 10). Such a pectate lyase produced by *Fusarium solani* f. sp. *pisi* (*Nectria haematococca*, mating type VI; hereafter referred to as *F. solani pisi*) was suggested to be involved in the infection of its host, *Pisum sativum* (8). Even though induction of the *pelA* gene by pectin was demonstrated (10), how the large polymer in the host cell wall would induce the synthesis of the encoded protein (pectate lyase A [PLA]) remains unclear. Oligomeric products generated by a low level of constitutively expressed pectin-degrading enzymes might be the real inducers. However, no constitutively expressed pectinase gene has been cloned and characterized from phytopathogenic fungi. We report the cloning of a novel constitutively expressed pectate lyase gene, *pelB*, from *F. solani pisi*, using the previously cloned *pelA* cDNA from this fungus as the probe (10). We have found that the 5′ flanking regions of *pelA* and

* Corresponding author. Mailing address: Neurobiotechnology Center, The Ohio State University, 206 Rightmire Hall, 1060 Carmack Road, Columbus, OH 43210. Phone: (614) 292-5682. Fax: (614) 292-5379. Electronic mail address: Kolattukudy.2@ohio.edu.

† Present address: Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de los Alimentos, 46010-Valencia, Spain.

*pelB* translationally fused to the β-glucuronidase (GUS) gene drive the marker gene expression. The results demonstrate that the expression of *pelB* is constitutive and is not repressed by glucose. We have also isolated *pelB* cDNA by reverse transcription-mediated PCR (RT-PCR), expressed it in *Pichia pastoris*, and characterized PLB as an endo lyase. Evidence for expression of both *pelA* and *pelB* on infected host tissues by *F. solani pisi* is also presented.

## MATERIALS AND METHODS

**Fungal and bacterial strains.** *F. solani pisi* field isolate T8 was obtained from H. D. Van Etten (University of Arizona). Single-spore isolates were routinely maintained on potato dextrose agar containing 1 g of finely ground pea stem per liter. *F. solani pisi* cultures were grown in mineral medium (14) containing 0.5 to 2% glucose on a rotary shaker at 200 rpm. *Escherichia coli* DH5α was used for construction of the genomic library from *F. solani pisi* and propagation of plasmids.

**Nucleic acid isolation and analysis.** *F. solani pisi* conidia ($10^7$) were inoculated in 100 ml of mineral medium containing 2% glucose and incubated for 48 to 72 h at 24°C with shaking. Mycelia were harvested by filtration through Whatman no. 1 filters and frozen in liquid nitrogen. The samples were kept at −80°C until needed. Genomic DNA was isolated from the mycelia as described before (22). For Southern hybridization, restriction enzyme-digested DNA was fractionated in 0.8% agarose gel, transferred to a nylon membrane by using a vacuum blotting system (LKB 2016 VacuGene), and hybridized to a $^{32}$P-labeled DNA probe.

**Genomic library construction and screening.** The genomic DNA of *F. solani pisi* was digested with *Xba*I and separated on 0.8% agarose gels. The gel segment corresponding to 2.5 to 20 kb of DNA was cut out, and the DNA was electro-eluted. The DNA was ligated to *Xba*I-digested pUC18 and used to transform *E. coli* DH5α cells. The library was screened by colony hybridization using labeled *pelA* cDNA under low-stringency hybridization conditions at 60°C in 6× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate [pH 7.6]) and 0.5% sodium dodecyl sulfate (SDS). The membrane was washed twice at 60°C for 30 min with 4× SSC and 0.1% SDS.

**DNA sequencing and sequence analysis.** A genomic clone, pX1311, containing a 6.4-kb *Xba*I fragment which had sequence homology with *pelA* cDNA, was identified from the *F. solani pisi* genomic library. A 2.6-kb *Sal*I fragment was subcloned from pX1311 to the *Sma*I site of pUC18, yielding pPLB. A series of nested deletion subclones was made from pPLB following treatment of the original plasmid with exonuclease III and nuclease S1, and the nucleotide sequences of these subclones were determined. DNA was sequenced by using a Sequenase 2.0 DNA sequencing kit from United States Biochemical as recommended by the supplier. DNA Strider 1.2 and the Genetics Computer Group packages were used to analyze and align DNA sequences.

**Amplification of *pelB* cDNA by RT-PCR.** *F. solani pisi* conidia ($10^7$) were inoculated in 100 ml of mineral medium supplemented with 2% glucose and incubated at 24°C with shaking. After 3 days, 10 ml of this culture was transferred to 100 ml of mineral medium containing 0.5% glucose and incubated for another 2 days. Mycelia were harvested by filtration through Whatman no. 1 filters and frozen in liquid nitrogen. Total RNA isolated with TRIzol reagent (Bethesda Research Laboratories [BRL]) as previously described (18) was treated with amplification grade RNase-free DNase (BRL) and reverse transcribed into cDNA with murine leukemia virus reverse transcriptase (BRL), using oligo(dT) primers. The first-strand cDNA was used directly for PCR amplification with a sense primer, 5′-GGG GAA TTC CAA TCA CCA TGA AGG CC-3′, and an antisense primer, 5′-GGG GAA TTC GCA AAG CTA GCG AAG CC-3′. The PCR procedure consisted of a denaturation step at 94°C for 5 min followed by 40 cycles of the following steps: denaturation at 94°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 2 min. A last elongation step was done at 72°C for 15 min. The product was purified from 0.8% agarose gel and cloned into pCRII vector (TA cloning kit; Invitrogen), yielding pTAB.

***P. pastoris* transformation and induction of protein expression.** *P. pastoris* growth, transformation, and induction of protein expression were done according to the user instructions provided with the Invitrogen expression kit. A 780-bp *Eco*RI fragment from pTAB, containing the cDNA of *pelB*, was cloned into the *Eco*RI site of *P. pastoris* expression vector pHILD2, generating pHILD2B. *P. pastoris* transformants were selected on minimal dextrose plates which did not contain histidine. The alcohol oxidase gene (*AOX-1*) disruption mutants were selected on the basis of their inability to grow on minimal methanol plates. Five transformants were randomly selected to inoculate 10 ml of buffered minimal glycerol medium. The cells were harvested after 2 days of growth with shaking (200 rpm) at 30°C, resuspended in 2 ml of buffered minimal methanol medium, and cultured for another 2 days. Pectate lyase activity in the culture supernatant was measured, and the transformant which produced the highest level of pectate lyase activity was used for large-scale expression.

**Pectate lyase purification.** *P. pastoris* culture fluid was harvested by centrifugation of a 1-liter culture. To the supernatant, $(NH_4)_2SO_4$ was added to 60% saturation with stirring, and the mixture was kept on ice for 1 h. The precipitate was removed by centrifugation, and the supernatant was 85% saturated with $(NH_4)_2SO_4$. The mixture was kept on ice for another 1 hour. The precipitate collected by centrifugation was dissolved in $H_2O$, dialyzed against $H_2O$, and lyophilized. The lyophilized enzyme was dissolved in 20 mM Tris-HCl (pH 7.0)–100 mM NaCl and loaded onto a Superdex G75 column for fast protein liquid chromatography. Pectate lyase activity of aliquots of each fraction was measured, and the fractions containing the most lyase activity were combined. The enzyme was stored at −80°C for future use.

**Pectate lyase assay.** Pectate lyase activity was routinely determined by measuring the change in $A_{235}$ with 3 mg of PGA (Sigma) per ml as the substrate in 50 mM Tris-HCl (pH 9.5) containing 1 mM $CaCl_2$ (8). Viscometric assays were performed with an Ostwald capillary viscometer as described previously (8). One unit of pectate lyase activity releases 1 μmol of unsaturated product per min at 30°C.

**Analysis of PGA degradation products by anion-exchange chromatography.** PGA (60 mg) was dissolved in 20 ml of 50 mM Tris-HCl (pH 9.5) containing 1 mM $CaCl_2$. Purified PLB (0.4 U) was added to the solution, the reaction mixture was incubated at room temperature for 1 h, and the reaction was stopped by boiling the mixture for 10 min in a water bath. The reaction mixture was diluted five times with $H_2O$, filtered through a 0.2-μm-pore-size filter, and loaded onto a mono Q HR 16/10 column which was equilibrated with 20 mM Tris-HCl (pH 7.0) containing 100 mM NaCl. The products were eluted with a 100 to 400 mM NaCl linear gradient as described previously (20).

**Electrophoresis, Western blotting (immunoblotting), and detection of glycosylation.** SDS-polyacrylamide gel electrophoresis (PAGE) was done by the method of Laemmli (26), with a 12% separating gel and a 4% stacking gel. A GlycoTrack kit (Oxford GlycoSystems) was used to detect carbohydrate moieties in protein after SDS-PAGE and transblotting onto an Immobilon P membrane as suggested by the manufacturer. For Western blotting, the electrophoresed proteins were transblotted onto a nitrocellulose membrane in 10 mM cyclohexylaminopropane sulfonic acid transfer buffer (pH 10.5)–15% methanol. The antibodies prepared against PLA which was purified from culture fluid of *F. solani pisi* (8) were diluted 1:500 for Western blot analysis. The blot was visualized by using horseradish peroxidase-protein A and an enhanced chemiluminescence system (Amersham) as recommended by the manufacturer.

**Inhibition of pectate lyase activities by antibody.** The immunoglobulin G (IgG) fraction of the PLA immune serum, obtained by $(NH_4)_2SO_4$ precipitation, was used for lyase activity inhibition assay. Pectate lyase (20 mU) was incubated with different concentrations of IgG in phosphate-buffered saline (pH 7.4) for 1 h at room temperature, and then the enzyme activity was measured as indicated above.

**Construction of transformation vectors and *F. solani pisi* transformation.** Plasmid pGUS.2 (30) was used to construct GUS expression vectors in *F. solani pisi*. A 1.0-kb *Sma*I-*Stu*I fragment from the 5′ flanking region of *pelB*, blunt ended by Klenow DNA polymerase, was ligated into the *Sma*I site of pGUS.2, yielding *pelB*/GUS. A 1.1-kb *Xba*I-*Bsp*HI fragment from the 5′ flanking region of *pelA* was blunt ended by Klenow DNA polymerase and ligated into the Klenow DNA polymerase-blunted *Bam*HI site of pGUS.2, yielding *pelA*/GUS. A hygromycin resistance gene (*hyg*) fused to a constitutive promoter from *Cochliobolus heterostrophus* (36) was cloned into the *Eco*RI sites of both constructs as the selection marker for *F. solani pisi* transformation. *F. solani pisi* conidia ($10^7$) were inoculated into 500 ml of mineral medium containing 2% glucose. After the culture was shaken for 24 h at 24°C, mycelia were harvested by filtration through a Whatman no. 1 filter and washed twice with 0.6 M $MgCl_2$. Protoplast preparation and transformation were done as described by Soliday et al. (34). The transformed protoplasts were plated on mineral medium containing 2% glucose, 1.2 M sorbitol, and 2% agar. Overlay of 1% agar containing hygromycin B (300 μg/ml) was added after 24 h of incubation.

**Assay for GUS activity.** For GUS activity analysis, conidia ($10^7$) of each transformant were inoculated in 100 ml of mineral medium supplemented with 0.5% glucose and incubated at 24°C until the glucose was completely depleted as determined by a glucose oxidase assay (27). Pectin and glucose were added to give a final concentration of 0.2% glucose, 0.2% pectin, or 0.2% pectin plus 2% glucose. The $CaCl_2$ concentration was adjusted to 1 mM. Fungal mycelia collected from the cultures by filtration through Whatman no. 1 filter were homogenized for 2 min in a Mini-Bead Beater apparatus with two 4.7-mm-diameter stainless steel beads. The homogenate was centrifuged at 12,000 × *g* for 5 min, and the supernatant was used for protein determination and GUS assay (30). GUS activity was determined as described before (19). Fluorescence was determined by using the Millipore CytoFluor 2350 fluorescence measurement system.

**Isolation of RNA from pea stems infected with *F. solani pisi* and RT-PCR using gene-specific primers.** Pea (variety Extra Early Alaska; Livingston Seed Co., Columbus, Ohio) seedlings were prepared as described before (32). Sections (2 to 2.5 cm long) were cut from the second internode and placed on moist filter paper in a petri dish. Conidia ($5 \times 10^5$) in 5 μl of $H_2O$ were placed on each pea stem. The stem sections were placed in a moist chamber and incubated at 24°C for the indicated periods. Total RNA was isolated from the infected plant tissue as previously described (18). RNA (1 μg) was treated with amplification-grade RNase-free DNase (BRL) and reverse transcribed into cDNA with murine leukemia virus reverse transcriptase (BRL), using oligo(dT) primers. The first-strand cDNA was used directly for PCR analysis using gene-specific primers. One pair of primers was synthesized for each gene on the basis of the least homologous regions flanking the first intron. The sense and antisense primers

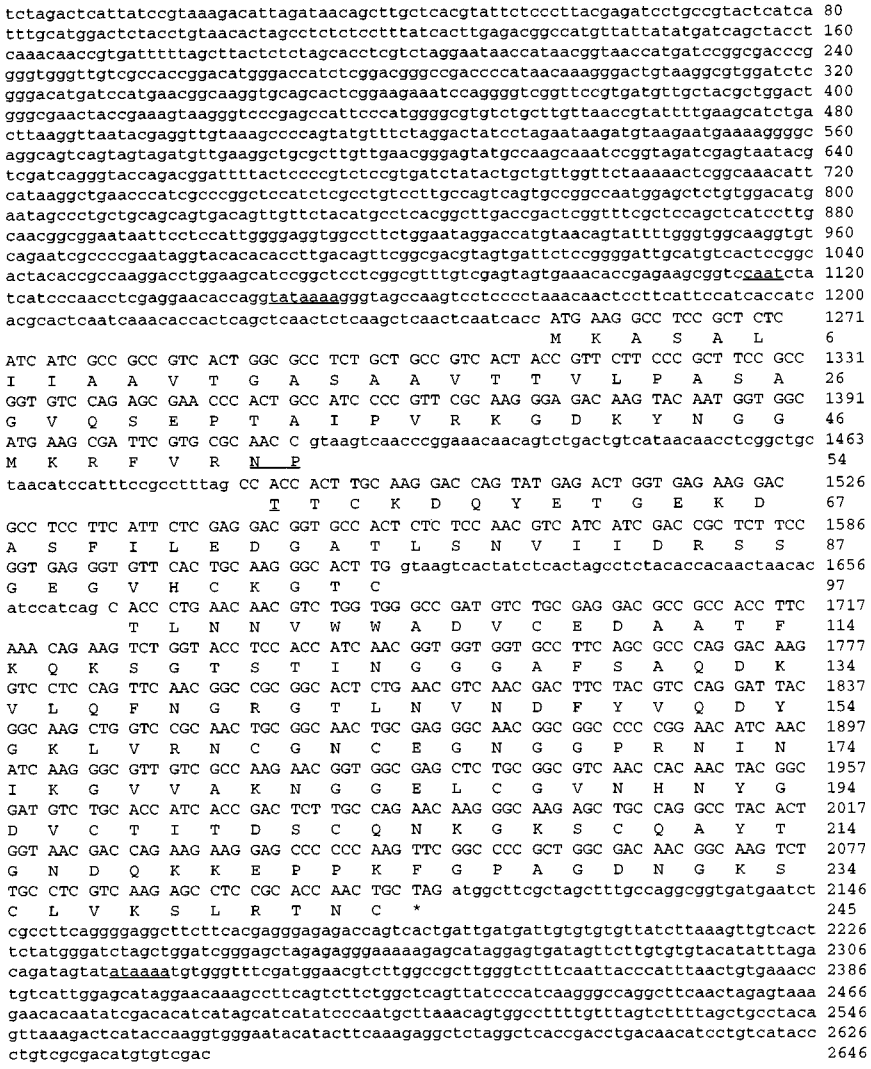

FIG. 1. Nucleotide sequence of the cloned DNA fragment showing the open reading frame coding for PLB. Sequences resembling the TATA box, CAAT motif, N-glycosylation site, and polyadenylation signal are underlined.

used were 5′-CAA GAC TCT CCC CAA GAG-3′ and 5′-GGC CTG GGA AGC ACC AAT-3′, respectively, for *pelA* and 5′-TAC CGT TCT TCC CGC TTC-3′ and 5′-ACC GGA AGA GCG GTC GAT-3′, respectively, for *pelB*. The PCR procedure consisted of a denaturation step at 94°C for 5 min followed by 40 cycles of the following steps: denaturation at 94°C for 30 s, annealing at 59°C for 45 s, and extension at 72°C for 45 s. A last elongation step was done at 72°C for 10 min. Finally, 5-μl aliquots of the PCR products were run on a 1.5% agarose gel; the products were isolated from the gel and cloned into pCRII vector. Plasmid DNA was prepared from randomly selected transformants and sequenced as described above.

**Nucleotide sequence accession number.** The nucleotide sequence for the *pelB* gene is in the GenBank database under accession number U13051.

## RESULTS

**Isolation and sequencing of *pelB*.** To search for pectate lyase genes related to the previously cloned *pelA*, a library made with 2.5- to 20-kb *Xba*I fragments of the genomic DNA from *F. solani pisi*, constructed in pUC18, was screened by using labeled *pelA* cDNA probe under low-stringency hybridization conditions. From the clone which showed hybridization and contained a 6.4-kb *Xba*I fragment, a 2.6-kb *Sal*I fragment which hybridized with *pelA* cDNA was subcloned (pPLB) and sequenced. On the basis of the sequence alignment with *pelA* and conserved intron border sequences of filamentous fungi (1, 10, 12), the coding region of the *pelB* was identified (Fig. 1).

The nucleotide sequence of *pelB* showed one open reading frame of 732 bp interrupted by two introns of 72 and 50 bp. The coding sequence of *pelB* predicts a 25.6-kDa protein product of 244 amino acid residues. The first 16 amino acid residues show characteristics of a signal peptide (37), indicating that *pelB* encodes an extracellular pectate lyase (PLB). The amino acid sequence of PLB showed 65% identity with that of PLA and 77% similarity when the conservative replacements are included. Based on the −3, −1 rule proposed by von Heijne (38), a possible cleavage site of the signal peptide was located between Ala-16 and Ala-17, resulting in a mature protein of 228 amino acids with a calculated molecular mass of 24.2 kDa.

The nucleotide sequences of both the 5′ and 3′ flanking regions of *pelB* were also determined. A TATAAAA box is found 107 nucleotides upstream from the first ATG start codon. A CAAT motif is found 33 nucleotides upstream from the predicted TATA box. The polyadenylation sequence AATAAA, which is frequently found in eukaryotic genes, is not found in the 536 nucleotides of the 3′ flanking region

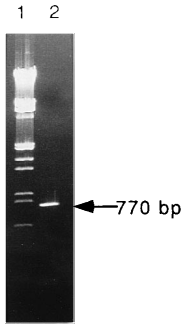


FIG. 2. PCR amplification of *pelB* cDNA. Lane 1, *Eco*RI-*Hin*dIII-digested λ DNA size marker; lane 2, PCR product.

sequenced. However, the sequence ATAAAA is identified 206 nucleotides downstream from the stop codon.

**RT-PCR and expression of the open reading frame of *pelB* in *P. pastoris*.** Total RNA isolated from *F. solani pisi* culture grown in the presence of 0.5% glucose as the sole carbon source was used as the template for RT-PCR. The PCR product that matched the expected size of *pelB* cDNA (Fig. 2) was cloned into pCRII. The DNA sequence of one randomly selected cDNA clone matched exactly that of the *pelB* genomic sequence except for the introns. Since the coding sequence of *pelB* has a putative leader peptide, the lyase product would be expected to be extracellular, and therefore this coding sequence with the *pelB* leader was introduced into the *P. pastoris* expression vector pHILD2, which does not contain a *P. pastoris* secretion signal. *P. pastoris* transformants harboring the vector were isolated, and expression of PLB was determined by measuring the pectate lyase activity in the culture supernatant. Five randomly selected pHILD2B transformants secreted enzymatically active pectate lyase (2.2 to 3.3 U/ml) into the culture medium, whereas the wild type and the control transformant expressing human serum albumin did not (data not shown). These results demonstrate that the putative signal sequence of *pelB* is functional.

**Purification and characterization of the recombinant PLB.** The recombinant PLB was purified to apparent homogeneity by a two-step process involving $(NH_4)_2SO_4$ precipitation followed by Superdex G75 gel filtration. A single Coomassie blue-staining band corresponding to a molecular mass of 29 kDa was found after SDS-PAGE (Fig. 3A). The molecular mass of the mature form of PLB calculated from the *pelB* coding sequences was 4.8 kDa smaller than the size of 29 kDa

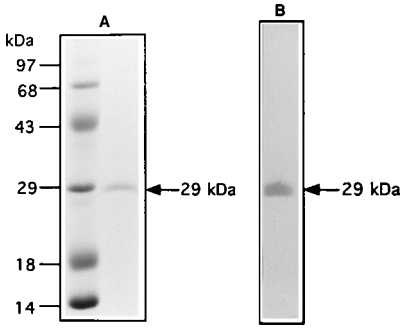


FIG. 3. (A) Coomassie blue-stained SDS-polyacrylamide gel of the recombinant PLB purified from *P. pastoris* culture supernatant. Positions of molecular mass markers are shown on the left. (B) Carbohydrate detection of the recombinant PLB (1 μg) by using a GlycoTrack kit.

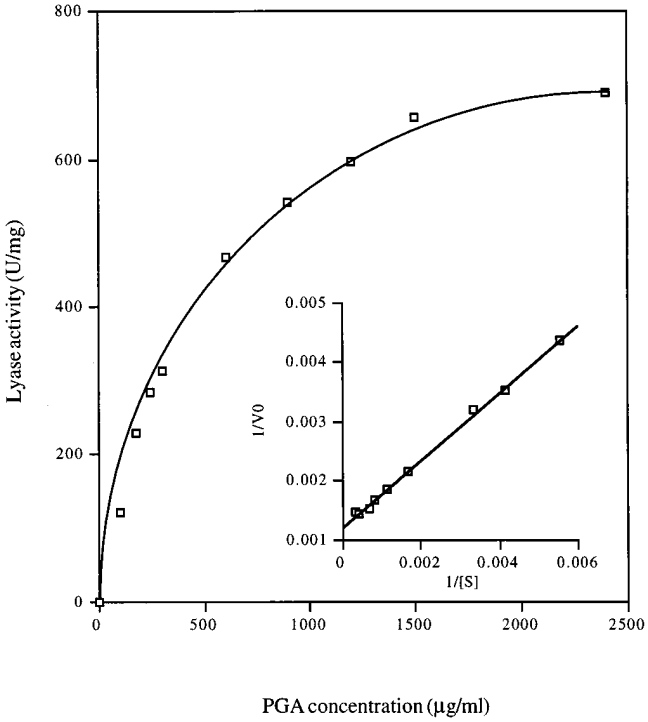


FIG. 4. Effect of PGA concentration on the rate of lyase action. The pectate lyase concentration was 0.05 μg/ml. The $[Ca^{2+}]$/[PGA] ratio was adjusted to its optimum at 0.11. Inset, double-reciprocal plot of the same data.

determined from SDS-PAGE. This difference could be due to glycosylation, as the recombinant PLB appeared to be a glycoprotein (Fig. 3B).

The purified recombinant pectate lyase showed a basic pH optimum of about 10.0, and the enzyme was two to three times more active with PGA as a substrate than with pectin (~70% methylated) (data not shown). The activity of PLB required $Ca^{2+}$. EGTA completely inhibited the lyase activity, and this inhibition could be fully reversed by $Ca^{2+}$. Optimal activity was observed at a $[Ca^{2+}]$/[PGA] molar ratio of 0.11. Monovalent cations, $Mg^{2+}$, $Co^{2+}$, and $Zn^{2+}$, could not substitute for $Ca^{2+}$. However, with $Mn^{2+}$, 11% of the activity detected with $Ca^{2+}$ was observed, as also found with PLC, the product of a homologous lyase gene from *F. solani pisi* (11). PLB showed a typical substrate saturation pattern with PGA resulting in a linear double-reciprocal plot (Fig. 4). The $K_m$ and $V_{max}$ were calculated to be 566 μg/ml and 1,000 U/mg enzyme, respectively.

To determine whether PLB had an endo or exo activity, the effect of PLB on the viscosity of a PGA solution was measured. The viscosity dropped rapidly, decreasing by 50%, when approximately 1.1% of the total glycosidic linkages were cleaved, indicating that it is an endo enzyme cleaving internal glycosidic linkages randomly (Fig. 5A). That PLB is an endo pectate lyase was confirmed by analysis of the PGA degradation products by anion-exchange chromatography. After approximately 10% of the available bonds were cleaved, the partially digested PGA was loaded to a Mono Q column. More than 10 peaks of oligomers could be resolved from the column when a linear NaCl gradient of 100 to 400 mM was applied to it (Fig. 5B).

To test whether PLB was immunologically related to PLA, Western blot analysis was done with the antibody raised against PLA. PLB could be recognized by the PLA antibody nearly as well as PLA (Fig. 6). The PLA antibody also inhibited

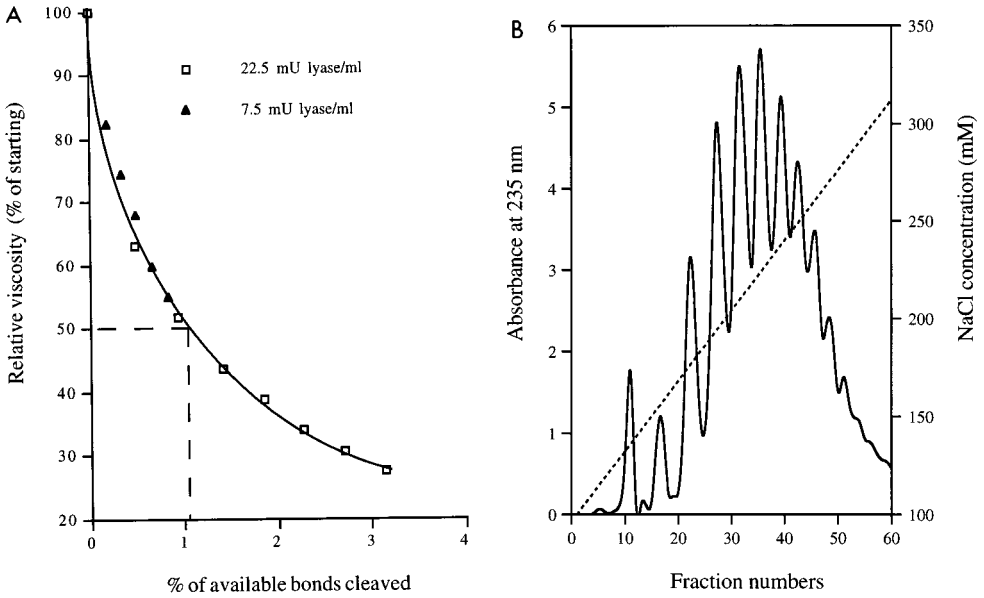


FIG. 5. (A) Change in viscosity of the PGA solution as a result of the action of pectate lyase. Viscosity was measured with an Ostwald capillary viscometer at room temperature. The dashed line indicates 50% decrease in viscosity at approximately 1.1% bond cleavage. (B) Anion-exchange chromatographic analysis of PGA degradation products generated by the action of pectate lyase when 10% of the available glycosidic bonds were cleaved. PGA degradation products were loaded onto a Mono Q 16/10 HR column and subjected to elution with a linear NaCl gradient from 100 to 400 mM in 20 mM Tris-HCl (pH 7.0). The percentage of available bonds cleaved was calculated as described before (8).

the lyase activity of PLB, although at IgG concentrations much higher than that required to inhibit PLA (Fig. 7).

**Regulation of *pelB* expression.** To study the regulation of *pelB* expression and to compare it with that of *pelA*, a 1.0-kb fragment from the 5′ flanking region of *pelB* and a 1.1-kb fragment from the 5′ flanking region of *pelA* were translationally fused to the GUS gene, yielding *pelB*/GUS and *pelA*/GUS, respectively (Fig. 8). Five *pelA*/GUS transformants and five *pelB*/GUS transformants of *F. solani pisi* were randomly selected for further analysis. Southern hybridization using the GUS coding region as the probe showed that all transformants had at least one copy of the GUS gene integrated into the genome (data not shown). After the 0.5% glucose in the culture media of the transformants was completely depleted, glucose, pectin, or the combination of glucose and pectin was added to the cultures, and GUS activity was measured at periodic intervals thereafter. Pectate lyase activities in the culture supernatants of all of the transformants were the same (data not shown). For *pelA*/GUS transformants, no GUS activity was detected when glucose was added as the carbon source (Fig. 9A). When pectin was added, GUS activity reached a high level 24 h after pectin addition and increased dramatically during the next 24 h (Fig. 9B). When 2% glucose was added with

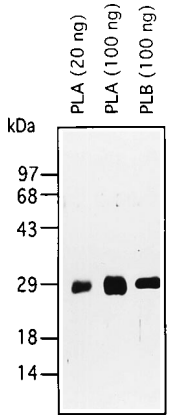


FIG. 6. Western blot analysis of *F. solani pisi* PLA and the recombinant PLB expressed in *P. pastoris*, using antibodies raised against PLA. Molecular masses are shown on the left. Purified PLA and PLB were used.

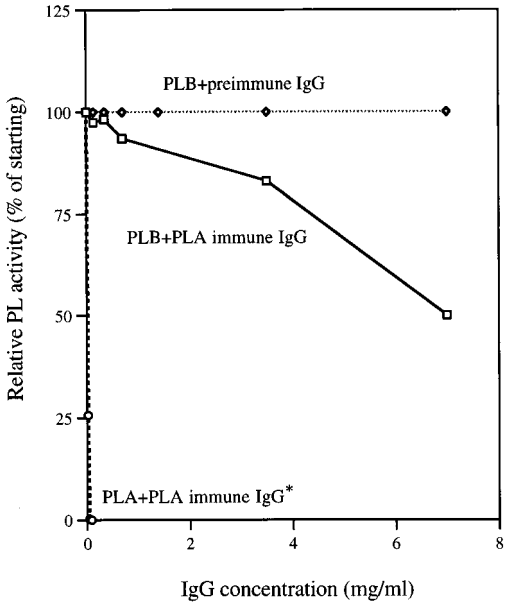


FIG. 7. Inhibition of *F. solani pisi* PLB lyase activity with antibodies raised against PLA. The lyase activity is expressed as a percentage of the value for the control without antibody. *, the lyase activity of PLA was extremely sensitive to inhibition by anti-PLA IgG, giving 75% inhibition at 0.022 mg of IgG per ml and 100% inhibition at 0.088 mg of IgG per ml.

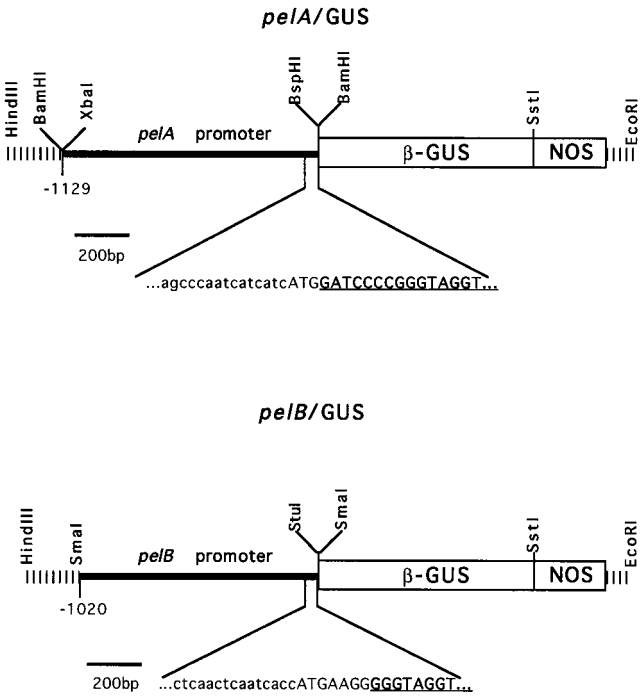


FIG. 8. Diagrammatic representation of the chimeric *pelA*- and *pelB* promoter-GUS fusions for *F. solani pisi* transformation. The nucleotide sequence of the region showing translational fusion of the pectate lyase gene to the GUS gene is given for each construct. The sequence of the GUS gene is in boldface and underlined. NOS, Nopaline synthase.

pectin, GUS activity was not detectable (Fig. 9C) but 0.5% glucose was not sufficient to cause repression (data not shown). In contrast, the low GUS activity levels in the *pelB*/GUS transformants were not affected by the presence of different carbon sources (Fig. 9).

**Expression of *pelB* and *pelA* in infected pea stems.** To determine whether *pelB* and *pelA* are expressed by *F. solani pisi* during infection of its host, conidia of *F. solani pisi* were placed on pea stems and infection was allowed to proceed in a humid chamber. RT-PCR was used to detect the presence of *pelA* and *pelB* transcripts in the infected pea tissues, using gene-specific primers. Electrophoretic analysis of the PCR products showed that they contained cDNA of the expected size corresponding to each of the genes (Fig. 10). Cloning and sequencing of the PCR products showed that their sequences matched perfectly those of the corresponding genomic sequences except for the intron regions, showing that both *pelA* and *pelB* are expressed during the infection process. The transcripts of *pelA* and *pelB* could be detected by 24 h after inoculation, although no transcript was found at 12 h after inoculation (Fig. 10).

## DISCUSSION

In this report we describe the cloning and sequence analysis of a novel constitutively expressed pectate lyase gene, *pelB*, from the phytopathogenic fungus *F. solani pisi*. To our knowledge, this is the first report on the isolation, sequencing, and characterization of a constitutively expressed pectic enzyme-encoding gene from a phytopathogenic fungus. The sequence of the full-length *pelB*, including part of the 5′ and 3′ flanking regions, is reported. This constitutively expressed *pelB* is closely related to *pelA*, as the predicted protein product, PLB, has 65% sequence identity with PLA. The 5′ and 3′ flanking regions of *pelB* have little in common with the untranslated regions in *pelA*. The currently available databases do not reveal any other genes or proteins showing significant homology to *pelB* or PLB. On the basis of pectate lyase gene sequences from *Erwinia chrysanthemi*, *E. carotovora*, and plants, Barras et al. defined three consensus sequences in the gene products: (i) (D/E)(G/S)-hDh--(A/G)(S/A)--hThS (where h stands for I, L, or V), (ii) h--R-P--R-G-hH--NN-Y, and (iii) (S/A/T)--hWVDH--h (2). These consensus sequences are also found highly conserved in pectin lyases of *E. carotovora* and therefore were suggested as signatures of pectate or pectin lyase activity (2). However, these sequences could not be identified in the predicted *pelB* product. Since structure-based alignments of pectate lyases could not accommodate the *pelA* gene from *F. solani pisi*, it was concluded that this lyase belongs to a different group (16, 17) and *pelB* must belong to that new group.

On the basis of consensus sequences for introns in filamen-

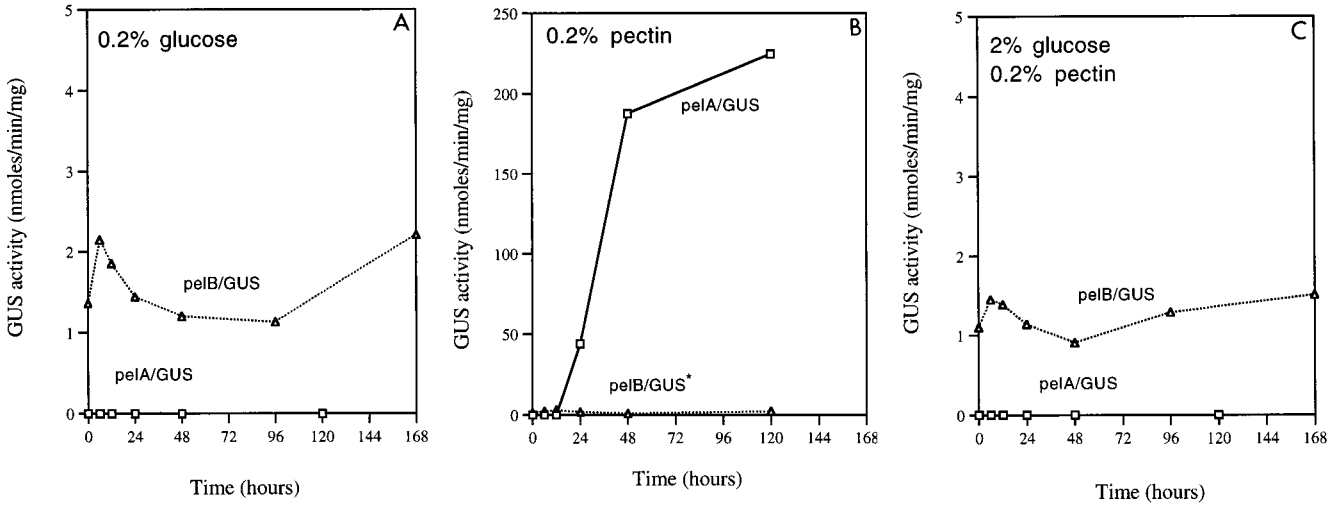


FIG. 9. Time course of activation of the *pelA* and *pelB* promoters in the GUS transformants of *F. solani pisi* when different carbon sources were added to final concentrations of 0.2% glucose (A), 0.2% pectin (B), and 0.2% pectin plus 2% glucose (C). $CaCl_2$ was added to a final concentration of 1 mM in each case. Each time point represents the mean value of five randomly selected transformants from each construct. *, the GUS activity of *pelB*/GUS was about 1.0 to 3.0 nmol/min/mg.

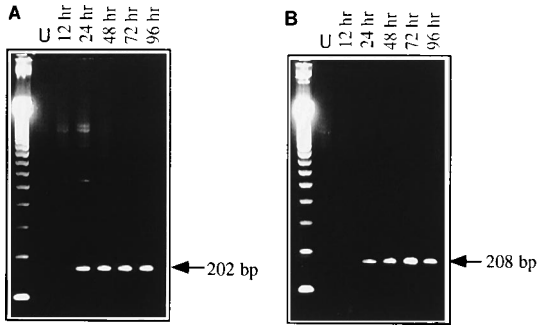


FIG. 10. RT-PCR products showing the expression patterns of *pelB* and *pelA* during the infection process on pea stems by *F. solani pisi*. First-strand cDNAs generated from total RNA isolated at different time points from the infected pea stems were used as templates. PCR was performed with these templates and gene-specific primers for *pelA* (A) and *pelB* (B) (Materials and Methods). Aliquots (5 μl) of the PCR products were run on an agarose gel with a 123-bp ladder marker (BRL). In both panels, U denotes RNA isolated from uninfected pea stems.

tous fungi, it appears that the putative *pelB* product would be encoded by three exons in *F. solani pisi*. The nucleotide sequence of the cDNA confirmed the presence of two introns in the genomic clone. The sizes of the introns (72 and 50 bp) in *pelB*, the 5′ and 3′ border sequences, and the putative lariat sequences are all typical of introns in genes of filamentous fungi (12). The positions of introns are also conserved in *pelA* and *pelB*. The introns of *pelB* are located at the same positions as the first two introns in *pelA*, while the third one found in *pelA* is absent in *pelB*. The similar positionings of the introns may suggest that *pelA* and *pelB* were derived from the same ancestral gene. The *pelB* gene, like *pelA*, shows a high codon bias; only 38 of 61 possible codons are used. In the wobble position, there is a high preference of pyrimidines, especially cytosine; adenosine is avoided at the third position. These features are found in genes which are constitutively or highly expressed in *Neurospora* spp. (12).

The predicted mature form of PLB contains 228 amino acids with a calculated molecular mass of 24.2 kDa, which is 4.8 kDa smaller than the size estimated on the basis of SDS-PAGE. Since there is one potential N-glycosylation site found in PLB at Asn-53–Pro-54–Thr-56, and O-linked glycosylation is possible, the difference in molecular mass probably represents glycosylation found in secreted proteins. In fact, a glycosylation test showed that the 29-kDa protein is a glycoprotein.

In recent years, several genes encoding pectinases have been cloned from phytopathogenic fungi (10, 31, 33, 35). However, how the expression of these genes is regulated is poorly understood. To study the regulation of pectate lyase gene expression in *F. solani pisi*, the 5′ flanking regions of both *pelA* and *pelB* were translationally fused to the GUS gene. The wild-type *F. solani pisi* showed no GUS activity. The time course of induction of the GUS gene by pectin and repression by glucose was studied. The expression of the GUS gene in the *pelA*/GUS transformants was found to be inducible by pectin, and this induction by pectin was completely repressed by the presence of glucose in the culture medium. Thus, the regulation of GUS gene expression by those *pelA*/GUS transformants truly reflects previously found regulation of native *pelA* expression (10). The GUS gene expression driven by the *pelB* promoter was constitutive. In GUS transformants, the *pelA* gene present in them was found to be inducible by pectin and repressed by glucose. Thus, the transformation process did not cause abnormalities in the inducible and repressible expression of lyase genes, and any difference in GUS activities among *pelA*/GUS and *pelB*/GUS transformants was due to the presence of different promoter activities conferred by the 5′ flanking regions of the lyase genes. The results from the GUS activity analyses suggest that the expression of *pelB* is constitutive and is not subject to glucose repression, whereas *pelA* is expressed only when induced by pectin and is suppressed by glucose.

How glucose mediates repression of pectate lyase expression in *F. solani pisi* is not clear. It has been suggested that the catabolic repression is mediated by cyclic AMP (cAMP) (10). Indeed, the 5′ flanking region of *pelA* contains the sequence GACGAGA (-191 to -185), which shows homology to the consensus cAMP-responsive element sequence, GT(T/G)ACG (A/T)CA, found in higher eukaryotes (5). The corresponding motif is not found in the 5′ flanking region of *pelB*. However, until detailed promoter analysis is done, the functional significance of the putative cAMP-responsive element found in inducible *pelA* but not in constitutively expressed *pelB* remains uncertain.

If and how the different genes encoding pectinolytic enzymes play a significant role in pathogenesis remain to be elucidated. *F. solani pisi* is known to produce different pectinolytic enzymes when grown in the presence of pectin (25). Two polygalacturonases and one pectate lyase were purified (8, 24). In addition to *pelB*, *pelA* and *pelC* have also been cloned (10, 11). Whether these genes play a significant role in pathogenesis has not been determined. Antibodies prepared against PLA protect pea stems against *F. solani pisi* infection, indicating that this enzyme may play a significant role in pathogenesis (8). However, disruption of *pelA* failed to decrease the virulence (unpublished results), suggesting that other lyases that immunologically cross-react with PLA antibody may be important in pathogenesis. If so, the antibodies would be able to protect the host by inhibiting such lyases. We show that in fact PLB immunologically cross-reacts with antibodies prepared against PLA. PLA antibodies could protect the host by inhibiting PLB and/or other immunologically related pectin-degrading enzymes, including those which might be produced only during infection of the host. Thus, the use of antibodies can protect the host against the effects of a variety of immunologically related plant cell wall-degrading enzymes, whereas disruption of a single gene out of many that can encode wall-degrading enzymes would not be expected to decrease virulence.

The differential regulation of expression of the different *pel* genes could have real biological functions. Constitutively expressed *pelB* could lead to generation of cell wall degradation products which in turn may induce the expression of genes that encode other pectin-degrading enzymes. PLA is induced by oligomers produced from pectin by endo pectate lyase (unpublished results), and here we demonstrate that PLB is an endo lyase. If this constitutively produced PLB is used to sense the contact with plant cell walls by releasing small amount of pectic fragments, the absence of PLB might prevent inducible expression of such pectinolytic enzymes. Disruption of the *pelB* gene to test this possibility is under way.

## ACKNOWLEDGMENTS

We thank Debra Gamble for assistance in preparing the manuscript and Clifford Beall for help in analyzing the sequence data.

This work was supported in part by National Science Foundation grant IBN-9318554.

## REFERENCES

1. **Ballance, D. J.** 1991. Transformation systems for filamentous fungi and an overview of fungal gene structure, p. 1–17. *In* S. A. Leong and R. M. Berka (ed.), Molecular industrial mycology: systems and applications for filamentous fungi. Mycology series, vol. 8. Marcel Dekker Inc., New York.

2. **Barras, F., F. V. Gijsegem, and A. K. Chatterjee.** 1994. Extracellular enzymes and pathogenesis of soft-rot Erwinia. Annu. Rev. Phytopathol. **32:**201–234.
3. **Bauer, W. D., D. F. Bateman, and C. H. Whalen.** 1977. Purification of an endo-β-1,4-galactanase produced by *Sclerotinia sclerotiorum*: effects on isolated plant cell walls and potato tissue. Phytopathology **67:**862–868.
4. **Bussink, H. J. D., F. P. Buxton, B. A. Fraaye, L. H. deGraaff, and J. Visser.** 1992. The polygalacturonases of *Aspergillus niger* are encoded by a family of diverged genes. Eur. J. Biochem. **208:**83–90.
5. **Choi, H., B. Li, Z. Lin, L. E. Huang, and A. Y.-C. Liu.** 1991. cAMP and cAMP-dependent protein kinase regulate the human heat shock protein 70 gene promoter activity. J. Biol. Chem. **266:**11858–11865.
6. **Collmer, A., and N. T. Keen.** 1986. The role of pectic enzymes in plant pathogenesis. Annu. Rev. Phytopathol. **24:**383–409.
7. **Cooper, R. M., and R. K. S. Wood.** 1975. Regulation of synthesis of cell-wall degrading enzymes by *Verticillium albo-atrum* and *Fusarium oxysporum* f. sp. *lycopersici*. Physiol. Plant Pathol. **5:**135–156.
8. **Crawford, M. S., and P. E. Kolattukudy.** 1987. Pectate lyase from *Fusarium solani* f. sp. *pisi*: purification, characterization, in vitro translation of the mRNA, and involvement in pathogenecity. Arch. Biochem. Biophys. **258:** 196–205.
9. **Dean, R. A., and W. E. Timberlake.** 1989. Regulation of the *Aspergillus nidulans* pectate lyase gene (*pelA*). Plant Cell **1:**275–284.
10. **González-Candelas, L., and P. E. Kolattukudy.** 1992. Isolation and analysis of a novel inducible pectate lyase gene from the phytopathogenic fungus *Fusarium solani* f. sp. *pisi*. J. Bacteriol. **174:**6343–6349.
11. **Guo, W., L. González-Candelas, and P. E. Kolattukudy.** Cloning of a new pectate lyase gene *pelC* from *Fusarium solani* f. sp. *pisi* (*Nectria haematococca*, mating type VI) and characterization of the gene product expressed in *Pichia pastoris*. Arch. Biochem. Biophys., in press.
12. **Gurr, S. J., S. E. Unkles, and J. R. Kinghorn.** 1987. The structure and organization of nuclear genes of filamentous fungi, p. 93–139. *In* J. R. Kinghorn (ed.), Gene structure in eukaryotic microbes. IRL Press, Oxford.
13. **Hahn, M. G., P. Bucheli, F. Cervone, S. H. Doares, R. A. O'Neill, A. Darvill, and P. Albersheim.** 1989. The roles of cell wall constituents in plant-pathogen interactions, p. 131–181. *In* T. Kosuge and E. W. Nester (ed.), Plant-microbe interactions. Molecular and genetic perspectives, vol. 3. McGraw-Hill, New York.
14. **Hankin, L., and P. E. Kolattukudy.** 1968. Metabolism of a plant wax paraffin (n-nonacosane) by a soil bacterium (*Micrococcus cerificans*). J. Gen. Microbiol. **51:**457–463.
15. **Harmsen, J. A. M., M. A. K. Someren, and J. Visser.** 1990. Cloning and expression of a second *Aspergillus niger* pectin lyase gene (pelA): indications of a pectin lyase gene family in *A. niger*. Curr. Genet. **18:**161–166.
16. **Heffron, S., B. Henrissat, M. D. Yoder, S. Lietzke, and F. Jurnak.** 1995. Structure-based multiple alignment of extracellular pectate lyase sequences. Mol. Plant-Microbe Interact. **8:**331–334.
17. **Henrissat, B., S. E. Heffron, M. D. Yoder, S. E. Lietzke, and F. Jurnak.** 1995. Functional implications of structure-based sequence alignment of proteins in the extracellular pectate lyase superfamily. Plant Physiol. **107:**963–976.
18. **Hwang, C.-S., M. A. Flaishman, and P. E. Kolattukudy.** 1995. Cloning of a gene expressed during appressorium formation by *Colletotrichum gloeosporioides* and a marked degrease in virulence by disruption of this gene. Plant Cell **7:**183–193.
19. **Jefferson, R. A.** 1987. Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. **5:**387–405.
20. **Jin, D. F., and C. A. West.** 1984. Characteristics of galacturonic acid oligomers as elicitors of casbene synthetase activity in castor bean seedlings. Plant Physiol. **74:**989–992.
21. **Jones, T. M., A. J. Anderson, and P. Albersheim.** 1972. Host-pathogen interactions. IV. Studies on the polysaccharide degrading enzymes secreted by *Fusarium oxysporum* f. sp. *lycopersici*. Physiol. Plant Pathol. **2:**153–166.
22. **Kämper, J. T., U. Kämper, L. M. Rogers, and P. E. Kolattukudy.** 1994. Identification of regulatory elements in the cutinase promoter from *Fusarium solani* f. sp. *pisi*. J. Biol. Chem. **269:**9195–9204.
23. **Keegstra, K., K. W. Talmadge, W. D. Bauer, and P. Albersheim.** 1973. The structure of plant cell walls. III. A model of the wall of suspension-cultured sycamore cells based on interconnections of the macromolecular components. Plant Physiol. **51:**188–197.
24. **Kolattukudy, P. E., G. K. Podila, B. A. Sherf, M. A. Bajar, and R. Mohan.** 1990. Mutual triggering of gene expression in plant-fungus interactions, p. 242–249. *In* H. Hennecke and D. P. S. Verma (ed.), Advances in molecular genetics of plant-microbe interactions, vol. 1. Kluwer Academic Publishers, Dordrecht, The Netherlands.
25. **Kolattukudy, P. E., J. Sebastian, W. F. Ettinger, and M. S. Crawford.** 1987. Cutinase and pectinase in host-pathogen and plant-bacterial interaction, p. 43–50. *In* D. P. S. Verma and N. Brisson (ed.), Molecular genetics of plant-microbe interactions. Martinus Nijhoff, Boston.
26. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
27. **Lin, T. S., and P. E. Kolattukudy.** 1978. Induction of a biopolyester hydrolase (cutinase) by low levels of cutin monomers in *Fusarium solani* f. sp. *pisi*. J. Bacteriol. **133:**942–951.
28. **Mankarios, A. T., and J. Friend.** 1980. Polysaccharide degrading enzymes of *Botrytis allii* and *Sclerotium cepivorum*: enzyme production in culture and the effect of the enzymes on isolated onion cell walls. Physiol. Plant Pathol. **17:**93–104.
29. **McNeil, M., A. G. Darvill, S. C. Fry, and P. Albersheim.** 1984. Structure and function of the primary cell wall of plants. Annu. Rev. Biochem. **53:**625–663.
30. **Mohan, R., A. M. Bajar, and P. E. Kolattukudy.** 1993. Induction of a tomato anionic peroxidase gene (*tap1*) by wounding in transgenic tobacco and activation of *tap1*/GUS and *tap2*/GUS chimeric gene fusions in transgenic tobacco by wounding and pathogen attack. Plant Mol. Biol. **21:**341–354.
31. **Reymond, P., G. Deléage, C. Rascle, and M. Fèvre.** 1994. Cloning and sequence analysis of a polygalacturonase-encoding gene from the phytopathogenic fungus *Sclerotinia sclerotiorum*. Gene **146:**233–237.
32. **Rogers, L. M., M. A. Flaishman, and P. E. Kolattukudy.** 1994. Cutinase gene disruption in *Fusarium solani* f. sp. *pisi* decrease its virulence on pea. Plant Cell **6:**935–945.
33. **Scott-Craig, J. S., D. G. Panaccione, F. Cervone, and J. D. Walton.** 1990. Endopolygalacturonase is not required for pathogenicity of *Cochliobolus carbonum* on maize. Plant Cell **2:**1191–1200.
34. **Soliday, C. L., M. B. Dickman, and P. E. Kolattukudy.** 1989. Structure of the cutinase gene and detection of promoter activity in the 5′-flanking region by fungal transformation. J. Bacteriol. **171:**1942–1951.
35. **Templeton, M. D., K. R. Sharrock, J. K. Bowen, R. N. Crowhurst, and E. H. A. Rikkerink.** 1994. The pectin lyase-encoding gene (pnl) family from *Glomerella cingulata*. Gene **142:**141–146.
36. **Turgeon, B. G., R. C. Garber, and O. C. Yoder.** 1987. Development of a fungal transformation system based on selection of sequences with promoter activity. Mol. Cell. Biol. **7:**3297–3305.
37. **von Heijne, G.** 1985. Signal sequences. The limits of variation. J. Mol. Biol. **184:**99–105.
38. **von Heijne, G.** 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14:**4683–4690.
39. **Wattad, C., A. Dinoor, and D. Prusky.** 1994. Purification of pectate lyase produced by *Collectotrichum gloeosporioides* and its inhibition by epicatechin: a possible factor involved in the resistance of unripe avocado fruits to anthracnose. Mol. Plant-Microbe Interact. **7:**293–297.