Rapid Responses of Cultured Carrot Cells and Protoplasts to an Elicitor from the Cell Wall of *Pythium aphanidermatum* (Edson) Fitzp.

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Suspension cultured cells of an anthocyanin-containing cell line (DCb) from *Daucus carota* L. ssp. *sativus* respond very rapidly to treatment with a soluble carbohydrate elicitor from the wall of the oomycete *Pythium aphanidermatum* by synthesizing 4-hydroxybenzoic acid which is incorporated into the plant cell wall. Enzymes of phenol metabolism, phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS), respond to elicitor treatment in different ways. Both the catalytic activity of PAL and its subunit concentration, measured by means of immunoblotting, show a transient increase upon elicitation, whereas CHS the initial enzyme of the flavonoid pathway, is inhibited after administration of the elicitor, and consequently anthocyanin accumulation ceases. In protoplasts derived from the cultured cells a very similar elicitor-induced response has been observed. Lacking a cell wall, the protoplasts secrete the 4-hydroxybenzoic acid into the culture fluid. The carrot protoplasts isolated by wall degrading enzymes retain their responsiveness to the fungal carbohydrate elicitor. The value of the protoplast system for studying the immediate events following elicitor treatment is discussed.

Introduction

Plants respond actively to fungal and bacterial infection by a complex of defense reactions. Among these are the accumulation of soluble low molecular weight compounds with antimicrobial activity [1], and additionally these may be the accumulation of phenolics in the plant cell wall [2, 3]. Determination of the rate of synthesis of mRNAs encoding enzyme proteins involved in the respective biosynthetic pathways for these compounds, phenylalanine ammonia-lyase and chalcone synthase in *Glycine max* [4] and phenylanine ammonia-lyase and hydroxycinnamoyl: CoA ligase in potato [5], strongly supports the hypothesis that the *de novo* synthesis of phytoalexins or other host plant reactions such as the synthesis of "pathogenesis related proteins" [6] are regulated by temporal gene activation [7]. The defence response is triggered not only by components of microbial walls but also by substances in the culture fluid of pathogenic microorganisms [8]. Such responses of the intact parsley plants [1] are also shown by model systems such as cell cultures [9] or protoplasts [10].

Apart from exogenous elicitors there are endogenous elicitors released by microbial enzymes from the walls of higher plants [8, 11, 12]. A very interesting current question in the transduction of the elicitor signal to the accumulation of phytoalexins is the discrimination of the appropriate signal by the plant cell. There is little information on this aspect of host-pathogen interactions. On the one hand, protoplasts would appear to be excellent systems for the study of the initial part of the signal sequence but on the other hand, soybean protoplasts respond to the isolation procedure by synthesizing phytoalexins to such a high extent that the elicitor from *Phytophthora megasperma* is unable to stimulate further synthesis of phytoalexins [13]. Binding studies with soybean protoplasts and microsomal membrane fractions demonstrate the existence of membrane-bound receptor sites [14, 15]. It is not yet clear whether the primary receptor is at the plasma membrane or the elicitor is taken up by the cell and binds to an intracellular receptor [16]. In soybean [17] and carrot [18] Ca^{2+} acts as a "second messenger" in signal transduction to the genes encoding for enzymes necessary for phytoalexin biosynthesis.

In carrots the pathogen-related induction of compounds involved in defense response is well-known since a long time [19–22]. Besides the well known isocoumarin, 6-methoxymellein, various other compounds such as scopoletin, falcarinol, methoxypsora-

Abbreviations: BCIP, 5-bromo-4-chloro-indolylphosphate; BSA, bovine serum albumine; DMSO, dimethylsulfoxide; GA_3, gibberellic acid.

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len and 4-hydroxybenzoic acid are induced on infection and other environmental stresses. In the present communication we describe a model system suitable for studying signal transduction during an elicitor-induced biosynthesis of phenol derivatives. Suspension-cultured cells from carrot and protoplasts derived from these cells respond to elicitors from the oomycete *Pythium aphanidermatum* by synthesizing large amounts of 4-hydroxybenzoic acid which accumulates in the walls of suspension cultured cells or is released into the culture medium from protoplasts.

Materials and Methods

Chemicals

[2-^{14}C]malonyl-CoA was supplied by Amersham-Buchler (Braunschweig, F.R.G.). Goat-anti-rabbit IgG (conjugated with alkaline phosphatase), BCIP, BSA, malonyl-CoA, and trypsin were from Sigma (Munich, F.R.G.). Ponceau S, Evans Blue, and Dowex 1×2 (200–400 mesh, Cl^-) were obtained from Serva (Heidelberg, F.R.G.). Pronase E (from *Streptomyces*) was from Merck (Darmstadt, F.R.G.). Cellulase "Onozuka" R 10 was purchased from Yakult (Tokyo, Japan) and Rhozyme HP 150 from Pollock and Pool (Reading, U.K.). Calcofluor White ST was from Cyanamid (Bound Brook, U.S.A.) and Aqualuma from Baker Chemicals (Deventer, The Netherlands). 4-Coumaroyl-CoA was synthesized according to [23]. All other chemicals and solvents were of analytical grade and obtained from Merck (Darmstadt, F.R.G.). Antisera against PAL and CHS were gifts from Dr. K. Hahlbrock (Cologne, F.R.G.).

Buffers and solutions

The isolation and culture medium for protoplasts (PC-6) was based on the I,2a-medium [24] with 660 mg·l^{-1} $CaCl_2 \cdot 2H_2O$, 1.5 g·l^{-1} sucrose, and 4.5 g·l^{-1} mannitol and sorbitol respectively; it contained 1 mg·l^{-1} 2,4-dichlorophenoxy acetic acid [25].

Cell cultures

The anthocyanin-containing cell line DCb from *Daucus carota* L. spp. *sativus* was cultivated as previously described [26].

Protoplast isolation

The cell culture (seven days after inoculation) was passed through a steel sieve (1 mm mesh size) and the cells were collected on Miracloth (Calbiochem, La Jolla, U.S.A.). From the filtered cells 5 g were transferred to a 250 Erlenmeyer flask. To 1 g fresh weight 10 ml of the isolation medium (1.5% cellulase Onozuka R10, 0.4% Rhozyme HP 150, 0.1% BSA in PC-6-medium pH 5.6) [25] were added. The suspension was incubated for 6 h at 26 °C on a gyratory shaker (125 rpm). Undigested cell clusters were removed by filtration through nylon nets (140 and 60 μm). The protoplasts were concentrated by centrifugation at 100 × *g* for 5 min and washed three times with PC-6 in order to remove the isolation medium. Protoplasts in the purified suspension were counted in a Fuchs-Rosenthal hematocytometer. The suspension was adjusted to a cell titer of $0.5 \cdot 16^6 \cdot ml^{-1}$. The protoplasts were cultivated in portions of 12 ml in petri dishes at 26 °C in the dark.

Cell wall preparations from Pythium aphanidermatum

Mycelia were grown as a batch liquid culture in a Gamborg B5 medium [27] without growth regulators [28]. The crude elicitor fraction was prepared from mycelium (10 g fresh weight) of a nine week old batch culture according to Ayers *et al.* [29]. The remaining acetone-dried wall material was stored in a desiccator.

Cellulase treatment of the fungal wall and release of the elicitor

The crude wall preparation (100 mg) was frozen in liquid nitrogen and ground in a mortar. The powder was resuspended in 40 ml 5 mM phosphate buffer (pH 5.5) and 10 mg cellulase (Onozuka R10), dissolved in 10 ml of the same buffer, were added. After stirring for 5 h at 26 °C the enzymic reaction was terminated by boiling for 5 min. The digest was centrifuged at 1500 × *g* for 10 min, the supernatant carefully removed and concentrated to 8 ml under reduced pressure. The resulting concentrated supernatant was centrifuged at 1500 × *g* for 10 min. The supernatant containing the elicitor was stored at 4 °C.

Determination of carbohydrate content of elicitor and plant wall fractions

The carbohydrate concentration was determined by the phenol-sulfuric acid method [30]. Samples of 10–50 μl were brought to a final volume of 1 ml

thoroughly mixed with 25 µl of 80% phenol (w/v) and then 2.5 ml of conc. H_2SO_4 were carefully added. After 20 min at room temperature the optical density was measured at 490 nm. Glucose was used as a standard. The elicitor concentrations are expressed as glucose equivalents.

Enzyme extraction

a) From protoplasts. From each petri dish the protoplasts were harvested by centrifugation at $100 \times g$, 0.15 g Dowex 1×2 (equilibrated with 0.2 M phosphate buffer pH 8) and 2 ml phosphate buffer containing 50 mM sodium hydrosulfite were added. The mixture was treated for 30 s at 70 W in a micro-tip sonifier (Branson, Danbury, Connecticut, U.S.A.). After centrifugation at $40{,}000 \times g$ the supernatant was distributed to Eppendorf reaction vials (0.5 ml each) frozen in liquid nitrogen and stored at −70 °C.

b) Suspension cells. Cells (1 g fresh weight) were mixed in 3 ml phosphate buffer (s.a.) with 0.5 g Dowex 1×2 and treated in a sonifier for $2 \cdot 30$ s and centrifuged for 10 min at $20{,}000 \times g$. The supernatant (0.5 ml portions) was frozen and stored at −70 °C.

Enzyme assays

a) Chalcone synthase (CHS). CHS activity was determined according to [31]. The standard assay mixture contained 150 µl of 100 mM phosphate buffer (pH 7.5, 1.4 mM 2-mercaptoethanol, 1% (w/v) BSA, 5 µl (1 nmol) 4-coumaroyl-CoA, 5 µl [2-^{14}C]malonyl-CoA (1.87 nmol, 139,000 dpm) and 50 µl enzyme extract. The mixture was incubated at 30 °C for 30 min and the reaction terminated by adding 20 µg naringenin in 20 µl methanol. The assay mixture was extracted with 250 µl ethylacetate and 100 µl portions of the organic phase counted in Aqualuma.

b) Phenylalanine ammonia-lyase (PAL). The extractable activity was determined according to [32].

SDS-PAGE

Crude protein extracts were subjected to SDS-PAGE according to Laemmli [33] with a 6% stacking gel and a 10% separation gel. Each slot contained equal amounts of protein. The relative molecular masses were determined using the low molecular weight calibration kit from Pharmacia (Uppsala, Sweden).

Western blotting

The proteins were transferred electrophoretically to nitrocellulose as described by Towbin *et al.* [34]. The transfer was carried out in the vertical trans-blot cell from Bio-Rad Laboratories (Richmond, C.A., U.S.A.) for 12 h ($8\ V \cdot cm^{-1}$) in 20 mM Tris, 150 mM glycine, 20% (v/v) methanol.

Immunostaining

For immunostaining the blot was blocked with 5% BSA (dissolved in 50 mM Tris, 150 mM NaCl, 0.05 Tween 80, and 0.02% sodium azide) for 1 h (washing buffer). After rinsing with washing buffer the blot was incubated with 60 ml washing buffer containing 2 µl CHS antiserum and 1 µl PAL antiserum both raised against enzymes from parsley. After about 6 h the blot was washed and incubated for another 6 h in 60 ml of washing buffer containing 5 µl of the second antibody (goat anti-rabbit IgG conjugated with alkaline phosphatase). After washing, the blot was developed in 0.05% BCIP, which was predissolved in DMSO and adjusted to 14 ml with 1 M Tris-HCl, 1 mM $MgCl_2$, and 0.02% sodium azide (pH 8.8).

Measurement of 4-hydroxybenzoic acid and high-performance liquid chromatography

a) From walls of suspension-cultured cells. The culture fluid was removed under suction. The cells were homogenized in acetone by ultrasonication for 1 min and then allowed to stand for 30 min. The extract was filtered through GF/C filters (Whatman, Maidstone, U.K.). The remaining fragments were washed twice with 8 ml 70% ethanol ($6000 \times g$ for 10 min). For further purification these crude extracts were treated successively for 15 min with 5 ml each of 90% DMSO, 1 M NaCl, 0.1% SDS, methanol/chloroform (1 : 1) and finally with acetone. The material was dried in an air stream. Wall bound 4-hydroxybenzoic acid was released by saponification with 1 M NaOH (5 volume equivalents per 1 g fresh weight) at 25 °C overnight followed by acidification with HCl and extraction (0.5 ml per ml extract) with 1-butanol. After centrifugation at $16{,}000 \times g$ for 5 min the organic solvent was used for high-performance liquid chromatography.

b) Protoplasts. Ten ml of supernatant after removal of protoplasts by centrifugation at $100 \times g$ was freeze dried. The residue was suspended in 1.5 ml of 1 M NaOH and the mixture allowed to stand for 2 h.

After acidification with HCl the aqueous phase was extracted with 1-butanol. The organic phase was used for HPLC analysis.

c) Acetone extracts from suspension cells and protoplasts. Filtered acetone extracts (GF/C-Filters) were evaporated to dryness under reduced pressure. The residue was dissolved in 4 ml *n*-hexane and 4 ml of 2 M NaOH and centrifuged at $6000 \times g$. The *n*-hexane phase was discarded and 1 ml of conc. HCl was added. The solution was extracted once more with 4 ml *n*-hexane. The resulting aqueous phase was extracted with 1-butanol (3 volume equivalents per 1 g fresh weight). After centrifugation the 1-butanol phase was stored at −18 °C prior to analysis by high-performance liquid chromatography.

The extracts were analyzed using a ODS Hypersil reverse-phase column (Shandon) with a linear gradient of H_2O/acetic acid (95 : 5) and methanol ranging from 0–30% methanol over 20 min. Phenolic acids were detected on a LKB-Pharmacia HPLC at 280 nm. The flow rate was 2 ml · min^{-1} at 50 °C.

Protein determination

Protein was determined by Bradford's method [35] using BSA as a standard.

Calcofluor White ST and Evans Blue staining

Calcofluor White ST was used to follow wall regeneration by freshly isolated protoplasts.

In order to determine the viability of protoplasts they were stained with 0.1% of Evans blue dissolved in PC-6 and counted in a Fuchs-Rosenthal hematocytometer.

Results

Fungal elicitors induced changes in the catalytic activities of both phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) in anthocyanin-containing carrot cells (DCb) and derived protoplasts. PAL showed a rapid and transient increase in its specific catalytic activity (Fig. 1). Maximum activity was reached 8–12 h after administration of the elicitor either to the cells or protoplast suspensions. In both systems the maximum values were more or less equal and the response was dose dependent. The responses of PAL at two elicitor concentrations (10 and 100 μg · ml^{-1}), measured in terms of glucose equivalents, are shown in Fig. 1. In suspension-cultured cells the PAL activity did not change during the period whereas in protoplasts a slow but continuous increase was observed. This could be due to a slight stress reaction from the isolation procedure, nevertheless there is a significant measure in the enzyme activity on elicitor treatment.

Chalcone synthase exhibited a different response pattern. In cells the catalytic activity decreased during 12 h (Fig. 2) and as a consequence the anthocyanin content leveled down to a basic value (not shown). In the case of the protoplast system also a dose-dependent inhibition was observed. The maximum and minimum specific activities of CHS were again very similar in cell suspensions and protoplasts.

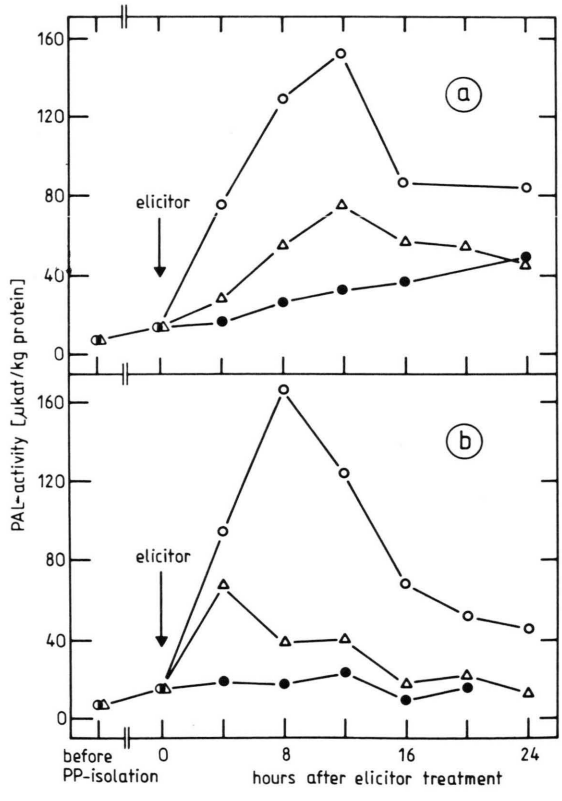

Fig. 1. Changes in the catalytic activity of phenylalanine ammonia-lyase in a) protoplasts and b) suspension-cultured cells of *Daucus carota* L. ssp. *sativus* ("black carrot") after treatment with an elicitor released from the wall of *Pythium aphanidermatum*. The elicitor was applied eight days after inoculation. Protoplasts were isolated from the same culture batch of suspension-cultured cells which were used in the experiment. The isolation procedure for protoplasts took place eight hours prior to the administration of the elicitor. ●——● Controls, △——△ elicitor (10 μg glucose equivalents/ml cell suspension), ○——○ elicitor (100 μg glucose equivalents/ml cell suspension).

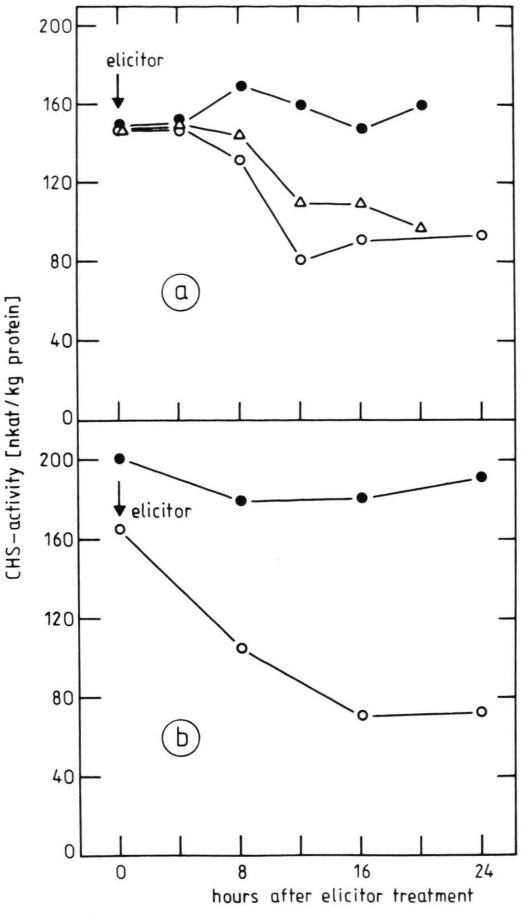

Fig. 2. Changes in catalytic activity of chalcone synthase in a) protoplasts and b) suspension-cultured cells after treatment with an elicitor from the cell wall of *Pythium aphanidermatum*. ●——● Controls, △——△ elicitor (10 µg glucose equivalents/ml cell suspension), ○——○ elicitor (100 µg glucose equivalents/ml cell suspension).

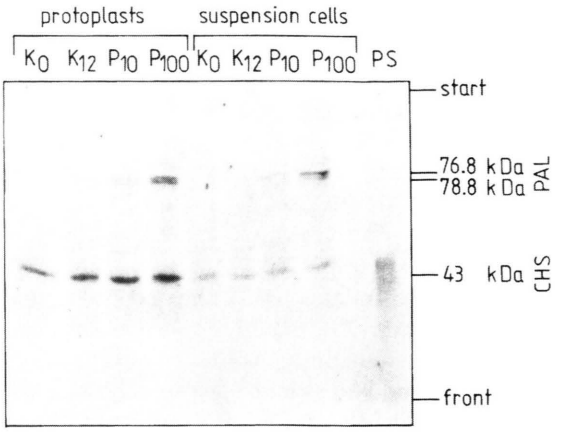

Fig. 3. Western blot analysis of PAL and CHS subunits from suspension-cultured cells and protoplasts derived from the same culture batch with cross-reactive antisera raised against parsley enzyme proteins. All samples contained the same amount of soluble protein. Protoplasts and suspension-cultured cells were treated with the elicitor 8 days after inoculation. The protein was extracted 12 h after administration of the elicitor with the only exception of K_0 which was taken at the end of isolation of the protoplasts. P_{10} = 10 µg glucose equivalents/ml cell suspension, P_{100} = 100 µg glucose equivalents/ml cell suspension, PS = subunits of CHS from parsley (43 kDa). For Western blotting and immunostaining see Materials and Methods. The relative molecular mass was estimated using the calibration kit from Pharmacia. The proteins were stained prior to the immunoreaction with Ponceau S.

Western blotting

Information on changes in the subunit concentrations of PAL and CHS in cells and protoplasts after treatment with the fungal elicitor was obtained by immunoblotting with cross-reactive antibodies for PAL and CHS from parsley cells. The subunits of PAL and CHS behaved in a different way (Fig. 3). 12 h after elicitor treatment (10 and 100 µg glucose equivalents per ml), PAL subunits showed a significant increase in concentration parallel to the changes in extractable enzyme activity (Fig. 1) in both intact cells and in protoplasts. There were two immunostained bands on the nitrocellulose membrane with relative molecular masses of 78.8 and 76.8 kDa. These may represent two isoenzymes of PAL, but it is also possible that the smaller polypeptide is a degradation product as discussed by Jones [36].

The immunostainable subunits of CHS co-migrated with CHS subunits from parsley with a relative molecular mass of 43 kDa. In spite of the decrease of the catalytic activity of the enzyme after elicitation the intensity of staining did not change.

Accumulation of 4-hydroxybenzoic acid

a) Cell cultures. Parallel to the transient increase of the extractable PAL activity in suspension cultured cells an intensive browning was visible which could be due to oxidation reactions of wall-bound phenolics as described by Vance *et al.* [2]. After saponification of the ester bonds of compounds such as phenols to wall polysaccharides 4-hydroxybenzoic acid was released. As shown in Fig. 4 an elicitor-induced accumulation of wall-bound phenols was ob-

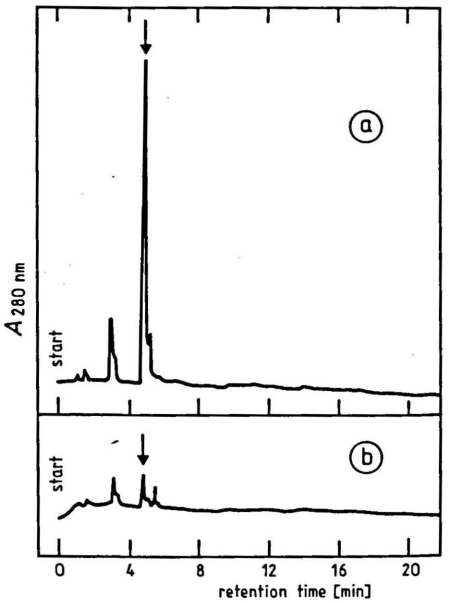

Fig. 4. Separation of extracts of wall-bound phenolic acids from a) elicitor-induced and b) control cells on high-performance liquid chromatography (ODS Hypersil, flow rate 2 ml·min^{-1}). 100 µg glucose equivalents/ml cell suspension were applied. Cells were harvested 24 h after elicitor treatment. The phenolic acids were released by saponification with 1 M NaOH and detected following HPLC at 280 nm. The position of 4-hydroxybenzoic acid is marked by an arrow.

served. The major component of this fraction was identified by co-chromatography on HPLC and thin-layer chromatography to be 4-hydroxybenzoic acid. The elution diagram showed no additional compounds absorbing at 280 nm. The time course of accumulated wall-bound 4-hydroxybenzoic acid is summarized in Fig. 5b. After a lag-phase of about 8 h the compound was steadily accumulated. In control cells the concentration of 4-hydroxybenzoic acid remained very low as measured in acetone extracts from the cells (Fig. 6b). However, the concentration of free 4-hydroxybenzoic acid was only doubled. This suggests that most of the 4-hydroxybenzoic acid was transferred to the cell wall after treatment of cells with the elicitor. The elicitor-mediated stimulation of the accumulation of the 4-hydroxybenzoic acid was clearly dose-dependent as was the catalytic activity of PAL and CHS.

b) Protoplasts. In contrast to cells protoplasts lacking a detectable wall secreted 4-hydroxybenzoic acid into the surrounding medium (Fig. 5a). The concentration of 4-hydroxybenzoic acid within elicitor-treated and control protoplasts was identical (Fig. 6a). Similar to the slight increase in PAL activity the control level of 4-hydroxybenzoic acid increased also slowly to a limited extent (Fig. 5a). This effect was due to stress of the protoplasts during isolation and possibly due to the release of an endogenous elicitor from the carrot wall during protoplasting.

Viability and wall regeneration

The elicitor had no influence on the viability of suspension cultured cells or protoplasts. Over a period of 24 h the viability determined by Evans blue staining was 92%. In addition to the viability the wall

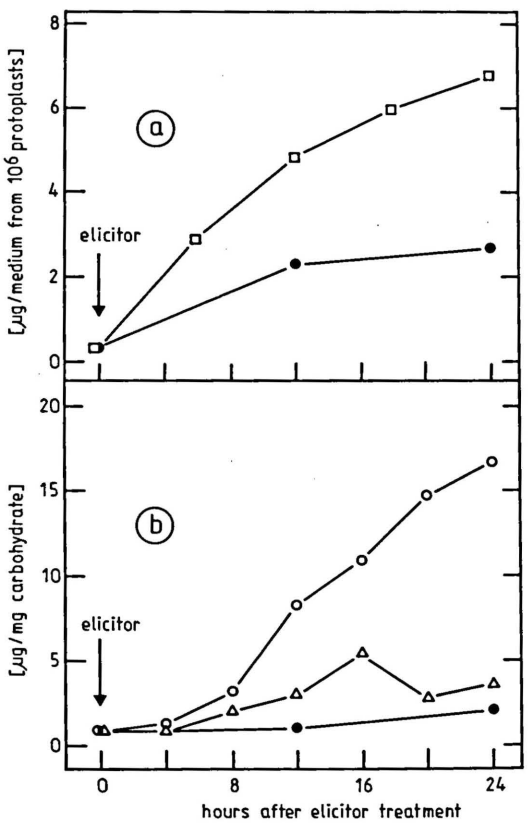

Fig. 5. Changes in 4-hydroxybenzoic acid content a) of the culture medium of protoplasts and b) the walls of suspension-cultured cells. The elicitor was applied eight days after inoculation. ●——● Control without elicitor, △——△ 10 µg glucose equivalents/ml cell suspension, ○——○ 100 µg glucose equivalents/ml cell suspension, □——□ 200 µg glucose equivalents/ml cell suspension.

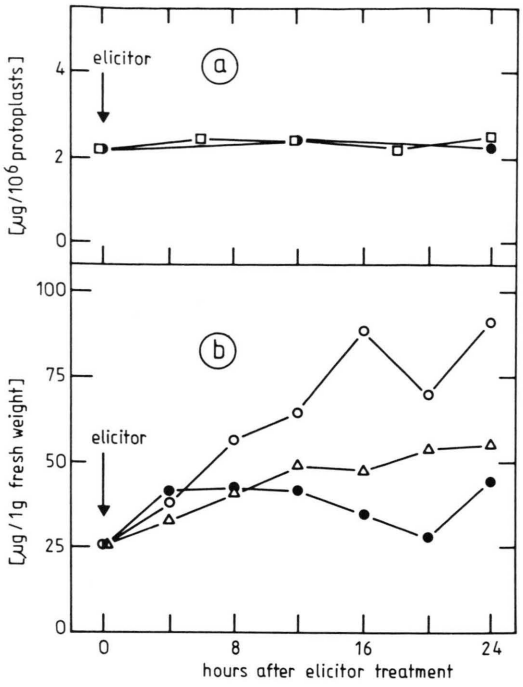

Fig. 6. 4-Hydroxybenzoic acid content in acetone extracts a) from protoplasts and b) suspension-cultured cells. Elicitor was added 8 days after inoculation. For HPLC analysis see Fig. 4. ●——● Control without elicitor, △——△ 10 µg glucose equivalents/ml cell suspension, ○——○ 100 µg glucose equivalents/ml cell suspension, □——□ 200 µg glucose equivalents/ml cell suspension.

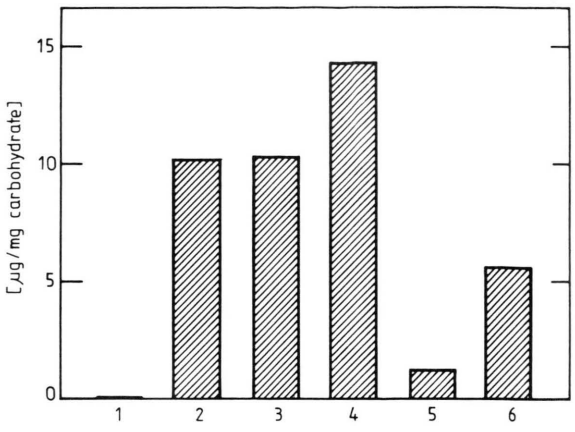

Fig. 7. Elicitor activity measured by release of 4-hydroxybenzoic acid from carrot walls with 1 M NaOH after treatment of the elicitor with protein-degrading enzymes (pronase E and trypsin). 1 = Buffer control, 2 = elicitor, 3 = elicitor + trypsin, 4 = elicitor + pronase, 5 = trypsin alone, 6 = pronase alone.

regeneration has been measured by Calcofluor White staining. Eight hours after the isolation of protoplasts a weak blue fluorescence was already observed. After 24 h nearly all protoplasts possessed a wall which can be stained by Calcofluor White. Another indicator of wall regeneration was the change from spherical shape of freshly isolated protoplasts to oval shaped cells.

Nature of the elicitor released from the wall of Pythium aphanidermatum

Wall fragments prepared by enzymic hydrolysis of the crude freeze-dried mycelial preparation were analyzed to ascertain whether the eliciting principle is a protein or a carbohydrate. Neither trypsin nor pronase E treatment (1.7 mg enzyme per mg glucose equivalents in phosphate buffer pH 7.5 for 5 h at 26 °C) influenced elicitor activity (Fig. 7). In addition after treatment with 25% TCA the elicitor remained soluble (data not shown). These observations are consistent with the hypothesis that the *P. aphanidermatum* elicitor is a carbohydrate.

Discussion

Carrot cell cultures and derived protoplasts respond in the same manner on treatment with an elicitor from the oomycete *Pythium aphanidermatum* with regard to extractable activities of PAL and CHS. Furthermore, the changes in the subunit patterns of both enzymes as analyzed by Western blots were also nearly identical in cells and protoplasts. Elicitor treatment results in an increase in PAL activity. This is a consequence of *de novo* synthesis as determined by puromycin inhibition [37]. Immunoblots of PAL displayed two stainable subunits (78.8 and 76.8 kDa, respectively). In contrast to the situation in legumes, where both enzymes are synthesized *de novo* upon elicitor treatment, CHS activity was inhibited in the carrot system. It is noteworthy that with regard to CHS a similar response is obtained if GA_3 is added to the culture medium [38]. In spite of the inhibition of the catalytic activity upon elicitor treatment or in the presence of GA_3, the staining intensity of the 43 kDa subunit of CHS did not change on Western blots [39]. This result strongly suggests a post-translational regulation of this CHS

species. In light-induced carrot cells, a second CHS form is present whose subunits have a relative molecular mass of 40 kDa; this CHS species seems to be regulated by its synthesis as determined by puromycin inhibition [40].

Cinnamic acid derivatives produced by PAL can be metabolized along at least two pathways. The flux along these pathways apparently changes under the influence of the *Pythium* elicitor. Anthocyanin biosynthesis was depressed whereas the general phenylpropanoid pathway is intensively increased. In parallel with the dramatic rise of PAL activity we observed an enhanced accumulation of 4-hydroxybenzoic acid which was incorporated into the wall. Carrot cells are known to respond to fungal infection by accumulating the fungitoxic 4-hydroxybenzoic acid [12, 21]. The fungitoxicity has been demonstrated by means of the *Botrytis cinerea* spore-biotest [21].

Accumulation of 4-hydroxybenzoic acid is a very good indicator of elicitor-induced metabolic events in suspension-cultured cells and protoplasts as well. In cell cultures this compound was incorporated into the walls, whereas protoplasts, lacking cell walls, secrete it into the culture medium. The role of trans-cinnamic acid intermediates in 4-hydroxybenzoic acid biosynthesis is not yet clear and the enzyme system involved remains to be investigated.

In parsley an elicitor derived from the wall of *Phytophthora megasperma* induces as the major compounds furanocoumarins after treatment of cell cultures and protoplasts. The active molecule from the fungal wall is a glycoprotein [41]. In cell cultures from the legume *Glycine max* the major elicitor-induced response is the biosynthesis of isoflavonoids such as glyceollin. In this system protoplasts in contrast to intact cells are unable to respond to exogenous elicitors since they are already induced to syntheses of phytoalexins following protoplast isolation so that the heptaglucoside [15] elicitor from *Phytophthora megasperma* is unable to stimulate further production of the phytoalexins [13]. These results with protoplasts have been explained by the release of endogenous elicitors from the host wall during enzymic digestion of the wall material.

The advantage of our carrot system is that intact cells and protoplasts respond in the same way to an exogenous elicitor like the parsley system. But with regard to the nature of the elicitor, we found similarities with the soybean system: several tests have shown that it may be a soluble carbohydrate. In contrast to soybean and carrot the active compound in the parsley system is a glycoprotein [41].

The difference between the soybean system at the one hand and parsley and carrot on the other hand might be explained by the different metabolic responses to exogenous and endogenous elicitors [42]. In carrot, pectinase treatment of the host cell wall leads to the accumulation of 6-methoxymellein an isocoumarin synthesized *via* the polyketide pathway, whereas wall fragments of the pathogen *Chaetomium globosum* induce the expression of the general phenylpropanoid pathway and the subsequent production of aromatic derivatives [42]. In our carrot system derived from the root of an Afghan cultivar of *Daucus carota* ("black carrot") both cells and protoplasts respond to exogenously applied elicitors. Thus *Daucus carota* offers an excellent system for studying the molecular mechanism of signal transduction from the plasma membrane to the nucleus.

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