Mycol. Res. **106** (10): 1211–1223 (October 2002). © The British Mycological Society
DOI: 10.1017/S0953756202006512 *Printed in the United Kingdom.*

Characterisation of *Phytophthora nicotianae* zoospore and cyst membrane proteins

Heidi J. MITCHELL[1], Kathryn A. KOVAC[1] and Adrienne R. HARDHAM*

Plant Cell Biology Group, Research School of Biological Sciences, Australian National University, Canberra ACT 2601, Australia.
E-mail: Hardham@RSBS.ANU.edu.au

Received 12 April 2002; accepted 27 July 2002.

Motile zoospores of species of oomycetes encyst rapidly to form walled cysts that germinate and infect host plants. Differences in the protein composition of the plasma membrane and endomembranes of zoospores and cysts of the oomycete *Phytophthora nicotianae* have been explored by comparing patterns of polypeptides in one- and two-dimensional gels of microsomal fractions. Against a backdrop of common components, this comparison revealed that at least 53 proteins were specific to, or occurred preferentially in, the microsomal fraction of one spore type or the other. In addition, proteins common to zoospore and cyst plasma membranes were further investigated by immunocytochemical labelling with a panel of 10 monoclonal antibodies raised against *P. nicotianae* spore components. The results of immunolocalisation studies and immunoblotting showed that the antibodies reacted with four different sets of membrane proteins, and were grouped accordingly. Group 1 antibodies bound preferentially to the bladder of the water expulsion vacuole or a region of the cell surface associated with it. Group 2 antibodies reacted with a protein of high relative molecular weight (>200 kDa) found in zoospores and cyst plasma membranes and in the cleavage membranes of sporangia. Group 3 antibodies reacted with a set of proteins occurring in the plasma membrane of zoospores and cysts, in the peripheral cisternae, in the spongiome membranes of the water expulsion vacuole, in the cleavage membranes of sporangia, and in the plasma membrane and apical vesicle membranes in hyphae and germinating cysts. Group 4 antibodies bound to a set of proteins present in the zoospore and cyst plasma membrane, in sporangial cleavage membranes and in the spongiome but not present in the peripheral cisternae, hyphae or germinating cysts. The results document the presence of proteins that are common to the plasma membrane of all stages of the asexual life cycle of *P. nicotianae*, of proteins that occur in the plasma membrane of the asexual spores but not of hyphae, and of proteins that occur in the membranes of either zoospores or cysts. The results also indicate that zoospore and cyst plasma membrane proteins may be present in specific subsets of other membranes within the zoospores.

INTRODUCTION

Phytophthora nicotianae is a plant pathogen belonging to the *Oomyceota*. It has a wide host range, infecting over 1000 species of plants (Erwin & Ribeiro 1996). Its main method of dispersal is by the production of large numbers of uninucleate, motile zoospores formed by asexual reproduction. The zoospores are released into soil water where they reach new host plants by chemotactically swimming towards substances in the root exudate. On reaching a potential host, they undergo the process of encystment, shedding their flagella and secreting an adhesive which attaches them to the root. Within 5–10 min they form a thin cellulosic cell wall. The newly-formed cysts germinate 10–20 min later and the germ tube invades the underlying root tissue. In susceptible plants, the pathogen colonises the root tissues and sporulates 2–3 d after the onset of infection. Multinucleate sporangia develop on the root surface and subsequently cleave to form uninucleate motile zoospores.

Molecules on the cell surface are known to play important roles in the transfer of information and nutrients, cell–cell recognition and communication (Simon 1992). In the case of a pathogen such as *P. nicotianae*, plasma membrane proteins are likely to be crucial for infection of the host. Receptors will be involved in zoospore chemotaxis (Cameron & Carlile 1981), induction of encystment (Hardham & Suzaki 1986) and triggering secretion of adhesive (Gubler & Hardham 1988,

* Corresponding author.
[1] Present address: Division of Plant Industry, CSIRO, Canberra, ACT 2601, Australia.

Hardham & Gubler 1990). Encysting zoospores also use plasma membrane-bound enzymes for the synthesis of cell wall microfibrils. Surface components such as these have been described as 'pathogenic determinants' as they are the primary factors initiating host-pathogen interactions and influencing pathogen behaviour and even host responses (Pendland & Boucias 1998). Characterisation of plasma membrane proteins of *Phytophthora* zoospores and cysts is clearly an important part of the development of effective methods of controlling these pathogens. In other pathogens, a number of strategies have been adopted to identify surface molecules associated with different stages of fungal growth. Subtractive hybridisation has been used to isolate cDNA clones present in germinating cysts and appressoria but not in zoospores of *P. infestans* (Görnhardt, Rouhara & Schmelzer 2000). These clones include genes encoding mucin-like proteins present in the cell wall of germlings but which disappear as soon as the germlings invade the host. The mucin-like proteins are thought to form a viscous, mucous layer which may protect the germling from desiccation and physical damage. Lectin labelling has been used in studies of *Peronospora parasitica* and *Puccinia coronata* to demonstrate differences in carbohydrates on the surface of infection structures normally occurring on the plant epidermis (Carzaniga, Bowyer & O'Connell 2001) and between infection structures on the surface and inside the plant (Mendgen, Lange & Bretschneider 1985). Lectin labelling has also shown changes in surface carbohydrates during encystment of *P. cinnamomi* zoospores (Hardham & Suzaki 1990).

Antibodies have been successfully used to detect specialisations of the plasma membrane, cell wall and extracellular matrices in a range of fungal and oomycete plant pathogens (Hardham & Mitchell 1998). Monoclonal antibodies, for example, have detected the presence of a proline-rich glycoprotein in the wall of intracellular hyphae but not in the walls of other infection structures of *Colletotrichum lindemuthianum* (Pain *et al.* 1994, Perfect *et al.* 1998). Antibodies have also identified a component specific for the wall of *Melampsora lini* haustoria (Murdoch, Kobayashi & Hardham 1998) and have localised an amino acid transporter, identified by differential screening of a cDNA library, restricted to the plasma membrane of *Uromyces fabae* haustoria (Hahn *et al.* 1997). In the present study, a number of approaches has been used to characterise zoospore and cyst plasma membrane proteins with a view to ultimately elucidating their function in the spores and their role in the infection process. Proteins present in microsomal membranes in zoospores and cysts have been compared using one- and two-dimensional gel electrophoresis, and many differences in the polypeptide complement of these two spore types were found. The binding of ten monoclonal antibodies raised against *P. nicotianae* spore components has been characterised, and four sets of proteins in the plasma membrane of *P. nicotianae* zoospores identified. The results lay the groundwork for future molecular analysis of a range of zoospore and cyst plasma membrane proteins.

MATERIALS AND METHODS

Culture

Phytophthora nicotianae (isolate H1111, Gabor *et al.* 1993; ATCC MYA-141) was isolated from *Nicotiana tabacum* and kindly supplied by Dxxxx Guest (his isolate M4951). Production of *P. nicotianae* zoospores followed Robold & Hardham (1998). Cysts were produced either by vigorously shaking the zoospore suspension in a large measuring cylinder or centrifuging at 1000 ***g*** for 3 min. Newly formed cysts were either used immediately or after 20 min. To produce germinating cysts, zoospores were incubated at room temperature for 25 min with 10 d old *Nicotiana tabacum* seedlings. Vegetative hyphae were produced by inoculating a 150 ml flask of V8 broth (Chen & Zentmyer 1970) with six 5 mm × 5 mm squares of mycelium and shaking at 150 rpm for 4 d at 22 °C in the dark. To prevent initiation of sporulation, the V8 broth was changed each day. The mycelium was harvested by filtering through Miracloth. To produce sporulating hyphae, the mycelium was grown as for vegetative hyphae and then shaken for a further 3 d without changing the V8 broth.

Zoospore and cyst microsomal fractions

Zoospores or 20 min cysts were added to osmoticum buffer at room temperature to give a final concentration of 0·2 M sorbitol, 50 mM MES (2-[*N*-morpholino]ethanesulphonic acid)-KOH (pH 6.5), 0.1% (w/v) polyvinylpolypyrrolidone-40 (PVP-40), 2 mM $MgSO_4$, 5 mM KCl and 1 mM ethylenediamine tetraacetic acid. The cells were immediately sonicated for 40 s (8 × 5 s bursts) to break the plasma membrane. Lysed cells were quickly transferred to precooled centrifuge tubes on ice, centrifuged at 6000 ***g*** for 15 min and the supernatant recentrifuged at 85 000 ***g*** for 1 h. For application to the sucrose gradient the pellet was solubilised in resuspension buffer containing 10 mM MES-KOH (pH 6.5), 2 mM $MgSO_4$, 0.3 M sucrose, 1 mM KCl, 0.1% (w/v) PVP-40, 1 mM dithiothreitol (DTT) and 1 mM phenylmethanesulphonyl fluoride (PMSF).

Separation of microsomal fractions

Sucrose step gradients were formed with 1.0 ml each of 28, 35, 42 and 49% sucrose buffered in 10 mM MES-KOH (pH 6.5), 1 mM KCl, 2 mM $MgSO_4$ and 0.1% (w/v) PVP-40. Approximately 1.0 ml of microsomal fraction (containing 1–2 mg protein) was carefully layered onto each gradient and centrifuged at 85 000 ***g*** for 2 h. Visible membrane bands at the 28/35, 35/42 and 42/49 interfaces were collected, diluted 10-fold with resuspension buffer and re-pelleted at 85 000 ***g*** for 45 min. Pellets were resuspended in 200 µl of resuspension buffer and stored at −80 °C.

SDS-PAGE and silver staining of proteins

Protein samples from the sucrose gradient (20 μg) were mixed with an equal volume of SDS sample buffer, boiled for 2 min and separated in a 7% SDS polyacrylamide gel (Laemmli 1970). Silver staining was performed according to Heukeshoven & Dernick (1985), with several modifications. Gels were fixed in three changes of 50% (v/v) ethanol/10% (v/v) acetic acid (15 min each) and then immersed in 0.5% (w/v) silver nitrate for 30 min, developer (2.5% (w/v) sodium carbonate/0.05% (v/v) formaldehyde) for 15 min, and Farmer's reducer for 5–45 min or until the background staining had been reduced to an appropriate level. The silver nitrate and developer steps were repeated for 20 min and 10 min, respectively, and colour development was stopped with the addition of 5% (v/v) acetic acid. Gels were rinsed with double distilled water between each step and incubated in 1% (v/v) glycerol for 20 min before drying.

Two-dimensional SDS-PAGE

Isoelectric focusing was carried out using a Pharmacia Biotech two-dimensional gel electrophoresis system. Zoospore and cyst microsomal fractions were phenol extracted using a method modified from Hurkman & Tanaka (1986). The microsomal pellets were resuspended in extraction buffer containing 0.7 M sucrose, 0.5 M Tris (Tris(hydroxymethyl)aminomethane), 30 mM HCl, 50 mM EDTA, 0.1 M KCl, 2% (v/v) β-mercaptoethanol and 2 mM PMSF then incubated for 10 min at 4 °. An equal volume of Tris-saturated phenol was added and the suspension was vortexed for 10 min and centrifuged at 13 000 ***g*** for 5 min. The phenol phase was re-extracted with an equal volume of extraction buffer, then five volumes of 0.1 M ammonium acetate in methanol were added to precipitate the protein. After overnight incubation at −20 °, the precipitate was centrifuged at 13 000 ***g*** for 20 min, washed three times with ammonium acetate and once with acetone, air-dried and stored at −20 °. The precipitated microsomal proteins were solubilised in sample buffer containing 9 M urea, 2% (v/v) β-mercaptoethanol, 2% (v/v) carrier ampholytes pH 3–10 and 0.5% (v/v) Triton X-100. Protein samples (200 μg) were applied to 180 mm pH 4–7 linear precast polyacrylamide gel strips and focused for approximately 55 h. The gel strips were rehydrated before use in a solution containing 8 M urea, 0.5% (v/v) Triton X-100, 13 mM DTT and 0.52% (v/v) carrier ampholytes pH 4–7. After isoelectric focusing, strips were equilibrated for 10 min in 0.5 M Tris–HCl (pH 6.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 6.5 mM DTT and then for 10 min in the same buffer containing 10 mM iodoacetamide instead of DTT. The strips were run on precast 12–14% polyacrylamide gels following the manufacturer's instructions using Novex molecular weight standards, and then silver stained.

Immunoblotting of zoospore proteins

Immunoblotting and immunocytochemistry (see below) used 10 monoclonal antibodies raised against *Phytophthora nicotianae* spores (Gautam, Cahill & Hardham 1999) that reacted with the zoospore surface. Samples of freeze-dried zoospores were solubilised in 8 M urea, centrifuged to remove insoluble material and assayed for protein concentration (Bradford 1976). Solubilised proteins (17 μg per lane) were separated in 5–15% SDS-polyacrylamide gels then transferred electrophoretically to Immobilon-P membrane (Millipore Corporation, MA). The membrane was probed with undiluted monoclonal antibody supernatants, followed by sheep anti-mouse immunoglobulin conjugated to alkaline phosphatase (Cope *et al*. 1996). Immunodot blots were performed using freeze-dried *P. nicotianae* zoospores solubilised in 6 M guanidine as described in Robold & Hardham (1998).

Immunofluorescence microscopy

Zoospores and newly formed cysts were fixed in suspension for 30 min in 4% (w/v) formaldehyde/0.2% (v/v) glutaraldehyde in 50 mM Pipes (piperazine-*N*,*N*′-bis(2-ethanesulphonic acid) buffer (pH 7.0). After washing, cells were air dried onto multiwell slides. For cryosectioning, vegetative or sporulating hyphae were fixed for 30 min at room temperature in 4% (w/v) formaldehyde in 50 mM Pipes buffer (pH 7.0). After rinsing in 50 mM Pipes buffer (pH 7.0), cells were frozen in Tissue Tek embedding compound (Miles Elkhart, IN) in plastic moulds by plunging in liquid nitrogen. Sections (12 μm) were cut on a Reichert–Jung 2800 Frigocut E cryotome and dried onto gelatine coated slides. Immunolabelling was as described previously (Hardham *et al*. 1991b). Fluorescence was detected using a Zeiss Axioplan microscope equipped with epifluorescence optics.

Immunogold labelling

For immunogold labelling, zoospores and germinating cysts were fixed in 1% (v/v) glutaraldehyde in 100 mM Pipes buffer (pH 7.0) for 2 h at room temperature, washed in 100 mM Pipes buffer (pH 7.0) and embedded in 2% (w/v) low gelling temperature agarose. The samples were dehydrated in a graded ethanol series at 4 ° until 50% (v/v) ethanol was reached and then at −20 ° for the remaining steps. The samples were embedded in Lowicryl K4M resin under N_2 at −20 °. Ultrathin sections were immunolabelled on gold grids at room temperature as described in Cope *et al*. (1996) with 10 nm gold conjugated goat anti-mouse antibodies (GAM-Au10, Amersham) as the secondary antibody. Grids were stained with 2% (w/v) uranyl acetate for 6 min followed by lead citrate for 2 min. Sections were viewed at 75 kV using a Hitachi 7100 transmission electron microscope.

RESULTS

One-dimensional gel analysis of proteins

Phytophthora nicotianae proteins from the microsomal fractions at the 28/35, 35/42 and 42/49% interfaces on the sucrose gradients were separated in an SDS polyacrylamide gel and stained with silver (Fig. 1). For both zoospore and cyst samples, the general pattern of bands was similar in the fractions from the three interfaces, but the intensity of the bands in the samples from the different interfaces varied considerably. Comparison of the zoospore and cyst samples at each interface showed many differences in relative intensity of bands between the two cells types, and several bands were visible in samples from one cell type only. Six different bands were unique to or more prominent in the zoospore fraction and 11 different bands were unique to or more prominent in the cyst fraction (Fig. 1).

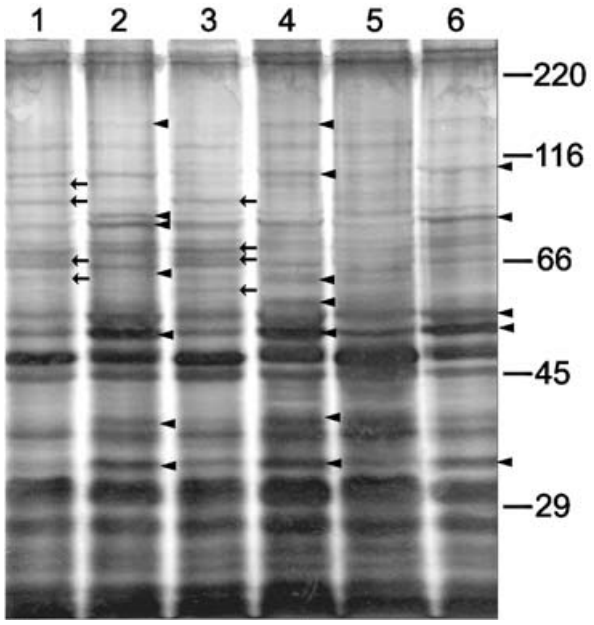


Fig. 1. SDS-polyacrylamide gel of *Phytophthora nicotianae* zoospore and cyst microsomal fractions separated on sucrose step gradients and silver stained. Lane 1, zoospore 28/35% interface; lane 2, cyst 28/35% interface; lane 3, zoospore 35/42% interface; lane 4, cyst 35/42% interface; lane 5, zoospore 42/49% interface; lane 6, cyst 42/49% interface. Bands that were unique to or more prominent in zoospore (arrows) or cyst (arrowheads) fractions are marked. The position of molecular weight standards is indicated on the right (kDa).

Two-dimensional gel analysis of proteins

To further compare the protein composition of the *Phytophthora nicotianae* zoospore and cyst preparations, solubilised proteins from microsomal fractions of both cells types were separated on two-dimensional gels and silver stained (Figs 2–3). The molecular weights of the majority of the polypeptides were evenly distributed through the size range 10–55 kDa, and most had isoelectric points of 4.5–5.5. Comparison of the two gels shows that many protein spots are either unique to, or more intense in, gels from one cell type than from the other. Five spots were present in zoospore fractions only; 23 spots were present in cyst fractions only; nine spots were more intense in zoospore fractions than in cyst fractions; and 16 spots were more intense in cyst fractions than in zoospore fractions.

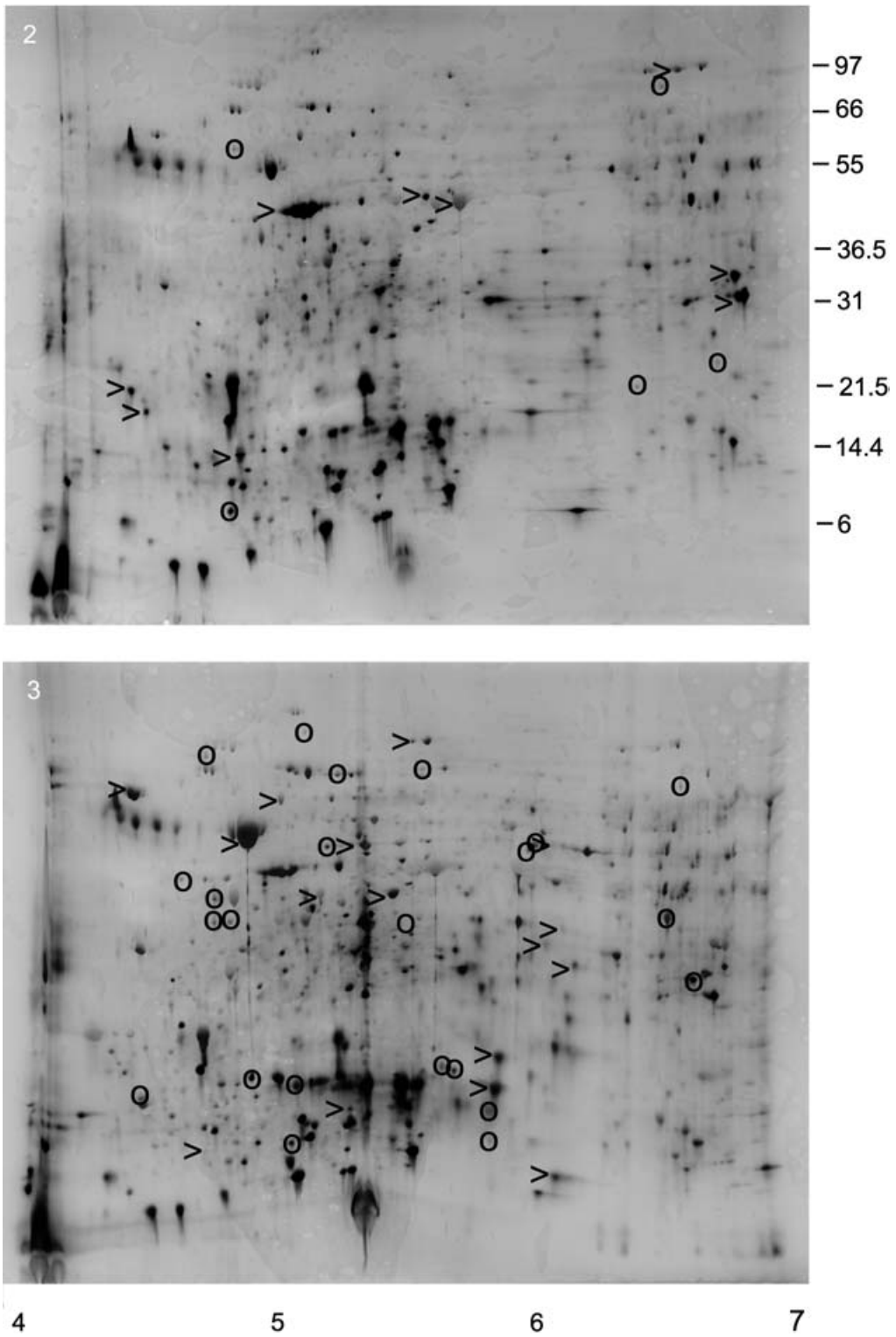
Characterisation of proteins using immunofluorescence microscopy

The protein composition of *Phytophthora nicotianae* zoospore and cyst membranes was investigated using immunocytochemical labelling. The reaction of ten monoclonal antibodies raised against *P. nicotianae* spores was tested with *P. nicotianae* cysts, zoospores, sporulating hyphae and vegetative hyphae. No labelling was observed in controls in which the primary antibody was omitted.

The mature cyst wall is a barrier to antibody access to the plasma membrane. In order to investigate reaction of the antibodies with the cyst plasma membrane, young cysts fixed within 5 min after the induction of encystment were used in immunofluorescent labelling experiments. In these cells, the cell wall is not fully formed and the antibodies are able to reach the underlying plasma membrane. All ten antibodies reacted with the surface of young cysts although the labelling intensity varied considerably (Fig. 4). The brighter cells were often not spherical, a feature indicative of immature walls (e.g. cell * in Fig. 4). Thus the fluorescence is consistent with the labelling of the plasma membrane rather than the cyst wall. Bright spots on the surface of some cells are probably due to labelling of the plasma membrane of detached and degenerating flagella.

The reaction of the antibodies with zoospores was investigated using cells fixed in a combined formaldehyde-glutaraldehyde solution that preserves the plasma membrane of the zoospores and allows surface labelling only (Hardham 1985). Based on the results of the immunofluorescence assay, the ten antibodies were divided into two groups. Pn3B7 and Pn10D4 reacted strongly with the water expulsion vacuole or an area of plasma membrane associated with it in the anterior end of the cell and weakly with the zoospore plasma membrane over the rest of the cell body (Fig. 5). The other eight antibodies, Pn3B3, Pn3C3, Pn3C6, Pn3F7, Pn4D6, Pn6G9, Pn7B5 and Pn11F3, all gave a uniform labelling of the zoospore plasma membrane, including the domains over the flagella, and associated with the water expulsion vacuole (Fig. 6).

Testing of the ten antibodies on cryosections of *P. nicotianae* hyphae led to further subdivision of the antibodies based on labelling patterns. Of the ten antibodies, only Pn3C6, Pn6G9 and Pn11F3 reacted positively with vegetative hyphae, labelling surface and cytoplasmic structures (Fig. 7). All the antibodies gave

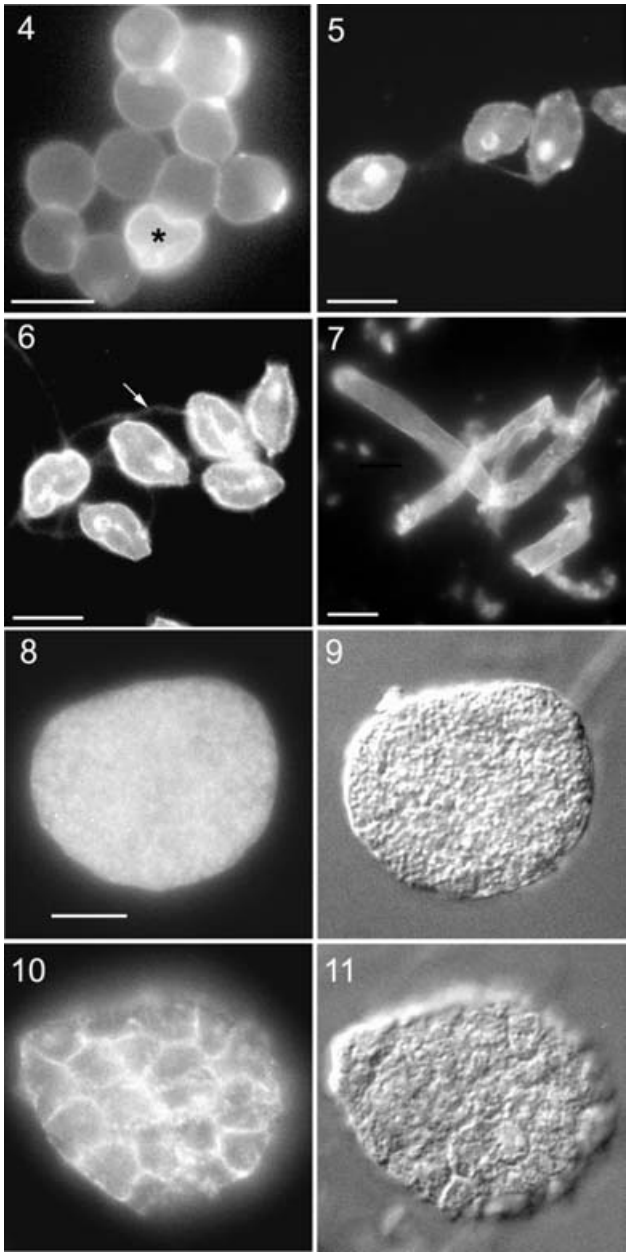


Figs 2–3. Two-dimensional SDS-PAGE of *Phytophthora nicotianae* zoospore (**Fig. 2**) and cyst (**Fig. 3**) microsomal fractions. Protein spots that are more intense in one gel than in the other are indicated with arrowheads and proteins unique to one gel are circled. The position of molecular weight standards is indicated on the right (kDa) and approximate pI is indicated beneath.

a diffuse fluorescence in the cytoplasm of uncleaved sporangia (Figs 8–9), and all except Pn3B7 and Pn10D4 labelled the cleavage membranes in sporangia undergoing cytokinesis during zoospore formation (Figs 10–11).

Ultrastructural localisation of membrane antigens

Four of the ten antibodies did not react with material processed for electron microscopy, but post-embedding immunogold labelling of *Phytophthora nicotianae*



Figs 4–11. Immunofluorescence labelling of *Phytophthora nicotianae* cells. Bars = 10 μm. **Fig. 4.** Labelling of the surface of newly encysted spores by Pn7B5. The cell indicated by the asterisk is not spherical and is likely to be a younger cyst than the other cells. **Fig. 5.** Strong labelling of the water expulsion vacuole and weak labelling of the plasma membrane of zoospores by Pn10D4. **Fig. 6.** Labelling of the plasma membrane of zoospores by Pn3B3. Arrow: flagellar labelling. **Fig. 7.** Labelling of hyphae by Pn3C6. **Figs 8–9.** Diffuse cytoplasmic labelling of uncleaved sporangia by Pn3C3. **Fig. 8.** Immunofluorescence image. **Fig. 9.** Differential interference contrast image. **Figs 10–11.** Labelling of cleavage membranes in sporangia by Pn4D6. **Fig. 10.** Immunofluorescence image. **Fig. 11.** Differential interference contrast image.

zoospores and germinating cysts with the other antibodies confirmed the grouping of Pn3C6, Pn6G9 and Pn11F3, and its distinction from a group including Pn3F7, Pn4D6 and Pn7B5. In zoospores, Pn3C6, Pn6G9 and Pn11F3 all labelled the plasma membrane, the underlying peripheral cisternae, the spongiome tubules of the water expulsion vacuole, and vesicular and tubular membranes in the zoospore cytoplasm (Figs 12–15). The bladder of the water expulsion vacuole, the endoplasmic reticulum and other organelle membranes were not labelled. In germinating cysts, these antibodies labelled the plasma membrane as well as small (100–470 nm) vesicles in the cyst and germ tube tip (Figs 16–17). In cross-section, these vesicles appeared either spherical or tubular.

The other three antibodies that gave positive reactions in the post-embedding labelling, Pn3F7, Pn4D6 and Pn7B5, also labelled the plasma membrane and the spongiome but not the bladder of the water expulsion vacuole (Figs 18–20). They did not label the plasma membrane or apical vesicles in germinating cysts. In addition, these antibodies did not appear to label the peripheral cisternae, which lies within a zone 30–60 nm inside the outer surface of the plasma membrane. To quantitatively assess this impression, the distance of gold particles (between 187 and 361 gold particles for each antibody) from the outer surface of the plasma membrane was measured in micrographs of zoospores labelled with the six antibodies (Fig. 21). For the first group of antibodies, Pn3C6, Pn6G9 and Pn11F3, 63, 64, and 72% of the gold particles, respectively, occurred within a 100-nm zone on the inside of the plasma membrane. However, for the second group of antibodies, Pn3F7, Pn4D6 and Pn7B5, the distribution of gold particles was approximately the same on either side of the plasma membrane, with 46, 44 and 46% of the gold particles, respectively, lying within a 100 nm zone on the inside of the plasma membrane.

Biochemical characterisation of the antigens

To determine the nature of the antigenic epitopes, the antibodies were tested in a dotblot assay using zoospores which had been freeze-dried and solubilised in 6 M guanadine. Eight of the nine antibodies tested showed a strong reaction in the dotblot assay. Solubilised antigens were pre-treated with either pronase, which degrades protein epitopes, or periodate, which oxidises carbohydrate epitopes, prior to antibody labelling (illustrated for Pn3C3 and Pn6G9 in Fig. 22). Labelling with Pn3B3, Pn3C3, Pn3F7, Pn4D6 and Pn7B5 was completely abolished by pronase digestion. Weak sensitivity to pronase digestion was seen for labelling with the water expulsion vacuole antibodies, Pn3B7 and Pn10D4, at 1 mg ml^{-1} pronase. Labelling with Pn3C6 and Pn6G9 showed no sensitivity to either pronase or periodate digestion.

Nine of the antibodies were tested on western blots of zoospore proteins separated by SDS-PAGE (Fig. 23). Pn3B3 and Pn3C3 both reacted with a similar sized protein of >200 kDa M_r. Pn3C6 and Pn6G9 both reacted with a protein at 40 kDa M_r. Pn6G9 also produced a smeared reaction between about 160 and 23 kDa M_r.

Pn3F7, Pn4D6 and Pn7B5 labelled a band >200 kDa M_r, but of lower M_r than the band seen for Pn3B3 and Pn3C3, and a smear of proteins. There was no reaction detected on western blots with Pn3B7 and Pn10D4, the two antibodies that preferentially label the water expulsion vacuole in immunofluorescence assays. The characterisation and grouping of the antibodies is summarised in Table 1.

DISCUSSION

It is widely recognised that plasma membrane proteins play important roles in the reception and transmission of signals to and from the environment and other cells, and in the maintenance of ionic homeostasis in the cytoplasm. While there is much known about a wide range of plasma membrane proteins including receptors, pumps, channels, transporters and cytoskeleton-associated proteins in animal cells, there is only limited information on plant or fungal plasma membrane proteins. For plants, the situation has improved over the last few years with the sequencing of polypeptides separated from plant plasma membrane preparations by high resolution two-dimensional gel electrophoresis (Rouquié *et al.* 1997, Santoni *et al.* 1998, 1999) and sequencing of cDNAs selected by immunoscreening (Shi *et al.* 1995, Galaud *et al.* 1999). These studies include investigations of developmental differences in the protein complement of the plasma membrane (Borgmann, Sinha & Frommer 1994, Masson & Rossignol 1995). Information on proteins in fungal plasma membranes has not been as rapidly forthcoming, although genes encoding an amino acid permease and a hexose transporter have recently been cloned and the proteins shown to reside in the plasma membrane of the haustorium of the biotrophic fungus, *Uromyces fabae* (Hahn *et al.* 1997, Voegele *et al.* 2001). At this stage, the H^+-ATPase still remains the most well-studied fungal plasma membrane protein (Monk & Perlin 1994, Struck, Hahn & Mendgen 1996) and has also been shown to occur in the plasma membrane of *Phytophthora nicotianae* zoospores (Mitchell & Hardham 1999). In the present study, one- and two-dimensional gel electrophoresis has been used to compare proteins in the microsomal fraction of *P. nicotianae* zoospores and cysts, and a panel of monoclonal antibodies directed towards components of the zoospore plasma membrane has been used to further characterise plasma membrane proteins.

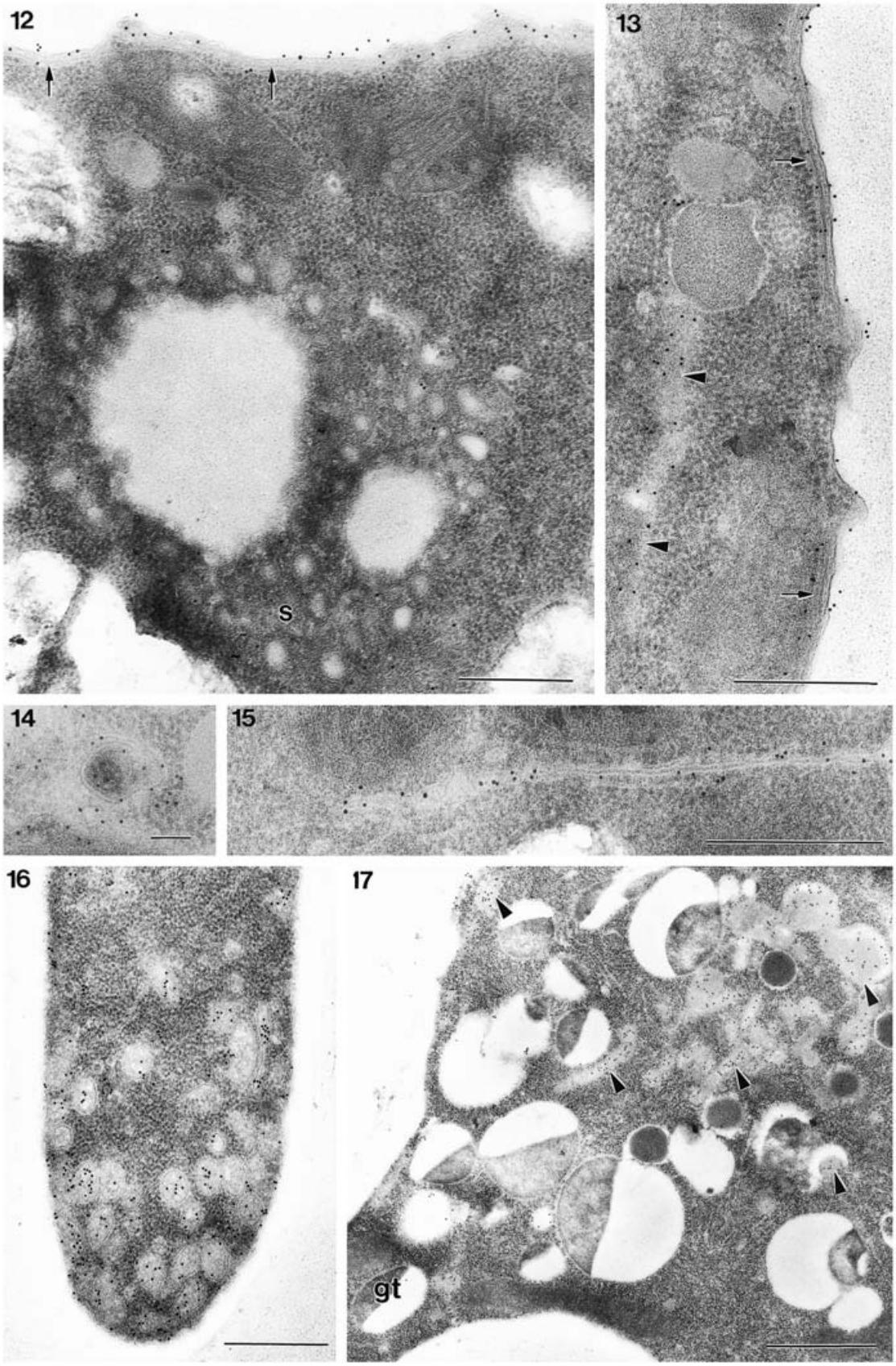
Changes in microsomal proteins during encystment

Encystment of motile, biflagellate oomycete zoospores to form walled cysts is a rapid process that involves a number of dramatic changes to cell structure and organisation. Within 5 min of the induction of encystment, the flagella are detached; the peripheral cisternae, which underlie the plasma membrane over the whole zoospore surface except the groove, vesiculate and apparently fuse with the plasma membrane; the contents of ventral and dorsal vesicles are secreted; basal bodies, mitochondria and large peripheral vesicles move away from the cell surface; the water expulsion vacuole complex disappears; and a cell wall containing cellulose microfibrils is formed (Bimpong & Hickman 1975, Paktitis, Grant & Lawrie 1986, Hardham 1989, Hardham *et al.* 1994). Within 5–10 min, the cell wall is sufficiently strong to allow the generation of cell turgor pressure.

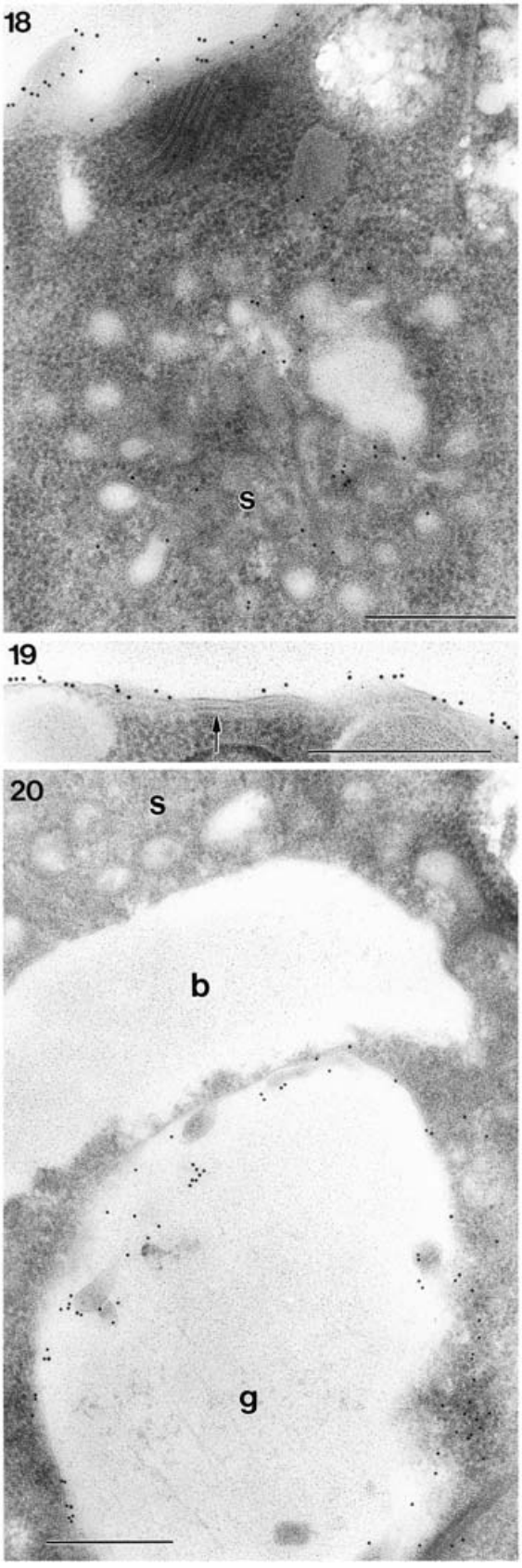
Many, but not all, of these changes are likely to contribute to differences in the protein complement of the microsomal fraction from the two cell types. Flagellar detachment, for example, will lead to loss of any proteins restricted to the flagellar plasma membrane. We have immunocytochemical evidence that at least one protein (labelled by monoclonal antibody Zf-1; (Hardham, Suzaki & Perkin 1986) is specific to this domain, and in all likelihood there are many others. On the other hand, proteins in the membranes of the peripheral cisternae, and ventral and dorsal vesicles may still be present in the cysts, despite the fusion of these organelles with the plasma membrane.

The electrophoretic analysis of the microsomal preparations from *P. nicotianae* zoospores and cysts revealed many differences in the spectrum of proteins from these two cell types. The similar appearance of the banding pattern in the different fractions of the sucrose gradient indicates that this procedure did not efficiently separate different types of membranes in the microsomal fraction. Nevertheless this analysis showed six polypeptides that decreased or disappeared during the encystment process and 11 polypeptides that appeared or increased in abundance. The two-dimensional gel analysis revealed even more extensive changes, with 14 polypeptides unique to, or more abundant in, the zoospore samples and 39 polypeptides unique to or more abundant in the cyst samples. This figure is likely to be an underestimate of the number of changes because only polypeptides with a M_r less than approximately 100 kDa are included, and it is also likely that some integral membrane proteins have not been solubilised by the detergent used, a technical problem which is currently being addressed (Molloy 2000).

The changes observed are remarkable considering the short time period (5 min) between the two developmental stages. Other studies that have used one- or two-dimensional gel electrophoresis to compare proteins produced during the differentiation of fungal infection structures have detected fewer changes despite longer time periods and extensive morphological changes. Using one-dimensional gels, the appearance of two or three new proteins has been detected during the differentiation of appressoria and substomatal vesicles in wheat rust (Wanner *et al.* 1985) and bean rust (Huang & Staples 1982), respectively. During the same developmental period in bean rust, analysis of two-dimensional gels revealed more changes with the appearance of eight new polypeptides, an increase in the amounts of several small polypeptides and a decrease in many polypeptides



Figs 12–17. Immunogold post-embedding labelling of *P. nicotianae* zoospores and cysts with Pn3C6, Pn6G9 and Pn11F3. **Fig. 12.** Zoospore labelled with Pn6G9. The antibody labels the plasma membrane, peripheral cisternae (arrows) and the spongiome (s) of the water expulsion vacuole. Bar = 500 nm. **Fig. 13.** Zoospore labelled with Pn3C6. The antibody reacts with the plasma membrane and peripheral cisternae (arrows) and with an elongated membraneous structure (arrowheads) in the cytoplasm. Bar = 500 nm. **Fig. 14.** Pn6G9 labels a circular membraneous structure in the cytoplasm of a zoospore. Bar = 100 nm.



greater than 69 kDa in size (Staples *et al.* 1986). The only previous study in the Oomycetes followed changes in the total protein complement during the development of infection structures in *P. infestans* (Krämer, Freytag & Schmelzer 1997). On comparison of hyphae, cysts, germinating cysts and appressoria, three polypeptides specific to hyphae and three to appressoria were identified, as well as changes in the levels of ten other proteins.

Immunocharacterisation of zoospore and cyst plasma membranes

Characterisation of the ten antibodies that bind to the zoospore and cyst plasma membranes has given evidence of four different sets of proteins in the plasma membrane of the *Phytophthora nicotianae* spores.

The two antibodies in Group 1 (Pn3B7 and Pn10D4) react predominantly with an area of the zoospore surface at the site of the water expulsion vacuole. This, purportedly contractile, vacuole functions to expel water entering the wall-less zoospore by osmosis (Patterson 1980). It consists of a central bladder surrounded by a reticulum of tubules and vesicles known as the spongiome. In the immunofluorescence assay, fixation of the zoospores with glutaraldehyde and formaldehyde allows only surface labelling (Hardham 1985). The distribution pattern of the Group 1 antibodies suggests that they are either labelling the bladder when it is open to the external medium or a domain on the plasma membrane overlying the water expulsion vacuole complex. Unfortunately, these antibodies do not react in post-embedding immunogold labelling so it is not possible to verify the binding site.

In addition to labelling the plasma membrane, the antibodies in Group 3 (Pn3C6, Pn6G9 and Pn11F3) and Group 4 (Pn3F7, Pn4D6 and Pn7B5) react in immunogold assays with the spongiome of the water expulsion vacuole but not with the bladder membrane. A previous study of *P. nicotianae* also showed that the two

Figs 18–20. Immunogold post-embedding labelling of *Phytophthora nicotianae* zoospores with Pn3F7, Pn4D6 and Pn7B5. **Fig. 18.** Zoospore labelled with Pn7B5. The antibody labels the plasma membrane and the spongiome (s) of the water expulsion vacuole. Bar = 500 nm. **Fig. 19.** Zoospore labelled with Pn3F7. Most gold particles lie over the plasma membrane but not over the peripheral cisternae (arrow). Bar = 100 nm. **Fig. 20.** Zoospore labelled with Pn4D6 showing reaction with the plasma membrane in the groove region (g) of the zoospore but not of the bladder (b) of the water expulsion vacuole. s, spongiome of the water expulsion vacuole. Bar = 500 nm.

Fig. 15. Pn11F3 labels an elongated membranous structure in the cytoplasm of a zoospore. Bar = 500 nm. **Fig. 16.** Germinating cyst labelled with Pn3C6. The antibody reacts with the hyphal plasma membrane and vesicles present in the hyphal tip. Bar = 500 nm. **Fig. 17.** Germinating cyst labelled with Pn3C6. The antibody reacts with the cyst plasma membrane and vesicles (arrowheads) present in the cyst. gt, germ tube. Bar = 1 μm.

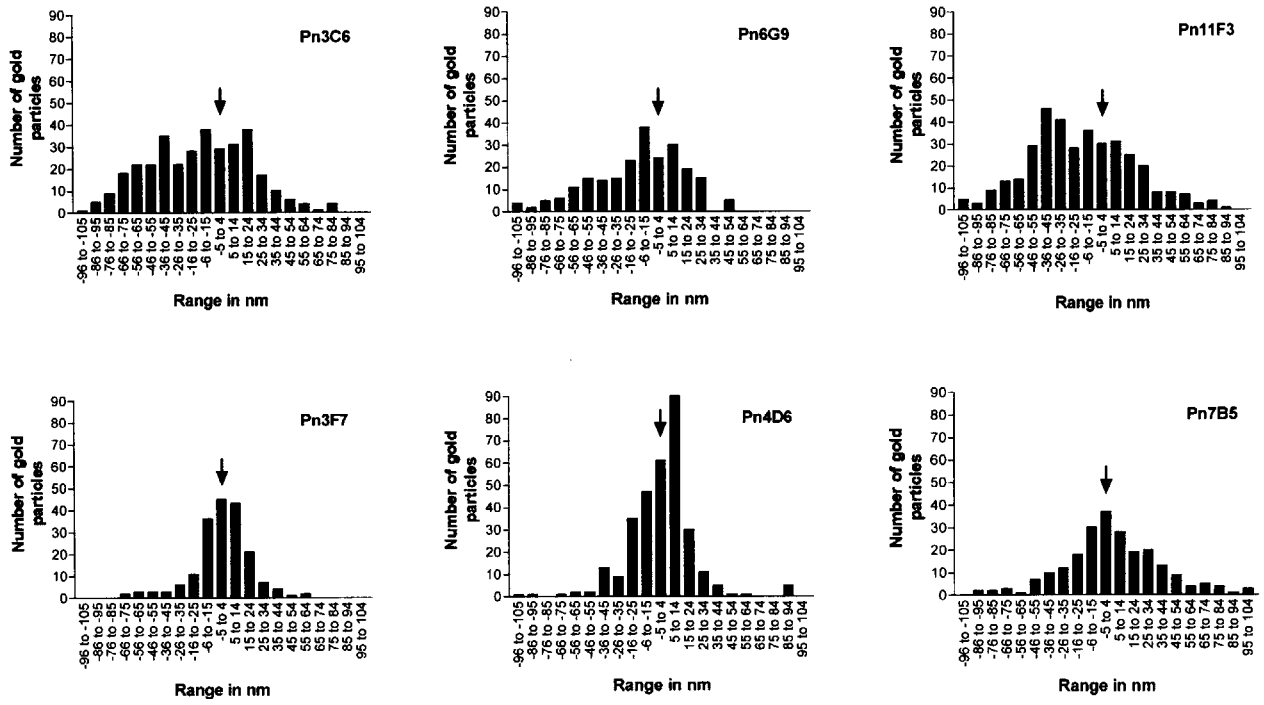


Fig. 21. Graphs showing analysis of the distance of gold particles from the outer surface of the plasma membrane of zoospores of *Phytophthora nicotianae* in micrographs labelled with Pn3F7, Pn4D6, Pn7B5, Pn3C6, Pn6G9 and Pn11F3. The arrow indicates the position of the plasma membrane. Negative values show distribution in the cell cortex, positive values show distribution outside the cell.

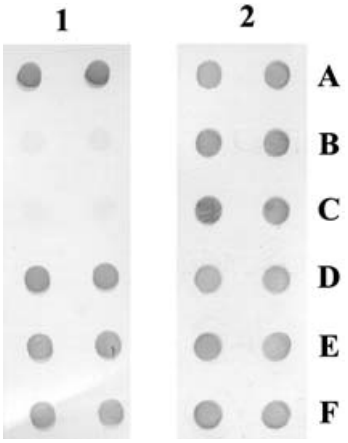


Fig. 22. Dotblot showing antibody reaction with *Phytophthora nicotianae* zoospore extracts after pronase or periodate treatment. Duplicated samples are labelled with Pn3C3 (1) and Pn6G9 (2). Rows A–C have been treated with TBS (A) 100 μg ml^{-1} pronase (B) or 1 mg ml^{-1} pronase (C). Rows D–F have been treated with acetate buffer (D) or 10 mM periodate (E) or 20 mM periodate (F). Labelling with Pn3C3 is completely abolished by pronase digestion. Labelling with Pn6G9 shows no sensitivity to either pronase or periodate digestion.

membrane systems of the water expulsion vacuole were distinct, as only the spongiome was labelled by an antibody specific for vacuolar type H$^+$-ATPase (Mitchell & Hardham 1999). A model for the operation of the water expulsion vacuole in *Phytophthora* zoospores proposes that the spongiome develops into the bladder which then fuses with the plasma membrane during the water expulsion phase (Cho & Fuller 1989a). In light of the immunocytochemical data showing differences in the protein content of the two membranes, for this model to be correct, conversion of the spongiome into the bladder would have to involve retrieval of at least the vacuolar type H$^+$-ATPase and the proteins targetted by the Group 3 and Group 4 antibodies.

The proteins labelled by the Group 3 antibodies are found in the plasma membrane of all stages of the asexual life cycle of *P. nicotianae*. Their presence in zoospore peripheral cisternae and cyst and hyphal apical vesicles is likely to be related to a role in the plasma membrane. Membranes of the peripheral cisternae are morphologically similar to the plasma membrane (Hardham 1987). Vesiculation and fusion of the peripheral cisternae with the plasma membrane could bring about rapid and wholesale changes to the composition of the plasma membrane during encystment.

It is generally accepted that apical vesicles in tip-growing cells such as fungal hyphae, pollen tubes and root hairs fuse with the plasma membrane to deliver new membrane, wall matrix materials and enzymes. In fungal hyphae, secreted enzymes may be responsible for wall modifications, for degradation of the host cell wall and for breakdown of molecules for nutrition. Apical vesicles in fungi have been divided into two categories: microvesicles which are less than 100 nm in diameter, and macrovesicles which are greater than 100 nm in diameter (Bartnicki-Garcia 1990). The vesicles in *P. nicotianae* labelled by the Group 3 antibodies range in

Table 1. Summary of antibody labelling reactions.

		Zoospore									
Group	Mab	PM	PC	B	S	Cyst	Hyphae	AV	CM	Dotblot	Western blot
1	Pn3B7	+/−	ND	+	ND	+	−	ND	−	Pronase	ND
1	Pn10D4	+/−	ND	+	ND	+	−	ND	−	Pronase	ND
2	Pn3B3	+	ND	ND	ND	+	−	ND	+	Pronase	⩾200 kDa
2	Pn3C3	+	ND	ND	ND	+	−	ND	+	Pronase	⩾200 kDa
3	Pn3C6	+	+	−	+	+	+	+	+	Neither	40 kDa
3	Pn6G9	+	+	−	+	+	+	+	+	Neither	40 kDa + smear
3	Pn11F3	+	+	−	+	+	+	+	+	ND	ND
4	Pn3F7	+	−	−	+	+	−	−	+	Pronase	>200 kDa
4	Pn4D6	+	−	−	+	+	−	−	+	Pronase	>200 kDa
4	Pn7B5	+	−	−	+	+	−	−	+	Pronase	>200 kDa

Abbreviations: Mab, monoclonal antibody; AV, apical vesicles; CM, sporangial cleavage membranes; PM, plasma membrane; PC, peripheral cisternae; B, water expulsion vacuole bladder; S, water expulsion vacuole spongiome; ND, not determined; +, labelled; −, unlabelled; +/−, weak labelling.

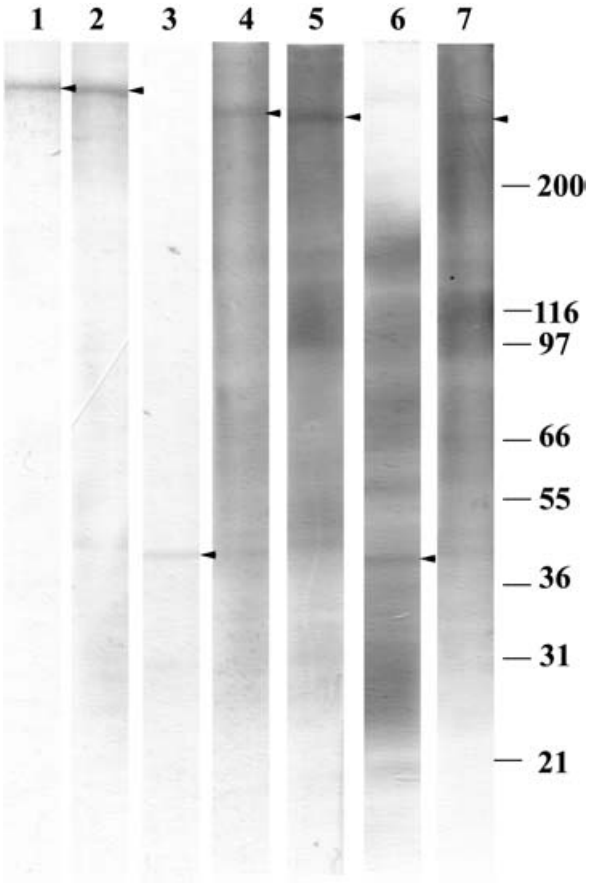


Fig. 23. Immunoblots of *Phytophthora nicotianae* zoospore proteins separated by SDS-PAGE. Lane 1, Pn3B3; lane 2, Pn3C3; lane 3, Pn3C6; lane 4, Pn3F7; lane 5, Pn4D6; lane 6, Pn6G9; lane 7, Pn7B5. Major bands recognised by the antibodies are indicated (arrowheads). The positions of molecular weight standards are indicated on the right (kDa).

size from 100–470 nm diam and thus fit into the macrovesicle category. Similar vesicles have been observed in *P. infestans* hyphal apices (Förster & Mendgen 1987) and in germinating cysts of *P. palmivora* prepared by freeze-substitution (Cho & Fuller 1989b). Apart from evidence of pectinesterase (Förster & Mendgen 1987) and cellulase enzymes (Nolan & Bal 1974), our knowledge of the contents of apical vesicles is still limited. As Bartnicki-Garcia (1990) points out, we do not know if macrovesicle populations are uniform or if different vesicles transport different molecules.

Labelling of *P. nicotianae* apical vesicles has also been observed with the monoclonal antibody Cpw-1. This antibody, which reacts with a smear of polypeptides greater than 60 kDa in size (Hyde *et al.* 1991), labels the peripheral cisternae (probably lumenal contents), the cyst wall (Hardham, Gubler & Duniec 1991a) and apical vesicles in germinating cysts (F. Gubler & A. R. Hardham, unpubl.). In this case, the labelling pattern is indicative of the antigens being wall polymers rather than plasma membrane components. Antibodies (Pn2F5, Pn3F2 and Pn9G7) that have been described in an earlier study of *P. nicotianae* spore surface components (Robold & Hardham 1998) appear to have a possibly similar binding distribution to those of Group 3 described in the present study. Pn2F5, Pn3F2 and Pn9G7 target a proteinaceous antigen in the plasma membrane and peripheral cisternae of *P. nicotianae* zoospores and, in cysts, in multivesicular bodies, isolated vesicles and the cell surface. The reaction of these antibodies with hyphae and germinating cysts has not been determined. Immunoblots, however, indicate that these antibodies and those in Groups 2, 3 and 4 in the present study bind to different sets of polypeptides and are thus distinct.

Group 4 antibodies, in contrast to those of Group 3, label the plasma membrane and spongiome but not the peripheral cisternae, apical vesicles or hyphal plasma membrane. Group 2 antibodies label the plasma membrane of zoospores and cysts and not the hyphal plasma membrane, but it has not been possible to determine if there are any intracellular binding sites because the antibodies do not react in immunogold labelling assays. It is of interest to note that, like the Pn2F5, Pn3F2 and Pn9G7 antibodies previously described (Robold & Hardham 1998), antibodies in Groups 2 and 4 target protein epitopes while binding of the two antibodies

tested from Group 3 (Pn3C6 and Pn6G9) is not inhibited by either pronase or periodate.

The five groups of monoclonal antibodies that label the zoospore plasma membrane and whose binding characteristics have been investigated in the present and earlier studies (Robold & Hardham 1998, Gautam *et al.* 1999) display a range of interactions with additional cell structures, as described above. While binding of a number of the antibodies to the peripheral cisternae and, in some cases, apical vesicles can be understood in the context of these components being direct precursors to the plasma membrane, cross-reaction with a protein in the spongiome of the water expulsion vacuole would seem to be in a different category. The proteins involved may serve a similar function in this distinct location to that in the plasma membrane. There may be many proteins common to the plasma membrane and spongiome membrane but absent from all other membranes in *Phytophthora* zoospores; possible candidates could include a member of the aquaporin superfamily (Maurel & Chrispeels 2001). A range of distributions of plasma membrane proteins is also apparent from the sequence analysis of plant plasma membrane proteins separated in two-dimensional gels (Santoni *et al.* 1998). In this latter study, many of the plasma membrane proteins that have sequence homologies with known proteins are also found in other subsets of membranes in the plant cell.

The investigations of *Phytophthora* spore plasma membrane components described will form the basis for future studies that complement the biochemical and cell biological approaches with molecular genetic techniques. Microsequencing of proteins separated in two-dimensional gels, and cloning and sequencing of genes encoding the antigens targetted by antibodies described here will help elucidate the nature and functions of plasma membrane proteins in these cells and will increase our understanding of their roles in *Phytophthora* spore biology and pathogenicity.

ACKNOWLEDGEMENTS

We thank Val Maclean for excellent technical assistance. Aspects of this study were undertaken while K.K. was enrolled in the Division of Biochemistry and Molecular Biology at the Australian National University.

REFERENCES

Bartnicki-Garcia, S. (1990) Role of vesicles in apical growth and a new mathematical model of hyphal morphogenesis. In *Tip Growth in Plant and Fungal Cells* (I. B. Heath, ed.): 211–232. Academic Press, San Diego.

Bimpong, C. E. & Hickman, C. J. (1975) Ultrastructural and cytochemical studies of zoospores, cysts, and germinating cysts of *Phytophthora palmivora*. *Canadian Journal of Botany* **53**: 1310–1327.

Borgmann, K., Sinha, P. & Frommer, W. B. (1994) Changes in the two-dimensional protein pattern and in gene expression during the sink-to-source transition of potato tubers. *Plant Science* **99**: 97–108.

Bradford, M. M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-binding dye. *Analytical Biochemistry* **72**: 248–254.

Cameron, J. N. & Carlile, M. J. (1981) Binding of isovaleraldehyde, an attractant, to zoospores of the fungus *Phytophthora palmivora* in relation to zoospore chemotaxis. *Journal of Cell Science* **49**: 273–281.

Carzaniga, R., Bowyer, P. & O'Connell, R. J. (2001) Production of extracellular matrices during development of infection structures by the downy mildew *Peronospora parasitica*. *New Phytologist* **149**: 83–93.

Chen, D.-W. & Zentmyer, G. A. (1970) Production of sporangia by *Phytophthora cinnamomi* in axenic culture. *Mycologia* **62**: 397–402.

Cho, C. W. & Fuller, M. S. (1989a) Observations of the water expulsion vacuole of *Phytophthora palmivora*. *Protoplasma* **149**: 47–55.

Cho, C. W. & Fuller, M. S. (1989b) Ultrastructural studies of encystment and germination in *Phytophthora palmivora*. *Mycologia* **81**: 539–548.

Cope, M., Webb, M. C., O'Gara, E. T., Philip, B. A. & Hardham, A. R. (1996) Immunocytochemical comparison of peripheral vesicles in zoospores of *Phytophthora* and *Pythium* species. *Mycologia* **88**: 523–532.

Erwin, D. C. & Ribeiro, O. K. (1996) *Phytophthora Diseases Worldwide*. American Phytopathological Society Press, St Paul, MN.

Förster, H. & Mendgen, K. (1987) Immunocytochemical localization of pectinesterases in hyphae of *Phytophthora infestans*. *Canadian Journal of Botany* **65**: 2607–2613.

Gabor, B. K., O'Gara, E. T., Philip, B. A., Horan, D. P. & Hardham, A. R. (1993) Specificities of monoclonal antibodies to *Phytophthora cinnamomi* in two rapid diagnostic assays. *Plant Disease* **77**: 1189–1197.

Galaud, J.-P., Carrière, M., Pauly, N., Canut, H., Chalon, P., Caput, D. & Pont-Lezica, R. F. (1999) Construction of two ordered cDNA libraries enriched in genes encoding plasmalemma and tonoplast proteins from a high-efficiency expression library. *Plant Journal* **17**: 111–118.

Gautam, Y., Cahill, D. M. & Hardham, A. R. (1999) Development of a quantitative immunodipstick assay for *Phytophthora nicotianae*. *Food and Agricultural Immunology* **11**: 229–242.

Görnhardt, B., Rouhara, I. & Schmelzer, E. (2000) Cyst germination proteins of the potato pathogen *Phytophthora infestans* share homology with human mucins. *Molecular Plant-Microbe Interactions* **13**: 32–42.

Gubler, F. & Hardham, A. R. (1988) Secretion of adhesive material during encystment of *Phytophthora cinnamomi* zoospores, characterized by immunogold labelling with monoclonal antibodies to components of peripheral vesicles. *Journal of Cell Science* **90**: 225–235.

Hahn, M., Neef, U., Struck, C., Göttfert, M. & Mendgen, K. (1997) A putative amino acid transporter is specifically expressed in haustoria of the rust fungus *Uromyces fabae*. *Molecular Plant–Microbe Interactions* **10**: 438–445.

Hardham, A. R. (1985) Studies on the cell surface of zoospores and cysts of the fungus *Phytophthora cinnamomi*: the influence of fixation on patterns of lectin binding. *Journal of Histochemistry and Cytochemistry* **33**: 110–118.

Hardham, A. R. (1987) Ultrastructure and serial section reconstruction of zoospores of the fungus *Phytophthora cinnamomi*. *Experimental Mycology* **11**: 297–306.

Hardham, A. R. (1989) Lectin and antibody labelling of surface components of spores of *Phytophthora cinnamomi*. *Australian Journal of Plant Physiology* **16**: 19–32.

Hardham, A. R., Cahill, D. M., Cope, M., Gabor, B. K., Gubler, F. & Hyde, G. J. (1994) Cell surface antigens of *Phytophthora* spores: biological and taxonomic characterization. *Protoplasma* **181**: 213–232.

Hardham, A. R. & Gubler, F. (1990) Polarity of attachment of zoospores of a root pathogen and pre-alignment of the emerging germ tube. *Cell Biology International Reports* **14**: 947–956.

Hardham, A. R., Gubler, F. & Duniec, J. (1991a) Ultrastructural and immunological studies of zoospores of *Phytophthora*. In *Phytophthora* (J. A. Lucas, R. C. Shattock, D. S. Shaw & L. R. Cooke, eds): 50–69. Cambridge University Press, Cambridge, UK.

Hardham, A. R., Gubler, F., Duniec, J. & Elliott, J. (1991b) A review of methods for the production and use of monoclonal antibodies to study zoosporic plant pathogens. *Journal of Microscopy* **162**: 305–318.

Hardham, A. R. & Mitchell, H. J. (1998) Use of molecular cytology to study the structure and biology of phytopathogenic and mycorrhizal fungi. *Fungal Genetics and Biology* **24**: 252–284.

Hardham, A. R. & Suzaki, E. (1986) Encystment of zoospores of the fungus, *Phytophthora cinnamomi*, is induced by specific lectin and monoclonal antibody binding to the cell surface. *Protoplasma* **133**: 165–173.

Hardham, A. R. & Suzaki, E. (1990) Glycoconjugates on the surface of the pathogenic fungus *Phytophthora cinnamomi* studied using fluorescence and electron microscopy and flow cytometry. *Canadian Journal of Microbiology* **36**: 183–192.

Hardham, A. R., Suzaki, E. & Perkin, J. L. (1986) Monoclonal antibodies to isolate-, species- and genus-specific components on the surface of zoospores and cysts of the fungus *Phytophthora cinnamomi*. *Canadian Journal of Botany* **64**: 311–321.

Heukeshoven, J. & Dernick, R. (1985) Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. *Electrophoresis* **6**: 103–112.

Huang, B.-F. & Staples, R. C. (1982) Synthesis of proteins during differentiation of the bean rust fungus. *Experimental Mycology* **6**: 7–14.

Hurkman, W. J. & Tanaka, C. K. (1986) Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. *Plant Physiology* **81**: 802–806.

Hyde, G. J., Lancelle, S., Hepler, P. K. & Hardham, A. R. (1991) Freeze substitution reveals a new model for sporangial cleavage in *Phytophthora*, a result with implications for cytokinesis in other eukaryotes. *Journal of Cell Science* **100**: 735–746.

Krämer, R., Freytag, S. & Schmelzer, E. (1997) *In vitro* formation of infection structures of *Phytophthora infestans* is associated with synthesis of stage specific polypeptides. *European Journal of Plant Pathology* **103**: 43–53.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

Masson, F. & Rossignol, M. (1995) Basic plasticity of protein expression in tobacco leaf plasma membrane. *Plant Journal* **8**: 77–85.

Maurel, C. & Chrispeels, M. J. (2001) Aquaporins. A molecular entry into plant water relations. *Plant Physiology* **125**: 135–138.

Mendgen, K., Lange, M. & Bretschneider, K. (1985) Quantitative estimation of the surface carbohydrates on the infection structures of rust fungi with enzymes and lectins. *Archives of Microbiology* **140**: 307–311.

Mitchell, H. J. & Hardham, A. R. (1999) Characterisation of the water expulsion vacuole in *Phytophthora nicotianae* zoospores. *Protoplasma* **206**: 118–130.

Molloy, M. P. (2000) Two-dimensional electrophoresis of membrane proteins using immobilized pH gradients. *Analytical Biochemistry* **280**: 1–10.

Monk, B. C. & Perlin, D. S. (1994) Fungal plasma membrane proton pumps as promising new antifungal targets. *Critical Reviews in Microbiology* **20**: 209–223.

Murdoch, L. J., Kobayashi, I. & Hardham, A. R. (1998) Production and characterisation of monoclonal antibodies to cell wall components of the flax rust fungus. *European Journal of Plant Pathology* **104**: 331–346.

Nolan, R. A. & Bal, A. K. (1974) Cellulase localization in hyphae of *Achlya ambisexualis*. *Journal of Bacteriology* **117**: 840–843.

Pain, N. A., O'Connell, R. J., Mendgen, K. & Green, J. R. (1994) Identification of glycoproteins specific to biotrophic intracellular hyphae formed in the *Colletotrichum lindemuthianum*–bean interaction. *New Phytologist* **127**: 233–242.

Paktitis, S., Grant, B. & Lawrie, A. (1986) Surface changes in *Phytophthora palmivora* zoospores following induced differentiation. *Protoplasma* **135**: 119–129.

Patterson, D. J. (1980) Contractile vacuoles and associated structures: their organization and function. *Biological Review* **55**: 1–46.

Pendland, J. C. & Boucias, D. G. (1998) Characterization of monoclonal antibodies against cell wall epitopes of the insect pathogenic fungus, *Nomuraea rileyi*: differential binding to fungal surfaces and cross-reactivity with host hemocytes and basement membrane components. *European Journal of Cell Biology* **75**: 118–127.

Perfect, S. E., O'Connell, R. J., Green, E. F., Doering-Saad, C. & Green, J. R. (1998) Expression cloning of a fungal proline-rich glycoprotein specific to the biotrophic interface formed in the *Colletotrichum*–bean interaction. *Plant Journal* **15**: 273–279.

Robold, A. V. & Hardham, A. R. (1998) Production of species-specific monoclonal antibodies that react with surface components on zoospores and cysts of *Phytophthora nicotianae*. *Canadian Journal of Microbiology* **44**: 1161–1170.

Rouquié, D., Peltier, J.-B., Marquis-Mansion, M., Tournaire, C., Doumas, P. & Rossignol, M. (1997) Construction of a directory of tobacco plasma membrane proteins by combined two-dimensional gel electrophoresis and protein sequencing. *Electrophoresis* **18**: 654–660.

Santoni, V., Doumas, P., Rouquié, D., Mansion, M., Rabilloud, T. & Rossignol, M. (1999) Large scale characterization of plant plasma membrane proteins. *Biochimie* **81**: 655–661.

Santoni, V., Rouquié, D., Doumas, P., Mansion, M., Boutry, M., Degand, H., Dupree, P., Packman, L., Sherrier, J., Prime, T., Bauw, G., Posada, E., Rouzé, P., Dehais, P., Sahnoun, I., Barlier, I. & Rossignol, M. (1998) Use of a proteome strategy for tagging proteins present at the plasma membrane. *Plant Journal* **16**: 633–641.

Shi, J., Dixon, R. A., Gonzales, R. A., Kjellbom, P. & Bhattacharyya, M. K. (1995) Identification of cDNA clones encoding valosin-containing protein and other plant plasma membrane-associated proteins by a general immunoscreening strategy. *Proceedings of the National Academy of Sciences, USA* **92**: 4457–4461.

Simon, M. I. (1992) Summary: the cell surface regulates information flow, material transport, and cell identity. *Cold Spring Harbor Symposium on Quantitative Biology* **57**: 673–688.

Staples, R. C., Yoder, O. C., Hoch, H. C., Epstein, L. & Bhairi, S. (1986) Gene expression during infection structure development by germlings of the rust fungi. *NATO ASI Series* **H1**: 331–341.

Struck, C., Hahn, M. & Mendgen, K. (1996) Plasma membrane H^+-ATPase activity in spores, germ tubes, and haustoria of the rust fungus *Uromyces viciae-fabae*. *Fungal Genetics and Biology* **20**: 30–35.

Voegele, R. T., Struck, C., Hahn, M. & Mendgen, K. (2001) The role of haustoria in sugar supply during infection of broad bean by the rust fungus *Uromyces fabae*. *Proceedings of the National Academy of Sciences, USA* **98**: 8133–8138.

Wanner, R., Förster, H., Mendgen, K. & Staples, R. C. (1985) Synthesis of differentiation-specific proteins in germlings of the wheat stem rust fungus after heat shock. *Experimental Mycology* **9**: 279–283.

Corresponding Editor: R. W. Roberson