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An Extracellular Glycoprotein from *Phytophthora megasperma* f. sp. *glycinea* Elicits Phytoalexin Synthesis in Cultured Parsley Cells and Protoplasts

Jane E. Parker, Wolfgang Schulte, Klaus Hahlbrock, and Dierk Scheel

Max-Planck-Institut für Züchtungsforschung, Abteilung Biochemie, D-5000 Köln 30, Germany.
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A potent elicitor of phytoalexin accumulation in cultured parsley cells and protoplasts has been purified from the culture filtrate of *Phytophthora megasperma* f. sp. *glycinea*. It is a glycoprotein with an apparent relative molecular mass of 42 kDa and contains an N-linked high mannose-type sugar. Deglycosylation and proteinase treatments established that elicitor activity resides in the protein portion but was not reduced by autoclaving. In parsley cells and protoplasts the pure glycoprotein stimulated synthesis of the full complement of furanocoumarin phytoalexins normally identified in infected leaves or after treatment of cultured cells with a crude mycelial wall preparation from *P. m.* f. sp. *glycinea*. The pure glycoprotein was also shown to induce accumulation of defense-related mRNAs in parsley protoplasts in a manner similar to the crude wall elicitor. Parsley leaves did not respond to the pure glycoprotein, although the crude wall and culture filtrate preparations from *P. m.* f. sp. *glycinea* stimulated phytoalexin production when injected into leaf disks. Polyclonal antisera were raised against the intact glycoprotein and the deglycosylated protein. The former antiserum cross-reacted with many components from *P. m.* f. sp. *glycinea*, whereas the latter was monospecific. With this antiserum the 42-kDa glycoprotein was also shown to be a constituent of the cell wall but not of the soluble fraction of *P. m.* f. sp. *glycinea*. In addition, cross-reacting proteins of slightly smaller molecular mass were found in culture filtrates and mycelial cell walls of *P. nicotianae* var. *parasitica* and *P. parasitica*. Several other phytopathogenic fungi, however, did not contain this glycoprotein.

Additional keywords: gene activation, *Petroselinum crispum*, plant defense, recognition.

Plants defend themselves against attack by potential pathogens with the rapid activation of a complex pattern of resistance reactions (Hahlbrock and Scheel 1987). Similar defense mechanisms appear to operate in nonhost- and host-incompatible interactions. Specific gene activation is involved in many of these responses (Hahlbrock and Scheel 1987, 1989; Dixon and Lamb 1990), whereas other reactions rely on allosteric enzyme activation (Kauss *et al.* 1989). The molecular basis of non-self recognition in plants is not understood but clearly depends on perception of appropriate signals by the plant cell. Non-self recognition appears to involve detection of components of the microbe as well as the plant surface, because induced defense reactions are elicited by compounds released from both fungal and plant cell walls (Darvill and Albersheim 1984; Ebel 1986). These elicitors may bind specifically to plant plasma membrane components and thereby initiate the signaling events required for the onset of defense responses (Scheel and Parker 1990; Ebel and Scheel, in press).

We are examining the primary events of the interaction between cultured parsley cells and elicitors derived from the fungus *Phytophthora megasperma* Drechs. f. sp. *glycinea* Kuan & Erwin. The reactions induced by a crude fungal wall-derived elicitor in cultured cells are very similar to the defense responses of parsley leaf cells to infection by *P. m.* f. sp. *glycinea* (Scheel *et al.* 1986; Jahnen and Hahlbrock 1988; Schmelzer *et al.* 1989). These reactions include transcriptional activation of defense-related genes of unknown function (Somssich *et al.* 1986, 1989) and of genes coding for enzymes involved in the biosynthesis of coumarin phytoalexins (Chappell and Hahlbrock 1984; Scheel *et al.* 1987). Because of their autofluoresence under UV light, the furanocoumarins, which are rapidly synthesized and excreted by elicitor-treated cells (Tietjen *et al.* 1983; Hauffe *et al.* 1986), are readily detected in the culture medium. Because, in addition, freshly prepared parsley protoplasts completely retain their responsiveness to the crude fungal wall elicitor (Dangl *et al.* 1987), they represent an ideal system for examining the primary interaction of fungal and plant molecules, which finally results in defense gene activation.

We have used the protoplasts to screen crude preparations of the *P. m.* f. sp. *glycinea* cell wall and culture filtrate for active elicitors. In previous studies we showed that proteinaceous components of the *P. m.* f. sp. *glycinea* wall material are responsible for its elicitor activity in parsley (Parker *et al.* 1988). Here we report the identification, purification, and partial characterization of a glycoprotein that has been isolated from the *P. m.* f. sp. *glycinea* culture filtrate and is a highly active elicitor in cultured parsley cells and protoplasts.

Current address of J. E. Parker: Sainsbury Laboratory, John Innes Institute, Colney Lane, Norwich NR4 7UH, UK.
Address correspondence to D. Scheel: Max-Planck-Institut für Züchtungsforschung, Abteilung Biochemie, Carl-von-Linné-Weg 10, D-5000 Köln 30, Germany.

MATERIALS AND METHODS

Materials. All chemicals were of analytical grade and were purchased from Sigma (Munich, Germany), Merck

(Darmstadt, Germany), or Serva (Heidelberg, Germany) unless stated otherwise.

Fungal culture and crude elicitor preparation. Races 1 and 3 of *P. m.* f. sp. *glycinea* were maintained as described previously (Kombrink and Hahlbrock 1986). Liquid cultures were harvested after 3–4 wk of growth on asparagine medium (Keen 1975). The mycelium of race 1 was processed to produce the crude *P. m.* f. sp. *glycinea* wall elicitor as described previously (Ayers *et al.* 1976). The culture filtrate was passed through a 0.2-μm cellulose nitrate membrane and concentrated in a 500-ml ultrafiltration unit by using either YM30 or YM10 membranes (Amicon, Witten, Germany). The crude *P. m.* f. sp. *glycinea* wall and culture filtrate materials, stored as freeze-dried preparations, were dissolved in water and autoclaved or filter-sterilized before use.

P. infestans (Mont.) de Bary (races 0, 1, 4, and 1,4), *P. nicotianae* van Breda de Haan var. *parasitica* (Dastur) Waterhouse, and *P. parasitica* Dastur were grown on vegetable juice agar (Rohwer *et al.* 1987); *Alternaria carthami* Chowdhury and *Fusarium solani* (Mart.) Sacc. on potato-dextrose agar (Difco, Detroit, MI); *Rhynchosporium secalis* (Oudem.) Davis on lima bean agar (Difco); and *Sclerotinia sclerotiorum* (Lib.) de Bary on malt extract-peptone agar (30 g/L malt extract, 3 g/L peptone from soybean, 15 g/L agar, pH 5.6). For liquid cultures *A. carthami* and *F. solani* were cultivated in Filner's medium (Tietjen *et al.* 1983), *P. nicotianae* and *P. parasitica* in asparagine medium (Keen 1975), *P. infestans* in Henninger medium (Henninger 1963), *R. secalis* in Fries medium no. 3 (Pringle and Sheffer 1963), and *S. sclerotiorum* in malt extract-peptone medium (30 g/L malt extract and 3 g/L peptone from soybean, pH 5.6). *A. carthami*, isolate 4, was kindly provided by U. Matern (Freiburg, Germany), *P. m.* f. sp. *glycinea*, races 1 and 3, by H. Grisebach (Freiburg), *P. infestans*, races 0, 1, 4, and 1,4, by H. R. Hohl (Zürich, Switzerland) and B. Schöber (Braunschweig, Germany), and *R. secalis* by W. Knogge (Köln). The other fungal strains were obtained from the German Collection of Microorganisms (Göttingen, Germany).

Elicitor treatment of cultured cells, protoplasts, and leaves from parsley. Cell suspension cultures of parsley (*Petroselinum crispum* (Miller) A. W. Hill) were maintained on modified B5 medium (Hahlbrock 1975) supplemented with 171 mg/L of $CaCl_2 \times 2\ H_2O$ and 1 mg/L of 2,4-dichlorophenoxyacetic acid as described previously (Kombrink and Hahlbrock 1986). Protoplasts were prepared from cultured cells 5 days after passage (Dangl *et al.* 1987). Elicitor treatment of cell cultures was as described previously (Parker *et al.* 1988), using a 10-ml cell suspension in 110-ml Erlenmeyer flasks. Protoplasts were treated in 4-ml volumes (Parker *et al.* 1988) or were pipetted in 100-μl aliquots (5×10^5 protoplasts per milliliter) into wells of a microtiter dish (Costar, Cambridge, MA) containing up to 8 μl of elicitor preparation per well. The production of furanocoumarins in 10-ml cell and 4-ml protoplast cultures was measured 24 hr after the onset of treatment by extraction and spectrophotometric analysis (Parker *et al.* 1988). Coumarin accumulation in 0.1-ml protoplast cultures was determined by measuring fluorescence of the medium at 410 nm with excitation at 350 nm in a Perkin Elmer LS-2 B fluorometer (Beaconsfield, U.K.). Thin-layer chromatography of coumarins was performed on precoated silica gel plates as described by Kombrink and Hahlbrock (1986).

The elicitor activity in leaves of greenhouse-grown parsley plants was tested by injecting 20 μl of the elicitor solutions into leaf disks (diameter 12 mm) cut with a cork borer. The disks, floating on 4 ml of the respective elicitor medium, were incubated in petri dishes for up to 50 hr. Aliquots of this solution were withdrawn and analyzed for furanocoumarins after various periods of incubation in the dark at 26° C.

Purification of glycoprotein elicitor. *P. m.* f. sp. *glycinea* culture filtrate (3–4 L) was concentrated in a 350-ml Amicon ultrafiltration unit with reservoir and a YM30 membrane. The concentrate was further washed with 1 L of 50 mM potassium phosphate buffer, pH 6.8, containing 5 mM EDTA and 1 mM phenylmethylsulfonylfluoride (PMSF) (buffer A) and loaded onto DE-cellulose (Whatman, Maidstone, U.K.) equilibrated in buffer A. The column was washed extensively and bound protein eluted with a gradient of 0–0.4 M KCl in a total volume of 120 ml of buffer A. Fractions with elicitor activity in the parsley 0.1-ml microassay (see above) were pooled and the buffer exchanged to 25 mM *N*-methylpiperazine, pH 5.7 (buffer B). The pooled eluate was loaded onto a Mono P column attached to a fast protein liquid chromatography (FPLC) system (Pharmacia, Freiburg) equilibrated in buffer B. The elicitor-active peak eluted at approximately pH 4 using a polybuffer gradient from pH 5.6 to 3.8. It was added to the same volume of 3 M $(NH_4)_2SO_4$ in 100 mM sodium phosphate buffer, pH 6.8, and loaded onto an Alkyl Superose column (FPLC) equilibrated with 1.5 M $(NH_4)_2SO_4$ in 50 mM sodium phosphate buffer, pH 6.8 (buffer C). Pure elicitor glycoprotein was eluted from the column at 50% buffer D (50 mM sodium phosphate, pH 6.8) using a linear gradient of 0–65% buffer D. The pure elicitor was washed with several volumes of buffer D in an Amicon centricon cartridge (YM30) to remove the $(NH_4)_2SO_4$, and was stored at −80° C. Sizing analysis of the elicitor-active DE-cellulose eluate was performed on a Superose 12 column (FPLC) in 50 mM potassium phosphate, pH 7.0.

Extraction of mycelial cell walls. Fungal mycelia (100 g fresh weight) were ground to powder in a mortar with liquid nitrogen, suspended in 200 ml of 50 mM Tris-HCl, pH 7.5, containing 10 mM EDTA and 0.5 mM PMSF, and centrifuged (8,000 × *g*, 20 min, 4° C), resulting in a crude cell-wall pellet of approximately 40 g fresh weight. After a second centrifugation (100,000 × *g*, 1 hr, 4° C), the supernatant was used as the *P. m.* f. sp. *glycinea* soluble fraction. The crude cell-wall preparation was extracted with 1 M LiCl, 3 M LiCl, 1 M guanidine hydrochloride, or 3 M guanidine hydrochloride (4 ml/g) overnight at 4° C, cleared by centrifugation (35,000 × *g*, 20 min, 4° C), and dialyzed against H_2O.

Extraction of parsley cells. A crude protein extract was prepared from cultured parsley cells by grinding freshly harvested cells in liquid nitrogen to fine powder, which was extracted with 20 ml of 0.1 M Tris-HCl, pH 7.5, containing 0.5 mM PMSF, and cleared by centrifugation

(20,000 × *g*, 20 min, 4° C).

Sodium dodecyl sulfate (SDS)-polyacrylamide gel and western blot analysis. Proteins were fractionated on 10–16% SDS-polyacrylamide slab gels and silver-stained by using the method of Blum *et al.* (1987). Proteins were electrophoretically transferred to nitrocellulose (Burnette 1981) and the efficiency of transfer determined by staining with Ponceau S (Muilerman *et al.* 1982). The nitrocellulose blots were probed with concanavalin A for detection of glycoproteins (Hawkes 1982) or with polyclonal antisera to detect specifically the glycoprotein elicitor (Dangl *et al.* 1987).

Proteinase and deglycosylation treatments. Aliquots of pure glycoprotein elicitor (50 μg/ml) were treated with equal amounts of pronase or trypsin as described previously (Parker *et al.* 1988). After treatment the autoclaved samples were added to parsley protoplasts and cultured cells at starting elicitor concentrations known to induce maximal furanocoumarin accumulation in parsley protoplasts. Chemical deglycosylation of 200 μg of pure elicitor was performed with trifluoromethanesulfonic acid according to the method of Sojar and Bahl (1987). Enzymatic deglycosylation of 600 μg of pure elicitor was conducted by using 20 units of glycopeptidase F (PNGase F, Boehringer, Mannheim, Germany) immobilized on CNBr-activated Sepharose 4B (2 ml of swollen gel, Pharmacia). Elicitor glycoprotein was heated for 10 min at 56° C in 50 mM potassium phosphate, pH 7.2, containing 10 mM EDTA, 1% (v/v) mercaptoethanol, and 0.1% (w/v) SDS. This was loaded onto the column and incubated at room temperature for 48 hr. The protein and cleaved sugar were eluted from the column with 6 ml of 50 mM potassium phosphate, pH 7.2, containing 0.1% (w/v) SDS and loaded directly onto a 3-mm-thick preparative SDS-polyacrylamide gel. The deglycosylated elicitor protein was eluted by grinding portions of the gel in phosphate-buffered saline (10 mM potassium phosphate, pH 7.2, 0.15 M NaCl) containing 0.1% (w/v) SDS and leaving the preparation overnight at 4° C before removing gel debris by centrifugation. Protein recovery, purity, and success of deglycosylation were assessed on SDS-polyacrylamide gels and western blots probed with concanavalin A.

Production of antisera. For the production of antiserum the elicitor glycoprotein was purified by chromatography on DE-cellulose and Superose 12, dissolved in 50 mM potassium phosphate buffer, pH 7.0, and mixed with equal volumes of complete (first injection) or incomplete Freund's adjuvant (second and third injections) before subcutaneous injection into rabbits. A second antiserum was obtained against the deglycosylated elicitor. In this case the glycoprotein eluted from the Superose 12 column was enzymatically deglycosylated and purified by gel electrophoresis. The immunization was performed as described for the intact glycoprotein. Both antisera were precipitated with 45% $(NH_4)_2SO_4$, dialyzed against phosphate-buffered saline, and stored in aliquots with 0.02% NaN_3 at −80° C.

RNA analysis. Protoplasts (8×10^6) were harvested and lysed as described by Dangl *et al.* (1987). The combined aqueous phases resulting from the phenol/chloroform extractions were mixed with 0.1 volumes of 3 M sodium acetate, pH 5.2, and 2.5 volumes of ethanol and incubated at 4° C for 20 min. The resulting precipitate was pelleted (10,000 × *g*, 15 min, 4° C), washed once in 70% ethanol, and redissolved in 400 μl of water. This was added to an equal volume of 4 M LiCl in 20 mM sodium acetate, pH 5.2, and RNA was precipitated on ice for 30 min. After centrifugation (10,000 × *g*, 15 min, 4° C), the pellet was redissolved in 400 μl of water, precipitated with ethanol as before, and taken up in a final volume of 200 μl of water. Yields of between 100 and 200 μg of total RNA were obtained. RNA samples were treated for dot blot hybridization as described by Dangl *et al.* (1987). Identical amounts of total RNA (1 μg) were used in all experiments. Preparation of ^{32}P-labeled cDNA probes by nick translation (Rigby *et al.* 1977) and hybridization conditions have been described by Somssich *et al.* (1986).

Analytical techniques. Total protein was measured by using the assay of Bradford (1976) with bovine serum albumin as the standard. Hexose sugars were assayed by the anthrone test (Dische 1962) with β-D-glucose used as the standard.

RESULTS

We have shown previously that parsley cells and protoplasts respond to protein components of the crude *P. m.* f. sp. *glycinea* wall elicitor whose activity does not depend on carbohydrate (Parker *et al.* 1988). We then attempted to purify a single active elicitor from this crude *P. m.* f. sp. *glycinea* wall preparation. For this purpose we developed a microassay to measure the response of parsley protoplasts to *P. m.* f. sp. *glycinea* elicitor in a total volume of 0.1 ml. This enables rapid fluorometric screening of active molecules during purification. Isolation of a single elicitor activity from the crude *P. m.* f. sp. *glycinea* wall preparation was not possible since the active proteins showed a disperse distribution on ion exchange, reverse-phase, and gel filtration chromatography columns (results not shown) and purification was not taken further.

Elicitor activity was also identified in the *P. m.* f. sp. *glycinea* culture filtrate. A large proportion of this activity had a molecular mass greater than 10 kDa, was sensitive to protease digestion, and was insensitive to chemical deglycosylation (results not shown). The total elicitor activity and amount of extracellular protein increased with *P. m.* f. sp. *glycinea* culture age up to the late log phase of growth (3–4 wk). We therefore harvested and concentrated culture filtrate material greater than 10 kDa from 3.5-wk-old cultures for further experiments. This is referred to henceforth as the crude culture filtrate.

Elicitor activity bound to DE-cellulose and was eluted as a discrete peak at approximately 0.2 M KCl. The activity profile correlated with the enrichment of a 42-kDa protein on SDS-polyacrylamide gels (Fig. 1, lane 2) and could be resolved into two separate peaks of activity on a gel filtration column (Fig. 2). The higher molecular weight peak (65–85 kDa) possessed a carbohydrate/protein ratio of 1:1 (w/w), did not contain the 42-kDa protein, and was poorly resolved on SDS-polyacrylamide gels (Fig. 1, lane 3). The lower molecular weight peak (25–35 kDa) had a carbohydrate/protein ratio of 0:4 (w/w) and contained the 42-kDa protein (Fig. 1, lane 4). The two activities were not interchangeable by rechromatography of each peak in

a higher salt buffer. We concentrated on the lower molecular weight peak, since it had the highest elicitor activity (see Fig. 2), which was possibly conferred by the 42-kDa protein. Further purification of this protein by chromatofocusing on an FPLC-Mono P column (Fig. 1, lane 5) and to homogeneity by hydrophobic interaction chromatography on Alkyl Superose (Fig. 1, lane 6) confirmed this. Approximately 300 μg of pure elicitor protein was obtained from 4 L of starting culture filtrate.

The pure 42-kDa protein elicited furanocoumarin synthesis in parsley protoplasts at very low concentrations (50% maximum at 4 ng/ml) and was at least 100 times more active than the crude culture filtrate or the *P. m.* f. sp. *glycinea* wall elicitor on a protein basis (Fig. 3). Its isoelectric point was determined as 5.2–5.4 by isoelectric focusing (results not shown). It is a glycoprotein (Fig. 4) that binds to concanavalin A on a protein blot (Fig. 4B) and contains 1.5% (w/w) anthrone-reactive sugar. We performed proteinase digestion and deglycosylation treatments of the pure glycoprotein to determine which portion is responsible for elicitor activity. Deglycosylation was conducted by chemical means with trifluoromethanesulfonic acid to destroy both N- and O-linked sugar chains (Sojar and Bahl 1987) and enzymatically using PNGase F, which cleaves N-linked high mannose-type sugars at the glycosylamine bond (Tarentino *et al.* 1985). Both treatments led to a shift in the mobility of the protein to approximately 40 kDa on an SDS-polyacrylamide gel (Fig. 4A) and a loss of concanavalin A reactivity of the blotted protein (Fig. 4B). The chemically deglycosylated protein was partially degraded, but the PNGase F-treated protein remained fully intact.

We experienced considerable difficulty in maintaining solubility of the deglycosylated protein after either treatment. Attempts to purify the enzymatically deglycosylated protein away from the PNGase F and the cleaved sugar moiety by anion exchange and concanavalin A affinity chromatography failed because of rapid loss of soluble protein. We therefore treated the glycoprotein with immobilized PNGase F in the presence of 0.1% (w/v) SDS. The processed protein was then separated from the sugar on a preparative SDS-polyacrylamide gel and eluted from the gel. The gel-purified and chemically deglycosylated protein and the proteinase-treated samples were tested in parsley protoplasts and cultured cells with starting concentrations of elicitor known to induce maximal furanocoumarin synthesis in the protoplasts (40 ng/ml protein,

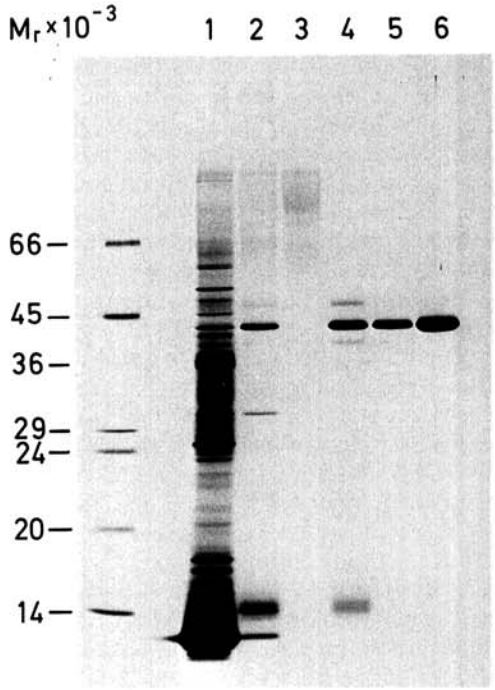

Fig. 1. Silver-stained sodium dodecyl sulfate-polyacrylamide gel showing stages in the purification of a 42-kDa glycoprotein elicitor from *Phytophthora megasperma* f. sp. *glycinea* culture filtrate. Lane 1, 2 μg of crude culture filtrate; lane 2, 1 μg of DE-cellulose eluate; lane 3, 0.6 μg of high molecular mass fraction from FPLC-Superose 12 column; lane 4, 0.3 μg of low molecular mass fraction from FPLC-Superose 12 column; lane 5, 0.3 μg of FPLC-Mono P eluate; and lane 6, 0.6 μg of FPLC-Alkyl Superose eluate.

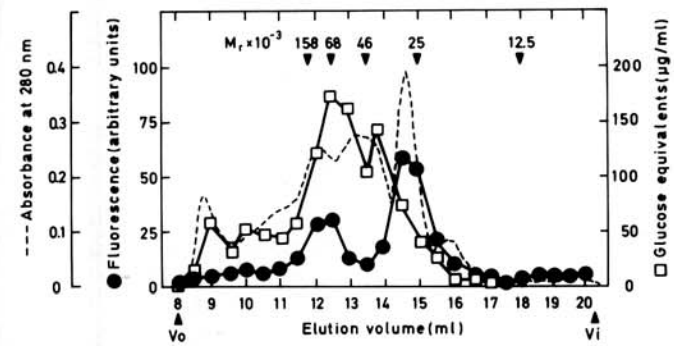

Fig. 2. FPLC-Superose 12 fractionation of elicitor-active *Phytophthora megasperma* f. sp. *glycinea* culture filtrate material eluted from a DE-cellulose column. Elicitor activity (●) in each fraction was determined by measuring fluorescence at 410 nm 24 hr after addition to 0.1 ml of a parsley protoplast suspension in a microtiter dish. The positions of molecular mass markers are shown. V_0 = void volume, V_i = inclusion volume.

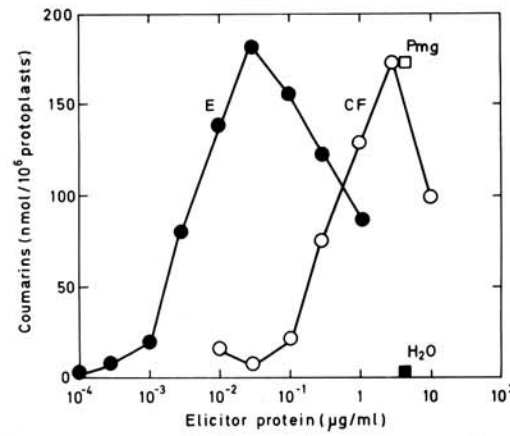

Fig. 3. Dose-response curve for *Phytophthora megasperma* f. sp. *glycinea* culture filtrate elicitor in parsley protoplasts. Increasing amounts of pure 42-kDa glycoprotein (E) or crude culture filtrate (CF) were added to protoplasts and coumarins extracted from the medium after 24 hr as a measure of elicitor activity. Results are from two separate experiments using two different elicitor preparations. Pmg = 5 μg/ml of *P. m.* f. sp. *glycinea* crude wall elicitor protein.

see Fig. 3). The results in Table 1 show that activity of the 42-kDa glycoprotein was unaffected by autoclaving the elicitor before treatment of both parsley protoplasts and cells. Treatment with the proteinases pronase and trypsin destroyed the activity in both protoplasts and cells, whereas enzymatic or chemical deglycosylation had no effect. Activities of the crude *P. m.* f. sp. *glycinea* culture filtrate and the crude wall elicitor were also tested at maximal concentrations established for parsley protoplasts (5 μg/ml protein, see Fig. 3). Both preparations elicited higher levels of furanocoumarins than the 42-kDa glycoprotein in cells but not in protoplasts (Table 1). The difference between the crude preparations and the purified elicitor was even more dramatic when tested in leaf disks. Parsley leaves synthesized equal amounts of furanocoumarin phytoalexins in response to injection of equivalent amounts (on a protein basis) of crude wall or culture filtrate elicitor. However, no coumarins were detectable after treatment with autoclaved or nonautoclaved pure glycoprotein elicitor at concentrations between 4 ng/ml and 4 μg/ml (results not shown).

We analyzed, by thin-layer chromatography, the qualitative pattern of furanocoumarins in the medium of parsley protoplasts and cells 24 hr after the addition of the pure 42-kDa elicitor, the crude *P. m.* f. sp. *glycinea* culture filtrate, or the *P. m.* f. sp. *glycinea* wall elicitor. The 42-kDa protein elicited the full complement of furanocoumarin products (Fig. 5). In cells the relative proportions of individual furanocoumarins differed between cultures treated with the pure elicitor and those treated with the crude *P. m.* f. sp. *glycinea* preparations (Fig. 5). Cells treated with the pure glycoprotein excreted mainly the coumarin umbelliferone, which is a precursor of furanocoumarins, but only low amounts of furanocoumarins.

To determine if the purified elicitor induces the same set of genes that are activated by the crude cell wall preparation, we compared their efficiencies in stimulating the accumulation of mRNA of elicitor-responsive genes previously isolated from cultured parsley cells (Somssich *et al.* 1986, 1989). These comprised mRNAs encoding 4-coumarate:CoA ligase of general phenylpropanoid metabolism, *S*-adenosyl-L-methionine:bergaptol *O*-methyl transferase specifically of the furanocoumarin biosynthetic pathway, and selected elicitor-induced mRNAs of unknown function (pathogenesis-related proteins 1 and 2 and elicitor-responsive genes 5 and 12). Total RNA was extracted from protoplasts at intervals up to 24 hr after the addition of the pure 42-kDa elicitor or crude *P. m.* f. sp. *glycinea* wall elicitor, and RNA dot blots were probed with the respective ^{32}P-labeled cDNAs. Results showed that the hybridizable levels of *P. m.* f. sp. *glycinea* wall elicitor-inducible mRNA species were also induced by the pure

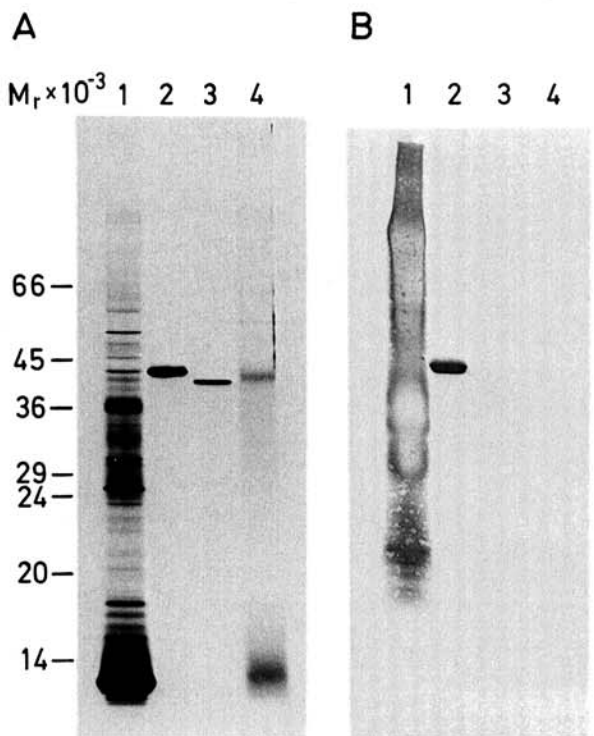

Fig. 4. A, Silver-stained sodium dodecyl sulfate-polyacrylamide gel, and **B,** protein blot probed with concanavalin A of 2 μg (**A**) and 20 μg (**B**) of *Phytophthora megasperma* f. sp. *glycinea* crude culture filtrate (lane 1), and 0.2 μg (**A**) and 1 μg (**B**) of untreated *Phytophthora megasperma* f. sp. *glycinea* 42-kDa glycoprotein (lane 2), PNGase F-treated 42-kDa glycoprotein (lane 3), and chemically deglycosylated 42-kDa glycoprotein (lane 4).

Table 1. Effect of proteinase and deglycosylation treatments on the *Phytophthora megasperma* f. sp. *glycinea* 42-kDa glycoprotein elicitor before its addition to parsley protoplasts and cells[a]

	Phytoalexin accumulation (% of maximum)[c]	
Sample[b]	**Protoplasts**	**Cells**
Elicitor only	100 (128)[d]	100 (710)[d]
Nonautoclaved elicitor	89	98
Buffer only	0	0
Pronase + elicitor	0	0
Autoclaved pronase + elicitor	95	95
Pronase only	0	0
Trypsin + elicitor	0	0
Autoclaved trypsin + elicitor	103	79
Trypsin only	2	0
Chemically deglycosylated elicitor	110	115
PNGase F-deglycosylated elicitor	56	82
Gel elution buffer + elicitor	59	111
Gel elution buffer only	0	0
P. m. f. sp. *glycinea* crude culture filtrate	96	150
P. m. f. sp. *glycinea* crude wall elicitor	114	151

[a] The response was measured by coumarin accumulation in the culture medium 24 hr after the application of 40 ng/ml of pure glycoprotein and 5 μg/ml of protein from each of the *P. m.* f. sp. *glycinea* crude culture filtrate and crude wall elicitors.

[b] All samples were autoclaved unless stated otherwise. The buffer was PBS (10 mM potassium phosphate, pH 6.5, 0.15 M NaCl). Deglycosylated samples contained amounts of protein equivalent to the untreated elicitor. The PNGase F-treated protein was purified through a sodium dodecyl sulfate (SDS)-polyacrylamide gel and eluted in PBS containing 0.1% (w/v) SDS (gel elution buffer). For proteinase digestions, the elicitor was treated with an equal weight of pronase or trypsin. Samples were then autoclaved to inactivate the proteinases.

[c] Results are from two separate experiments using two different elicitor preparations.

[d] Values in parentheses are amounts of furanocoumarins in nanomoles per 10^6 protoplasts and nanomoles per gram fresh weight of cells.

42-kDa protein (Fig. 6). It is noted that the degree of mRNA accumulation was lower in the 42-kDa elicitor-treated protoplasts and the maximum several hours later than in protoplasts treated with the crude elicitor. However, furanocoumarin production after 24 hr was similar in both treatments (187 nmoles/10^6 protoplasts with *P. m.* f. sp. *glycinea* crude wall elicitor, 158 nmoles/10^6 protoplasts with the 42-kDa glycoprotein). High mRNA levels of constitutively expressed gene 2 and very low amounts of UV light-inducible chalcone synthase mRNA were demonstrated in all mRNA samples.

Polyclonal antisera were raised in rabbits against both the pure 42-kDa glycoprotein and its carbohydrate-free protein portion. Both antisera recognized the 42-kDa glycoprotein as well as the deglycosylated elicitor on western blots and showed no cross-reaction with parsley proteins (Fig. 7). The antiserum raised against the complete glycoprotein cross-reacted with many components from *P. m.* f. sp. *glycinea*, whereas that directed against the deglycosylated protein was monospecific. The latter serum was used to detect the elicitor protein in fractionated extracts from *P. m.* f. sp. *glycinea* mycelia (Fig. 8). No elicitor was detectable in soluble cell fractions, whereas all cell wall extracts contained the glycoprotein, although in variable amounts. The most efficient method for extraction was treatment of *P. m.* f. sp. *glycinea* cell walls with 3 M guanidine hydrochloride.

The same antiserum detected cross-reacting cell-wall proteins of slightly smaller apparent molecular mass in *P. n.* var. *parasitica* and *P. parasitica*, whereas no cross-reaction was found with cell-wall extracts from *A. carthami*, *F. solani*, *P. infestans*, *R. secalis*, and *S. sclerotiorum* (Fig. 9). Identical results were obtained with culture filtrates of these fungi (results not shown).

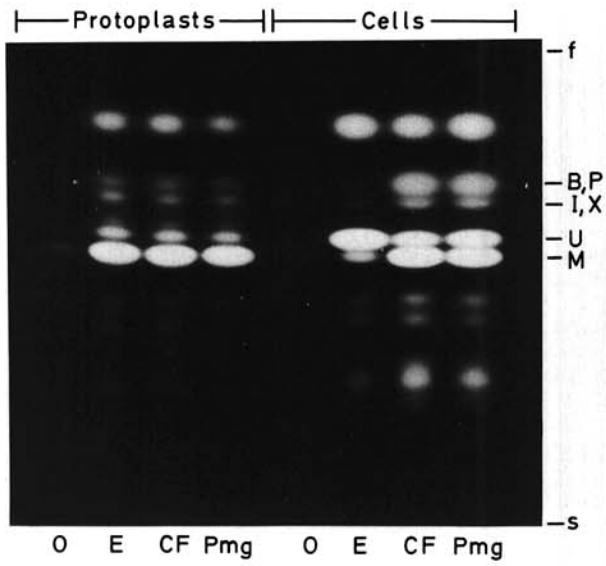

Fig. 5. Thin-layer chromatography of coumarin derivatives from the media of parsley protoplasts and suspension-cultured cells after 24 hr of incubation with water (0), 40 ng/ml of *Phytophthora megasperma* f. sp. *glycinea* 42-kDa glycoprotein elicitor (E), and 5 μg/ml each of *P. m.* f. sp. *glycinea* crude culture filtrate protein (CF) and *P. m.* f. sp. *glycinea* crude wall elicitor protein (Pmg). Protoplasts (4 ml) were treated with 100- and cultured cells (20 ml) with 500-μl volumes of water or the respective elicitor solutions to give final protein concentrations as indicated. Co-chromatography of authentic compounds is indicated (B = bergapten, P = psoralen, I = isopimpinellin, X = xanthotoxin, U = umbelliferone, M = marmesin, s = start, and f = front).

DISCUSSION

Elicitor preparations of varying degrees of purity have been valuable tools in determining the biochemistry and molecular biology of induced plant defense reactions in systems of reduced complexity (Ebel 1986). The isolation of individual elicitor-active molecules requires a simple assay for the detection of elicitor activity. Representative components of the defense response, such as development of local necrosis or accumulation of phytoalexins, have been successfully applied for this purpose (for review see Ebel and Scheel, in press).

We have identified and purified a 42-kDa glycoprotein from the *P. m.* f. sp. *glycinea* culture filtrate, which stimulates furanocoumarin synthesis in parsley protoplasts and cultured cells at very low concentrations. Western blot analysis clearly shows that the glycoprotein is a constituent

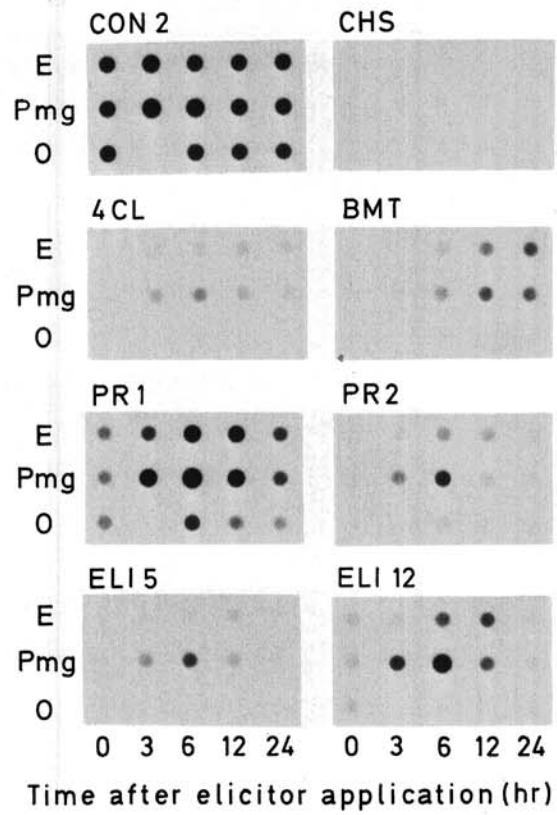

Fig. 6. Dot blot time course analysis of accumulation of mRNAs in parsley protoplasts after treatment with 40 ng/ml of *Phytophthora megasperma* f. sp. *glycinea* 42-kDa glycoprotein (E), 5 μg/ml of *P. m.* f. sp. *glycinea* crude wall elicitor protein (Pmg), or water (0). Protoplasts (4 ml) were treated with 100 μl water or elicitor solutions, respectively. One microgram of total RNA was loaded in each sample and the blots probed with ^{32}P-labeled cDNA probes as indicated (CON2 = constitutively expressed gene 2, CHS = chalcone synthase, 4CL = 4-coumarate:CoA ligase, BMT = *S*-adenosyl-L-methionine:bergaptol *O*-methyltransferase, PR1 and PR2 = pathogenesis-related proteins 1 and 2, and ELI5 and ELI12 = elicitor-responsive genes 5 and 12).

of the fungal cell wall, which is released into the culture medium during fungal growth. Although elicitor activity is conferred solely by the protein portion, the N-linked carbohydrate moiety may be important in protein conformation and stability, since deglycosylation leads to a loss of soluble protein. In addition, the sugar may have vital recognition or extracellular targeting functions in the fungus.

Only a few descriptions of purified proteinaceous elicitors exist in the literature (for review see Ebel and Scheel, in press). They can be divided into two classes. One group consists of pathogen-derived enzymes that release endogenous elicitors from plant cell walls (Darvill and Albersheim 1984). The second class comprises non-enzymatic elicitors that act directly on the plant cell by an unknown mechanism. No consensus structure appears to exist among these molecules, which elicit development of necrosis or phytoalexin accumulation in different plants (Scheel and Parker 1990; Ebel and Scheel, in press). The 42-kDa glycoprotein isolated from *P. m.* f. sp. *glycinea* is likely to belong to the second group.

Our previous results established that proteins and not carbohydrates in the crude *P. m.* f. sp. *glycinea* wall elicitor are active in parsley protoplasts and cells (Parker *et al.* 1988). However, our attempts to purify a single active component from the crude preparation were unsuccessful. This may be due to the rigorous treatment of the wall fraction (Ayers *et al.* 1976), which could lead to considerable protein breakdown with many different degradation products retaining some elicitor activity in parsley. Extraction of the cell wall with guanidine hydrochloride, for example, released the intact 42-kDa glycoprotein, which was not detectable on western blots of the crude wall elicitor. The *P. m.* f. sp. *glycinea* culture filtrate was processed in a much gentler fashion under conditions that minimized protein degradation and has proven to be a more suitable starting material for purification of a single active elicitor molecule. It is interesting in this context that elicitor activity of the pure 42-kDa glycoprotein as well as the crude *P. m.* f. sp. *glycinea* wall and culture filtrate preparations was not reduced by autoclaving. The 42-kDa glycoprotein formed stable high molecular weight aggregates that retained activity (J. E. Parker and D. Scheel, unpublished results). This is so far unexplained but suggests that elicitor activity is a property of amino acid sequence rather than native structure.

We assume that the primary target site of the elicitor lies on the plant plasma membrane, since freshly prepared protoplasts are fully responsive (Dangl *et al.* 1987). The glycoprotein, therefore, seems to be able to penetrate the plant cell wall as an intact molecule, as has been demonstrated for even larger molecules (Tepper and Taylor 1981), unless it is partially cleaved by plant proteinases before contact.

The 42-kDa glycoprotein is an extracellular component of *P. m.* f. sp. *glycinea*. Antibodies against this protein detected proteins of similar size in mycelial cell walls of two other *Phytophthora* species. Therefore, the glycoprotein is probably not unique to *P. m.* f. sp. *glycinea* but is by no means common to all fungal cell walls, since it was not present in cell walls of several other phytopathogenic fungi that contained potent elicitors of phytoalexin

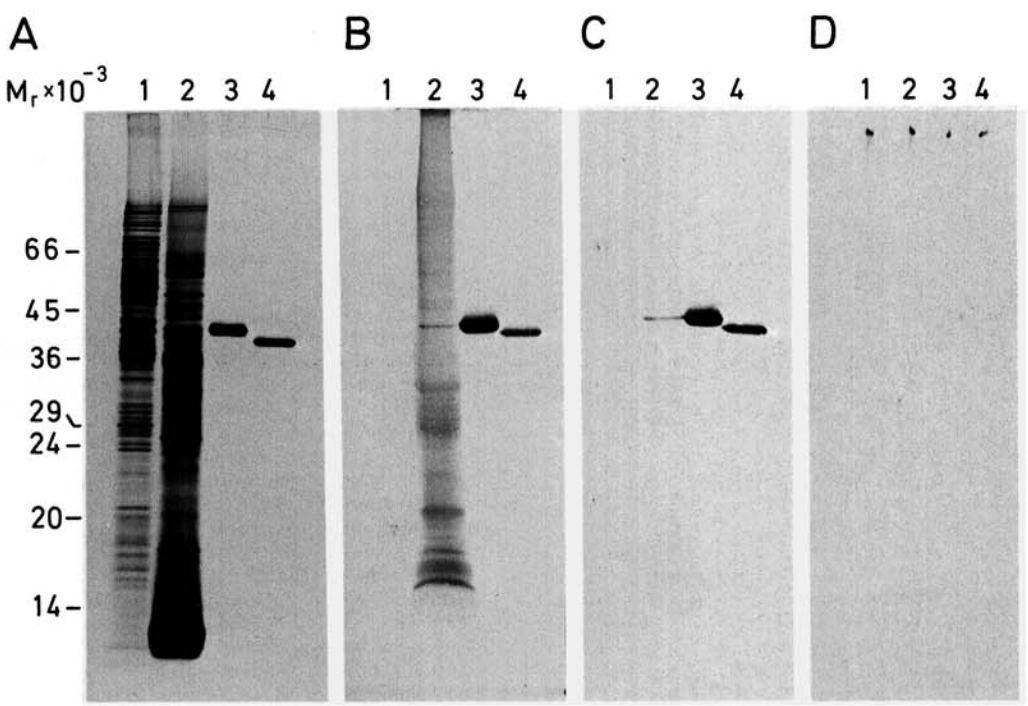

Fig. 7. A, Silver-stained sodium dodecyl sulfate-polyacrylamide gel, and **B–D,** protein blots of a crude protein extract from cultured parsley cells (lane 1, 2 μg in **A**; 20 μg in **B–D**), crude *Phytophthora megasperma* f. sp. *glycinea* culture filtrate (lane 2, 2 μg in **A**; 20 μg in **B–D**), untreated (lane 3, 0.2 μg in **A**; 1 μg in **B–D**), and PNGase F-treated *P. m.* f. sp. *glycinea* 42-kDa glycoprotein (lane 4, 0.2 μg in **A**; 0.5 μg in **B–D**). The blots were probed with polyclonal antisera raised either against the intact (**B**) or the enzymatically deglycosylated 42-kDa glycoprotein (**C**), or with preimmune serum (**D**).

accumulation in parsley (Scheel *et al.* 1989).

The 42-kDa glycoprotein is not the only *P. m.* f. sp. *glycinea* culture filtrate elicitor of furanocoumarin accumulation in parsley protoplasts. A more disperse, higher molecular mass fraction was identified, which we did not study further. It is noted, however, that the crude elicitor preparations induced higher levels of furanocoumarins in the cultured cells than the pure elicitor, while both stimulated the formation of similar amounts of phytoalexins in protoplasts. Moreover, parsley leaves responded to the crude elicitors but not to the pure glycoprotein. The intact cells and the leaves may react to components of the crude material that have no effects in equivalent concentrations on the protoplasts or are even deleterious for them. It is also possible that molecules removed from the pure preparation facilitate penetration of the plant cell wall by the pure elicitor, although this has not been tested.

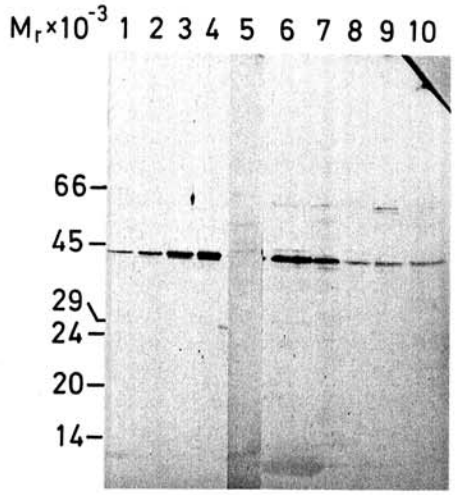

Fig. 8. Protein blot of a sodium dodecyl sulfate-polyacrylamide gel of a soluble *Phytophthora megasperma* f. sp. *glycinea* cell extract (lane 5), *P. m.* f. sp. *glycinea* culture filtrate (lane 6), and different mycelial cell-wall extracts from *P. m.* f. sp. *glycinea* (lane 7, 3 M guanidine hydrochloride; lane 8, 1 M guanidine hydrochloride; lane 9, 3 M LiCl; lane 10, 1 M LiCl) probed with a polyclonal antiserum raised against the enzymatically deglycosylated 42-kDa glycoprotein from *P. m.* f. sp. *glycinea*. Equal amounts of protein (15 μg) were applied to lanes 5–10. Increasing amounts of the pure 42-kDa glycoprotein were loaded in lanes 1–4 (lane 1, 50 μg; lane 2, 100 μg; lane 3, 200 μg; lane 4, 300 μg).

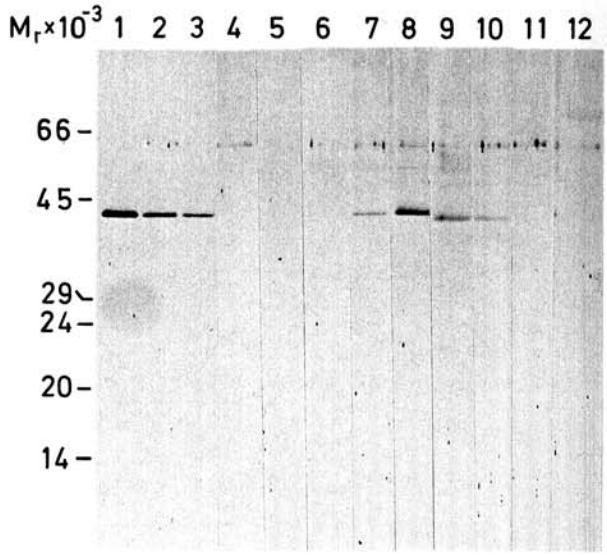

Fig. 9. Protein blot of a sodium dodecyl sulfate-polyacrylamide gel of cell-wall extracts (3 M guanidine hydrochloride) from mycelia of different phytopathogenic fungi probed with a polyclonal antiserum raised against the enzymatically deglycosylated 42-kDa glycoprotein from *Phytophthora megasperma* f. sp. *glycinea*. Decreasing amounts of the pure 42-kDa glycoprotein were loaded in lanes 1–3 (lane 1, 200 ng; lane 2, 100 ng; lane 3, 50 ng). Equal amounts of protein (15 μg) were applied to lanes 4–12, which had been extracted from mycelial cell walls of *Alternaria carthami* (lane 4), *Fusarium solani* (lane 5), *P. infestans*, race 4 (lane 6), *P. m.* f. sp. *glycinea*, race 1 (lane 7), *P. m.* f. sp. *glycinea*, race 3 (lane 8), *P. nicotianae* var. parasitica (lane 9), *P. parasitica* (lane 10), *Rhynchosporium secalis* (lane 11), and *Sclerotinia sclerotiorum* (lane 12).

The stimulation of furanocoumarin synthesis in parsley by the crude *P. m.* f. sp. *glycinea* wall elicitor is due to specific transcriptional activation of the genes encoding biosynthetic enzymes (Chappell and Hahlbrock 1984). This process is always accompanied by increased run-on transcription rates of a typical set of defense-related genes of unknown function (Somssich *et al.* 1986, 1989). The accumulation of the respective mRNAs in parsley protoplasts in response to treatment with the pure glycoprotein elicitor suggests that the whole set of defense-related genes can be activated by one elicitor molecule. A single recognition event at the cell surface may either initiate multiple secondary signals, specifically switching on different genes, or induce a unique transduction process that activates all elicitor-responsive genes. The pure elicitor may now be used as a ligand to identify and isolate its target site(s) on the plant cell surface. It also provides a means by which possible early changes in plasma membrane potential (Pelissier *et al.* 1986), cytosolic pH (Strasser *et al.* 1983; Ojalvo *et al.* 1987), and ion fluxes (Kurosaki *et al.* 1987; Stäb and Ebel 1987; Scheel *et al.* 1989) can be unambiguously associated with elicitation of the defense response.

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LITERATURE CITED

Ayers, A. R., Ebel, J., Valent, B., and Albersheim, P. 1976. Host-pathogen interactions X. Fractionation and biological activity of an elicitor isolated from the mycelial walls of *Phytophthora megasperma* var. *sojae*. Plant Physiol. 57:760-765.

Blum, H., Beier, H., and Gross, H. J. 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8:93-99.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Burnette, N. 1981. "Western blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. 112:195-203.

Chappell, J., and Hahlbrock, K. 1984. Transcription of plant defense genes in response to UV light or fungal elicitor. Nature (London) 311:76-78.

Dangl, J. L., Hauffe, K. D., Lipphardt, S., Hahlbrock, K., and Scheel, D. 1987. Parsley protoplasts retain differential responsiveness to u.v. light and fungal elicitor. EMBO J. 6:2551-2556.

Darvill, A. G., and Albersheim, P. 1984. Phytoalexins and their elicitors–A defense against microbial infection in plants. Annu. Rev. Plant Physiol. 35:243-275.

Dische, Z. 1962. General color reactions. Methods Carbohydr. Chem. 1:478-512.

Dixon, R. A., and Lamb, C. J. 1990. Molecular communication in interactions between plants and microbial pathogens. Annu. Rev. Plant Physiol. Plant Mol. Biol. 41:339-367.

Ebel, J. 1986. Phytoalexin synthesis: The biochemical analysis of the induction process. Annu. Rev. Phytopathol. 24:235-264.

Ebel, J., and Scheel, D. Elicitor recognition and signal transduction. In: Plant Gene Research. Genes Involved in Plant Defense, Vol. 8. T. Boller and F. Meins, eds. Springer, New York. In press.

Hahlbrock, K. 1975. Further studies on the relationship between the rates of nitrate uptake, growth and conductivity changes in the medium of plant cell suspension cultures. Planta 124:311-318.

Hahlbrock, K., and Scheel, D. 1987. Biochemical responses of plants to pathogens. Pages 229-254 in: Innovative Approaches to Plant Disease Control. I. Chet, ed. John Wiley & Sons, New York.

Hahlbrock, K., and Scheel, D. 1989. Physiology and molecular biology of phenylpropanoid metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. 40:347-369.

Hauffe, K. D., Hahlbrock, K., and Scheel, D. 1986. Elicitor-stimulated furanocoumarin biosynthesis in cultured parsley cells: S-adenosyl-L-methionine:bergaptol and S-adenosyl-L-methionine:xanthotoxol *O*-methyltransferases. Z. Naturforsch. 41c:228-239.

Hawkes, R. 1982. Identification of concanavalin A-binding proteins after sodium dodecyl sulfate-gel electrophoresis and protein blotting. Anal. Biochem. 123:143-146.

Henninger, H. 1963. Zur Kultur von *Phytophthora infestans* auf vollsynthetischen Nährsubstanzen. Z. Allg. Mikrobiol. 3:126-135.

Jahnen, W., and Hahlbrock, K. 1988. Cellular localization of nonhost resistance reactions of parsley (*Petroselinum crispum*) to fungal infection. Planta 173:197-204.

Kauss, H., Waldmann, T., Jeblick, W., Euler, G., Ranjeva, R., and Domard, A. 1989. Ca^{2+} is an important but not the only signal in callose synthesis induced by chitosan, saponins and polyene antibiotics. Pages 107-116 in: Signal Molecules in Plants and Plant-Microbe Interactions. B. J. J. Lugtenberg, ed. Springer, Berlin.

Keen, N. T. 1975. Specific elicitors of plant phytoalexin production: Determinants of race specificity in pathogens? Science 187:74-75.

Kombrink, E., and Hahlbrock, K. 1986. Responses of cultured parsley cells to elicitors from phytopathogenic fungi. Plant Physiol. 81:216-221.

Kurosaki, F., Tsurusawa, Y., and Nishi, A. 1987. The elicitation of phytoalexins by Ca^{2+} and cyclic AMP in carrot cells. Phytochemistry 26:1919-1923.

Muilerman, H. G., Ter Hart, H. G. J., and Van Dijk, W. 1982. Specific detection of inactive enzyme protein after polyacrylamide gel electrophoresis by a new enzyme-immunoassay method using unspecific antiserum and partially purified active enzyme: Application to rat liver phosphodiesterase I. Anal. Biochem. 120:46-51.

Ojalvo, I., Rokem, J. S., Navon, G., and Goldberg, I. 1987. ^{31}P NMR study of elicitor treated *Phaseolus vulgaris* cell suspension cultures. Plant Physiol. 85:716-719.

Parker, J. E., Hahlbrock, K., and Scheel, D. 1988. Different cell-wall components from *Phytophthora megasperma* f. sp. *glycinea* elicit phytoalexin production in soybean and parsley. Planta 176:75-82.

Pelissier, B., Thibaud, J. B., Grignon, C., and Esquerré-Tugayé, M. T. 1986. Cell surfaces in plant-microorganism interactions. VII. Elicitor preparations from two fungal pathogens depolarize plant membranes. Plant Sci. 46:103-109.

Pringle, R. B., and Sheffer, R. P. 1963. Purification of the selective toxin of *Periconia circinata*. Phytopathology 53:785-789.

Rigby, P. W. J., Dieckmann, M., Rhodes, C., and Berg, P. 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. 113:237-251.

Rohwer, F., Fritzemeier, K.-H., Scheel, D., and Hahlbrock, K. 1987. Biochemical reactions of different tissues of potato (*Solanum tuberosum*) to zoospores or elicitors from *Phytophthora infestans*. Planta 170:556-567.

Scheel, D., and Parker, J. E. 1990. Elicitor recognition and signal transduction in plant defense gene activation. Z. Naturforsch. 45c:569-575.

Scheel, D., Hauffe, K. D., Jahnen, W., and Hahlbrock, K. 1986. Stimulation of phytoalexin formation in fungus-infected plants and elicitor-treated cell cultures of parsley. Pages 325-331 in: Recognition in Microbe-Plant Symbiotic and Pathogenic Interactions. B. Lugtenberg, ed. Springer, Berlin.

Scheel, D., Dangl, J. L., Douglas, C., Hauffe, K. D., Hermann, A., Hoffmann, H., Lozoya, E., Schulz, W., and Hahlbrock, K. 1987. Stimulation of phenylpropanoid pathways by environmental factors. Pages 315-326 in: Plant Molecular Biology. D. V. Wettstein and N.-H. Chua, eds. Plenum, New York.

Scheel, D., Colling, C., Keller, H., Parker, J., Schulte, W., and Hahlbrock, K. 1989. Studies on elicitor recognition and signal transduction in host and non-host plant/fungus pathogenic interactions. Pages 211-218 in: Signal Molecules in Plants and Plant-Microbe Interactions. B. J. J. Lugtenberg, ed. Springer, Berlin.

Schmelzer, E., Krüger-Lebus, S., and Hahlbrock, K. 1989. Temporal and spatial patterns of gene expression around sites of attempted fungal infection in parsley leaves. Plant Cell 1:993-1001.

Sojar, H. T., and Bahl, O. P. 1987. A chemical method for the deglycosylation of proteins. Arch. Biochem. Biophys. 259:52-57.

Somssich, I. E., Schmelzer, E., Bollmann, J., and Hahlbrock, K. 1986. Rapid activation by fungal elicitor of genes encoding "pathogenesis-related" proteins in cultured parsley cells. Proc. Natl. Acad. Sci. USA 83:2427-2430.

Somssich, I. E., Bollmann, J., Hahlbrock, K., Kombrink, E., and Schulz, W. 1989. Differential early activation of defense-related genes in elicitor-treated parsley cells. Plant Mol. Biol. 12:227-234.

Stäb, M. R., and Ebel, J. 1987. Effects of Ca^{2+} on phytoalexin induction by fungal elicitor in soybean cells. Arch. Biochem. Biophys. 257:416-423.

Strasser, H., Tietjen, K. G., Himmelspach, K., and Matern, U. 1983. Rapid effect of an elicitor on uptake and intracellular distribution of phosphate in cultured parsley cells. Plant Cell Rep. 2:140-143.

Tarentino, A. L., Gomez, C. M., and Plummer, T. H. 1985. Deglycosylation of asparagine-linked glycans by peptide:*N*-glycosidase F. Biochemistry 24:4665-4671.

Tepper, M., and Taylor, I. E. P. 1981. The permeability of plant cell walls as measured by gel filtration chromatography. Science 213:761-763.

Tietjen, K. G., Hunkler, D., and Matern, U. 1983. Differential response of cultured parsley cells to elicitors from two non-pathogenic strains of fungi. I. Identification of induced products as coumarin derivatives. Eur. J. Biochem. 131:401-407.