Purification, Elicitor Activity, and Cell Wall Localization of a Glycoprotein from *Phytophthora parasitica* var. *nicotianae*, a Fungal Pathogen of Tobacco

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ABSTRACT

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A glycoprotein of 34 kDa (GP 34) was solubilized at acidic pH from the mycelium of *Phytophthora parasitica* var. *nicotianae* and was purified by ion exchange and gel permeation chromatography. Whole tobacco plants treated with GP 34 through their roots showed an enhanced lipoxygenase activity as well as hydroxyproline-rich glycoprotein accumulation, indicating that this molecule had elicitor properties. An antiserum raised against the pure glycoprotein allowed localization of GP 34 by immunogold-labeling on the cell surface of the mycelium when the fungus was grown in vitro. In the wall-less zoospores, GP 34 was limited to the flagellum surface. It was then abundantly synthesized at the onset of encystment. During infection of tobacco plants, labeling was very faint at early stages of colonization, particularly in the susceptible host cultivar. It appeared earlier in the resistant host cultivar and was restricted to the living fungus, declining with mycelium cell death.

Additional keywords: host-pathogen interactions.

At early stages of the interaction between plants and fungal pathogens, the most critical events underlying attachment, recognition, and pathogenicity require cell surface molecules. Some of them have the ability to elicit defense responses in plants. Fungal elicitors may originate from the cell wall or be secreted into the extracellular space of the mycelium. Cell wall elicitors are best illustrated by the hepta-beta glucan fragment from *Phytophthora sojae* that was released from not yet identified larger polymers by mild acid hydrolysis of the cell wall (2) or by enzymic degradation (14). Secreted molecules have also been purified from a few fungi. Among them, the peptide elicitors of *Cladosporium fulvum* and *Rynchosporium secalis* are particularly interesting, because they have been shown to be the products of avirulence genes (13,18,34); knowledge of these peptides and of the corresponding genes, notably the *avr4* and *avr9* genes of *C. fulvum*, has greatly contributed to the understanding of race-cultivar specificity at the molecular level (1). Other elicitors, active on nonhost plants, have been isolated from various *Phytophthora* species. The elicitins that pertain to this class of elicitors are a family of low molecular weight peptides that have the interesting property of inducing necrosis and defense in tobacco, while being produced only by *Phytophthora* species that are not pathogenic on tobacco (26). Two glycoproteins of 42 and 32 kDa, active on parsley and tobacco, respectively, have been isolated from *P. sojae*, the pathogen of soybean (3,25). These molecules induce necrosis and pathogenesis-related proteins in these nonhost plants.

Although tobacco has often been used as the recipient plant in elicitor bioassays, only a few studies have employed elicitors derived from tobacco pathogens. As part of our studies on the tobacco-*P. parasitica* var. *nicotianae* system, we are interested in the molecules underlying cell surface interactions between the two partners. The pathogenic strains of this tobacco pathogen do not secrete elicitins. Farmer and Helgeson (10) showed that the culture filtrate of the fungus contains a glycoprotein of 46 kDa that elicits the synthesis of phytoalexins in tobacco callus. Cell wall-derived fragments with elicitor properties have been prepared and partly characterized in our laboratory (28). Analysis of their chemical composition suggests that they derive from cell wall glycoproteins. Very few proteins have been isolated from the cell wall of fungal pathogens. The Oomycetes, notably *Phytophthora*, possess a cell wall whose composition is chemically related to that of some algae and of higher plants (4). Thus, the techniques employed for isolation of plant cell wall glycoproteins were used in this work. In this paper, we describe the isolation, partial characterization, and immunocytolocalization of a glycoprotein solubilized from *P. parasitica* var. *nicotianae* race 0. The presence of this molecule was investigated during in vitro culture of the fungus and during its parasitic stage in planta after inoculation of susceptible or resistant isolines of the host plant.

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MATERIALS AND METHODS

Biological material. *P. parasitica* Dastur var. *nicotianae* (Breda de Haan) Tucker race 0 was maintained on oatmeal agar at 25°C in the dark. For liquid culture of the fungus, the mycelium was grown in the dark at 25°C for 14 days on the synthetic medium as described (28). The mycelium was harvested by gentle filtration, washed, and frozen at –20°C. Zoospores were initiated from a 7-day-old culture of mycelium on oatmeal agar by starvation and cold shock according to Gooding and Lucas (12).

Tobacco plants (*Nicotiana tabacum* L.) of near-isogenic lines 46-8 and 49-10 were grown on vermiculite in a growth chamber with a photoperiod of 12 h of light at 140 μE m^{-2} s^{-1} and 25°C, and 12 h of dark at 18°C.

To follow fungal colonization of the host plant during infection, the inoculation protocol described by Fournier et al. (11) was adapted to young seedlings. For this purpose, tobacco seeds were sown in petri dishes on Miracloth discs (Calbiochem-Behring, La Jolla, CA) lying on glass beads and fed with a nutrient solution. They were germinated in a growth chamber under the above conditions of light and temperature. Three weeks after germination, the seedlings were removed from the petri dishes and subjected to root inoculation by dipping their root system in 1.5 ml of a suspension of 10^6 zoospores per milliliter in small test tubes. They were left in the growth chamber under the same conditions as above and, after complete absorption of the solution, incubation continued in nutrient medium.

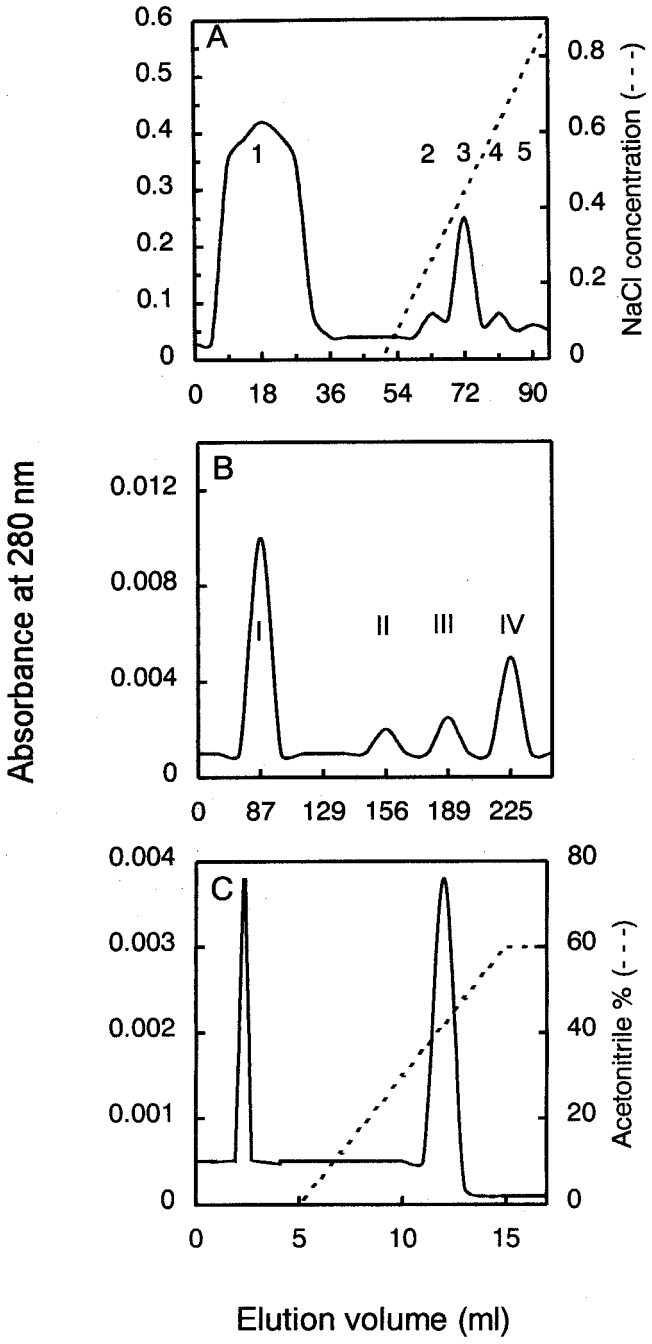

Fig. 1. Chromatographic purification of GP 34. Absorbance of the material was monitored at 280 nm. **A,** Chromatography on CM-Sepharose (Pharmacia) at acidic pH of the crude extract prepared from *Phytophthora parasitica* var. *nicotianae* race 0. Elution was performed with a NaCl gradient. **B,** Gel permeation chromatography on Biogel P30 of CM-Sepharose fraction 3. **C,** High-pressure liquid chromatography on a Nucleosil 300 Å-C4 column of the fractions corresponding to peak I of Figure 2B. The first peak corresponds to salt elution.

Protein solubilization and purification. Extraction and purification of proteins were adapted from a protocol described by Mazau et al. (22). The frozen mycelium (100 g) was ground in liquid nitrogen with a Dangoumau grinder (Seli, Toulouse, France). The obtained powder was suspended in 150 ml of a mixture of ethanol-1.25 N HCl (3:1, vol/vol) containing 4 mM sodium metabisulfite, and the slurry was kept under continuous stirring at 4°C for 48 h. The slurry was then cleared by centrifugation at 2,000 × *g*. Carbohydrates and proteins of the supernatant were precipitated with three volumes of cold acetone for 24 h. After centrifugation at 3,700 × *g* for 10 min, the pellet was resuspended in 50 ml of 100 mM sodium acetate (pH 3.8), and the resulting extract was dialyzed for 24 h against cold distilled water (Visking, dialysis bag, molecular weight cut-off of 8,000; Polylabo, Paris) and lyophilized. The obtained powder was solubilized in 65 ml of 50 mM sodium acetate, pH 3.8. The proteins contained in this crude extract were purified by anion exchange chromatography on a column (1 × 10 cm) of carboxymethyl-Sepharose (CM-Sepharose; Pharmacia, Orsay, France) equilibrated in the same buffer. Elution was achieved with 100 ml of a linear gradient of 0 to 1 M NaCl. The column eluate was monitored at 280 nm and recovered in fractions of 1.8 ml. Fractions corresponding to the third absorption peak were pooled, loaded on a gel filtration column (1 × 70 cm, Biogel P30; Bio-Rad Laboratories, Paris), and equilibrated with 50 mM sodium acetate, pH 3.8, containing 500 mM NaCl. Elution was achieved with the same buffer. The column eluate was monitored at 280 nm, and the 1.8-ml fractions corresponding to the exclusion volume were pooled and dialyzed against cold distilled water. An aliquot of 300 µl of the material contained in the dialyzed extract was lyophilized, and the resulting powder was solubilized in 10 µl of 0.1% trifluoroacetic acid and subjected to chromatography on a Nucleosil 300 Å-C4 hydrophobic column (Société Francaise ChromatoColonne Shandon, Eragny, France). Elution was performed with a gradient from 0 to 60% acetonitrile in 0.1% trifluoroacetic acid.

Cell wall extract. Cell walls were prepared from lyophilized mycelium that was ground in liquid nitrogen with a Dangoumau grinder (Seli), essentially as described by Ayers et al. (2).

Elicitor bioassay. The chromatographic fractions of interest were pooled, dialyzed against distilled water at 4°C for 24 h, and subsequently lyophilized. They were then checked for their ability to elicit lipoxygenase (LOX) in bioassays performed on whole plants. Seven-week-old seedlings were allowed to absorb 350 µl of a solution containing 2.14 mg of dry weight material per milliliter through their root system. After complete absorption of the solution, incubation continued for the indicated times in nutrient solution. The treated seedlings were then ground in liquid nitrogen, and the powder was suspended in 3 ml/g of a solution composed of 250 mM sodium phosphate buffer, pH 6.5, and 5% polyvinylpyrrolidone. This crude extract was centrifuged at 20,000 × *g*, and the supernatant was recovered for polarographic LOX activity measurements according to Fournier et al. (11). For hydroxyproline-rich glycoprotein (HRGP) elicitation, whole plants were treated as described above and collected after 24 h of incubation.

Protein measurement. The protein content of the samples was measured according to Lowry et al. (21) with bovine serum albumin (BSA) as the standard protein. Measurements were carried out at 750 nm.

Amino acid and sugar analysis. The amino acid composition of the material to be analyzed was determined after hydrolysis of a sample (corresponding to 15 µg of protein) in 1.5 ml of 6 N HCl at 110°C for 24 h. After removal of the acid by evaporation, amino acids were analyzed with a HP 1090 AminoQuant analyzer (Hewlett-Packard Co., Rungis, France) according to Blakenship et al. (8).

The sugar composition of the same material was determined after hydrolysis of a sample (200 μg of protein equivalent) in 2 ml of 2 N trifluoroacetic acid at 120°C for 1 h. After removal of the acid, the sample was taken up into 3 ml of H_2O, and its sugar composition was analyzed by pellicular anion exchange chromatography and pulsed amperometric detection with a Dionex high-performance liquid chromatography (HPLC) system (Dionex Corp., Sunnyvale, CA) according to Lee (20). Elution was performed with an isocratic gradient of 15 mM NaOH (flow rate of 1 ml/min).

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Schägger and von Jagow (29) in a Tris-Tricine discontinuous buffer system. Standard molecular weight proteins were from Pharmacia (low molecular weight = 94,000, 67,000, 43,000, 30,000, and 14,400). Proteins (1 to 3 μg per lane) were stained with silver nitrate according to Oakley et al. (23).

For glycoprotein detection, proteins were transferred on nitrocellulose membrane after migration. Glycoprotein staining was performed according to Stromqvist and Gruffman (32).

Antibody preparation. Rabbit immunization was performed by Eurogentec SA (Seraing, Belgium), following a classical procedure. Three injections, 20 μg each, of a protein sample whose homogeneity was assessed by HPLC were carried out at 3-week intervals. Two weeks after the third injection, the serum was collected and titrated with an immunodot assay on nitrocellulose membranes as described by Parent et al. (24).

Western blots. Samples containing 20 μg of protein or corresponding to 50 μg of dried cell walls resuspended in the loading buffer were subjected to SDS-PAGE and transferred to a nitrocellulose membrane in a Biorad Transblot SD apparatus (Bio-Rad Laboratories), containing a 48 mM Tris-HCl, 39 mM glycine, and 1.3 mM SDS, pH 9.2, running buffer at 1.8 mA/cm^2 of membrane for 30 min. The strip containing M_r standards was cut off and directly stained in Coomassie brilliant blue. The rest of the membrane was incubated for 30 min in the blocking buffer composed of 10 mM Tris-HCl and 250 mM NaCl (Tris-buffered saline, TBS) (pH 7.4), and 5% skimmed milk. It was then incubated for 2 h at room temperature in the anti-GP 34 antiserum diluted 1/10,000 in TBS, rinsed once in TBS and twice in TBS-Tween for 15 min each, and incubated for 1 h at room temperature in a goat anti-rabbit immunoglobulin G (IgG) conjugate diluted 1/3,000 in TBS. After rinsing in TBS-Tween (× 1) and in TBS (× 2), for 15 min each time, the presence of bound horse radish peroxidase was detected according to recommendations of the conjugate supplier (Sigma Aldrich, Saint Quentin, Flavier, France).

Ultrastructural immunogold-labeling. Preparation of the samples and labeling were performed essentially according to Benhamou et al. (7). The fungal material, as well as pieces of infected tobacco plants, were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.5, for 2 h at room temperature. Embedding of mycelium and zoospores was performed in LR White (London Company Ltd., Pelanne Instruments, Toulouse, France), after stepwise dehydration in a graded series of solutions comprised from 20 to 100% ethanol. Embedding of elicited or infected tobacco plants was carried out in Epon 812 (Euromedex, Soussel Werysheim, France), after the same dehydration procedure as above, followed by an additional dehydration step in propylene oxide. Ultrathin sections were performed with a Reichert microtome OM U3 (Leica, Rueil-Malmaison, France) and collected on collodion-coated gold grids. For immunogold-labeling with the anti-GP 34 antiserum, the ultrathin sections were first preincubated for 30 min in a drop of goat anti-rabbit antiserum diluted in 10 mM sodium phosphate, pH 7.4, and 150 mM NaCl (phosphate-buffered saline, PBS) containing 0.5% BSA. They were then transferred in the anti-GP 34 antiserum diluted to 1/1,000 in the same buffer and incubated for 2 h at 37°C in a moist chamber. After rinsing in PBS-BSA 0.5% solution, the grids carrying the ultrathin sections were incubated in a drop of goat anti-rabbit IgG conjugated to 15-nm gold particles, diluted 1/20 in 50 mM Tris-HCl (pH 8.2), 50 mM NaCl, and 1% BSA for 1 h at room temperature. They were thoroughly washed in the same buffer, and then washed in distilled water before being stained with 2% aqueous uranyl acetate for 10 min and then lead citrate for 5 min. They were examined in a Phillips electron microscope (Phillips, Eindhoven, the Netherlands) at 80 kV.

Immunolabeling of HRGPs in elicited plants was performed with a rabbit antiserum raised against melon HRGP (22). The antiserum was diluted 1/100 in PBS-BSA buffer.

Specificity of the labeling was assessed by replacing the antiserum with a nonimmune serum and by preincubation of the antiserum with the antigen.

RESULTS

Protein solubilization. In an attempt to solubilize material related to the cell surface of the fungus, we adapted a protocol that was formerly shown to allow the selective extraction of plant cell wall glycoproteins (22). A crude extract was first prepared by incubation of the ground mycelium at acidic pH in cold ethanol, followed by acetone precipitation, and this extract was then purified by column chromatography on CM-Sepharose (Pharmacia). Most of the material did not bind to the column and was recovered in fraction 1 (Fig. 1A). Subsequent elution of the retained compounds with a linear gradient of NaCl resulted in four peaks of UV-absorbing material. The fractions corresponding to peaks 2 to 5 (Fig. 1A) were pooled and analyzed by SDS-PAGE. Peak 1 was heterogeneous and contained proteins of high M_r; in the other peaks, a major protein of apparent M_r 34,000, together with other small peptides, was observed (data not shown). Peak 3, in which the 34-kDa protein was the most abundant (Fig. 2A, lane 1), was retained for further purification of this molecule.

Purification of that protein called GP 34 was carried out by gel exclusion chromatography on Biogel P30 (Bio-Rad Laboratories) at pH 3.8 in the presence of NaCl. The loaded material was sep-

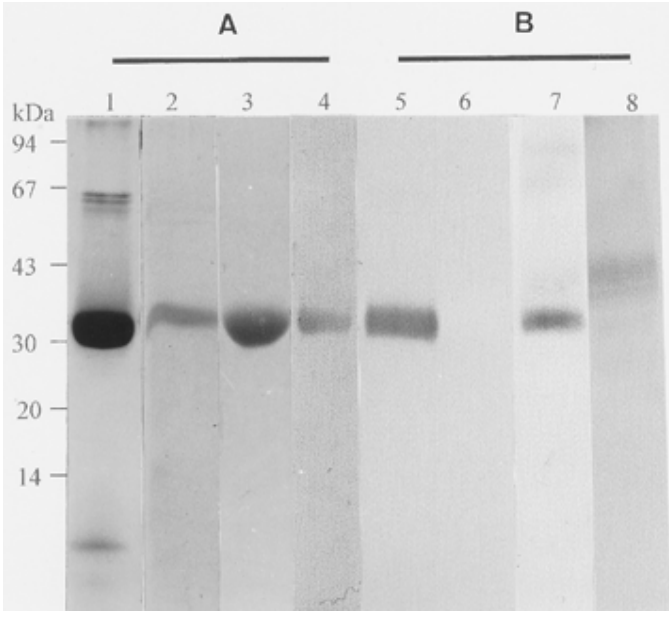

Fig. 2. Electrophoretic analysis of proteins from *Phytophthora parasitica* var. *nicotianae*. **A,** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the proteins recovered during purification: lane 1, fraction 3 of CM-Sepharose chromatography (Pharmacia); lane 2, fraction I recovered from Biogel P30 chromatography; and lanes 3 and 4, protein fractions obtained after high-performance liquid chromatography (HPLC) of peak I from Biogel P30. Gels were stained with silver nitrate (lanes 1 to 3) or Schiff reagent (lane 4). **B,** Western blot analysis of samples corresponding to the pure GP 34 recovered after HPLC (lane 5), a control performed on GP 34 with the preimmune serum (lane 6), fungal cell walls (lane 7), and culture filtrate (lane 8). The position of molecular size markers (kDa) is shown on the left.

arated into four peaks (Fig. 1B). Peak I, corresponding to the exclusion volume of the column, was shown to solely contain GP 34 by SDS-PAGE analysis (Fig. 2A, lane 2). Peaks II to IV (Fig. 1B) also contained GP 34, but other small peptides were contaminating them (data not shown). To assess the purity of GP 34 in the fractions corresponding to peak I, these fractions were pooled and subsequently subjected to HPLC on a Nucleosil 300 Å-C4 column (Société Francaise ChromatoColonne Shandon). SDS-PAGE analysis of the single peak that was recovered during elution showed that GP 34 was already pure after Biogel chromatography (Bio-Rad Laboratories) (Fig. 2A, lane 3). The whole procedure yielded 0.5 µg of GP 34 per gram of dry mycelium. The band of M_r 34,000 was positively stained with the Schiff reagent (Fig. 2A, lane 4),

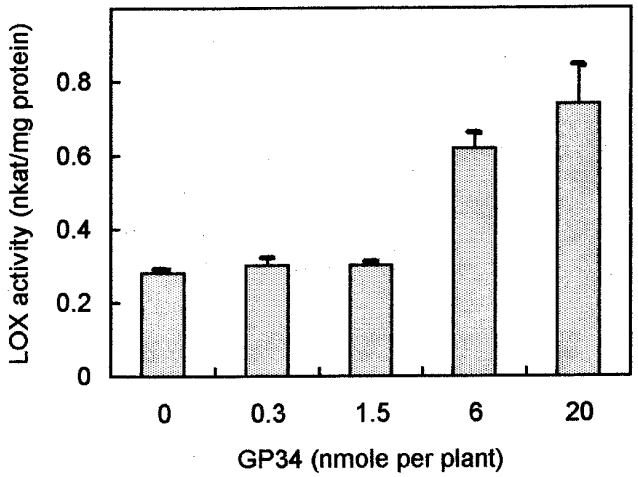

Fig. 3. Dose response study of the effect of GP 34 on lipoxygenase activity in tobacco plants (line 46-8, resistant to race 0). Lipoxygenase activity was measured by polarography on extracts prepared from tobacco plants treated with GP 34 for 48 h. Control plants were treated with water. Values are the mean of duplicate plants from two independent experiments ± standard deviation.

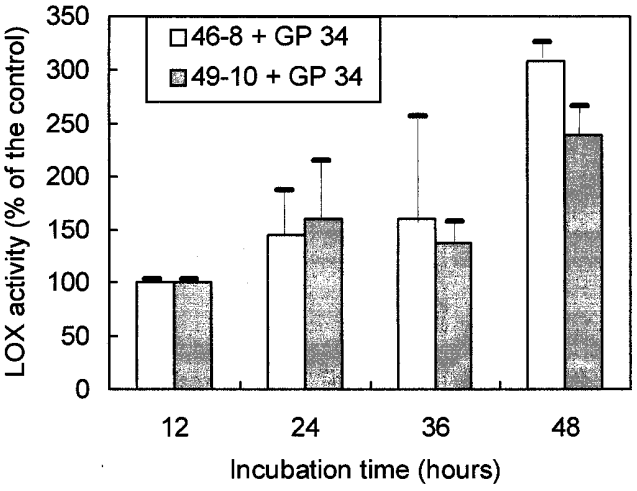

Fig. 4. Time-course measurement of lipoxygenase activity in tobacco plants treated with GP 34. Tobacco seedlings, lines 46-8 and 49-10, were allowed to absorb 20 nmoles of GP 34. Control seedlings were treated with water. Plants were harvested at different time intervals after the beginning of elicitor treatment, and lipoxygenase activity was measured by polarography. Values are expressed in percent of the controls and are the means of three independent experiments ± standard deviation. Lipoxygenase activity of 46-8 and 49-10 controls at the beginning of the experiment were 0.231 ± 0.015 and 0.261 ± 0.033 nkat/mg of protein, respectively. After 48 h of incubation in distilled water, control values were 0.508 ± 0.17 and 0.457 ± 0.16 nkat/mg of protein for 46-8 and 49-10 plants, respectively. Lipoxygenase activity was significantly induced in plants of the two lines treated with elicitor (F was significant at $P = 0.05$, with a two-way analysis of variance test).

indicating the glycoprotein nature of this compound. An antiserum was raised against the pure glycoprotein. The titer of the antiserum was determined on 0.5 µg of GP 34 and was found to be 1/30,000. This antiserum was used to detect GP 34 by Western blot analysis (Fig. 2B, lane 5). A control performed with the nonimmune serum was negative (Fig. 2B, lane 6). The specificity of the antiserum was assessed by Western blot analysis of the culture filtrate of the fungus and of a cell wall extract. Figure 2B (lane 7) shows that the antiserum revealed one single band of M_r 34,000 in the cell wall extract. In the culture filtrate (Fig. 2B, lane 8), compounds showing M_r of about 40,000 and 46,000 reacted with the antiserum. This might reflect common epitopes with GP 34, possibly sugar residues; indeed, extracellular proteins are often glycosylated. These experiments showed that the antiserum had an excellent titer and was specific for GP 34 in the fungal cell wall.

Partial chemical characterization of GP 34. The glycoprotein nature of GP 34 was confirmed by amino acid and sugar analysis. The sugar moiety accounts for 55% of the molecule and is mainly composed of glucose (93%); it also contains small amounts of mannose, galactose, and glucosamine. In the peptide moiety, serine, threonine, and asparagine/aspartic acid (Asx) are the most abundant amino acids, representing 36.5% of the total amino acids. Some of these residues might be involved in *O*-glycosylation, *N*-glycosylation, or both of the molecule. In addition, GP 34 contains 9% hydroxyproline, a rare amino acid only found in the cell wall of this class of fungi, in the cell wall of green algae and higher plants, and in animals (30). The overall composition of GP 34, notably the presence of glucan and hydroxyproline, is consistent with the chemical composition of the cell walls of *Phytophthora* (4).

Biological activity of GP 34. To look for possible elicitor activity of GP 34, whole tobacco seedlings were allowed to absorb this molecule through their root system. This mode of treatment was used, because it closely reflects the natural mode of infection by the fungus. In this system as well as in other host-pathogen interactions, the LOX pathway is supposed to be involved in a transduction cascade leading from pathogen or elicitor perception to plant defense gene activation (31). Indeed, LOX gene expression is highly induced in elicitor-treated tobacco cells and is expressed earlier during incompatibility of isoline 46-8 to race 0 of the pathogen than during compatibility of isoline 49-10 to the same race (35).

A dose-response experiment showed a significant increase of LOX activity in tobacco seedlings of line 46-8 treated with GP 34 at amounts ranging from 6 to 20 nmoles per plant (Fig. 3). Within the range of GP 34 amounts used, maximum induction (× 2.5) was obtained with 20 nmoles per plant.

The activity of GP 34 was assessed on the two near-isogenic lines of tobacco, resistant or susceptible to race 0. Time-course measurements of LOX were carried out after treatment with 20 nmoles of GP 34 per plant and expressed in percent LOX activity measured in control plants incubated with water. These controls showed only a weak increase of enzymic activity throughout the assay. Induction of LOX was observed at 24 h and increased until the end of the experiment, at 48 h of incubation (Fig. 4). No sig-

TABLE 1. Labeling density of hydroxyproline-rich glycoproteins (HRGPs) in tobacco plants treated with GP 34[a]

	HRGP labeling (gold particles/µm^2)			
	46-8		49-10	
	Control	+ GP 34	Control	+ GP 34
Root vessels	6.1 ± 3.2	48.3 ± 2.3	5.2 ± 2.0	93.5 ± 23
Background	1.3 ± 0.5	6.6 ± 1.8	1.1 ± 0.3	17.4 ± 4.0

[a] Tobacco plants treated with GP 34 for 24 h were prepared for immunogold-labeling and electron microscopy. Control plants were incubated with distilled water. Labeling density was estimated on six sections from three independent elicitation assays and was expressed as number of gold particles per square micrometer.

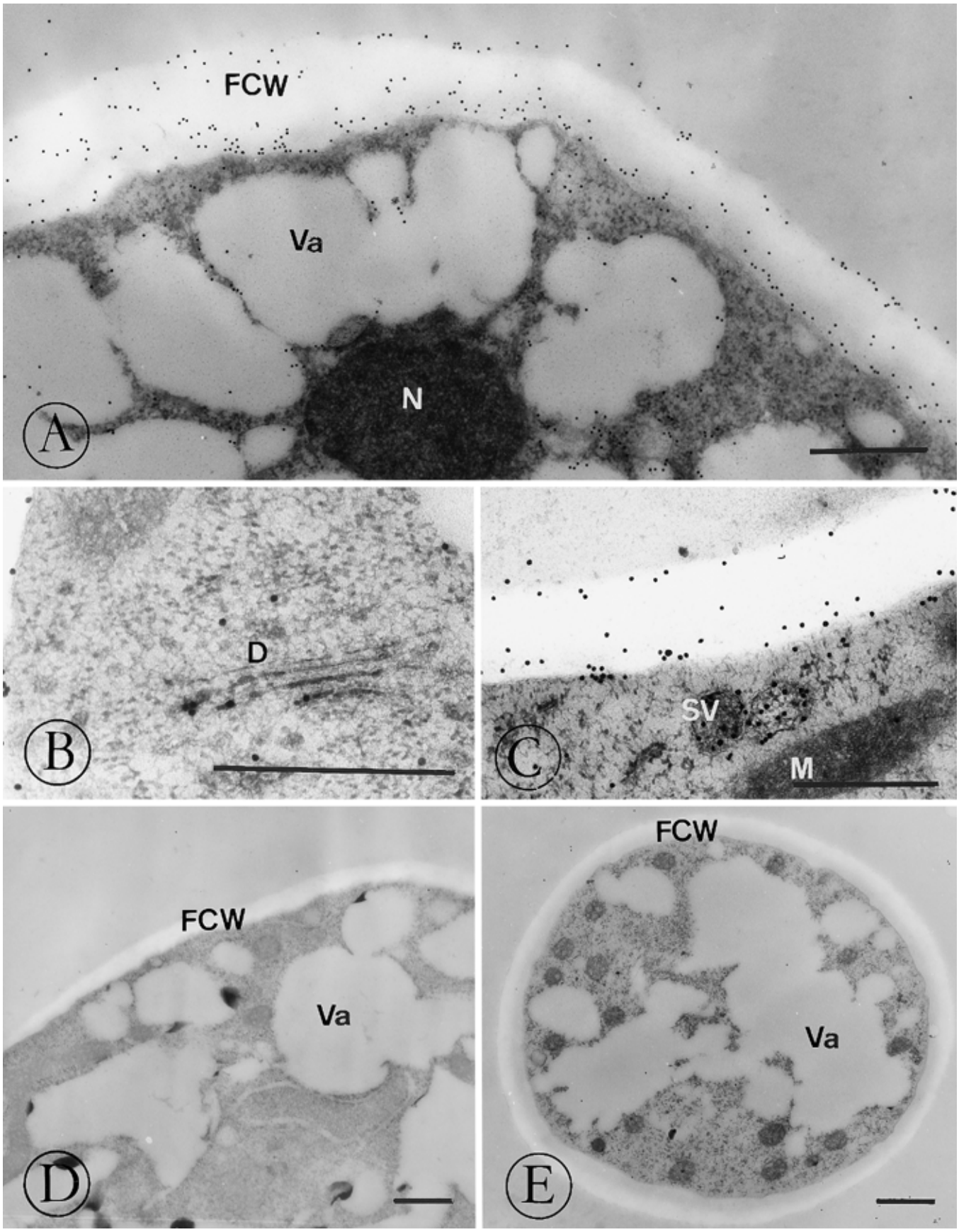

Fig. 5. Immunocytolocalization of GP 34 in the mycelium of *Phytophthora parasitica* var. *nicotianae* grown in vitro. **A,** Subapical zone of hypha. Note gold particles associated with the fungal cell wall (FCW) and vacuoles (Va). Nucleus (N) was devoid of labeling (×45,000). **B,** Immunolabeling of Golgi cisternae (×81,000). **C,** GP 34 labeling associated with small vesicles (SV) and absent from mitochondria (M) (×54,000). **D and E,** Controls performed with the nonimmune serum or with the antiserum presaturated by GP 34 prior to labeling (×22,000). Bar = 0.5 µm. D = dictyosome.

nificant difference in response intensity could be observed between the two lines in this bioassay. Next, accumulation of HRGPs was studied by immunocytochemistry using an anti-HRGP rabbit antiserum (22). Labeling density was estimated by counting the number of gold particles per square micrometer according to Bendayan (5) (Table 1). GP 34 treatment consistently induced HRGP labeling in root vessels of both susceptible and resistant tobacco isolines. Increase in background labeling was associated with this response, a feature generally observed when strong positive immunogold-staining is obtained. This phenomenon gave a bias to the statistical significance of HRGP induction by GP 34 in isoline 49-10. However, covariance analysis of the data indicated that induction was significant at $P = 0.05$ in the 46-8 isoline.

Immunocytolocalization of GP 34. The anti-GP 34 antiserum was used for ultrastructural immunocytolocalization studies of GP 34, first on the fungus grown in vitro and then on ultrathin sections of infected tobacco seedlings.

Examination of the mycelium grown in vitro was performed on the peripheral zone of a colony obtained on solid medium that contained apical and subapical hyphae (Fig. 5). The subapical region was characterized by the occurrence of internal membranes and organellar structures. In Figure 5A, immunogold-labeling was located mainly on external and internal layers of the cell wall and in vacuoles. A weak labeling of GP 34 was associated to Golgi cisternae (Fig. 5B), probably due to GP 34 precursors. Labeled cytoplasmic vesicles were also observed; they might originate from the Golgi apparatus, since they were typically concentrated at the tip of hyphae, where they secrete their content after fusion with the plasma membrane (Fig. 5C). Controls performed with the nonimmune serum and the anti-GP 34 antiserum saturated with the antigen prior to labeling were negative (Fig. 5D and E, respectively).

Immunogold-labeling was also undertaken on sporocysts and zoospores, which represent the asexual mode of reproduction and the infectious stage of the fungus. A strong labeling of the thick cell wall of the sporocyst was observed (data not shown). In the young wall-less zoospore, a very faint labeling was observed in the vicinity of the plasmalemma (Fig. 6A). An enlargement of this area showed that the labeling was related to residual flattened vesicles that underlied the plasma membrane over the whole cell, except in the groove region (Fig. 6B), and also to other small peripheral vesicles. Immunogold-labeling was more pronounced in the flagellum area, associated in this case to the plasma membrane (Fig. 6C). The flagellum microtubules were devoid of labeling (Fig. 6D). The position of this flagellum in the groove suggests it is the tinsel flagellum, in spite of the lack of mastigonemes that might result from the fixation procedure used for immunogold studies.

In the encysting zoospore, labeling paralleled the formation of a new cell wall. Gold particles were observed in small secretion vesicles and in the external and internal layers of the wall (Fig. 6E). At this stage, the water expulsion vacuole and the flattened vesicles observed during former stages had disappeared. Because encystment is required for attachment to the host plant, one may hypothesize that GP 34 is associated with the very first contact between the host and pathogen.

Electron microscopy analysis was then performed on inoculated seedlings. Following root inoculation with zoospores, macroscopic symptoms became visible at the level of the collar of susceptible plants about 3 days after inoculation. Black shank disease progressed throughout the susceptible plants (isoline 49-10), while resistant plants (isoline 46-8) showed no macroscopically detectable lesions, necrosis, browning, or wilting.

A thorough microscopic examination of resistant seedlings showed that the fungus entered the roots at only rare locations. For this reason, it was not detected at stages earlier than 48 h postinoculation. At this time, it was found at intercellular and intracellular locations in the cortical cell layers (Fig. 7A and B, respectively). The ultrastructure of the hyphae was essentially as reported by Hemmes and Hohl (17), with a dense cytoplasm, numerous vacuoles, and a thin cell wall. The cytoplasm of host cells surrounding intercellular-colonizing hypha was disorganized. When the fungus was intracellular, an amorphous electron-opaque deposit resembling papilla occurred along the host cell wall. The fungal cell was highly vacuolized with a cytoplasm devoid of organelles. Three days after inoculation, fungal growth had almost ceased. Infected cells and uninfected ones adjacent to the hyphae were collapsed (Fig. 7C). Intercellular fungal cells were still alive, while intracellular hyphae were dead (Fig. 7C and D, respectively). Massive fungal cell death occurred 5 days postinoculation, and highly vacuolized hyphae were observed at both inter- or intracellular locations (data not shown).

Immunogold-labeling of GP 34 in intercellular hypha 48 h after inoculation of the resistant isoline was associated with the fungal cell wall (Fig. 7A). No labeling was visible on the host cell wall, indicating that fungal and plant cells do not share common epitopes. Moreover, the labeling of GP 34 outside of the hypha was always very weak, thus confirming that the glycoprotein was not significantly secreted into the extracellular medium. In intracellular hypha (Fig. 7B), immunogold-labeling of GP 34 was randomly distributed throughout the vacuolized cytoplasm and was less markedly associated with the cell wall. Counting of gold particles on 11 sections of inter- and intracellular hyphae gave a value of 20.8 ± 1.9 gold particles per square micrometer of hyphae.

By 72 h after inoculation, GP 34 was localized in the cell wall and vacuoles of intercellular live fungal cells (Fig. 7C). However, when the fungal cell was intracellular, complete vacuolization of the hypha was observed, with immunogold-labeling distributed more or less randomly around the fungal cell wall (Fig. 7D). Labeling was slightly increased (26.6 ± 13.8 gold particles/μm^2 of hyphae) compared with the density observed at 48 h postinoculation.

The events observed during colonization of susceptible plants by *P. parasitica* var. *nicotianae* race 0 were essentially as described by Benhamou and Côté (6). During the first 2 days, the fungus grew intercellularly (Fig. 8B), occasionally entering host cells by means of haustorium-like bodies (Fig. 8A). Extensive alteration of cortical cells was observed, characterized by a complete disorganization of the host cell cytoplasm (Fig. 8A). By 72 h postinoculation, the fungus had spread intercellularly and intracellularly and developed extensively, except in the xylem. During intracellular growth, the hyphae exhibited considerable differences in morphology. They were strongly vacuolized and often surrounded by papilla-like structures (Fig. 8C). Thus, papilla production was not restricted to the resistant phenotype. From 72 to 120 h postinoculation, an increase in the number of invading hyphae was observed, and the fungus finally sporulated. By 120 h postinoculation, host cells were markedly altered with complete disruption of the cytoplasm in which the organelles were no longer visible. The plant cell wall showed some alterations (Fig. 8D, arrows). The fungal cell wall appeared thicker, the plasma membrane was convoluted, and the cytoplasm was dense with numerous vacuoles (Fig. 8D).

Immunogold-labeling of GP 34 in fungal cells was very faint during the first 2 days of colonization of susceptible plants. Labeling density was 3.6 ± 3 gold particles per square micrometer of hyphae (Fig. 8A and B). When present, it was mainly associated with vesicles (Fig. 8B).

By 72 h postinoculation, labeling increased markedly and was almost exclusively confined to the cell wall, regardless of the morphology of the fungus. Figure 8C shows the cell wall labeling of an intercellular hypha and of a vacuolized intracellular fungal cell. At this stage, labeling density was 48.6 ± 19 gold particles per square micrometer of hyphae.

By 120 h postinoculation, GP 34 labeling was still very high; gold particles were associated with the convoluted plasma membrane and randomly distributed throughout the cell wall (Fig. 8D). Labeling was neither observed in the fungal cell cytoplasm nor in the vacuoles.

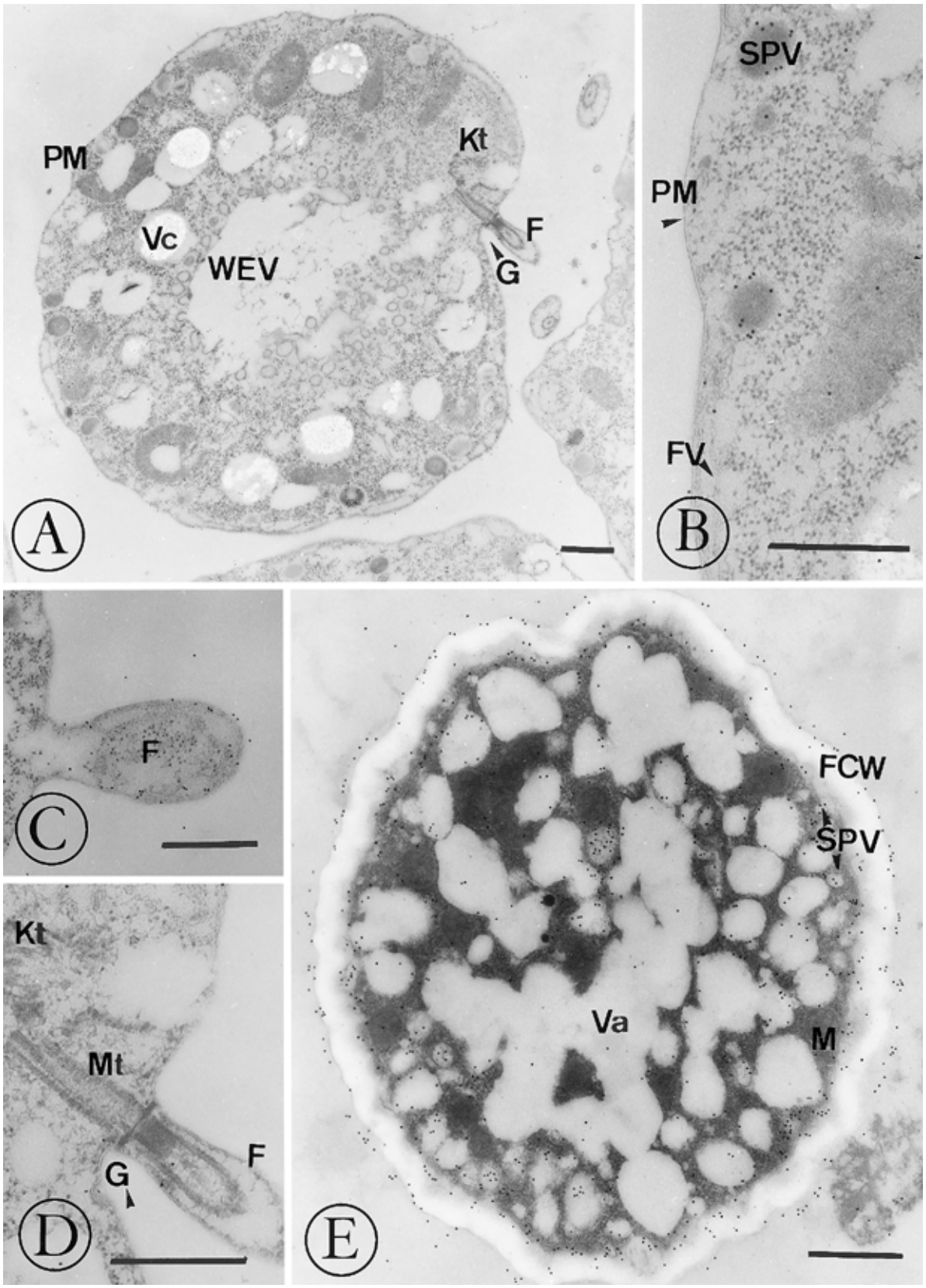

Fig. 6. Immunocytolocalization of GP 34 in a zoospore and in a cyst. **A,** Immunogold-labeling on a young flagellate zoospore (×18,000). **B,** Labeling density was weak and associated with small peripheral vesicles (SPV) and flattened vesicles (Fv) (×48,000). **C,** Surface section of a flagellum (F) showing that labeling of GP 34 is associated with the plasma membrane (PM) (×32,000). **D,** No labeling was observed on the microtubules (Mt) of the flagellum (×46,500). **E,** Immunocytolocalization of GP 34 on a cyst: gold particles are associated with small peripheral vesicles and to the newly formed fungal cell wall (FCW) (×33,000). Bar = 0.5 µm. Other abbreviations: G, groove; Kt, kinetosome; M, mitochondria; Vc, crystalline content vesicles; Va, vacuole; and WEV, water expulsion vacuole.

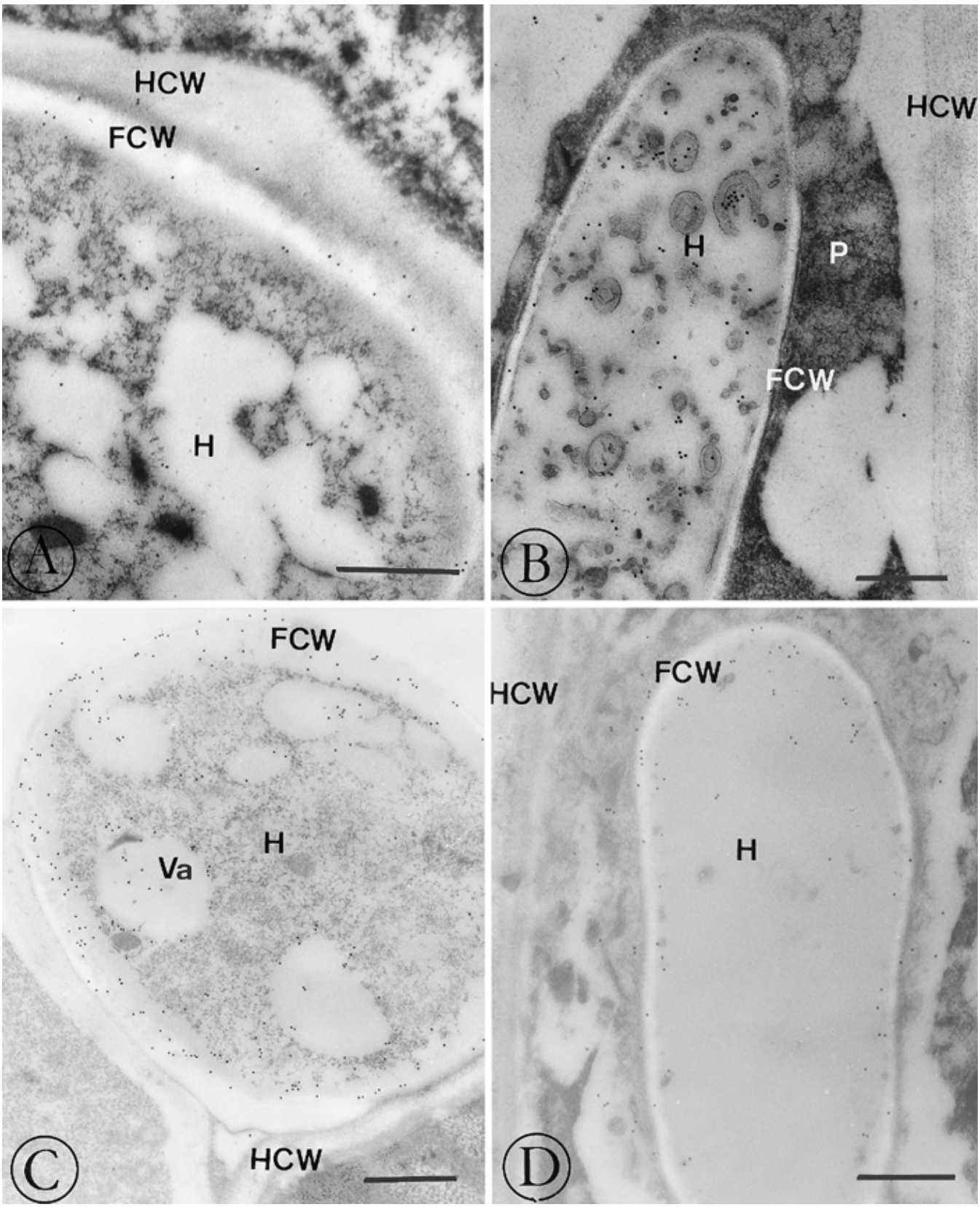

Fig. 7. Immunocytolocalization of GP 34 in infectious hyphae of *Phytophthora parasitica* var. *nicotianae* race 0 in resistant plants (isoline 46-8). **A,** Labeling of GP 34 on the cell wall 48 h postinoculation showing a weak labeling of the cell wall of an intercellular hypha (H) (×45,000). **B,** GP 34 labeling was mainly located in the collapsed cytoplasm of an intracellular hypha. Note the electron-opaque papilla-like material surrounding the fungal cell (×35,500). **C,** Intercellular localization of fungal hypha 72 h postinoculation; immunogold-labeling was associated with the cell wall and vacuoles (Va). Note the aggregated and electron-opaque aspect of the cytoplasm of adjacent host cells (×35,500). **D,** An intracellular, highly vacuolized hypha 72 h postinoculation, in which immunogold-labeling was randomly distributed in the surroundings of the cell wall. Bar = 0.5 μm. Other abbreviations: FCW, fungal cell wall; HCW, host cell wall; and P, papilla.

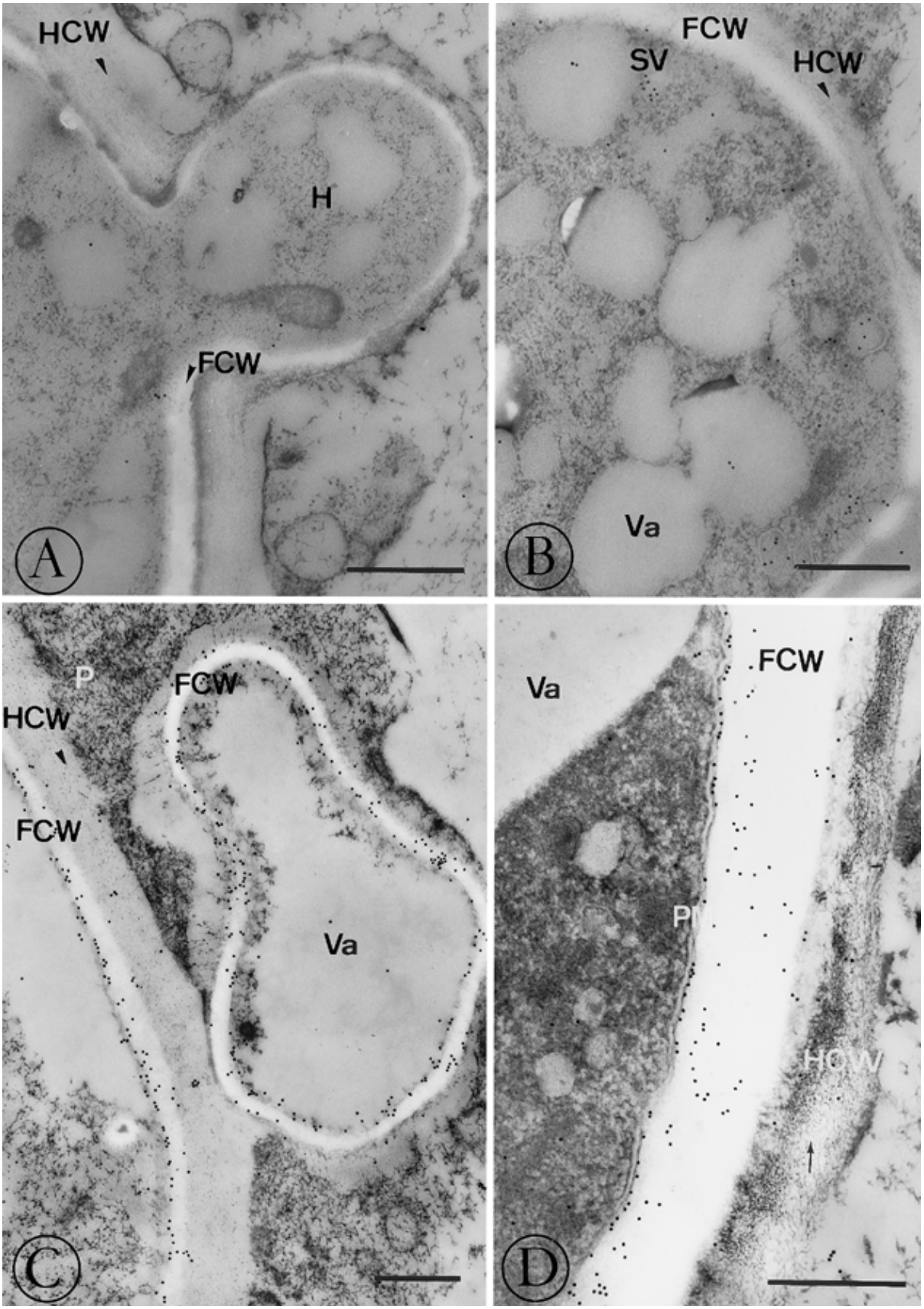

Fig. 8. Immunocytolocalization of GP 34 on infectious hyphae (H) of *Phytophthora parasitica* var. *nicotianae* race 0 in susceptible plants (isoline 49-10). **A,** Section of a haustorium-like body of a hypha penetrating a tobacco cell 48 h postinoculation. The host cell cytoplasm was collapsed, and the host cell wall (HCW) was devoid of labeling (×45,000). **B,** An intercellular hypha 48 h postinoculation. A very weak labeling was occasionally observed in small cytoplasmic vesicles (SV) (×45,000). **C,** Intercellular and intracellular hyphae 72 h postinoculation. The intracellular fungal cell was highly vacuolized and surrounded by a papilla-like material. A strong immunogold-labeling was exclusively associated to the fungal cell wall (FCW) in both cells (×35,000). **D,** Intercellular fungal growth of the fungus 120 h postinoculation. Note the thick cell wall and convoluted plasma membrane. Host cell wall was altered (arrow); GP 34 labeling was associated with the plasma membrane (PM) and the cell wall of *Phytophthora* (×50,000). Bar = 0.5 μm. Abbreviations: P, papilla and Va, vacuole.

DISCUSSION

To isolate elicitor-active molecules from the cell surface of *P. parasitica* var. *nicotianae*, we adapted a protocol that was previously used for the solubilization of plant cell wall glycoproteins. This allowed isolation of a glycoprotein of apparent M_r 34,000 whose sugar and amino acid composition are consistent with the chemical composition of *Phytophthora* cell walls.

The biological activity of GP 34 was assayed on whole plants. When introduced through the root system, it elicited LOX activity, a marker associated with disease resistance in this interaction (35). Elicitor activity was substantiated by its ability to induce HRGP accumulation in tobacco seedlings, a well known defense response (31). Quantitative immunogold-staining revealed that HRGP increased in both susceptible and resistant isolines. No macroscopic hypersensitive reaction was observed, which is consistent with the fact that resistance of inoculated tobacco plants to this pathogen is not characterized by externally visible lesions. GP 34 proved very active, since nanomole amounts were sufficient to significantly induce LOX in whole seedlings.

Purification to homogeneity of the elicitor allowed us to raise antibodies for further studies. Antiserum against GP 34 confirmed that this glycoprotein is located in the cell wall of the mycelium.

The overall data show that the obtained elicitor exhibits several novel characteristics. At the biochemical level, it differs from other cell wall-derived elicitors such as the hepta-β-glucan isolated from *P. sojae* (2,14) and the preparation obtained from *P. parasitica* var. *nicotianae* by autoclaving (28), in that it is a macromolecule and not an oligosaccharide or glycopeptide fragment. Moreover, with the exception of a glycoprotein isolated from the cell wall of urediospore germ tubes of *Puccinia graminis* (19), native elicitors described until now were isolated from the extracellular culture medium of fungi or from intercellular liquid fluids of infected plants. The elicitor-active glycoprotein described here does not seem to be constitutively secreted in the medium, because no molecule of such size could be identified in the culture filtrate of *P. parasitica* var. *nicotianae*. However, the possibility that molecules related to GP 34 are secreted into the extracellular medium cannot be ruled out, since components of higher M_r that cross-react with the anti-GP 34 antiserum were found in the culture filtrate. These molecules might share common glucan epitopes with GP 34. The possibility that the glycan portion of GP 34, which is mainly composed of glucose, might contain hepta-β-glucan-like elicitor fragments remains to be determined.

The antiserum against GP 34 was subsequently used for immunocytolocalization studies. There have been a number of such studies on fungal pathogens of plants, but the antibodies were most often raised against uncharacterized antigens present in culture filtrates, fungal extracts, or dead cells (9). To improve the specificity of the antisera, monoclonal antibodies have been obtained in a few instances and some of them have proved useful, notably for labeling the cell surface of Oomycetes (36) and visualizing encystment (15). The fact that GP 34 is highly immunogenic and corresponds to a glycoprotein with elicitor activity adds to our knowledge of fungal cell surface determinants. Thus, immunogold-labeling and electron microscopy performed on the fungus grown in vitro showed that GP 34 is mainly observed in the outer and inner layers of the cell wall, although the weakness of internal labeling of the cell wall may result from antigen inaccessibility (37). In the young wall-less zoospore, labeling was only observed at the level of the flagellum plasma membrane and in the small vesicles that underlie the cell surface. GP 34 was then abundantly secreted to the cell wall at the onset of encystment, which is consistent with the formation of a new cell wall. Hardham and Suzaki (16) have used monoclonal antibodies to follow the developmental stages of zoospore formation and encystment in *P. cinnamomi*. Although the functions of the corresponding antigens remain unknown, some interesting parallels can be made between their observations and our data. Thus, the labeling of GP 34 that was observed in cytoplasmic vesicles and large vacuoles during encystment of *P. parasitica* var. *nicotianae* might correspond to the material contained in the small peripheral vesicles and protein storage vacuoles, respectively, found in *P. cinnamomi* zoospores during encystment. These vesicles have been described to be involved in secretion of adhesion factors to the host plant (15). The location and kinetics of GP 34 deposition in the cyst cell wall suggest that this glycoprotein might be involved in recognition or adhesion phenomena.

At early stages of colonization of the host plant, i.e., 48 h postinoculation, GP 34 was only consistently observed during the incompatible interaction and was restricted to the cell wall. Labeling was less pronounced than on the fungus grown in vitro, possibly due to changes of the cell surface in response to growth conditions encountered in planta. There was no labeling of plant cell walls with the antiserum. At later stages, GP 34 was observed in the compatible interaction as well and reached high levels, while labeling diminished and ceased in the dying fungus within the resistant cultivar. Such a differential labeling is consistent with a role of GP 34 as an elicitor of early defense reactions during incompatibility. Indeed, LOX and defense genes are induced earlier during incompatibility of tobacco to race 0 than during compatibility (35). The fact that elicitation of LOX activity and HRGP accumulation was not clearly cultivar-specific in our bioassays might simply result from incubation of the two isolines with equal amounts of the glycoprotein at the same time. Arachidonic acid is another example of an elicitor that does not show any specificity in bioassays, but that is released earlier during the incompatible interaction of potato with *P. infestans* than during the compatible one (27). The differential labeling of GP 34 within the same race as a function of the host genetic background points to an effect of host signals on GP 34 synthesis. Future work will be aimed at studying this effect.

Besides GP 34, two other elicitors have been located in planta: the endopolygalacturonase of *Colletotrichum lindemuthianum* in infected bean seedlings (7) and the Pgt elicitor from *Puccinia graminis* f. sp. *tritici* in infected wheat leaves (33). Since these studies have been performed only on compatible interactions, nothing is known about the behavior of such elicitors in resistant seedlings. To our knowledge, our work provides the first complete study of the localization of a fungal elicitor at all stages of the life cycle of the fungus, i.e., during saprophytic growth as well as during colonization of susceptible or resistant cultivars. Whether this elicitor might serve a function such as adhesion to the host plant will be investigated in the future.

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