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An Extracellular Protein from *Phytophthora parasitica* var *nicotianae* Is Associated with Stress Metabolite Accumulation in Tobacco Callus

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ABSTRACT

The most abundant extracellular protein produced by *Phytophthora parasitica* var *nicotianae* at early stages of rapid growth in culture has a molecular weight of 46 kilodaltons and has been designated Ppn 46e. Culture conditions for the production of this protein have been optimized and the protein has been purified by gel filtration and ion-exchange chromatography. Ppn 46e is a soluble, acidic protein (pI 4.67). The amino acids Asx (aspartic acid or asparagine), alanine, glycine, Glx (glutamic acid or glutamine), and serine are the most abundant at 13.4%, 12.3%, 12.1%, 9.3%, and 9.3% of the residues, respectively. The purified protein is, by weight, 1.8% glucose, 1.6% mannose, and 0.5% galactose. A bioassay for Ppn 46e based on tobacco callus has been developed. In this assay as little as 20 nanograms (4.3×10^{-13} mole) Ppn 46e causes the accumulation of the sesquiterpenoid phytoalexin, capsidiol, as estimated by gas chromatography. Levels of capsidiol of 25 micrograms per gram fresh weight were elicited by 80 nanograms Ppn 46e per callus piece. Pretreatment of the protein with either pronase or by boiling resulted in a loss of elicitor activity. Periodate treatment, which inactivates glucan elicitors, did not affect the ability of Ppn 46e to cause capsidiol accumulation. Monospecific antibodies to Ppn 46e were raised in mice. Western blotting experiments employing these antibodies showed that Ppn 46e was present in infected tobacco plants. Dot blotting experiments revealed the presence of the Ppn 46e epitope(s) in *Phytophthora megasperma, P. cactorum, P. cinnamomi,* and *P. infestans* but not in *Fusarium.*

One of the ways in which plants respond to stress is through the production of stress metabolites, some of which are inhibitory to microorganisms and are termed phytoalexins (8). While the identities of many stress metabolites are now known, less is known of the molecules which elicit their accumulation. However, major advances in recent years, most notably in the laboratories of Albersheim and West, have led to the elucidation of the structures of several elicitors of phytoalexin accumulation (*cf.* Ref. [8] for review). For example, the precise structure of a hepta β-glucoside from the mycelial walls of *Phytophthora megasperma* is known (27). As little as 0.5 ng (about 0.4 pmol) of this molecule can elicit significant biosynthesis of the pterocarpan phytoalexin, glyceollin, in soybean cotyledons. Another class of biological molecules known to possess activity as elicitors are some pectic enzymes produced by plant pathogens (4, 9, 30). These enzymes release biologically active pectic fragments from the plant cell wall. In turn, the released fragments elicit the production of phytoalexins. Thus, the elicitation of phytoalexin biosynthesis by high specific activity elicitors can be mediated by oligosaccharides (8).

In the present work, we have studied a phytoalexin elicitor activity associated with an extracellular protein from *Phytophthora parasitica* var *nicotianae* (Ppn). This fungus causes black shank disease in tobacco. Members of the fungal genus *Phytophthora* are responsible for a number of the world's most devastating losses in crops such as potato, soybean, cocoa, tobacco, apple, and *Eucalyptus*. Despite extensive studies, the reasons that *Phytophthora* is such a successful genus are not clear. Whereas with several other genera of fungal plant pathogens, putative pathogenicity factors such as toxins and wilt-inducing substances have been identified, no such substances have been characterized in *Phytophthora*. Some excellent work on phytoalexin accumulation in whole plants infected with *Phytophthora* has been published (*e.g.* 13).

Tobacco is a useful plant in which to study *Phytophthora*-host interaction, and there is a large amount of literature concerning the biology and physiology of this plant. A number of sesquiterpenoid stress metabolites have been characterized in tobacco (6, 7, 15, 33). Nearly isogenic tobacco lines with (line '46-8') or without (line '49-10') dominant monogenic resistance to Ppn race 0 are available, and expression of this resistance has been demonstrated in tissue cultures (16; *cf.* 14 for review). We chose to investigate the extracellular molecules produced by Ppn in culture. A preliminary search for both low and high mol wt materials with phytotoxic activity was unsuccessful, as was a search for factors inhibiting *in vitro* mRNA translation in a wheat germ system (EE Farmer, JS Marshall, JP Helgeson, unpublished data). However, it was noted that a protein-rich fraction from gel filtration sometimes caused blackening when applied to tobacco callus tissue. Although not a highly reproducible phenomenon, further experiments showed that the blackening was associated with the most abundant extracellular protein produced by Ppn. Low levels of this extracellular protein, designated Ppn 46e, invariably caused the accumulation of phytoalexins in tobacco callus. This paper describes the purification and characterization of the protein and its activity in a tobacco callus assay for the accumulation of the sesquiterpenoid phytoalexin, capsidiol.

Having characterized a putative elicitor, it is important to establish that the molecule is present *in planta*. This is the first step in quantifying the relative contribution of various elicitor-active molecules to phytoalexin accumulation and is necessary because a wide array of crude fractions from mycelial walls and culture medium are often active as elicitor preparations (5). To

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this end we have raised antibodies to Ppn 46e and examined this protein's occurrence in infected tobacco plants.

MATERIALS AND METHODS

Dialysis tubing was from Union Carbide[2] and had a cut-off of 16,000 D. It was boiled in 1 mM Na_2 EDTA before use. Protein estimation was by the method of Smith *et al.* (28) with BSA as a standard. Milli-Q (Millipore) water was used in all analytical procedures. Freund's adjuvants were from Sigma.

Culture of *Phytophthora parasitica* var *nicotianae* (Ppn) Race 0. Defined liquid medium used for the culture of Ppn contains (per L): 15 g sucrose, 2 g L-asparagine, 0.2 g $MgSO_4 \cdot 7\ H_2O$, 1 mg $FeSO_4 \cdot 7\ H_2O$, 1 mg thiamin-HCl, 2.6 g $K_2HPO_4 \cdot 3\ H_2O$, 0.8 g KH_2PO_4, 0.2 mg $CuSO_4 \cdot 5\ H_2O$, 0.02 mg $Na_2MoO_4 \cdot 2\ H_2O$, 0.02 mg $MnCl_2$, 10 mg $CaCl_2 \cdot 2\ H_2O$, 20 mg β-sitosterol (added in CH_2Cl_2 prior to autoclaving.) No $CaCO_3$ was added to the medium. After autoclaving the pH of the medium was 7 and it appeared slightly opaque. The fungus was maintained on oatmeal agar containing 78 g oats (Quaker) per L. Single (1 cm^2) portions of 1-week-old mycelial mats were transferred to liquid medium (75 ml) in 250-ml Erlenmeyer flasks and shaken (50 rpm, 2.5 cm orbit) at 23°C. After 5 to 6 d, spent growth medium was drained from the colonies (diameter about 5 cm) the colonies were tipped into 1 L fresh growth medium in a Fernbach flask (three colonies per flask) and shaken as above for a further 5 d at which time the colonies had a diameter of about 7.5 cm. The colorless spent culture fluid was collected by filtering through three layers of cheesecloth. Periodically, the fungus was tested for pathogenicity on susceptible tobacco plants.

Purification of 46 kD Protein. Buffer 1: 50 mM potassium phosphate (pH 7.8) with 0.02% NaN_3 (w/v) and 1 mM Na_2EDTA. Buffer 2: 25 mM potassium phosphate (pH 7.8). All stages of purification were carried out at 4°C. Occurrence of 46 kD protein was monitored by SDS-PAGE on minigels. Growth medium (3 L) was concentrated by freeze-drying and taken up in a minimum volume (about 50 ml) of distilled water. The medium was dialyzed against three changes of buffer 1 and then freeze dried again. The residue was then taken up in 10 ml buffer 1, centrifuged to remove debris, and applied to a 4 × 2 cm column of DEAE-Trisacryl M (LKB) preequilibrated in buffer 1. Flow-rate was 10 ml/h. Unbound fractions were retained and concentrated by vacuum dialysis against three 500 ml changes of buffer 1. The concentrated protein solution (approximately 2 ml) was centrifuged to remove precipitates and applied to a 100 × 1.9 cm column of Ultrogel AcA 34 (LKB) preequilibrated in buffer 1. The column was developed with buffer 1 at a flow-rate of 8 ml/h and 8 ml fractions were collected. Fractions 21 through 24 (Fig. 1), containing the 46 kD protein as detected by SDS-PAGE, were pooled and concentrated by vacuum dialysis against buffer 2. At this stage the concentrate sometimes appeared pale yellow. The dialyzed protein was applied to a 9 × 1.2 cm column of DEAE-Sepharose CL-6B which had been preequilibrated in buffer 2. The column was developed with a 0 to 0.2 M KCl gradient (60 ml) in buffer 2 at a flow-rate of 7 ml/h. Fractions of 4.2 ml were collected (Fig. 2). Fractions containing 46 kD protein were concentrated by vacuum dialysis against 10 mM sodium phosphate (pH 7.2) to about 0.8 mg/ml and stored at −20°C.

Electrophoresis and Isoelectric Focusing. Electrophoresis on 10% polyacrylamide SDS minigels (Hoeffer) was performed according to Laemmli (21). Isoelectric focusing under nondenaturing conditions was carried-out according to Reid and Collmer (26) with ampholines (pH 4–6, 80% and pH 5–7, 20%) from Brinkmann. After focusing, the gels were washed overnight with several changes of 50% (v/v) methanol before silver staining according to Morrissey (24). The following standards (from Sigma) were employed; soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.13), carbonic anhydrase B (pI 5.85), and myoglobin (pI 6.76 and 7.16).

Amino Acid Analysis. The amino acid composition of Ppn 46e was established by the hydrolysis (106°C for 23 h) of either purified protein or stained protein bands in polyacrylamide gels (11). Amino acids were quantitated by post-column derivatization with ninhydrin on a Beckman 118 BL amino acid analyzer.

Carbohydrate Analysis and Periodate Treatment of Ppn 46e. Ppn 46e (between 50–300 μg) was treated with 2 M trifluoroacetic acid at 110°C for 2 h. The resultant solution was dried under N_2 and derivatized according to Blakeney *et al.* (3). Samples were analyzed on a Perkin-Elmer 8500 gas chromatograph with a 30 m DB225 megabore column (J & W Scientific, Folsom, CA) run isocratically at 215°C. The carrier (He) flow rate was 12 ml/min. The internal standard employed was myo-inositol which was included prior to TFA hydrolysis. Data were corrected for flame response but not for losses during hydrolysis which were within experimental error. To establish whether sugars were covalently linked to Ppn 46e the protein (300 μg) was subject to SDS-PAGE in an 8% polyacrylamide gel. The gel was stained with cold 0.25 M KCl and the stained band excised and extracted according to Sreekrishna *et al.* (31). Extracted Ppn 46e was dialyzed against water before TFA hydrolysis. Ppn 46e (5 μg) was incubated at 30°C for 3.5 h in 0.07 M $NaIO_4$ in a total volume of 20 μl. The reaction was terminated by the addition of 2 μl 1 M $Na_2S_2O_3$ and the mixture diluted up to 1 ml. A positive control contained the same mixture except that $Na_2S_2O_3$ was added to the Ppn 46e prior to the addition of $NaIO_4$. A further control containing elicitor active glucan from Ppn was included to ensure that periodate oxidation proceeded to completion. Note that $NaIO_4$ and $NaIO_3$, which are destroyed by $Na_2S_2O_3$, were found to be very toxic to the callus cells.

Bioassay. To further reduce the possibility that trace contaminants which had co-purified with Ppn 46e were introduced into the bioassay, Ppn 46e was dialyzed against the following buffer: 50 mM Na acetate (pH 5.7), containing 1 mM $CaCl_2 \cdot 2\ H_2O$, 1 mM $MnCl_2$, and 1 mM $MgCl_2 \cdot 6\ H_2O$. Dialyzed protein (250 μg) was then passed through a 300 μl column of Con A[3] Sepharose 4B (Sigma). Unbound material was collected and concentrated against 10 mM Na phosphate (pH 7.2). Callus from *Nicotiana tabacum* L., line 46-8, was derived from pith tissue and maintained as previously described (16). Only 5- to 7-week-old, tight, white callus was used. Older, drier callus is generally not responsive to elicitors. Filter sterilized samples to be tested for their ability to cause capsidiol accumulation were applied to the surface of callus tissue in 50 μl of 10 mM Na phosphate buffer (pH 7.2). As an alternative to filter sterilization, carbenicillin (50 μg/ml) can be included in the buffer. The applied drop was kept in place by a sterile tygon ring (i.d. 7 mm) as previously described (16). To investigate the effects of proteolysis on the biological activity of Ppn 46e, a solution of Ppn 46e (2 μg/ml) in 10 mM Na phosphate (pH 7.2) was incubated with pronase (0.008 units/ml) at 30°C for 14 h. The resulting solution was filter sterilized and applied to callus.

Incubation of samples with the callus was at 26 to 28°C in the

[2] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

[3] Abbreviations: Con A, concanavalin A; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; ELISA, enzyme-linked immunosorbent assay; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; NBT, nitroblue tetrazolium; NC, nitrocellulose; PMSF, phenylmethane sulfonyl fluoride; Glx, glutamic acid or glutamine; Asx, aspartic acid or asparagine.

dark at a minimum of 70% RH. After incubation with the sample, the surface of the callus usually appeared somewhat yellow due to the soaking-in of the sample. A piece of this yellow area (approximately 1 cm × 0.6 cm × 2 mm thick) was excised and trimmed to a known weight of about 150 mg. The tissue was then placed in an Eppendorf tube and 300 μl of 40% (v/v) ethanol containing C20 or C12 fatty acid methyl ester (0.2 mg/ml, 10 μl, as an internal standard) was added. The sample was ground with a glass rod and frozen in a dry ice-ethanol bath, then allowed to thaw. The tube was centrifuged (10,000*g* for 5 min) and the pellet reextracted with a further 300 μl of 40% ethanol. The ethanolic supernatants were then combined and the ethanol was removed under reduced pressure. The aqueous sample was then extracted three times each with 200 μl ethyl acetate. The combined ethyl acetate fraction was dried under nitrogen and stored at −20°C. Note that since performing many of the analyses presented herein the extraction procedure has been simplified such that freeze-dried callus is simply extracted with CH_2Cl_2 containing an internal standard, the extract is dried, taken up in ethanol, and injected into the GC. The data in Table III referring to periodate-treated Ppn 46e were obtained by this method.

Gas Chromatography of Capsidiol. Before chromatography, dried samples from the equivalent of 150 mg fresh callus tissue were taken up in 50 μl ethanol. The sides of the Eppendorf tube were carefully rinsed with the ethanol, the ethanol was removed under nitrogen, and the sample again taken up in 20 to 40 μl ethanol. Capsidiol was quantitated on a cross-linked dimethyl silicone capillary column (10 m × 0.25 mm i.d., Hewlett Packard) installed in a Hewlett Packard 5790 A chromatograph. Injection of samples was in the split mode (80:1); carrier (He) flow-rate was 2 ml/min; and the program was 60°C for 0.5 min, 60 to 240°C at 10°C/min, and 240°C for 5 min. Authenticity of capsidiol was confirmed by co-chromatography of the extracted sample with purified capsidiol (provided by A. D. Budde). Derivatization of samples and of authentic capsidiol was carried out with a 1:1 mixture of chloromethyldimethylsilane and bis-chloromethyltetramethyldisilane in ethyl acetate/diethylamine (60:1,6). Recovery of authentic capsidiol from untreated tobacco callus samples was 60 to 70% efficient.

Enzyme Assays. The following activities were assayed: β-fructofuranosidase (invertase), EC 3.2.1.26: Ppn 46e was incubated for 1 h at 30°C with 2 mM sucrose in 50 mM sodium phosphate buffer (pH 7). After incubation, reducing sugars were determined in the Nelson-Somogyi assay (29). β-D-Glucopyranosidase, EC 3.2.1.21: the substrate *p*-nitrophenyl-β-D-glucoside (4 mg/ml) was incubated with Ppn 46e at 30°C for 1 h in 0.1 M sodium acetate buffer (pH 5.5). The reaction was terminated by the addition of an equal volume of 1 M Na_2CO_3 and the absorbance at 400 nm was measured. The following procedures were used to assay pectate transeliminase, EC 4.2.2.2 (1); pectin methyl esterase, EC 3.1.1.11 (12); exopolygalacturonase, EC 4.2.2.9 (23); and triacylglycerol lipase, EC 3.1.1.3 (25). Asparaginase, EC 3.5.1.1, was assayed as follows: Ppn 46e was incubated with 20 mM Asn in 50 mM potassium phosphate buffer (pH 7.5) for 1 h at 25°C. The reaction mixture, along with the standards Asn and Asp, was applied to a cellulose TLC plate and developed in *n*-butanol/acetic acid/water, 4:1:1 and then sprayed with 0.01% ninhydrin in methanol then briefly baked at 60°C. Additionally, to test for the possibility that Ppn 46e caused capsidiol accumulation through a serine protease/esterase activity, the protein (100 ng) was incubated in the tobacco callus bioassay for 20 h in the presence of 1 mM PMSF.

Preparation of Tobacco Cell Walls. The midrib and lateral veins were removed from 250 g of large, healthy leaves from *N. tabacum* line 46-8. The leaf material was cut into 1 cm^2 pieces, suspended in 25 mM Na phosphate buffer (pH 7.5), and homogenized (polytron) at or below 5°C. The homogenized lamina was passed through a 1 mm^2 metal sieve. The material that did not pass through the sieve was further homogenized in a Corning ground-glass homogenizer then passed through the 1 mm^2 sieve. All material that had passed through the sieve was washed by centrifuging (6000*g* for 30 min) in 500 ml of 25 mM Na phosphate (pH 7.5). The supernatant was discarded and the pellet (cell wall) resuspended in buffer, washed two times in buffer, and then twice in distilled water (500 ml). The pellet was stored overnight at 4°C in ethanol and then boiled in 1 L ethanol for 1 h. The ethanol was removed by filtration and the cell walls were again boiled in a further 1 L ethanol for 1 h. After boiling, the ethanol was again removed by filtration, the walls were washed with cold ethanol (0.5 L), methanol/$CHCl_3$ (1:1, 1 L), and finally with ether (0.4 L). The wall powder was air dried for 2 d at roomtemperature then dried for a further 1 h at 40°C. The cell wall powder appeared off-white and was stored desiccated at room temperature. Overall yield in weight was 3.4% of the starting material.

Incubation of Ppn 46e with Tobacco Cell Wall. Cell wall samples (1 mg in Eppendorf tubes) were hydrated in buffer A (25 mM Na acetate buffer [pH 5.5] containing 1 mM $MgCl_2 \cdot 6H_2O$, 2 mM $CaCl_2 \cdot 2H_2O$, 50 μg/ml carbenicillin, and 1% Tween 20). The hydrated wall material was then centrifuged at 10,000*g* for 10 min and the supernatant discarded. The precipitate was resuspended in 1 ml of buffer B (identical to buffer A except that it did not contain Tween 20). The resuspended cell wall preparation was washed twice each with 1 ml of buffer B and centrifuged (10,000*g*, 10 min) to remove washing buffer. Ppn 46e protein (0.8 μg) in 400 μl of buffer B was incubated with the cell walls for 20 h at 30°C. Controls without Ppn 46e were also included. After incubation, the wall material was precipitated by centrifugation at 10,000*g* for 15 min and the supernatant was carefully filtered through glass wool to remove particulate matter. The filtrate was then stored frozen at −20°C. The filtrate was tested for hexose sugars by the anthrone assay (10) with glucose as a standard; the absorbance of the supernatants at 235 and 280 nm was also measured. In a similar experiment the filtrate was tested for reducing sugars (22).

Antisera. Four 7-week-old Balb/c mice were injected with purified Ppn 46e protein in the following regime: d 1, 120 μg protein in FCA, intraperitoneal; d 31, 100 μg protein in FIA, intraperitoneal; d 56, 60 μg protein in FIA, intraperitoneal; d 74, 3 μg protein in FIA, intramuscular booster; d 76, tails bled for ELISA; d 81, 4 μg protein in FIA, intramuscular booster; d 83, sacrifice mice to collect serum. Mice were bled into an equal volume of 1% (w/v) heparin.

Serum from an uninjected mouse of the same age, which had been housed with the other mice, was used as a control in ELISA. Western blotting ELISA according to a procedure published by Boehringer Mannheim Biochemicals was employed to establish the titre of anti-Ppn 46e antibody in the mouse serum after an initial boost. Detection of mouse antibody was with goat anti-mouse anti IgG linked to alkaline phosphatase (Sigma) and the fluorogenic substrate 4-methylumbelliferone (Sigma).

Inoculation of Tobacco Plants with Ppn and Extraction of Protein from the Plant and from Fungi. Ten-week-old plants of *Nicotiana tabacum* L. line 49-10 (susceptible to Ppn race 0) were inoculated with Ppn, race 0, by placing a small piece of mycelium in a 0.5 cm vertical slit in the stem approximately 5 cm above the crown. As a control, other plants were slit but not inoculated with the fungus. After 6 d, a black necrotic region, about 4 cm long, on the inoculated plants was clearly visible. In some cases, where the point of inoculation was near a leaf, the lesion spread into the midrib. A transverse section of stem approximately 3 mm thick was cut form the leading edge of the lesion. Other similar sections of stem were taken from a similar region of the

uninoculated plants (which did not show a necrotic lesion). The buffer used for extraction of tobacco tissues and fungal mycelium was 50 mM Tris-HCl (pH 7.6) containing 10% (v/v) glycerol, 5 mM DTT, 1 mM Na_2EDTA, and 0.02% (w/v) NaN_3. All operations were performed on ice or at 4°C. Weighed plant tissue was dropped into liquid N_2 and ground with sand for 5 min in a mortar with a pestle. The powder was then stirred for 15 min in extraction buffer, centrifuged (10,000*g* for 10 min), and 50 mg preequilibrated Dowex 1 X2 (Sigma) per ml of extract was immediately added. The mixture was stirred for 10 min before centrifugation (as above). The Dowex step was repeated since infected tissues contain considerable quantities of phenolics. Protein from the fungus was extracted by grinding known weights of water-washed mycelium in a mortar with sand at liquid N_2 temperature. The ground mycelia were then stirred with extraction buffer for 20 min at 4°C before removing debris by centrifugation (10,000*g* for 15 min).

Western Blotting and Dot Blotting. The method employed was similar to that of Towbin *et al.* (32). Electrophoresis was carried out according to Laemmli (21). After electrophoresis, gels were washed first with Milli-Q water then with transblot buffer before electroblotting onto nitrocellulose (Schleicher and Schuell) at 0.2 A for 90 min. After electroblotting, the NC was blocked overnight at 4°C in blocking buffer (17). Primary antibody, *i.e.* serum from mouse 2, was diluted 1- to 1000-fold in blocking buffer without NP-40 and incubated with the NC for 90 min at room temperature. The NC was then washed four times over 15 min in blocking buffer before a 90 min incubation with the secondary antibody, alkaline phosphatase conjugated-goat anti-mouse anti-IgG (Sigma) diluted 1 to 1000 in blocking buffer without NP-40. After this incubation, the NC was washed two times in blocking buffer for 10 min and then two times in blocking buffer containing 0.05% (w/v) SDS for 10 min. The blots were developed by the method of Knecht *et al.* (20) in a solution of NBT and the alkaline phosphatase substrate BCIP. Dot blots of mycelial protein from various fungi were developed in the same way as Western blots. The source of fungi used were: *Pythium ultimum*, NY 225; *Phytophthora cinnamomi*, Pc 40; *P. infestans* race 0, Cornell 128; *P. megasperma* f. sp. *glycinea* race 1, NY 160; *P. cactorum*, NY 188.

RESULTS

Growth of Ppn. A chemically defined growth medium was developed that allows rapid, uniform growth of Ppn. Shaking the liquid cultures at 50 rpm resulted in tight, round colonies and a rapid growth rate (Fig. 1, inset). Efficient production of Ppn 46e, as judged by gel electrophoresis, was achieved. Attempts were made to alter the quantity of Ppn 46e produced by the fungus. Extracts of tobacco plants or addition of tobacco cell wall did not affect Ppn 46e production nor did the substitution of mannitol for sucrose as the major carbon source (data not given). The time at which the growth medium of Ppn was harvested for Ppn 46e purification was critical. Once the organism entered the rapid growth phase, extracellular polysaccharide quickly accumulated as did new proteins and degraded forms of other proteins including Ppn 46e. The inset to Figure 1 shows the optimal time for harvesting Ppn 46e from the growth medium of Ppn race 0. At the time of harvest, the growth medium, after exhaustive dialysis, typically contained about 150 μg/ml glucose equivalents (anthrone assay). Protein levels were difficult to estimate, probably due to interfering carbohydrates; before TCA precipitation the medium contained about 550 μg/ml protein. Precipitation of protein with 8% TCA prior to assay yielded a value of 80 to 100 μg/ml protein in the medium.

Purification of Ppn 46e. By examining protein profiles from gel electrophoresis or gel filtration, it was apparent that Ppn 46e was approximately 20% of extracellular protein at the stage when

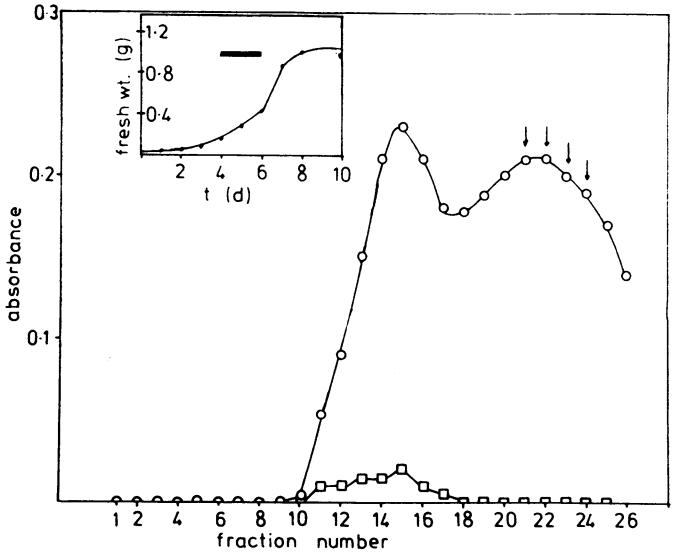

FIG. 1. Chromatography of extracellular protein from Ppn race 0 on an Ultrogel AcA 34 column (100 × 1.9 cm) at a flow rate of 8 ml/h. Fractions of 8 ml were collected; (○), *A* at 280 nm; (□), *A* at 490 nm. Arrow shows the fractions rich in Ppn 46e that were pooled for subsequent purification. Inset, growth, as fresh weight, of Ppn (●) in chemically defined medium; 250-ml Erlenmeyer flasks containing 75 ml medium were inoculated with 1 cm mycelial mat from 1-week-old cultures on oatmeal agar. Duplicate flasks were taken for each time point and the results given are averages. The solid bar represents the optimum time for the harvest of medium for Ppn 46e purification as determined by gel electrophoresis.

the medium was harvested for purification. Since no enzyme activity for Ppn 46e was known, purification was monitored on minigels. Initial purification on DEAE-Trisacryl M was useful because it removed most of the extracellular polysaccharide, much of which appeared to be acidic. Chromatography on AcA 34 (Fig. 1) removed much high mol wt material from Ppn 46e. Fractions from the AcA 34 column were analyzed on SDS-PAGE minigels and only those fractions containing the lowest levels of contaminants were retained (four fractions in the case of Fig. 1). An alternative gel filtration medium which yields better resolution of Ppn is Biogel P-150 (BioRad). However, the faster flow characteristics of Ultrogel AcA 34 were better suited to the current purification scheme. A further purification step on DEAE-Sepharose CL-6B (Fig. 2) was necessary to remove glycoprotein and some co-purifying elicitor-active glucan. It was important to equilibrate the column carefully at this step as Ppn 46e was only weakly bound. Generally, only the leading half of the eluting peak containing Ppn 46e was retained, since the trailing half of the peak sometimes contained a contaminant of 74 to 80 kD. The purification scheme is summarized in Table I. Large losses of Ppn 46e occur during purification, particularly during chromatography on DEAE-Sepharose. A reason for these losses is discussed below. Glycosyl analyses of Ppn 46e showed that a small amount of glucose (1.0–1.5%, by weight, of the protein) copurified with the protein but was not covalently linked.

Criteria of Purity. The following factors were taken as criteria of purity: only one band visible on gels stained with Coomassie blue and one band on moderately loaded, silver-stained gels (some minor bands were sometimes detectable with silver stain), and a single band in isoelectric focusing.

Chemical Properties of Ppn 46e. Ppn 46e was TCA precipitable and was stained by both Coomassie blue and silver stain (Fig. 3). Ppn 46e had a discrete mol wt of 46 kD with an absorption coefficient, $A^{1\%}_{1cm}$ of 14.7 at 278 nm. The purified molecule had

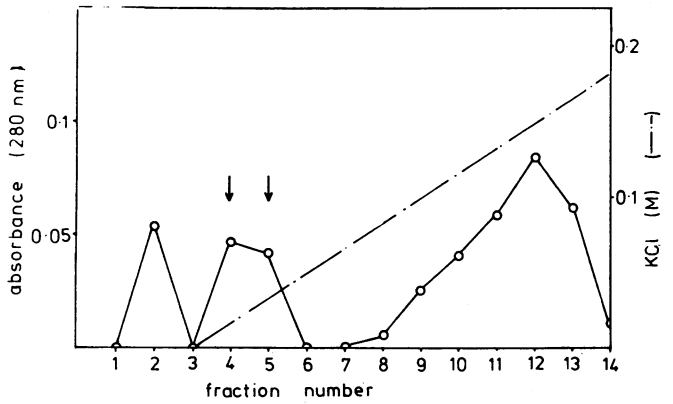

FIG. 2. Chromatography of pooled fractions from an Ultrogel Aca 34 column on a DEAE-Sepharose CL-6B column measuring 9 × 1.2 cm. The column was developed with a 0 to 0.2 M KCl gradient (60 ml) at a flow rate of 7 ml/h. Arrows indicate the fractions (4.2 ml) that were retained.

Table I. *Purification of Ppn 46e Protein from 3 L Growth Medium*

Stage	Volume	Total Protein
	ml	*mg*
Dialyzed and concentrated medium	7	178[a]
DEAE-Trisacryl M and concentrated	1.4	35
AcA 34 and concentrated	5	6.5
DEAE-Sepharose CL-6B and concentrated	1.4	0.49

[a] Protein estimation after TCA precipitation. All other samples were not precipitated before protein estimation.

a single pI of 4.67 under nondenaturing conditions (Fig. 4). and approximately 5.1 under denaturing conditions (data not shown). Ppn 46e consisted of amino acids and was protease-sensitive. Thus, it is concluded that Ppn 46e is a protein. Of the 16 amino acids quantitated (Table II) the most abundant were Asx, Ala, Gly, Glx, and Ser. The protein also contained Met but Cys was not detected. In the ninhydrin detection of amino acids, no unaccountable peaks were observed, suggesting that all amino acids were accounted for. The composition is unexceptional apart from a fairly high level of acidic residues. Glycosyl analysis of Ppn 46e extracted from an SDS polyacrylamide gel showed that the molecule contained 1.8% glucose, 1.6% mannose, and 0.5% galactose as determined by quantitative alditol acetate analysis.

Biological Activity of Ppn 46e. As little as 20 ng (4.3×10^{-13} mol) of Ppn 46e protein caused a significant accumulation of capsidiol in tobacco callus (Fig. 5). Capsidiol levels of between 15 and 20 μg/g fresh weight callus accumulated in response to 80 ng Ppn 46e. Figure 6 shows the time course of capsidiol accumulation in response to 80 ng Ppn 46e protein. Capsidiol was detectable 12 h after application of Ppn 46e. A similar quantity of boiled Ppn 46e elicited the accumulation of very little capsidiol (Fig. 6). Table III shows the effect of various treatments of Ppn 46e on the accumulation of capsidiol in tobacco callus. Pronase-treated or boiled Ppn 46e were poor elicitors of capsidiol accumulation, as was BSA in levels 1000 times higher than the effective concentration of Ppn 46e. However, periodate treatment did not destroy the elicitor activity of Ppn 46e. Ppn 46e was not associated with any of a number of hydrolytic activities that one might expect a phytopathogenic fungus to produce. Activities tested included glycosidases, lipase, and pectinolytic activities. When purified Ppn 46e was incubated with tobacco cell wall at pH 5.5 in the presence of divalent

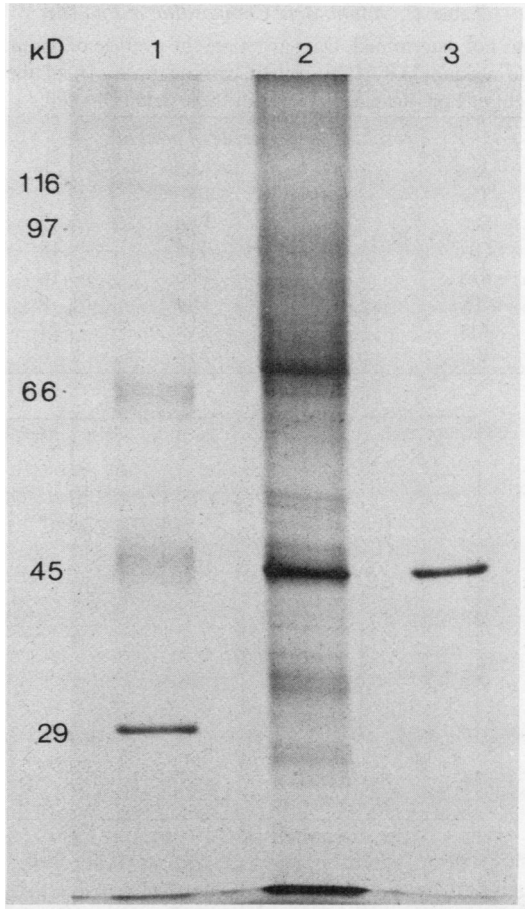

FIG. 3. Silver-stained SDS-PAGE (10% polyacrylamide) of protein from Ppn race 0. Lane 1, mol wt markers, numbers are mol wt in kD. Lane 2, total, dialyzed, extracellular protein (about 5 μg) from Ppn harvested after 5 d growth in liquid medium. Lane 3, Ppn 46e (0.55 μg) after purification.

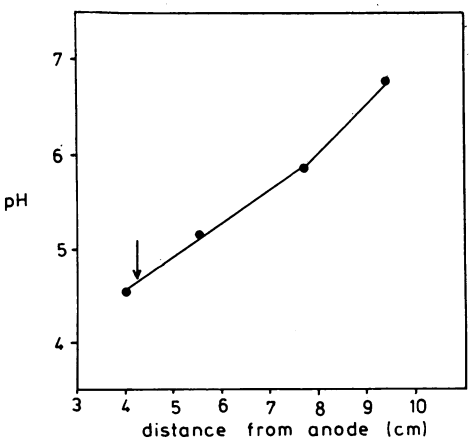

FIG. 4. Isoelectric focusing of Ppn 46e under nondenaturing conditions. Arrow shows the position of Ppn 46e relative to marker proteins.

cations, no significant release of reducing sugars and hexoses was noted. Furthermore, no increase in the absorbance of the cell-wall incubation buffer at 235 and 280 nm (the absorbance of unsaturated products of pectin/pectate transelimination and of aromatic groups, respectively) was noted. PMSF had no significant effect on capsidiol accumulation in tobacco callus treated with Ppn 46e (Table III).

Detection of Ppn 46e in Plant Tissue. Antibodies to purified

Table II. *Amino Acid Composition of Ppn 46e*

Trp was not determined. Data given are the average of 6 analyses and takes into account 3.89% by weight carbohydrate. M_r of the protein component of Ppn 46e calculated from these data is 44,199.

	residues per molecule of protein		
Asx	57	Met	8
Thr	29	Ile	7
Ser	39	Leu	27
Glx	39	Tyr	18
Pro	16	Phe	16
Gly	51	His	8
Ala	52	Lys	27
Val	22	Arg	6

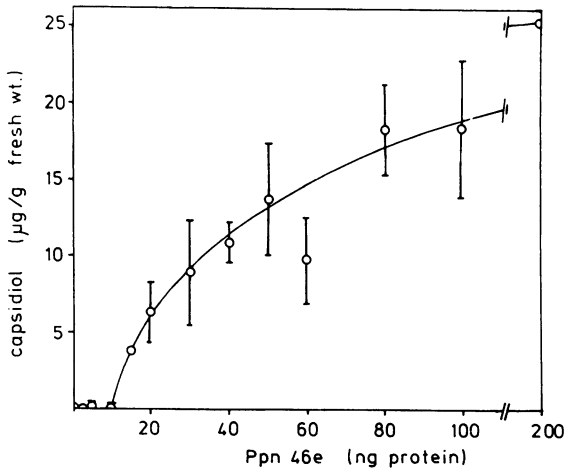

FIG. 5. Capsidiol accumulation plotted against the amount of Ppn 46e added to tobacco callus. Callus was incubated with the protein for 16 h. Data points are the average of four callus pieces (±SE); those points without error bars are the average of duplicate callus pieces.

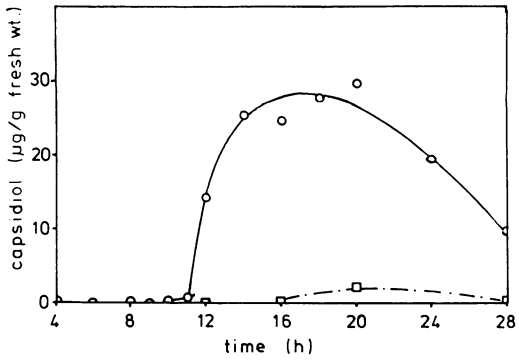

FIG. 6. Capsidiol accumulation in tobacco callus treated with 80 ng Ppn 46e protein (O), as compared to Ppn 46e protein which had been boiled for 4 min (□). Data points are averages of two callus pieces. In this experiment, 50 µg/ml carbenicillin was included in the bioassay buffer.

Ppn 46e protein were raised in mice. In our ELISA, three mice tested gave titers of 8000, 4000, and 4000, respectively. The uninjected 'control' mouse serum contained no Ppn 46e antibody. Western blotting of dialyzed extracellular macromolecules from Ppn race 0 grown for 5 d *in vitro* showed that the antisera of all three mice were monospecific for Ppn 46e (not shown). Western blotting of intracellular Ppn proteins revealed three bands (Fig. 7, lane 1); one of these migrated identically to purified Ppn 46e. The intracellular Ppn epitopes that cross-reacted with the antiserum were present in fairly low quantity; protein from

Table III. *Capsidiol Accumulation in Tobacco Callus in Response to Various Treatments*

The callus was extracted 16 h after treatment, data are the average of two or more callus pieces. Details of various treatments are given in the text.

Treatment	Capsidiol Relative to Unmodified Ppn 46e
	%
Ppn 46e (80 ng)	100.0
Ppn 46e (80 ng) boiled 4 min	2.4
BSA (25 µg)	0.0
BSA (50 µg)	8.9
Pronase treated Ppn 46e (100 ng)	0.4
Ppn 46e (100 ng) and PMSF (1 mM)	84.0
Periodate treated Ppn 46e (250 ng)[a]	125.5

[a] Subsequent boiling destroyed elicitor activity.

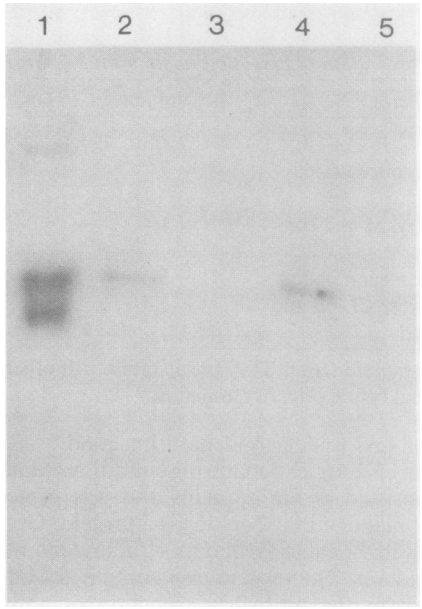

FIG. 7. Western blots developed with antibody to Ppn 46e. Lane 1, intracellular protein from Ppn race 0. Lane 2, extract of a tobacco stem from a plant infected with Ppn race 0. Lane 3, extract of a tobacco stem from a plant that had not been infected with the fungus. Lane 4, extract from a leaf midrib from another tobacco plant infected with Ppn race 0. Lane 5, extract of a leaf midrib from an uninfected plant.

the equivalent of 25 mg mycelium was necessary to reach the detection limit of the assay.

The presence of Ppn 46e in tobacco stems and leaf midribs infected with Ppn race 0 was also demonstrated by Western blotting. Only one band was visible on such blots (Fig. 7, lanes 2 and 4), and this migrated identically to purified Ppn 46e, suggesting that the protein is not substantially modified *in planta*. The fact that only one band was observed suggests that the blots detected only the extracellular Ppn 46e protein, since three antigenic bands were detected in ground mycelial extracts. No Ppn 46e protein was detectable in control tobacco plants (Fig. 7, lanes 3 and 5). Ppn 46e was detectable within and at the leading edge of the necrotic stem lesions (where the tobacco tissues showed no macroscopic evidence of damage). However, Ppn 46e was not detectable further than 6 mm away from the leading edge of the lesion.

Cross-Reaction of Ppn 46e Antiserum with Other Fungi. Figure 8 shows the result of a dot blotting experiment designed to

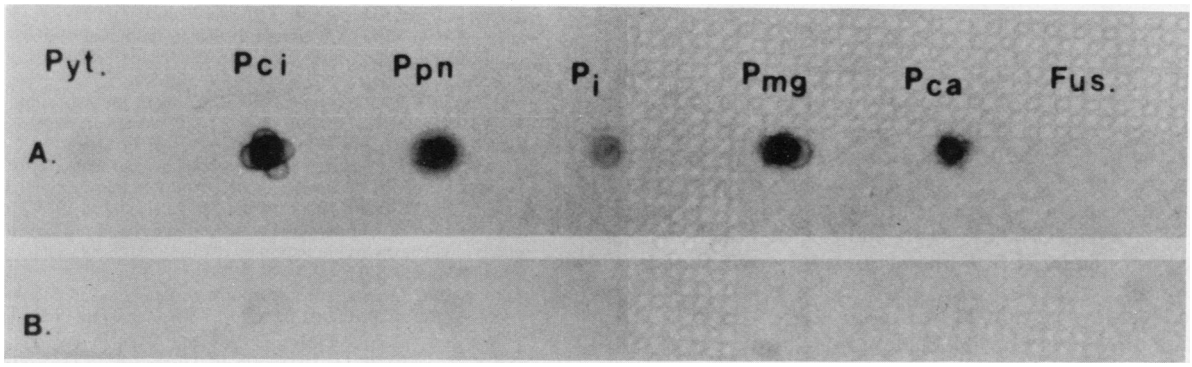

FIG. 8. Cross-reaction of 0.88 μg mycelial protein from various fungi with Ppn 46e antiserum (panel A) or with preimmune serum (panel B). Abbreviations: Pyt, *Pythium ultimum*; Pci, *Phytophthora cinnamomi*; Ppn, *P. parasitica* var *nicotianae* race 0; Pi, *P. infestans* race 0; Pmg, *P. megasperma* f. sp. *glycinea* race 1; Pca, *P. cactorum*; Fus, *Fusarium oxysporum* f. sp. *pisi* race 1.

detect Ppn 46e epitope. All the *Phytophthora* species tested gave a positive reaction, although *P. infestans* gave a noticeably weaker cross-reaction than did the other species. *Fusarium* extracts did not cross-react with the antiserum; a weak cross-reaction with *Pythium* extracts was only evident when very high levels of mycelial protein from this fungus were probed (data not shown). Race 1 of Ppn also produced material that cross-reacted with the antiserum (data not shown).

DISCUSSION

Purification of Ppn 46e. Despite the relative abundance of Ppn 46e, the protein was extremely difficult to purify, since in many purification schemes this protein bound irreversibly to certain chromatographic media. This problem was greatest when Ppn 46e was chromatographed on Sephacryl S-200 after DEAE-cellulose chromatography. We thus recommend that the purification scheme presented herein is followed. The reason for losses of Ppn 46e during purification may be due, in part, to its low pI (and thus strong association with anion exchange resins at high pH). It is essential that Ppn 46e be bound to a stationary phase (in this case, DEAE-Sepharose CL-6B) at least once during the purification. This is necessary to remove a glucan fraction of high mol wt.

Bioassay for Elicitor Activity. The bioassay utilizes tobacco callus and is a modification of a previously described system (16). Samples to be applied to the callus can first be filter-sterilized. However, filter sterilization of samples for the bioassay is not necessary if carbenicillin (50 μg/ml) is included in the buffer. Ppn 46e is a highly active elicitor of capsidiol accumulation in tobacco callus. However, because of differences in the various bioassays used, it is difficult to compare precisely the biological activity of Ppn 46e with other characterized elicitors of fungal origin. The *Rhizopus* endopolygalacturonase elicitor (4, 30) at levels of 0.15 μg/ml dramatically increases the activity of the enzyme involved in phytoalexin biosynthesis in split castor bean seedlings. A concentration of Ppn 46e of 0.4 μg/ml (in 50 μl) is biologically active in our assay. However, since applied drops tend to seep into the callus tissue during the assay, we express the quantity of Ppn 46e applied to callus pieces in terms of weight of protein. In molar terms, Ppn 46e is extremely active; 0.43 pmol of protein (20 ng) causes the accumulation of capsidiol in tobacco callus. This is comparable to the (molar) activity on soybean cotyledons of the hepta β-glucoside purified by Sharp *et al.* (27) (*cf.* Ref. 8 for review). The time-course of capsidiol accumulation in response to Ppn 46e (Fig. 6) is similar to that published by Ayers *et al.* (2) for the accumulation of pterocarpan phytoalexins in soybean hypocotyls treated with crude glucan elicitors. In the tobacco callus/Ppn 46e system, the accumulation of capsidiol is very abrupt (Fig. 6) suggesting that the increase in levels of this phytoalexin is tightly coordinated. The data in Figure 6 also suggest that capsidiol levels subside after about 24 h. However, in a similar experiment no such decline was observed and its previous appearance can probably be attributed to variability. The rapid accumulation of capsidiol in response to Ppn 46e might make the system an attractive experimental model for the study of gene expression during the stress response. Capsidiol levels resulting from Ppn 46e treatment of tobacco callus can exceed 25 μg/g fresh weight; a capsidiol concentration of 15 μg/ml has been shown to inhibit growth of Ppn *in vitro* (7).

Mechanism of Action of Ppn 46e. A major concern in interpreting the results from tobacco callus bioassays is that contaminating fungal glucans (which are active elicitors in *Nicotiana*) might be responsible for stress metabolite accumulation. The following evidence argues strongly against this: Ppn 46e preparations lose their ability to elicit capsidiol when boiled or treated with pronase but are unaffected by periodate treatment (Table III). We thus conclude that the elicitor activity of Ppn 46e is dependent on the structure of the protein. The protein could either be an enzyme which releases an active elicitor from the plant cell walls or might act by some other mechanism. In contrast to the high specific activity proteinaceous elicitors which have been previously characterized and found to be pectinolytic enzymes (4, 9, 30), Ppn 46e has not been associated with an enzyme activity. The failure to find an enzyme activity in Ppn 46e is interesting but much more work needs to be done on this aspect. Some other glycoprotein preparations from *Phytophthora* have been found to have either stimulatory (18) or inhibitory (34) effects on phytoalexin accumulation. In these cases it was shown that the carbohydrate portion of the glycoproteins was responsible for their biological activity.

When *Phytophthora* is grown in liquid medium, the quantity and type of extracellular macromolecules produced vary dramatically and continually throughout the culture time. In the case of Ppn, the first detectable extracellular macromolecules appear to be proteins; in later stages of growth, large quantities of macromolecular carbohydrate are produced (EE Farmer, unpublished data). This is one reason that makes it difficult to accurately estimate the specific activity of a given elicitor relative to the total extracellular milieu. Also, as in all other systems observed, no one molecule appears responsible for the elicitation of stress-metabolite accumulation. In our bioassay, the minimum amount of protein necessary to elicit capsidiol accumulation to at least 10 μg/g fresh weight in tobacco callus was about 20 ng Ppn 46e and about 10 ng protein (estimated after TCA precipitation) from crude growth medium. This latter figure is likely to be an underestimate of protein in the sample and does not take into account carbohydrate in the medium. Finally, except in the case of Ppn 46e, we do not know if all the extracellular macro-

molecules produced by Ppn *in vitro* are also produced *in planta*. Preliminary tests of the elicitor activity of Ppn 46e *in planta* have been carried out by injecting the protein (15 μl, 6 μg/ml) into the laminae of both susceptible (49-10) and resistant (46-8) lines of tobacco and extracting the leaves 7 h after injection. Low levels of capsidiol (1.5–3.7 μg/g fresh weight leaf with no correction for extraction efficiency) were measured in both tobacco lines, whereas control injection (buffer alone) did not lead to capsidiol accumulation. These results indicate that the protein is not a specificity factor in the interaction of Ppn with tobacco. Ppn 46e causes capsidiol production in callus derived from 49-10 tobacco plants (data not shown).

Detection of Ppn 46e in Planta. Ppn 46e can be detected in infected tobacco plants and thus is not an artifact of culture *in vitro*. An approximate minimum estimate of the Ppn 46e protein present *in planta* is 59 to 590 ng protein per g fresh weight. This is based on the observed detection limit of the Western blots. (This limit is about 1–10 ng Ppn 46e but is somewhat variable due to factors such as variations in electroblotting conditions between runs.) The minimum amount of tissue from which the protein can be detected was 17 mg fresh weight. Since this represents a minimum estimate and the protein is quite likely to be localized in the tobacco tissue, the localized concentration *in planta* may be considerably higher. An advantage of working with elicitor-active proteins is that they can be readily detected in plant tissue. Free fungal glucans are rapidly metabolized *in planta* (19).

The above results do not confirm a role for Ppn 46e in the black shank disease of tobacco but indicate that the protein may be a factor involved in the elicitation of stress responses *in planta*. The characterization of the protein and the demonstration of its existence in infected tobacco plants should allow further studies of the biochemistry of *Phytophthora* pathogenesis and of the mechanisms by which molecules of fungal origin trigger the stress responses of plants. Additionally, the presence of the epitope in other species of *Phytophthora* raises the possibility that the protein may be generally important to this genus.

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Note added in proof. We have recently found β (1-4) endoxylanase activity in Ppn 46e preparations but do not know whether or not elicitor activity and xylanase activity reside in the same protein. Y. Fuchs, A. Saxena, R. Gamble and J. D. Anderson (Co-purification of an ethylene inducing protein and a xylanase from cellulysin 1987 Plant Physiol 83: s141) have reported that a protein that stimulates ethylene production in tobacco cells co-purifies with endoxylanase activity.

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