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In vitro formation of infection structures of *Phytophthora infestans* is associated with synthesis of stage specific polypeptides

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Abstract

Efficient and synchronized production of infection structures of *Phytophthora infestans*, the causal agent of late blight of potato, was established on an artificial membrane without the host plant. Microscopic comparison of the *in vitro* and the *in planta* formed fungal structures revealed a high degree of similarity. *In vitro* development of infection structures enabled detailed cytological and biochemical investigations. By video microscopy the highly dynamic phenomenon of cytoplasmic migration was monitored within the living fungus. At four distinct developmental stages, hyphae, cysts, germinating cysts and appressoria, all grown *in vitro*, protein synthesis was analysed by comparative two-dimensional SDS-polyacrylamide gel electrophoresis. On two-dimensional gels of protein extracts of the four developmental stages a number of polypeptides were identified that showed stage-specific differences in their relative amounts. The *de novo* synthesis of proteins was investigated by *in vivo* labelling experiments. A number of polypeptides showed development-dependent expression. The majority of changes in protein synthesis occurred during germination of cysts and development of the germ tubes. In particular, at the stage of appressoria formation, the actual start of the infection process, several major polypeptides were newly synthesized.

Introduction

Many obligate biotrophic plant pathogens from various genera, preferentially causing mildew and rust diseases (Mendgen and Deising, 1993), as well as oomycetes such as *Phytophthora infestans* (Mont.) de Bary, the causal agent of the late blight disease of potato (*Solanum tuberosum* L.), form elaborate structures enabling invasion and colonisation of the plant tissue (Hardham, 1992). The morphological aspects of the development of these structures, in particular of *P. infestans*, have been intensively studied (Hohl and Suter, 1976; Coffey and Wilson, 1983; Coffey and Gees, 1991). The developmental stages during which this pathogen spreads in the field are sporangia and zoospores. Within a sporangium 6–8 biflagellate zoospores are produced and released into water. The mobile wall-less zoospores need to encyst prior to germination. Encystment is brought about by a series of rapid structural changes, of which the most dramatic ones are retraction or sometimes shed of the flagella and formation of a cell wall. The spherical cyst attaches to solid surfaces by secretion of adhesive material (Bartnicki-Garcia and Wang, 1983) and starts to germinate with a cylindrical germ tube. At an appropriate site the germ tube apex differentiates into a vesicular structure, the appressorium. From the appressorium an infection tube emerges and penetrates the epidermal cell wall. At the same time, the cyst and the major part of the germ tube become devoid of cytoplasm. Inside the host cell, the pathogen forms a vesicular structure termed 'infection vesicle' (Shimony and Friend, 1975). From this structure one or two secondary hyphae germinate and grow into either a

mesophyll cell or the intercellular space. The further intracellular development of the fungus includes the formation of haustoria. The triggers inducing formation of infection structures of *P. infestans* as well as the molecular basis for the control of these processes are largely unknown. One prerequisite for the understanding of pathogenicity of this wide-spread pathogen is the identification and characterization of infection related proteins.

We present here an experimental design facilitating detailed investigations of the dynamic aspects of the cellular changes as well as of the underlying molecular processes that are required for the formation of infection structures and, hence, for pathogenicity of *P. infestans*. We found *P. infestans* capable of developing, like rust fungi (Staples et al., 1986; Freytag et al., 1991), infection structures on artificial membranes, i.e. in the absence of the host plant. Microscopic comparison of the structures formed *in vitro* or *in planta* revealed similar morphology. In the living fungus, migration of the cytoplasm during development of the appressorium and the infection tube was monitored by video microscopy. To analyse in detail protein synthesis during the formation of infection structures and to identify infection related polypeptides, we used two-dimensional polyacrylamide gel electrophoresis. *In vitro* grown fungal structures of 4 developmental stages, hyphae (grown on agar plates), cysts, germinating cysts and appressoria served as starting material.

Materials and methods

Ampholines were from Pharmacia, Freiburg, Germany; (^{35}S)methionine from Amersham, Dreieich, Germany. The dyes DAPI, $DIOC_6$, and Nile Red were purchased from Molecular Probes inc., Eugene, OR., USA.

Cultivation of fungus and production of zoospores

Mycelium of races 1 and 4 of *Phytophthora infestans* were obtained from Biologische Bundesanstalt, Braunschweig, Germany. Preservation and storage was on agar pieces in liquid N_2 according to Hohl and Iselin (1986). For propagation, pieces of mycelia were thawed, transferred to vegetable juice-agar (Rohwer et al., 1987) and grown at 17 °C in the dark. Virulence was maintained by passage through tuber slices of respective susceptible potato cultivars. To induce formation of sporangia the fungus was grown on rye-agar at 15 °C and 95% humidity for about 12 days. For release of zoospores the mycelium grown on a rye-agar plate (ø 90 mm) was submerged with 10 ml sterile deionised water pre-cooled to 4 °C and incubated at 4 °C for at least 4 h. The zoospore suspension was collected from the dishes and encystment was induced by vigorous shaking (Vortex). Hyphae and premature sporangia were removed by filtration through 3 layers of Miracloth (Calbiochem, USA). Subsequently the cysts were sedimented by centrifugation at 500 × g for 4 min and gently resuspended in sterile tap water. The titer was determined using a Fuchs-Rosenthal counting chamber and adjusted to 1×10^6 cysts × ml^{-1}.

Enrichment of fungal developmental stages

Hyphae were grown in Petri dishes with vegetable juice-agar covered with cellophane foil that had been cut to appropriate size, boiled for 5 min in dest. water and autoclaved between filter paper. 14 days after inoculation the cellophane foil together with mycelium was stripped off, the mycelium was carefully removed from the foil, frozen in liquid N_2 and stored at −20 °C.

Cysts were isolated as described above, centrifuged, frozen in liquid N_2 and stored at −20 °C. Germination of cysts was achieved by incubation of a cyst suspension in sterile tap water at 17 °C for 3–4 h. The fungal structures were sedimented by centrifugation at 500 × g for 5 min, frozen in liquid N_2 and stored at −20 °C.

For formation of appressoria, cysts were sprayed on polypropylene foil (Plastibrand®, Brand, Germany) that had been spread on glass plates as support. Foils were placed into racks in plastic containers and incubated for 3–4 h at 17 °C. Care was taken to keep humidity inside containers near 100%. After incubation, foils were frozen in liquid N_2 and lyophilized. The dried fungal structures were scraped off the foils and carefully collected in a tube using a fine brush.

To test the suitability of other surfaces to induce appressoria formation, cysts were sprayed on glass plates, Petri dishes, Parafilm and Kollodium. Kollodium foil was prepared by homogeneous distribution and subsequent drying of a mixture of Kollodium/ethanol/diethylether (1:1:3, v/v/v) and 5 μl × ml^{-1} paraffin oil on glass Petri dishes.

Video microscopy and cytochemical staining

For video microscopy fungal cysts were grown on polypropylene foil. A small incubation chamber was obtained by placing a piece of the plastic foil and some droplets of a cyst suspension in water between two thin glass plates (0.8 mm thickness) spaced by a silicon ring (1 mm thickness). Germination and growth of individual fungal cysts was observed using a 63 × or 100 × oil-immersion objective (1.2 Na; Zeiss Incorporated, Göttingen, Germany) and differential interference contrast (Nomarski). The microscopic image was recorded on video tape with a S-VHS video recorder (Panasonic AG-6720, Matsushita Electric Industrial Co., Osaka, Japan) using the time lapse function (80x) via a CCD video camera (Hamamatsu 2400, Hamamatsu Photonics, Herrsching, Germany), mounted to the microscope. The video image was improved by analog contrast enhancement. Individual frames of relevant scenes recorded on tape were converted into digital information by a frame grabber board (VS 100, Imaging Technology Incorporated, Woburn, Mass., USA) and stored in computer memory (PC 386 AT). To improve quality, contrast of the images was digitally enhanced and edges of objects in the images were accentuated by a sharpening filter operation using appropriate image processing software (BioScan Optimas, BioScan Incorporated, Edmonds, U.S.A.). Pictures were displayed on a high resolution monitor and photographed on Agfapan 400 or Kodak Ektachrome 400.

Fungal structures, grown on plastic foil, were stained with either 5 μg ml^{-1} DAPI, or 5 μg ml^{-1} $DIOC_6$ (stock solution 1 mg ml^{-1} in ethanol), or 5 μg ml^{-1} Nile Red (stock solution 1 mg ml^{-1} in aceton) in water for 5 min. After washing briefly in water, specimens were examined under the epifluorescence microscope (excitation wave length for DAPI 365 nm, for $DIOC_6$ and Nile Red 450–560 nm).

Two-dimensional polyacrylamide gel electrophoresis

Extraction of proteins from the various fungal structures was performed according to a modified method described by Mayer et al. (1986). To approximately 4 × 10^7 fungal structures, 100 μl of extraction buffer (300 mM NaCl, 100 mM DTT, 1 mM EDTA, 1 mM EGTA, 5mM Ascorbate, 2% (v/v) Triton X-100, 2% (v/v) Ampholines, pH 3,5–10, 10 μg ml^{-1} phenylmethylsulfonylfluoride, 10 μg ml^{-1} Leupeptin was added. The fungal structures were disrupted by sonication for 2 × 20 sec at 10 W, interval mode 25%, on ice, using a Branson Sonifier 450 (Branson). For precipitation of nucleic acids, 1 mg/ml Protamin sulfate was added and extracts were stirred at room temperature for 10 min. After centrifugation at 15 000 × g for 10 min the supernatants were mixed with solid urea to a final concentration of 9.5 M till all urea crystals were solubilised. Protein concentration in extracts was determined as described by Ramagli and Rodriguez (1985). When extracts were tested for protease activity using the method of Gallagher et al. (1986), no activity was detected by this test.

For two-dimensional gel electrophoresis the procedure of O'Farrell (1976) was modified for separation of small protein amounts. Isoelectric focusing (IEF) was performed in glass capillaries of 1.5 mm in diameter and 6.5 cm in length. 20 μg total protein of extracts per capillary was loaded on the IEF-gel (9 M urea, 7% (w/v) acrylamide, 0.19% (w/v) bis-acrylamide, 2% Nonidet P 40 (v/v), 1% (v/v) Ampholines pH 3.5–10, 0.3% (v/v) TEMED), overlaid by a solution containing 9 M urea and 1% (v/v) Ampholines, pH 3.5–10 and run for 20 min at 200 V, then 20 min at 400 V and finally 9 h at 900 V. Buffers and gel were prevented from heating up by continuous cooling of the electrophoresis divice. Focusing was from cathode (buffer: 10 mM ethylene diamine) to anode (buffer: 10 mM iminodiacetate). After completion of focusing, rod gels were equilibrated in two steps for 15 min each in 100 mM DTT, 10% glycerol (v/v), 4% (w/v) SDS, 0.05% Bromophenolblue, 125 mM Tris/HCl, pH 6.8 and in 100 mM DTT, 10% glycerol (v/v), 2.3% (w/v) SDS, 0.05% Bromophenolblue, 60 mM Tris/HCl, pH 6.8. Polypeptides were separated in the second dimension by SDS-PAGE on 12.5% slab gels. IEF-gels were placed on top of the stacking gels and electrophoresis occurred at 15 mA per gel. Separation quality of isoelectric focusing was controlled by staining the rods with Coomassie Brilliant Blue 250 G according to Neuhoff et al. (1988). Slab gels were silver stained according to Blum et al. (1987).

In vivo *labelling*

De novo synthesized proteins were pulse-labelled *in vivo* with (^{35}S) methionine. For labelling of cysts 40 μCi of (^{35}S)methionine were added to 1.5 ml of a zoospore suspension (1 × 10^6 zoospores × ml^{-1}). The suspension was incubated for 20 min at room temperature and then thoroughly mixed (Vortex mixer) for 1 min to induce encystment. Cysts were collected by

centrifugation (5 min, 800 × g) and washed two times in 100 mM L-methionine, 10 mM Tris/HCl, pH 7. A similar procedure was used for labelling of germinating cysts. 1 ml of a cyst suspension (1×10^6 cysts $\times$ ml^{-1}) in sterile tap water was incubated for 2 h at 17 °C before 20 μCi of (^{35}S)methionine were added. The suspension was then incubated for a further 20 min. The fungal structures were centrifuged and washed as described above for cysts. Appressoria were produced on polypropylene foil as described above. After 3 h of growth of the fungus on the plastic foil, 40 μCi of (^{35}S)methionine in water was sprayed onto a foil (30 × 20 cm) containing 4×10^6 cysts. After 20 min of incubation the foil was carefully washed with sterile water, frozen in liquid N_2 and subsequently lyophilized. Freeze dried fungal structures were removed from the foil using an ice scraper, collected in 500 μl of dest. water and again freeze dried. To label hyphal proteins, freshly grown mycelium on an agar plate (vegetable juice agar) was submerged with 40 μCi of (^{35}S)methionine in 5 ml sterile water and incubated for 20 min at 17 °C. The mycelium was removed from the agar using forceps and washed 3 times as described above for cysts. Proteins were extracted from fungal structures as described above and two dimensional electrophoresis was carried out with $0.7 - 1 \times 10^5$ cpm per gel. For fluorography gels were fixed in 50% methanol (v/v), 12% acetic acid (v/v) for 1 h, infiltrated with Enlightning (Amersham) and dried.

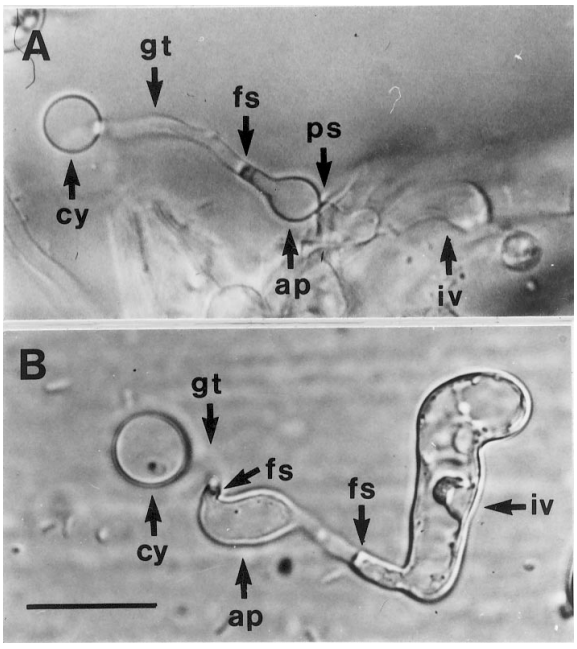

Figure 1. Morphology of fungal structures formed *in planta* and *in vitro*. Leaves of potato plants (*Solanum tuberosum* cv. Datura) were inoculated with zoospores of *Phytophthora infestans* race 1. 24 h after inoculation, detached leaflets were cleared, fixed and used for microscopic inspection of fungal infection structures (A). For development *in vitro*, fungal cysts were sprayed on polypropylene foil and grown for 3–4 h (B). ap, appressorium; cy, cyst; fs, false septum; gt, germ tube; iv, intracellular vesicle or *in vitro* formed vesicle-like structure; ps, penetration site. Bar represents 20 μm.

Results

Growth and development of infection structures in vitro

Experimental conditions were established to obtain efficient formation of appressoria and further hyphal and vesicular structures of the fungus *in vitro*, i.e. in the absence of the plant. Fungal cysts were grown at 17 °C and 100% relative humidity on several artificial surfaces. If the surface displayed topographic microdiscontinuities, like polypropylene foil or parafilm or Kollodium, a major proportion of the cysts (65–70%) differentiated appressoria within 3–4 h. Less appropriate was the surface of Petri dishes (only 45% appressoria). On plain surfaces, like glass, cysts germinated but appressoria were never observed. When cysts were kept in suspension, the germination rate reached maximal values of about 60% after an incubation of 3–4 h. Prolonged incubation only led to elongation of germ tubes without any formation of appressoria.

A light microscopical comparison of the morphology of the structures formed on polypropylene foil (*in vitro*) and *in planta* (Figure 1A,B) showed a high degree of similarity. The morphology of the germlings developing on polypropylene foil was clearly distinct from vegetative hyphae growing on agar plates and from elongated germ tubes on non-inducive surfaces or in suspension. It rather resembled the characteristic structures formed *in planta* (germ tube, appressorium, infection hypha, intracellular vesicle). Furthermore, sealing of the cytoplasm from empty distal structures by false septa at specific locations were similar *in vitro* and *in planta* during continuous translocation of the cytoplasm into the apical part of the growing structures.

Dynamic aspects of growth and development of infection structures on polypropylene foil, in particular the translocation of cytoplasm out of the cyst, were examined by video microscopy in the living fungus

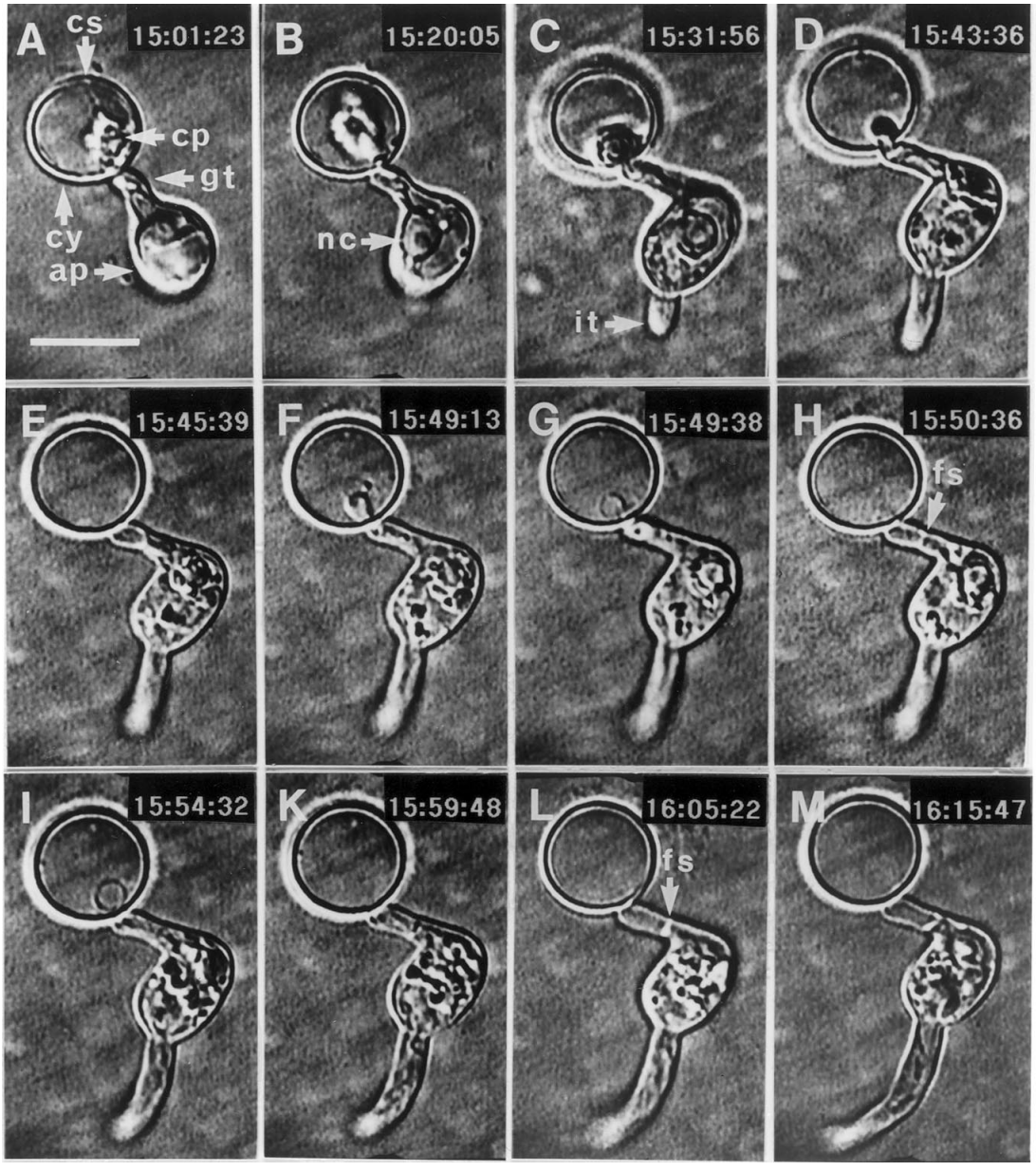

Figure 2. Cytoplasmic migration within the living fungus. Development of infection structures on polypropylene foil was recorded by video microscopy on tape. Individual video frames from a representative scene were digitized by a frame grabber board and stored in computer memory. Images were improved by digital contrast enhancement and a sharpening filter operation. The label in the upper right of the pictures indicates time (h:min:s). ap, appressorium; cy, cyst; cs, cytoplasmic strand; cp, cytoplasm; fs, false septum; gt, germ tube; it, *in vitro* formed infection tube-like structure; nc, nucleus. Bar represents 10 μm.

(Figure 2). Translocation became obvious when the proportion of the cytoplasm located within the cyst withdrew from the fungal wall and moved towards the opening of the germ tube, whilst occasionally cytoplasmic strands extended to the wall throughout the part of the cyst already devoid of cytoplasm (Figure 2A). At this stage the nucleus was located within the appressorium and the infection tube started to emerge from the appressorium (Figure 2B,C). Along with growth of the infection tube, more and more of the cytoplasm moved out of the cyst until it appeared to be empty (Figure 2C–E). At this stage, when the translo-

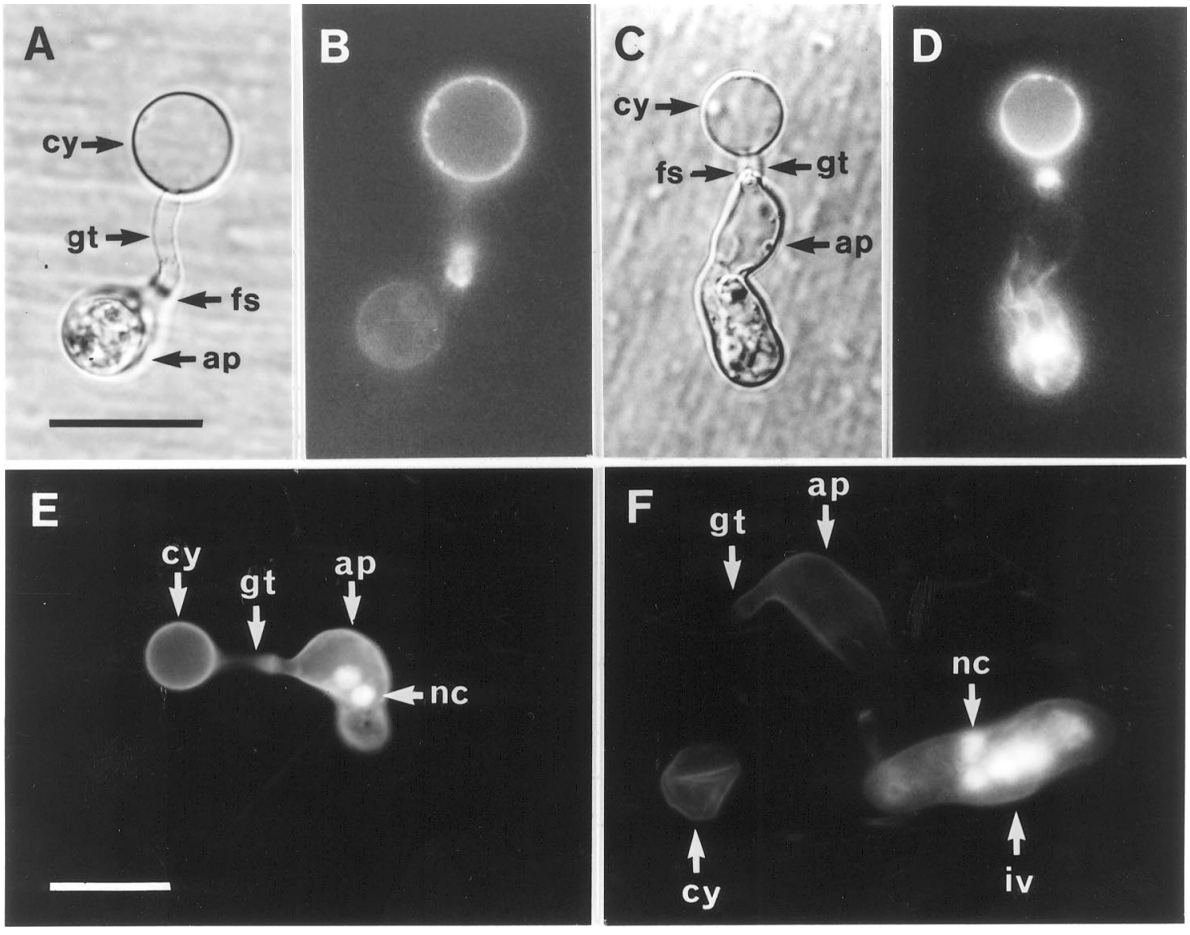

Figure 3. Staining of lipids and nuclei in *in vitro* grown structures. Fungal germlings grown on polypropylene foil were stained either with Nile red (A,B) or $DIOC_6$ (C,D) or DAPI (E,F). Pictures A and C were taken under differential interference contrast (Nomarski), all others under epifluorescence light. ap, appressorium; cy, cyst; fs, false septum; gt, germ tube; nc, nucleus; iv, *in vitro* formed vesicle-like structure. Bar represents 20 μm.

cation process seemed to be completed, often a small proportion of the cytoplasm suddenly flipped from the germ tube into the cyst and back again over the course of a few seconds (Figure 2F–H). Sometimes this pulsation occurred even twice, as shown in Figure 2I,K, until the false septum, already visible in Figure 2H, was fully developed (Figure 2L,M).

Both the lipid stain Nile red and the lipophilic dye $DiOC_6$ visualized a residual lipid layer covering the inner side of the wall and the false septum of the otherwise empty appearing part of fungal structures grown on polypropylene foil (Figure 3B,D). $DiOC_6$ stained, in addition, rod like structures within the cytoplasm that are mitochondria and endoplasmic reticulum (Figure 3D). Within the appressorium often 2 nuclei and at later stages up to 4 nuclei were detected in the *in vitro* grown structures by DAPI staining (Figure 3E,F).

Stage specific protein synthesis

Of four developmental stages, hyphae, cysts, germinating cysts and appressoria, all grown *in vitro*, differences in protein content were analysed by comparative two-dimensional SDS-polyacrylamide gel electrophoresis. When protein extracts from the four stages were separated, a total of 105–120 polypeptides per gel were identified. The majority of polypeptides had isoelectric points within the pH range of 5–8, yet, some focused at rather basic (pH 10) or acidic (pH 3,5) pI values. Comparing two independent experiments

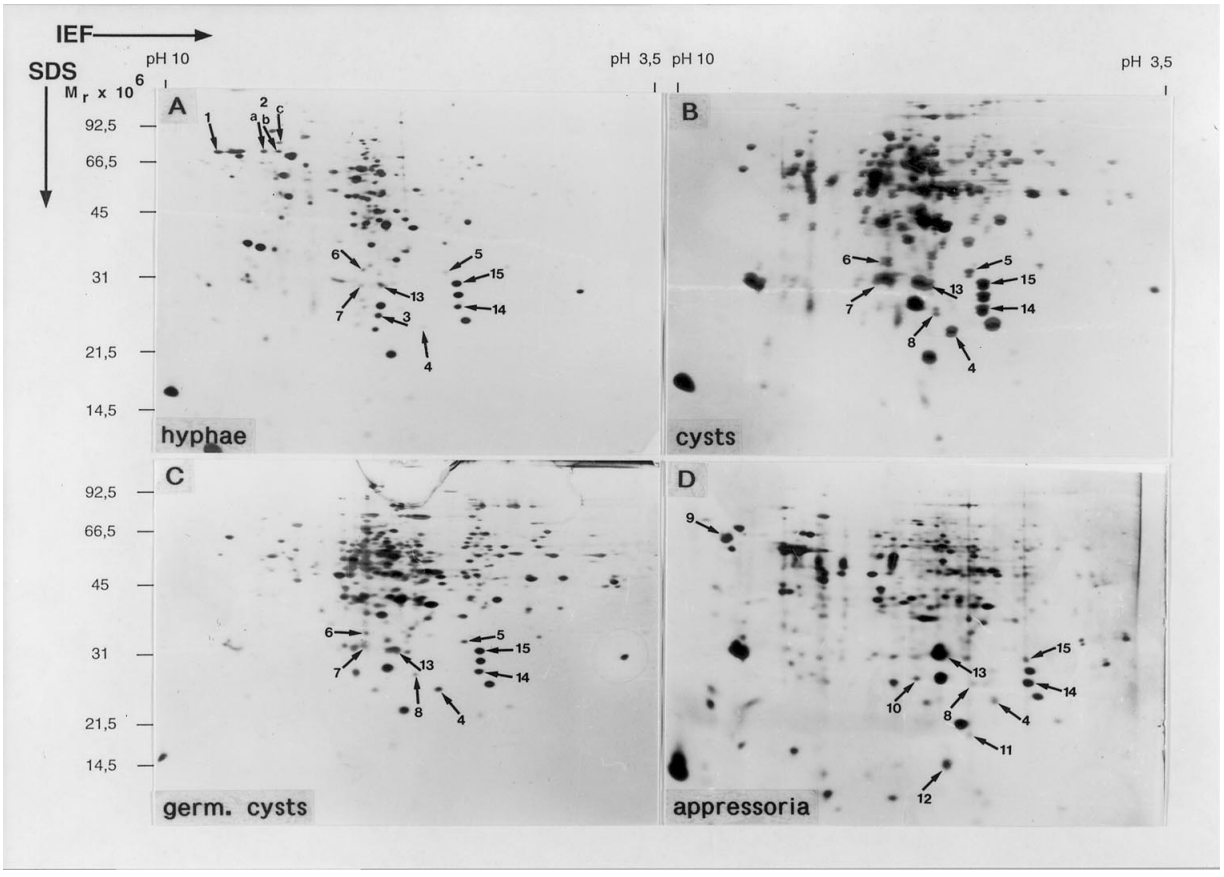

Figure 4. Two dimensional patterns of silver stained proteins. From 4 developmental stages, hyphae (A), cysts (B), germinating cysts (C), and appressoria (D), proteins were extracted, separated on two dimensional polyacrylamide gels and stained with silver. Polypeptides changing their occurrence during development are marked with arrows and labeled with numbers.

the relative position and staining intensity of reproducibly obtained polypeptides was analysed. In Figure 4 two-dimensional protein patterns from extracts of all 4 stages are shown. Striking differences in protein content between each developmental stage are apparent. However, exact identification of each single polypeptide in the area between pH 6–7.5 and 40–60 kDa was difficult due to the large number of polypeptides present in this region. This area was therefore excluded from further evaluation. Clearly identifiable polypeptides that either showed significant changes in staining during fungal differentiation or appeared or disappeared completely, were marked with numbers 1–15 in Figure 4. These proteins were subjected to assessment (Table 1). Polypeptides No. 1, 2a, b, c and 3 appeared only in hyphal extracts. Proteins No. 4, 5, 6, 7 and 8 are dominant in cysts, whereas No. 9, 10, 11 and 12 appeared exclusively in the extracts of appressoria. During germination and appressoria formation polypeptides 13 and 14 increased in intensity, whereas No. 15 decreased.

The *de novo* synthesis of proteins was investigated by *in vivo* labelling experiments. A short time pulse labelling with [^{35}S]methionine prior to extraction of proteins led to efficient incorporation. Interestingly, zoospores induced to encyst at the end of the incubation period, incorporated only 1–2% of the label of germinating cysts. In Figure 6, fluorograms of the two-dimensional patterns of *in vivo* labelled proteins of cysts, germinating cysts, appressoria and hyphae are shown. Similar to the silver-stained protein patterns many of the polypeptides labelled *in vivo* were found in the range of pH 6–8 and molecular weights of 40–65 kDa. After an exposition time of 6 days, 49 polypeptides could be identified clearly. Longer exposition times allowed the visualization of additional polypeptides but led to overexposure of stronger signals and increase of unspecific background. The most

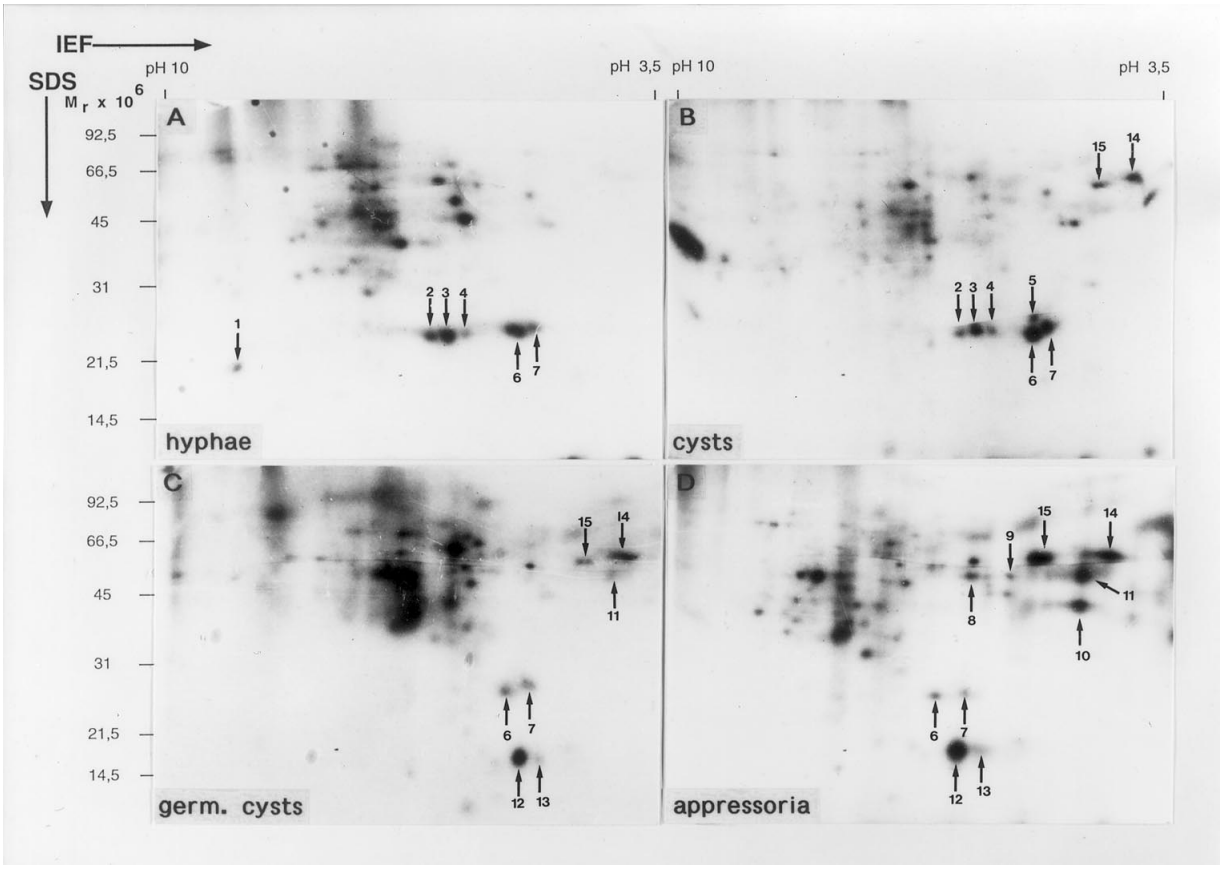

Figure 5. Two-dimensional patterns of *in vivo* labelled proteins. Fungal structures growing *in vitro* were pulse labelled with (^{35}S)methionine, proteins were extracted and subjected to two-dimensional polyacrylamide gel electrophoresis. Labelled polypeptides on the gels from hyphae (A), cysts (B), germinating cysts (C), and appressoria (D) were visualized by fluorography. Differentially synthesized polypeptides are marked with arrows and are labeled with numbers.

pronounced differences were observed between the protein patterns of cysts and germinating cysts. The protein patterns of cysts and hyphae on the one hand, and of germinating cysts and appressoria on the other hand showed a higher degree of similarity. Again, only clearly identifiable polypeptides from two reproducible experiments were marked in Figure 5. The rate of synthesis of only 2 polypeptides (No. 6 and 7) varied throughout all four stages. The others were detected either at particular stages only or were completely absent at one or two of the other stages. For example, polypeptides 1 and 5 appear exclusively in hyphae or cysts, respectively, whereas peptides 2, 3 and 4 are present in both. Peptides No. 8, 9 and 10 are visible only in the extracts form appressoria. During germination and appressoria formation peptides No. 11–15 increased in intensity.

Discussion

Most critical for the successful colonization of a host plant and, hence, propagation of the fungal pathogen appears to be the phase immediately before penetration of the first plant cell. In this view, the appressorium must be regarded as the most important infection structure. It needs not only to be timely developed and properly anchored but also to be positioned at an appropriate site. For *P. infestans* the preferential penetration site on potato leaves is the anticlinal wall of epidermal cells immediately adjacent to stomatal guard cells (Wilson and Coffey, 1980; Gees and Hohl, 1988; Cuypers and Hahlbrock, 1988). Other fungi favour different locations for appressorium formation, for instance, many rust fungi, precisely over stomates (Hoch and Staples, 1987). Obviously pathogenic fungi are able to sense topographic features (Wynn, 1976). In case

Table 1. Changes in protein content during development of infection structures. Comparing two-dimensional protein patterns of hyphae, cysts, germinating cysts and appressoria the relative staining intensity of proteins numbered with 1–15 in Figure 4 was estimated and marked with + (weak), ++ (medium), +++ (strong) or – (not detectable)

Protein number	Mainly present in	Hyphae	Cysts	Germinating cysts	Appressoria
1	Hyphae	++	–	–	–
2a,b,c	Hyphae	++	–	–	–
3	Hyphae	+++	–	–	–
4	Cysts	+	+++	++	+
5	Cysts	+	+++	++	–
6	Cysts	+	++	+	–
7	Cysts	+	+++	+	–
8	Cysts	–	++	+	+
9	Appressoria	–	–	–	+++
10	Appressoria	–	–	–	++
11	Appressoria	–	–	–	+
12	Appressoria	–(+)	–(+)	–(+)	++
13	Appressoria	+	++	++	+++
14	Appressoria	++	++	++	+++
15	All, weaker in appressoria	+++	+++	+++	++

of *Uromyces appendiculatus*, a ridge of an optimum height of 0.5 μm, associated with the stomatal lip of the guard cells of the respective bean host, functions as topographic signal for infection structure differentiation (Hoch et al., 1987). Our observations imply that topographic features may also play an important role in induction of appressorium formation of *P. infestans*, although we have not characterized their exact shape and dimensions. Moreover, it remains open if topographic features are the only type of signal triggering differentiation, or if additional signals, for instance of chemical nature, have to be considered. However, the dramatic changes in protein content may give evidence, that no additional stimulus is required during fungal differentiation. On the other hand, *P. infestans* does not only sense the groove above anticlinal cell walls on the leaf surface, but also orients the growth of its germ tube tip towards epidermal cells immediately adjacent to stomatal guard cells. Thus, only the combination of topographic signals and, possibly, chemical attractants may trigger both, the differentiation and orientation of the fungal structures. It is very clear however, that appressorium development is not influenced by factors involved in the race/cultivar specificity and resistance response. Just like rust and mildew fungi (Bushnell, 1982), *P. infestans* forms appressoria on susceptible as well as resistant hosts (Pristou and Gallegly, 1954; Hohl and Suter, 1976; Gees and Hohl, 1988; Freytag et al., 1994) and even on nonhost plants (Gross et al., 1993).

During the development of infection structures the most striking phenomenon was the migration of the cytoplasm into the appressorium and the subsequent formation of a false septum; this process had never been monitored before in the living fungus. It had been only observed previously that after completion of the appressorium, cyst and germ tube are devoid of cytoplasm (Hohl and Suter, 1976; Coffey and Gees, 1991) and false septa had been described as 'cellulose wall ingrowths' in *P. parasitica* (Gooday and Hunsley, 1971) and as 'electron-dense plugs' in *P. infestans*, where they were detected within the infection tube at the level of entry into the first host cell (Hohl and Suter, 1976; Coffey and Gees, 1991). At a homologous location, between the appressorium and the subsequent vesicular structure, we usually observed a false septum also in the structures grown *in vitro* (see Figures 1 and 2). In addition, we found another false septum inserted within the germ tube between cyst and appressorium. In any case, a false septum was inserted as border between the apical part of a fungal germling containing cytoplasm and the distal part devoid of cytoplasm. From our observations we support the view of Hohl and Suter (1976), that during the prepenetration phase

there is no significant increase in cytoplasmic mass. Therefore, the cytoplasm steadily concentrates within a region proximal to the growing tip of the fungal structure leaving empty appearing parts behind. A lipid layer seems to remain within these parts, as revealed by staining with $DIOC_6$ and Nile red. The mechanism of cytoplasmic migration within this fungus is largely unknown. It is possible, that portions of the plasmamembrane are left behind when the bulk of cytoplasm is moving forward.

For rust fungi it is known that nuclear divisions occur during formation of infection structures, the first mitosis correlating with appressorium development (Mendgen and Deising, 1993). Mitosis also takes place in infection structures of *P. infestans* as we could detect this during growth on polypropylene foil. Since we often found two nuclei in appressoria, the first mitotic event may happen, like in rust fungi, at this developmental stage. During further growth, up to two other mitotic events occur resulting in the maximal number of four nuclei at a stage equivalent to the intracellular vesicle *in planta*.

Spores or cysts of biotrophic fungi must possess a high metabolic capacity. Without the availability of nutrients during the prepenetration phase by utilizing endogenous metabolite stores, they are able to built up a huge mass of wall material and to manage such energy-consuming processes as nuclear divisions. We analysed in detail another capability of fungal germlings, the rapid synthesis and turnover of proteins. For rust fungi it had been shown that the synthesis of a broad spectrum of proteins is changing upon differentiation of fungal germlings and that some proteins are synthesized *de novo* (Huang and Staples, 1982; Staples et al., 1986; Deising et al., 1991). We have demonstrated here that also in the oomycete *P. infestans* striking differences are detectable when the two-dimensional patterns of silver stained proteins from several developmental stages are analysed. Compared to hyphae, in cysts 7 polypeptides were present in increased amounts and one polypeptide was missing. As one would expect, hyphae and cysts are apparently very different developmental stages. With germination of cysts and formation of appressoria some other proteins were found to increase or decrease in their amounts but most strikingly, 3 proteins are specifically found only in appressoria. Those three polypeptides may be regarded as infection related and may play a key-role in fungal pathogenicity.

Direct informations about changes in the actual protein synthesis during development of infection structures were obtained by *in vivo* labelling experiments. From the analysis of the two-dimensional patterns of proteins labelled *in vivo* the following major conclusions can be drawn. With germination of cysts drastic changes in protein synthesis are commencing that continue throughout the formation of germ tube and appressorium. Polypeptides synthesized exclusively at one of the four studied developmental stages can be regarded as stage specific such as polypeptide 10 in appressoria. Our data indicate that a complex program of gene activation and repression is initiated with cyst germination. Evidence for the necessity of protein synthesis for cyst germination of *P. infestans* has been shown by Clark and Melanson (1987) since they were able to inhibit this process with cycloheximide. This gene expression program may account for development of infection structures and for penetration of the first host cell and the respective gene products may either be directly involved in or at least related to this.

Recently, several genes were identified and cloned that are expressed by *P. infestans* specifically during colonization of a susceptible potato host cultivar (Pieterse et al., 1991; Pieterse et al., 1993; Pieterse et al., 1994). The strategy was to differentially screen a genomic library of *P. infestans* using as probes cDNA from heavily infected potato leaves and from *P. infestans* grown on agar plates. We think that *in vitro* growth of *P. infestans* on polypropylene foil, where as we have demonstrated the fungus develops infection structures, may be an alternative system to differentially clone infection-related genes and to study their expression. Recently, *in vitro* grown appressoria of *Colletotrichum gloeosporoides* have been used to successfully clone a gene expressed during this developmental stage (Huang et al., 1995). Disruption of this gene resulted in a marked decrease in virulence.

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