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Cellular localization of nonhost resistance reactions of parsley (*Petroselinum crispum*) to fungal infection

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Abstract. The response of parsley seedlings (*Petroselinum crispum*) inoculated with zoospores of the soybean-pathogenic fungus, *Phytophthora megasperma* f. sp. *glycinea,* ranged from "immunity" to "physiological susceptibility" depending on the post-inoculation environmental conditions. Typical nonhost resistance reactions, hypersensitive cell death and the formation of small local lesions, occurred under high relative humidity and 16 h illumination per day. Localized biochemical reactions were monitored using fluorescence microscopy combined with histochemical and immunohistochemical methods. The rapid accumulation of furanocoumarin phytoalexins, wall-bound phenolics and callose was detected around infection sites. Indirect antibody staining of frozen tissue sections demonstrated the local accumulation of phenylalanine ammonia-lyase, a key enzyme of general phenylpropanoid metabolism, and S-adenosyl-L-methionine:bergaptol O-methyltransferase, a specific enzyme of the furanocoumarin pathway. The results indicate that phenylpropanoid derivatives are synthesized de novo at infection sites.

Key words: Callose – Cell wall (phenolics) – Furanocoumarin – *Petroselinum* – Phenylalanine ammonia-lyase – *Phytophthora* – S-adenosyl-L-methionine:bergaptol O-methyltransferase – Phytoalexin (localization).

Introduction

Plants resist microbial attack by preformed as well as rapidly activated defense mechanisms. "Active defense" reactions (Bell 1981) include cell-wall modifications and the accumulation of phytoalexins, lytic enzymes and pathogenesis-related proteins (Hahlbrock and Scheel 1987). Activation of defense reactions occurs in all disease-resistance responses irrespective of the plant-pathogen combination.

An important distinction is made between genetically defined host and nonhost-microbe interactions (Heath 1980). Depending on the genetic constitution of the two organisms, a host-microbe interaction can be compatible or incompatible. A nonhost-microbe interaction is incompatible by definition; the plant is resistant under normal conditions. Nonhost resistance is a highly appreciated trait in economically important plants. Limited hyphal growth, accumulation of phenolic compounds near the infection site and hypersensitive cell death are characteristic of many nonhost-fungus interactions (Fernandez and Heath 1986).

We have studied the biochemical responses of cultured parsley cells to treatments with crude or partially purified cell-wall preparations from *Phytophthora megasperma* f. sp. *glycinea*, a fungal pathogen of soybean. This elicitor preparation efficiently induced the accumulation of furanocoumarin phytoalexins (Tietjen et al. 1983; Hauffe et al. 1986) and other putative components of the disease-resistance response in parsley (Kombrink and Hahlbrock 1986). The system has been used for studies of elicitor-mediated gene activation and for the elucidation of the furanocoumarin-biosynthetic pathway.

Using elicitor-treated cell cultures, several probes have been generated for the in-situ localization of increased defense-related metabolic activities in parsley plants infected with *P. megasperma* (Kombrink et al. 1986; Hahlbrock et al. 1986; Scheel et al. 1986). In this study, antisera against two enzymes, phenylalanine ammonia-lyase (PAL, general phenylpropanoid metabolism) and S-adenosyl-L-methionine:bergaptol O-methyltransferase (BMT; furanocoumarin pathway), together with

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Abbreviations: BMT = S-adenosyl-L-methionine:bergaptol O-methyltransferase; PAL = phenylalanine ammonia-lyase; PBS = phosphate-buffered saline

an antiserum recognizing *P. megasperma* (see also Moesta and Grisebach 1983), were used to localize PAL, BMT and the fungus in infected parsley tissue. The additional localization of furanocoumarins and wall-bound phenolics enabled the detection of local changes in metabolite concentrations. This had not been possible using averaging extraction procedures on uninfected tissue already containing high levels of substances accumulating at infection sites (Knogge et al. 1987).

Material and methods

Plant. Parsley seeds (*Petroselinum crispum* cv. Hamburger Schnitt) were surface-sterilized in 70% ethanol for 2 min and in commercial bleach diluted fourfold for 20 min, rinsed four times in sterile distilled water and germinated on 0.6% aqueous agar (DIFCO, Detroit, Mich., USA) in the dark at 25° C. On day 7, seedlings were transferred to aseptic hydroponic culture and maintained under a 16-h light (10^5 lx, 20° C)/8-h dark (17° C) regime. Plants were fertilized with Levatit hydroculture fertilizer (Bayer, Leverkusen, FRG) and watered with autoclaved tap water.

Fungus. Phytophthora megasperma f. sp. *glycinea,* race 1, was grown on Lima bean agar (DIFCO) in the dark at 25° C. Zoospores were isolated from confluent 9-cm Petri-dish cultures. They were flooded four times with 20 ml sterile Petri salt solution (2.5 mmol·l^{-1} $Ca(NO_3)_2$, 0.6 mmol·l^{-1} $MgSO_4$, 1.1 mmol·l^{-1} KH_2PO_4, 0.8 mmol·l^{-1} KCl) over 3 h followed by 20 ml sterile distilled water for 15 min and further incubated in 10 ml sterile distilled water for 10 h. Zoospores released into the final solution were routinely counted with a hemocytometer after staining with acid fuchsin/lactophenol (Clark 1981).

Inoculation. Ten-day-old hydroculture plants, 17 d after sowing, were inoculated at different sites, without wounding, with small droplets of fungal zoospore suspension (5–10 µl, 10^3 zoospores) using a micropipette. High (100%) relative humidity was maintained post inoculation by covering the plant containers with a Plexiglas hood.

Microscopy. Light microscopy was performed with an upright or inverted microscope using bright-field, differential interference contrast or epifluorescence optics. Autofluorescence was observed in unfixed, uncovered specimens with Zeiss filter set 02 (ultraviolet), 18 (violet) or 09 (blue; Zeiss, Oberkochen, FRG).

Histochemical staining. Leaves were fixed, extracted with methanol and stained with decolorized Aniline Blue (Eschrich and Currier 1964) for fluorescence microscopy, or with Toluidine Blue at pH 4.4 (Feder and O'Brien 1968), phloroglucinol/HCl, chlorine/sulfite (Sherwood and Vance 1976) or Sudan III (Jensen 1960). Vital staining of the fungus was accomplished by mounting infected plant tissue in a solution of fluorescein diacetate (0.001% in 50 mmol·l^{-1} sodium-phosphate buffer, pH 7.4). Mounts were viewed under epifluorescence blue-light excitation.

Scanning electron microscopy. Pieces of tissue were fixed in formaldehyde (prepared from paraformaldehyde, 4% in 50 mmol·l^{-1} sodium phosphate, pH 7.4) for 2 h on ice, dehydrated through a graded ethanol series, transferred to acetone, critical-point-dried with carbon dioxide, gold-sputtered and viewed with a Cambridge Stereoscan Mark IV scanning electron microscope (Cambridge, U.K.) at 20 kV acceleration voltage.

Coumarins. Infection droplets were washed off by immersing the aerial parts of approx. 20 plants in 20 ml distilled water. Coumarin derivatives were extracted and analyzed according to published methods (Kombrink and Hahlbrock 1986).

Preparation of antisera. Zoospores of *P. megasperma* ($5 \cdot 10^5$ ml^{-1}) were heat-inactivated and mixed in equal parts with Freund's incomplete adjuvans. Two ml of the emulsion were injected subcutaneously into a rabbit. After four weeks the rabbit was booster-injected with 1 ml of the same mixture and bled 14 d later. The use of crude antisera for immunohistochemical studies often resulted in nonspecific staining. Therefore, all antisera were routinely assayed by protein-blot analysis and fractionated when necessary. The protein-A-binding fraction (mainly immunoglobulin G) of PAL antiserum (Schröder et al. 1976) was purified according to Miller and Stone (1978). Antiserum of BMT (K. D. Hauffe, Max-Planck-Institut für Züchtungsforschung, Köln, FRG) was fractionated by precipitation with $(NH_4)_2SO_4$ (1.6 mol·l^{-1}), and *P. megasperma* antiserum was used without further purification. Pre-immune serum and antisera against *P. megasperma*, PAL and BMT were tested for cross-reaction with buffer-soluble plant extracts by protein-blot analysis following the procedure of Burnette (1981). Protein bands were visualized by indirect peroxidase staining using the color reagent, 4-chloronaphthol (Merck, Darmstadt, FRG).

Immunofluorescence. Infected tissue was fixed in formaldehyde (4% in 50 mmol·l^{-1} sodium phosphate), incubated first with PBS (25 mmol·l^{-1} sodium phosphate, 150 mmol·l^{-1} NaCl, pH 7.4) containing 10% newborn calf serum (PBS/NCS) for 15 min at room temperature, then with *P. megasperma* antiserum (diluted 200-fold in PBS/NCS) at 37° C for 30 min, washed three times in PBS, 3 min each, and finally incubated with fluorescein-labelled secondary antibody (Dakopatts, Hamburg, FRG; diluted 50-fold in PBS/NCS) at 37° C for 20 min. After washing three times in PBS, 3 min each, the tissue was mounted in 90% (v/v) glycerol/PBS containing 0.1% *n*-propyl gallate to prevent fading of the fluorescence (Giloh and Sedat 1982).

Immunoperoxidase staining. Infected tissue was fixed in formaldehyde (see above) or in a mixture of formaldehyde (2%) and glutaraldehyde (0.25%) for 2 h on ice. The fixed samples were infiltrated with 0.3 mol·l^{-1} and 0.5 mol·l^{-1} sucrose for 1 h each, transferred to Tissue Tek O.C.T. embedding compound (Miles, München, FRG), placed in gelatine capsules, rapidly frozen in cold *n*-hexane (−80° C) and stored at −20° C. Frozen sections of 15 µm thickness were cut at −15° C using a cryostat (Frigocut 2800 E; Reichert-Jung, Nussloch, FRG) and lifted off the knife with pre-cooled, chromalaune-gelatine coated slides (Clark 1981). Frozen sections were thawed, air-dried and scanned for autofluorescent infection sites under a fluorescence microscope. The sections were fixed to the slides for 20 min (see above) and dehydrated in a graded ethanol series up to 80%. Endogenous peroxidase activity was blocked by a 20-min incubation with 3% H_2O_2 in 80% aqueous methanol. After rehydration of the sections, nonspecific protein binding was saturated by a 10-min incubation with either PBS/NCS or PBS containing 1% bovine serum albumin. Primary antibodies were applied as *P. megasperma* antiserum (crude serum, diluted 500-fold in PBS/NCS), PAL antiserum (Protein-A-binding fraction, 60 µg·ml^{-1}) or BMT antiserum [$(NH_4)_2SO_4$-precipitated fraction, 80 µg·ml^{-1}]. The tissue sections were incubated with

primary antibody for 1 h at 37° C in a moist chamber. Unbound antibody was removed with two PBST washes (25 mmol·l^{-1} sodium phosphate, 0.5 mol·l^{-1} NaCl, 0.5% Tween 20, pH 7.4) and one PBS wash, 3 min each. This procedure was followed by a 30-min incubation in 100-fold-diluted peroxidase-labelled secondary antibody (Bio-Sys, Compiègne, France) at 37° C. Sections were rinsed, as above, and stained with the color reagent, diamino benzidine tetrahydrochloride (Polysciences, Warrington, USA) for up to 5 min (Sternberger 1979). After rinsing in distilled water the sections were mounted and viewed with bright-field and fluorescence microscopy.

Results

Conditions for infection. Plant inoculation was performed without wounding. Parsley plants, inoculated with *P. megasperma* zoospores and kept at 70% relative humidity and 16 h illumination per day, showed no visible symptoms of infection two weeks post-inoculation. Although zoospores were still visible by their faint blue autofluorescence under UV light, they did not cleave the vital stain, fluorescein diacetate, and were apparently dead. In plants kept at 100% relative humidity and 16 h illumination per day, infection and local lesion formation occurred reproducibly. Extensive fungal growth and finally sporulation was observed on inoculated plants kept at 100% relative humidity in the dark. These plants were water-soaked 7 d post-inoculation and eventually died.

Standard conditions used for all of the following infections were 100% relative humidity and 16 h illumination per day. A small portion of the applied zoospores germinated under these conditions (Fig. 1 A) and lay mostly over anticlinal walls (Fig. 1 B). At 50 spores per µl, approx. 1–5% of the zoospores produced individual lesions. Although a similar infection rate was observed at higher spore density (250 spores per µl), individual lesions were not always distinguishable and tended to fuse into large necrotic areas (see below).

Fluorescence microscopy. A rabbit polyclonal antiserum, raised against heat-inactivated *P. megasperma* zoospores, made visible the fungal cell walls, flagellae and spore contents on acetone-fixed, air-dried spore mounts (Fig. 2). Using this antiserum, fluorescence microscopy allowed both the fungus and infected, autofluorescent plant cells to be detected simultaneously. Fungal hyphae entered the plant tissue through stomata or anticlinal walls with no apparent preference. Intercellular vesicles or haustoria were never observed. Occasionally appressoria-like structures were formed at sites of attempted or successful penetration (Fig. 1 B). The size of local lesions ranged from one affected cell (Fig. 3 A), either a stoma, epidermal or mesophyll cell, to a few cells of either the same or different types (Fig. 3 B). When massive spore accumulation occurred as a result of clumping, individual lesions were no longer separate but appeared as large necrotic spots comprising dozens of cells (Fig. 3 C). In all cases, the fungus grew only intercellularly.

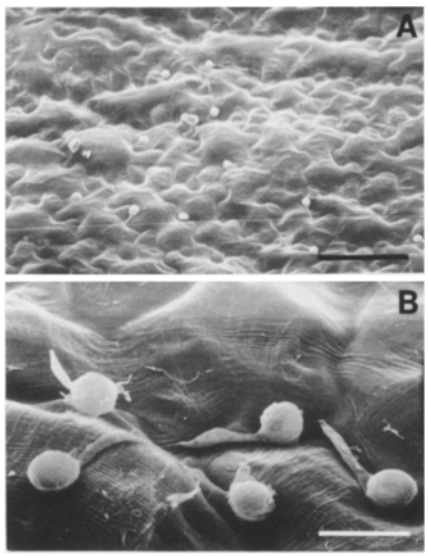


Fig. 1 A, B. Scanning electron micrographs of parsley cotyledons 12 h post-inoculation with zoospores of *Phytophthora megasperma* f. sp. *glycinea*. *Bars* 100 µm (**A**) and 20 µm (**B**)

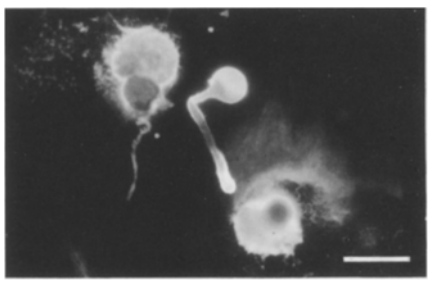
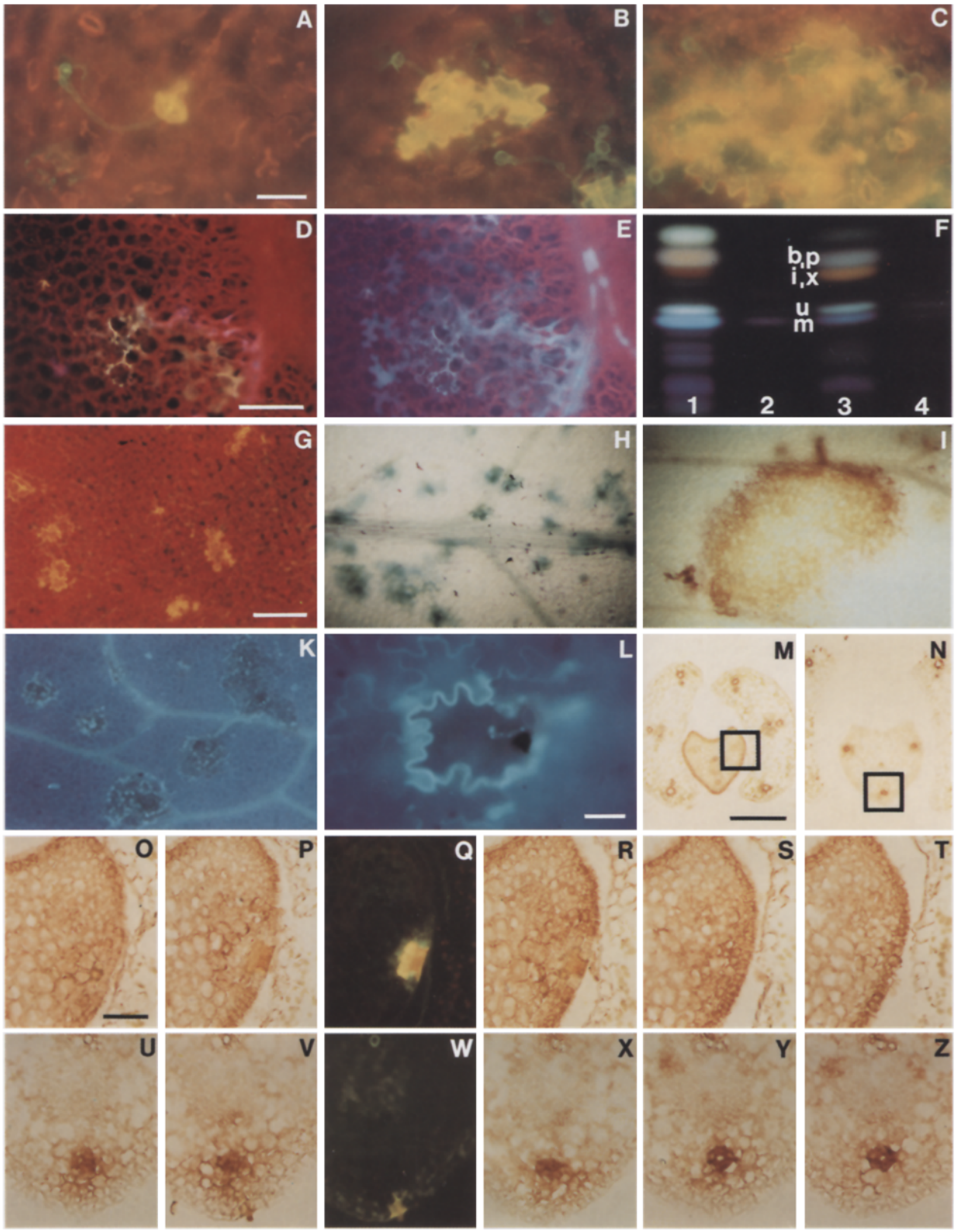


Fig. 2. Indirect immunofluorescence staining of *P. megasperma* zoospores and hyphae after 12 h of germination, using rabbit polyclonal antiserum against the heat-inactivated zoospores. Shown are an intact spore with germ tube (*center*) and broken spores with protruding contents. *Bar* = 20 µm

Fluorescence microscopy of unfixed, uncovered specimens yielded particularly well-resolved pictures of infection sites. At wavelengths above ultraviolet (UV) (violet: excitation maximum 395–425 nm/dichroic mirror 425 nm/barrier filter 450 nm; blue: 450–490 nm/510 nm/520 nm), infected cells appeared on a red background of chlorophyll autofluorescence and showed strong yellow fluorescence of cell walls and cytoplasm (Fig. 3 D). The cytoplasm of these cells had collapsed, and the cells were apparently dead. The yellow fluorescent material was not removed by a 40-min treatment with hot alkali (0.5 mol·l^{-1} NaOH, 80° C).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Under UV light (365 nm/395 nm/420 nm), the infection droplet and the area surrounding dead cells showed strong blue autofluorescence (Fig. 3E). The blue-fluorescing compounds were washed off with water. Chloroform extracts of infection droplets and the water wash contained, among other compounds, the coumarin umbelliferone and the furanocoumarin psoralen (Fig. 3F). Psoralen, one of the previously identified parsley phytoalexins (Scheel et al. 1986; Knogge et al. 1987), also accumulated rapidly in elicitor-treated parsley cell cultures (Hauffe et al. 1986). Analysis of these extracts, employing high-performance liquid chromatography and mass as well as nuclear-magnetic-resonance spectroscopy, elucidated the chemical nature of the major phytoalexins in the infection droplet (data not shown).

Histochemistry. A high density of UV-fluorescent spots 24 h post-inoculation demonstrates the efficiency of infection under the conditions used (Fig. 3G). Treatment at this time with Toluidine Blue at pH 4.4 gave green-stained areas around infection sites (Fig. 3H). The green color was observed in both plant protoplasts and cell walls. Sudan-III staining revealed the accumulation of lipids in necrotic cell walls. Phloroglucinol/HCl staining produced a red color 5 d post-inoculation, when necrotic areas comprised several hundred cells (Fig. 3I). The chlorine/sulfite test for lignin (Ride 1975) was negative in this tissue.

Staining with decolorized Aniline Blue demonstrated the presence of callose around the sites of attempted or successful penetration. Neighbouring, uninfected cells produced callose at the anticlinal cell walls (Fig. 3K, L). Five days post-inoculation a ring of callose was formed by healthy tissue surrounding necrotic areas (Fig. 3K). Occasionally it appeared that the fungus was stained positively. Treatment of cultured mycelial discs with decolorized Aniline Blue yielded positively stained material in hyphal septae and sporangia, and patchy staining of hyphae.

Immunohistochemistry. Indirect immunoperoxidase staining localized PAL and BMT in infected tissue. The *P. megasperma* antibodies did not cross-react with soluble plant proteins on protein blots. To test the sensitivity of antigen detection, pure PAL and BMT proteins were separately immobilized on nitrocellulose, treated with several fixatives and assayed by indirect immunoperoxidase staining. Both proteins were visible at levels as low as 150 ng per cm^2. Prior formaldehyde (4%) fixation did not alter the immunological detectability of the proteins. Formaldehyde (2%) in combination with glutaraldehyde (0.25%) or glutaraldehyde (1%) alone reduced the signal strength by a factor of 2. The signals were stronger in formaldehyde-fixed tissue sections than in other fixatives tested. However, mixtures of formaldehyde and glutaraldehyde improved tissue preservation and local retention of the antigens. Fixatives were used as indicated in the figure legends.

Treatment of serial sections of infected plant tissue with PAL antiserum and peroxidase-labelled secondary antibody showed the presence of PAL in all tissues. Relatively large amounts occurred in the epidermal cell layers and oil ducts. The enzyme PAL was most concentrated in the epidermis of the primary leaf petiole (Fig. 3M). Pre-immune serum did not appreciably stain the sections. At infection sites, PAL levels were above background in those cells surrounding necrotic areas. In a complete serial section of a small lesion (Fig. 3O–T) PAL was most abundant in uninfected cells. Infected cells displayed a yellowish-brown color clearly distinguishable from the dark-brown perox-

Fig. 3A–Z. Analysis of *P. megasperma* infections in parsley. **A–C:** Indirect immunofluorescence staining of infected parsley cotyledons fixed in formaldehyde. Local lesions are visible as a result of autofluorescence of cell walls. Lesion size 36 h post inoculation varied with inoculum density: **A**, one stoma; **B**, different cell types; **C**, several cells. *Bar* = 50 μm. **D–E:** Autofluorescence of unfixed, unprocessed infected parsley cotyledons 40 h post-inoculation. **D**, Violet excitation: red chlorophyll autofluorescence and yellow cell-wall fluorescence; **E**, UV light excitation: blue fluorescence of soluble compounds. *Bar* = 200 μm. **F:** Thin-layer chromatography of coumarin derivatives (*lane 1*, parsley cell culture media 30 h after *Pmg* elicitor treatment; *lane 2*, H_2O-treated cell culture control; *lane 3*, infection droplets 30 h post-inoculation; *lane 4*, H_2O-inoculated plant control). Identified components are psoralene (*p*), bergapten (*b*), isopimpinellin (*i*), xanthotoxin (*x*), umbelliferone (*u*) and marmesine (*m*). **G–L:** Histochemical staining of infected parsley cotyledons. **G**, blue-light autofluorescence of cell walls 60 h post-inoculation; **H**, Toluidine Blue (24 h post-inoculation); **I**, phloroglucinol-HCl; **K, L**, Aniline Blue fluorescence (**I, K, L:** 5 d post-inoculation). *Bars* = 200 μm (**G–K**) and 50 μm (**L**). **M–Z:** Indirect immunoperoxidase staining of frozen sections of infected parsley seedlings. **M** and **O–T**, Primary leaf petiole, 12 h post-inoculation, fixed with formaldehyde and stained with PAL antiserum (**M**, whole cross-section; **O–T**, framed area in **M** under visible light; **Q** = **P** shown under blue light for autofluorescence of infected cells. Yellow color of infected cells (**P, R**) was visible without immunostaining). *Bars* = 500 μm (**M**) and 50 μm (**O–T**). **N** and **U–Z**, Infected primary leaf petiole 13 h post-inoculation, fixed with a mixture of formaldehyde and glutaraldehyde and stained with BMT antiserum (**N**, whole cross-section; **U–Z**, framed area in **N** under visible light; **W** = **V** shown under UV light for autofluorescence of infected cells). *Bars* = 500 μm (**N**) and 50 μm (**U–Z**)

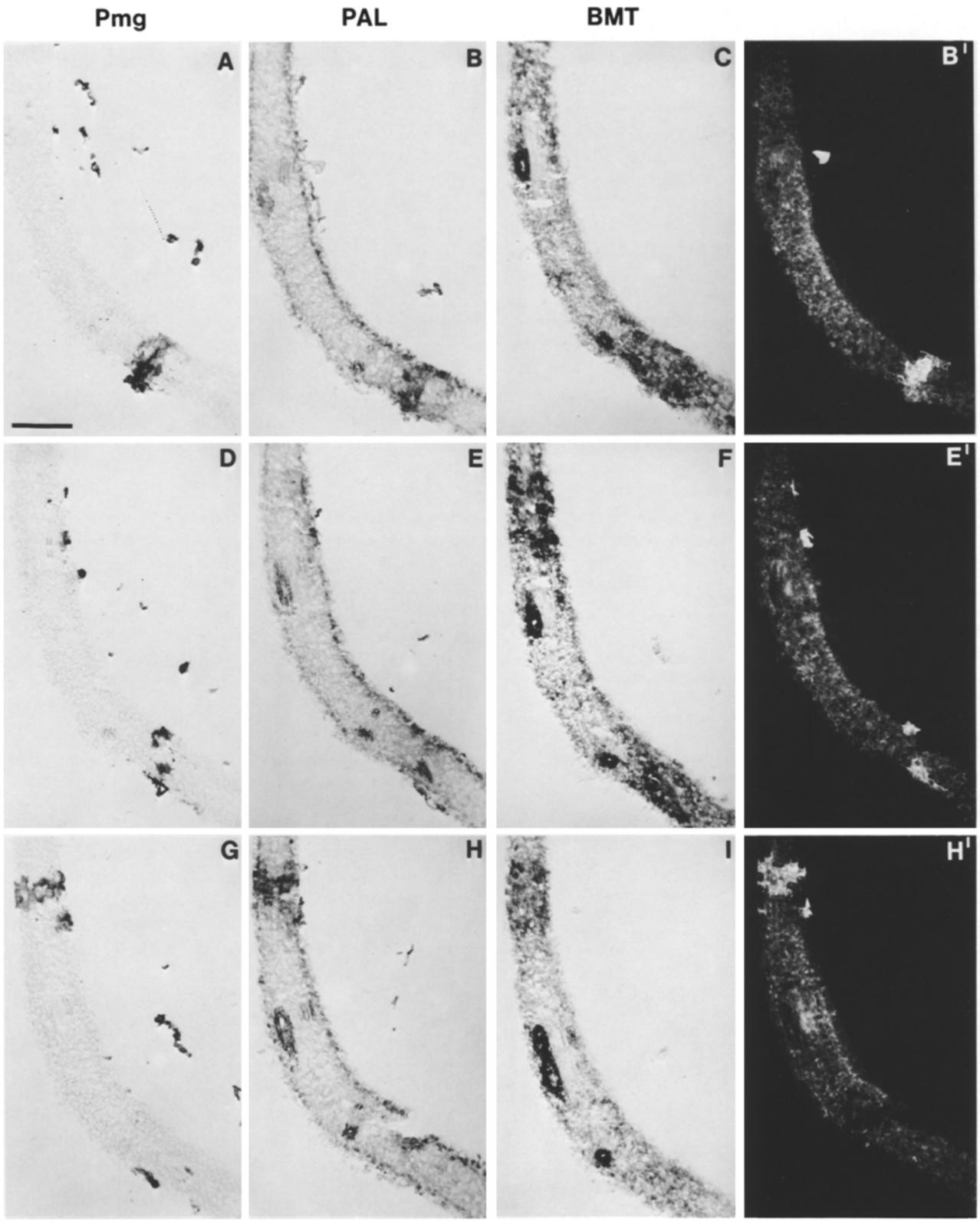

Fig. 4 A–H'. Indirect immunoperoxidase staining of frozen serial sections of *P. megasperma*-infected parsley leaves. Primary leaves were fixed in a mixture of formaldehyde and glutaraldehyde 36 h after inoculation, freeze-sectioned, alternatingly placed on three different microscopic slides and stained with antisera against *P. megasperma* (**A, D, G**), PAL (**B, E, H**), or BMT (**C, F, I**). Autofluorescence of sections **B, E** and **H** is shown in **B', E'** and **H'**. *Bars* = 100 μm

idase reaction product (Fig. 3P–R). Yellow-pigmented areas showed the characteristic autofluorescence of dead cells (Fig. 3Q). The normally positive-staining layer of epidermal cells was interrupted and replaced by a new PAL-rich cell layer deeper in the pith tissue. In the following section, this new layer was visible in a surface view (Fig. 3S). Finally, the original arrangement of PAL-positive epidermal cells was again visible (Fig. 3T). Thus, the strongly fluorescing cells in the center were surrounded by one or two layers of cells containing no detectable levels of PAL, followed by a layer of cells high in PAL.

In a similar series of sections taken from a different infection site, BMT antiserum reacted with most parts of the tissue (Fig. 3N, U–Z). The epidermis did not contain more BMT than the pith tissue, whereas the concentration in oil-duct cells was much higher than elsewhere in the tissue. Like PAL, BMT accumulated in the vicinity of necrotic cells (Fig. 3V–Y). However, the areas of BMT accumulation at the infection sites investigated at this early time after inoculation appeared less clearly structured than in the case of PAL.

In an additional experiment using leaf tissue, sections of the same infection sites were alternately treated with *P. megasperma*, PAL and BMT antisera. Figure 4 shows an uninterrupted section series of two neighbouring, yet distinct, local lesions of an infected primary leaf. This black-and-white presentation does not display the color differences between yellowish-brown dead cells (Fig. 3Q, W) and brown positive-staining cells, nor is the cellular distribution at this lower magnification and later stage of infection resolved to the same extent as shown in Fig. 3M–Z.

The *P. megasperma* antiserum efficiently visualized fungal structures, zoospores as well as hyphae, in the frozen sections. For the most part, cross sections of hyphae or other fungal structures were encountered (Fig. 4A, D, G). Longitudinally sectioned hyphae (not shown) demonstrated that the fungus grew only intercellularly and followed the shape of individual cells in the plant tissue. Diffuse staining of fungal structures indicated the release of antigenic material during fungal colonization or after fungal cell lysis (see also Fig. 3A–C).

Again, PAL (Fig. 4B, E, H) and BMT (Fig. 4C, F, I) were concentrated at infection sites. We have shown this through *P. megasperma*-positive staining in neighbouring sections (Fig. 4A, D, G) and autofluorescence under UV light (Fig. 4B', E', H') of those sections used for the localization of PAL. In uninfected tissue, PAL was most abundant in epidermal and oil-duct cells, whereas BMT was concentrated in oil-duct cells.

Discussion

The influence of environmental conditions on the interaction between many plants and microbes has been established (Bailey 1983). We have shown that the parsley-*P. megasperma* interaction is typical in this regard. The behavior of the inoculated plant ranged from one extreme, total "immunity" (no interaction), to the other, total "physiological susceptibility." This is despite the fact that parsley is genetically "nonhost resistant" to *P. megasperma*. We have chosen carefully controlled conditions for a clearly detectable interaction and nonhost resistance to be expressed as hypersensitive cell death leading to the formation of small local lesions.

Parsley proved to be a particularly suitable plant for our studies. The two major classes of phenylpropanoid derivatives accumulating at infection sites, furanocoumarin phytoalexins and wall-bound, structurally unidentified phenolics, were easily detected and distinguished by their strong differential UV/blue fluorescence. As the wall-bound compounds were not solubilized with hot alkali, they were probably non-esterified, polymerized or otherwise firmly bound components of the cell wall. The positive staining with Toluidine Blue and phloroglucinol/HCl does not allow conclusions to be drawn as to the precise chemical nature of these compounds (Vance et al. 1980). However, their accumulation along with Sudan-III-positive material indicates the formation of the mixed phenol-lipid polymer, suberin (Jensen 1960). Callose, another typical component of a newly formed structural barrier at infection sites (Aist 1976), was also found to accumulate rapidly at the same sites in parsley tissue infected with *P. megasperma*.

The local accumulation of furanocoumarins at infection sites confirms their putative role as phytoalexins in parsley (Tietjen et al. 1983; Knogge et al. 1987). The presence of these compounds in uninfected tissue (Knogge et al. 1987), predominantly in oil ducts (Jahnen 1986), might indicate they are translocated rather than synthesized de novo following infection. However, our results strongly support the hypothesis that furanocoumarins are newly synthesized at the site of infection. Hauffe et al. (1986) have demonstrated that BMT is highly specific for end-product formation in the furanocoumarin pathway, and our immunocytological data clearly indicate the accumulation

of this enzyme around necrotic areas caused by *P. megasperma* infection. Thus, we believe that furanocoumarins were synthesized where they were detected in situ.

The simultaneous occurrence of increased PAL and BMT levels at infection sites might indicate that the induction of both enzymes is related to the synthesis of furanocoumarins. In contrast to BMT, however, PAL is likely to have other biosynthetic functions as well. The rapid accumulation of wall-bound phenolics is one additional, possible reason for the observed accumulation of PAL. Beyond this connection, however, the results obtained thus far do not allow us to associate PAL with individual branch pathways of phenylpropanoid metabolism.

In conclusion, we have made two new observations at the cellular level. We have demonstrated the accumulation of furanocoumarins and wall-bound phenolics at the sites of infection of parsley tissue with *P. megasperma* and have provided evidence for the de-novo synthesis of these compounds by localizing increased amounts of one common and one furanocoumarin-specific enzyme at the infection sites.

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