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Production of monoclonal antibodies against peripheral-vesicle proteins in zoospores of *Phytophthora nicotianae*

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Summary. A coimmunisation protocol using microsomal fractions from *Phytophthora nicotianae* cells has enhanced the production of monoclonal antibodies directed towards proteins produced during asexual sporulation. Over 40% of the antibodies targeted three categories of zoospore peripheral vesicles. Five antibodies label the contents of dorsal vesicles, with three of these reacting with two *P. nicotianae* polypeptides with a relative molecular mass of approximately 100 kDa. Two antibodies label the contents of large peripheral vesicles and react with two very high-molecular-weight polypeptides in extracts of *P. nicotianae* cells. These antibodies cross-react with the contents of large peripheral vesicles in *P. cinnamomi* zoospores. Ten antibodies label the contents of *P. nicotianae* zoospore ventral vesicles and react with a single polypeptide with a relative molecular mass of 230 kDa. A number of these antibodies against the contents of ventral vesicles in *P. nicotianae* zoospores cross-react with ventral-vesicle proteins in *P. cinnamomi* cells in immunofluorescence and immunoblot assays. The study illustrates the value of the coimmunisation protocol and has produced antibodies that could be instrumental in the cloning of genes encoding peripheral-vesicle proteins.

Keywords: Cortical vesicle; Microsomal fraction; Monoclonal antibody; *Phytophthora nicotianae*; Zoospore.

Introduction

The members of the class Oomycetes are organisms whose morphology and mode of growth and nutrient acquisition is similar to that of fungi but whose phylogeny is quite distinct from that of true fungi (Dick 1989). Many species of Oomycetes, including those in the genus *Phytophthora*, cause major diseases of plants, leading to widespread economic losses in agriculture and extensive damage to natural ecosystems (Erwin and Ribeiro 1996, Schwinn and Staub 1995). Asexual, biflagellate zoospores are produced by many species of Oomycetes and are capable of swimming chemotactically towards suitable infection sites on a potential host. The zoospores are formed by the cleavage of multinucleate sporangia which develop in large numbers under favourable conditions at the apex of hyphae. Under the right circumstances, including the proximity to a host surface, the wall-less zoospores are triggered to encyst, a process of rapid differentiation into a walled cyst. Encystment is accompanied by a number of dramatic changes in the ultrastructure of the cells. The flagella are detached, the basal bodies move away from the plasma membrane, and the structure and composition of organelles in the cortical cytoplasm is altered. The cysts subsequently germinate and invade the adjacent host. Motile zoospores are the major infective agent for many species of *Phytophthora* and the closely related genus *Pythium*, and thus a better understanding of their biology promises to form a basis for the development of novel control strategies for this important group of plant pathogens. In addition, oomycete zoospores represent a valuable system in which to investigate fundamental aspects of eukaryotic cell biology, including cell motility, the function of the cytoskeleton, and regulated secretion.

During sporulation, proteins and cell components important for zoospore structure and function are synthesised in hyphae and transported into developing sporangia. Studies of *Phytophthora cinnamomi* have shown that amongst these components are three types of vesicles that become distributed with a distinct polarity within the zoospore cortical cytoplasm (Hardham 1995). Members of one category, called large peripheral vesicles, occur throughout most of the peripheral cytoplasm except close to the groove that runs along

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the ventral surface of the zoospores. During encystment, the large peripheral vesicles move away from the plasma membrane and become randomly distributed in the cyst cytoplasm (Gubler and Hardham 1990). Vesicles in the second category, the dorsal vesicles, have a distribution similar to that of the large peripheral vesicles but they are smaller and their contents are secreted during encystment (Gubler and Hardham 1988). Vesicles in the third category, the ventral vesicles, have a size similar to that of the dorsal vesicles and their contents are also secreted during encystment but they occur predominantly along the groove on the ventral surface (Hardham 1995, Hardham and Gubler 1990).

The existence and developmental regulation of these three categories of peripheral vesicles in *P. cinnamomi* zoospores has been revealed through the production and use of monoclonal antibodies directed towards their contents (reviewed in Hardham et al. 1991). The antibodies have aided studies of the synthesis of vesicle proteins, the formation of the vesicles during sporulation, and their fate during encystment (Dearnaley et al. 1996, Dearnaley and Hardham 1994, Hardham 1995). During the first few hours after cyst germination, the large peripheral vesicles disappear and their contents are believed to serve as sources of protein that support early germling development (Gubler and Hardham 1990). The material secreted from the dorsal vesicles forms a mucilage-like layer over the cysts. Proteins secreted from the ventral vesicles form a pad of material between the cyst and the underlying surface, and the timing of their secretion coincides with the acquistion of stickiness of the cells. Material in the ventral vesicles is thus believed to be the adhesive that attaches the spores to the substratum (Hardham and Gubler 1990).

Immunoblots show that each of the three types of peripheral vesicles contains high-molecular-weight proteins or glycoproteins. Lpv-1, the monoclonal antibody that labels the large peripheral vesicles in *P. cinnamomi* zoospores, reacts with a set of three polypeptides with a relative molecular mass greater than 500 kDa (Gubler and Hardham 1988, Marshall et al. 2001). Cpa-1, a monoclonal antibody that labels the dorsal vesicles in *P. cinnamomi* zoospores, reacts with three polypeptides considerably greater than 200 kDa in molecular mass (Gubler and Hardham 1988). Vsv-1, the monoclonal antibody that labels the ventral vesicles in *P. cinnamomi* zoospores, reacts with a single polypeptide with a molecular mass of approximately 220 kDa (Hardham and Gubler 1990).

Antibody screening of a cDNA expression library made from mRNA isolated from *P. cinnamomi* sporulating hyphae has led to the cloning of the 3′ half of a gene encoding one of the Lpv proteins (Marshall et al. 2001). RNA blotting shows that there are three copies of this gene, ranging from 11 to 14 kb in size in the *P. cinnamomi* genome. In the C-terminal half of the protein there are different numbers of a 178-amino-acid repeat. The majority of antibodies (Cpa-1 through Cpa-12; Hardham et al. 1986) that react with proteins in the dorsal vesicles recognise carbohydrate epitopes and are thus unsuitable for immunoscreening of cDNA libraries. The epitope recognised by Vsv-1 is proteinaceous; however, screening of cDNA expression libraries with Vsv-1 has so far failed to identify the gene encoding the Vsv antigen (J. S. Marshall, Australian National University, Canberra, A.C.T., unpubl. obs.).

The aim of the present study was to produce new monoclonal antibodies that recognise peripheral-vesicle proteins in immunoblots of Phytophthora zoospore proteins, with a view to using these antibodies to screen cDNA expression libraries and isolate genes encoding proteins in each of the three peripheral vesicles. *Phytophthora nicotianae* was chosen as the study organism because an isolate of this species produces about ten times more zoospores than any other species or isolate we have tested. It also makes an interesting comparison with *P. cinnamomi* as it is phylogenetically divergent from *P. cinnamomi* (Cooke et al. 2000). The approach followed was to use a coimmunisation protocol (Barclay and Smith 1986) and microsomal fractions from *P. nicotianae* cells as the inoculum. This approach proved to be highly effective in producing antibodies that react with the contents of large peripheral, dorsal, and ventral vesicles in *P. nicotianae* zoospores both in immunocytochemical and immunoblotting assays. These antibodies promise to be a valuable resource with which to isolate genes encoding the vesicle antigens.

Material and methods

Culturing of Phytophthora and Pythium species

Phytophthora nicotianae (isolate H1111 [Gabor et al. 1993], ATCC MYA-141) was isolated from *Nicotiana tabacum* and kindly supplied by Dr. David Guest (his isolate M4951). Production of zoospores followed the method described in Robold and Hardham (1998). Encystment was induced by following one of three methods: (i) the zoospore suspension was shaken vigorously in a glass measuring cylinder for approximately 30 s, (ii) 27% (v/v) cleared rye medium (6% organically grown rye kernels) containing 2% sucrose and 1 M mannitol was added to the zoospore suspension, or (iii) a drop of zoospore suspension was placed onto a tobacco seedling on a microscope slide. Vegetative hyphae were grown in still cultures in 20 ml of V8 broth in petri dishes for 5 days in the dark at 25 °C, harvested by collecting them into a double layer of Kimwipes wiper (Kimberly-Clark Australia Pty. Ltd., Milsons Point, N.S.W., Australia) and rinsed with distilled water. Sporulating hyphae were obtained by rinsing vegetative hyphae in mineral salts solution (Chen and Zentmyer 1970) and incubating them overnight at 23 °C in the light on a horizontal shaker.

Zoospores of *P. cinnamomi* (isolate H1000 [Gabor et al. 1993], ATCC 200982) were produced as described in Hardham et al. (1991). Zoospore encystment was induced by placing vials containing zoospore suspension against a vortex mixer for 30 s.

Pythium aphanidermatum (isolate H200 [Gabor et al. 1993], ATCC 200983) was maintained on V8 agar. For the production of zoospores, seven agar plugs containing hyphae were plated per petri dish (diameter, 90 mm) containing V8 agar and incubated for 5 days at 23 °C in the dark. The cultures were rinsed in water and 15 ml of distilled water added and incubated overnight during which time zoospores were released.

Plant material

Tobacco (*Nicotiana tabacum*) or *Eucalyptus sieberi* seeds were surface sterilized with 5% sodium hypochlorite for 5 min, rinsed briefly with distilled water, disinfected in 70% (w/v) ethanol for 5 min, and rinsed for 5 min in distilled water. They were transferred onto 2% water agar in petri dishes and incubated for 7 or 10 days at 23 °C in the dark.

Preparation of microsomal fractions

The microsomal fraction was prepared from *P. nicotianae* zoospores as described in Mitchell et al. (2002). Briefly, zoospores were lysed by sonication and centrifuged at 6,000 **g** for 15 min and the supernatant was recentrifuged at 85,000 **g** for 1 h. The hyphal microsomal preparation was obtained by vacuum infiltration of freshly harvested vegetative hyphae in osmoticum buffer on ice for 5 min (Giannini et al. 1988), homogenisation with mortar and pestle on ice with grinding sand and filtering through Kimwipes wiper. The preparation was centrifuged at 13,000 **g** for 15 min and the supernatant was recentrifuged for 40 min at 100,000 **g**. The resulting microsomal pellet was resuspended in Tris–morpholineethanesulfonic acid–sucrose and stored at −80 °C.

Samples of zoospore microsomal preparations were partially resuspended in 2% agarose in osmoticum buffer, fixed in 1% glutaraldehyde in osmoticum buffer, and then either osmicated in 1% OsO_4 in 25 mM phosphate buffer, dehydrated, and embedded in Spurr resin or dehydrated and embedded in Lowicryl K4M resin as described in Cope et al. (1996).

Production of monoclonal antibodies to P. nicotianae zoospore antigens

For the generation of monoclonal antibodies an approach known as coimmunisation was followed (Barclay and Smith 1986). Two BALB/c mice were immunised with the microsomal preparation from *P. nicotianae* vegetative hyphae (approximately 50 μg of protein per immunisation). After five booster immunisations, serum (referred to as anti-hyphae serum) was obtained and tested for a positive reaction in immunofluorescence assays on vegetative hyphae as detailed below. The anti-hyphae sera from the two mice were then mixed with the microsomal fraction from zoospores and used to immunise a third BALB/c mouse. Hybridoma cells were produced and cultured according to standard protocols (Goding 1983). Hybridoma supernatants were screened initially by immunofluorescence microscopy assays and secondarily by immunoblotting.

Immunofluorescence microscopy

Zoospores and cysts were fixed in suspension in either 4% formaldehyde in 50 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) buffer, pH 7.0, or 4% formaldehyde–0.2% glutaraldehyde in 50 mM PIPES buffer, pH 7.0, and labelled for immunofluorescence microscopy as described previously (Robold and Hardham 1998). For immunofluorescence assays on vegetative and sporulating hyphae, mycelia were harvested and either fixed in 4% formaldehyde in 50 mM PIPES buffer, pH 7.0, and washed with 100 mM PIPES buffer, pH 7.0, or used fresh. The hyphae were embedded in TissueTek embedding compound (Miles, Elkhart, Ind., U.S.A.) in plastic moulds, and plunge frozen in liquid nitrogen. The samples were stored at −20 °C until use. Sections 12 μm in thickness were cut on a Reichert-Jung 2800 Frigocut E cryotome at −20 °C, collected on gelatine-coated multiwell slides and labelled as for the zoospores. Hybridoma supernatants were used undiluted, and the sheep anti-mouse secondary antibody conjugated to fluorescein isothiocyanate was diluted 1:30 in phosphate-buffered saline containing 1% bovine serum albumin and 0.1% gelatin (PBSBG). In negative controls, the hybridoma culture medium (Dulbecco modified Eagle medium [ICN Biomedicals Inc., Irvine, Calif., U.S.A.]) and PBS were used in place of the primary antibody.

Roots of *E. sieberi* were infected with *P. cinnamomi* by immersion in a suspension of zoospores for 5 min. The infected roots were fixed for 2 h in 4% formaldehyde in 50 mM PIPES buffer and embedded in Tissue Tek for cryosectioning and immunolabelling as described above.

Immunogold labelling

In preembedding labelling experiments, *P. nicotianae* zoospores were fixed in 4% formaldehyde in 50 mM PIPES buffer, pH 7.0, for 30 min. They were washed for 5 min each in 100 mM PIPES buffer, pH 7.0, once with PBS and once with PBSBG. The samples were incubated at room temperature in 300 or 500 μl of primary antibody on a shaker for 60 or 70 min. PBS was used as a negative control. Cells were washed twice in PBS (5 min each) before incubation for 45–60 min in 120 or 150 μl of goat anti-mouse antibody conjugated to 10 nm gold diluted 1 : 15 in PBSBG. After rinsing twice in PBS (5 min each), the zoospores were re-fixed in 1% glutaraldehyde in 100 mM PIPES buffer for 40 min, rinsed three times with PIPES buffer (5 min each), embedded in 2% low-melting-point agarose (Sigma type VII) in 100 mM PIPES buffer, and stored overnight in buffer at 4 °C. They were fixed for 1 h in 1% OsO_4 in 100 mM PIPES buffer, dehydrated in a graded acetone series and embedded in Spurr resin. Ultrathin sections were cut on a Reichert Ultracut microtome with a Diatome diamond knife, collected onto gold grids coated with formvar, and stained for 30 min with uranyl acetate and 10 min with lead citrate.

For postembedding immunogold labelling, suspensions of zoospores or cysts were fixed in 1% glutaraldehyde in 100 mM PIPES buffer, embedded in Lowicryl K4M, and labelled as described previously (Robold and Hardham 1998). PBSBG was used as a negative control. Hybridoma supernatants were used undiluted; purified Vsv-1 (Hardham and Gubler 1990) was used at a concentration of 20 μg/ml.

Immunoblotting

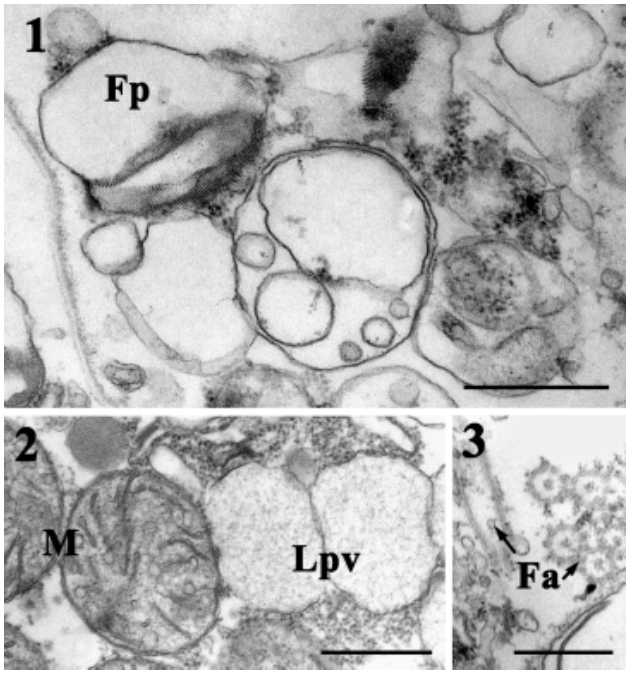
Proteins from freeze-dried zoospores (containing up to 10% cysts), vegetative hyphae, or sporulating hyphae were solubilized in 8 M urea. The preparations were homogenized and then centrifuged at 13,000 **g** for 3 min, and the supernatant was loaded onto 6% or 10% polyacrylamide gels and immunoblotted as described previously (Robold and Hardham 1998). Between 20–200 μg of protein were loaded per lane or up to 500 μg were loaded per curtain gel. Membranes were incubated for 1 h at room temperature in undiluted hybridoma culture supernatant on clean Nescofilm (Azwell Inc., Ozaka, Japan) or in 1 ml of hybridoma culture supernatant diluted in 1 ml of Tris-buffered saline containing 0.05% Tween 20 on a shaker. The secondary antibody was a sheep anti-mouse antibody conjugated to alkaline phosphatase diluted 1 : 10,000 in Tris-buffered saline and the incubation was for 45–60 min.

The sensitivity of antibody binding to pronase or periodate digestion of the antigen was determined on immunodot blots as described previously (Robold and Hardham 1998) except that 5 μg of protein from freeze-dried zoospores were solubilized in 200 μl of 6 M guanidine.

Results

Composition of zoospore microsomal fraction used for immunisations

Microsomal fractions were prepared by homogenising or sonicating *P. nicotianae* vegetative hyphae or zoospores,



Figs. 1–3. Transmission electron micrographs of *P. nicotianae* zoospore microsomal preparation. The appearance of the two vesicles in Fig. 2 is similar to that of large peripheral vesicles. *Fa* Flagellar axonemes; *Fp* fingerprint vesicle; *Lpv* putative large peripheral vesicles; *M* mitochondria. Bars: 0.5 μm

respectively, pelleting wall material and most large organelles with a low-speed spin, and then pelleting the membrane fraction with a high-speed spin. Ultrastructural examination of the zoospore microsomal fraction showed that it contained ribosomes, membranes, large and small vesicles, mitochondria, and flagella (Figs. 1–3). The finely lamellate contents of some of the vesicles indicated that they were fingerprint vesicles which contain mycolaminarin carbohydrates (Bartnicki-Garcia and Wang 1983).

Generation of monoclonal antibodies directed towards P. nicotianae spore components

Monoclonal antibodies directed towards *P. nicotianae* spore components were raised by a coimmunisation procedure (Barclay and Smith 1986). Firstly, two mice were immunised with the microsomal preparation from vegetative hyphae of *P. nicotianae.* After several booster immunisations, polyclonal serum was obtained from these mice, and a third mouse was immunised with the microsomal preparation from *P. nicotianae* zoospores mixed with the serum from the first two mice. The aim of this strategy was to reduce the generation of antibodies directed towards components common to the microsomal fractions from vegetative hyphae and zoospores and to enhance the production of antibodies directed towards components that occur only in the zoospores. Of the 364 hybridoma cell lines initially tested, 90 remained positive after several rounds of immunofluorescence screening. The supernatants of 40 that gave strong fluorescence were selected for more detailed analysis. Antigen distribution was investigated in *P. nicotianae* zoospores, cysts, vegetative hyphae, and sporulating hyphae by immunofluorescence and immunogold labelling. The antigen molecular weight was tested in immunoblots of zoospore proteins and the cross-reaction of some antibodies was assessed against *P. cinnamomi* antigens.

Localisation of zoospore and cyst antigens in immunofluorescence assays

Zoospores and cysts fixed either with formaldehyde only or with glutaraldehyde-formaldehyde were labelled for immunofluorescence microscopy. Fixation in formaldehyde alone allows antibodies to have access to the cytosol and the contents of organelles, including that of the peripheral vesicles, due to poor preservation of the plasma membrane and other membranes (Hardham 1985). The addition of 0.2% glutaraldehyde to the fixative leads to much better preservation of the plasma membrane, and antibodies are generally unable to penetrate into the zoospores, although some vesicles still rupture. The presence of the cell wall inhibits antibody penetration into mature cysts following either fixation protocol.

Immunofluorescence assays with the 40 antibodies revealed eight different labelling patterns on the *P. nicotianae* spores (Table 1). One antibody, Pn9F8, labelled a cytoplasmic reticulum in formaldehyde-fixed zoospores and did not label any other developmental stage (data not shown). The labelling pattern was similar to that shown by antibody 2B11 raised against *P. cinnamomi* (Hardham et al. 1991) and was suggestive of labelling of the endoplasmic reticulum. Three antibodies, Pn3B4, Pn6E9, and Pn9C2, labelled part of the ventral groove of zoospores; Pn3B4 and Pn6E9 also labelled sporulating hyphae and sporangia. One antibody, Pn11G7, labelled the anterior flagellum of formaldehyde-glutaraldehyde-fixed zoospores and was negative on the other developmental stages. Three monoclonal antibodies, Pn4D2, Pn5B9, and Pn17B3, reacted strongly with the water expulsion vacuole in formaldehyde-fixed zoospores (Fig. 4) and Pn17B3 also labelled the water expulsion vacuole in cryosections of mature sporangia (Fig. 5). Fifteen of the 40 antibodies reacted strongly with the entire surface of zoospores and cysts (Figs. 6 and 7) and were designated as being in a PnZCp group. With the exception of Pn10E7,

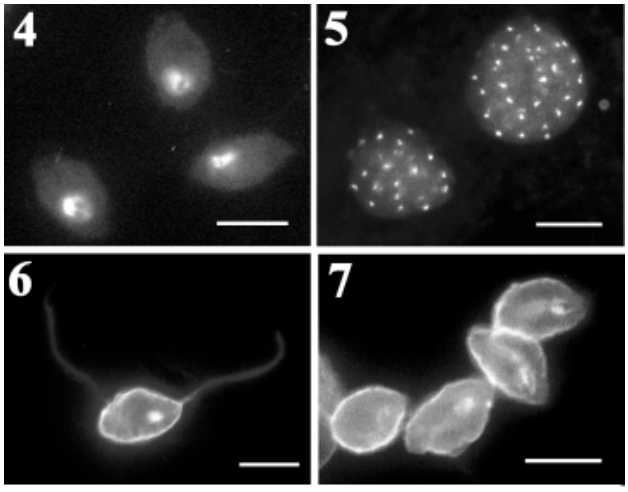
Table 1. Immunofluorescence and immunoblot labelling data with *P. nicotianae* cells and proteins

Group	MAb	Immunofluorescence labelling of:[a]				Polypeptide(s) recognized in immunoblots[b]
		Zoospore	Cyst	VH[c]	SH[d]	
1	Pn9F8	reticulum	−	−	−	−
2	Pn3B4	groove	−	−	+	−
	Pn6E9	groove	−	−	+	−
	Pn9C2	groove	−	−	+	−
3 (PnZg)	Pn11G7	anterior flagellum	−	−	−	−
4 (PnZw)	Pn4D2	WEV[e]	−	−	−	−
	Pn5B9	WEV	−	−	−	−
	Pn17B3	WEV	−	−	−	−
5 (PnZCp)	Pn1B4	surface	surface	+	+	wide range; many bands
	Pn2E5	surface	surface	+	+	wide range; many bands
	Pn3B3	surface	surface	+	+	wide range; many bands
	Pn7D8	surface	surface	+	+	wide range; many bands
	Pn7D9	surface	surface	−	+	>200 kDa; one band
	Pn10E7	surface	surface	−	−	−
	Pn11B2	surface	surface	−	+	>200 kDa; one band
	Pn12E2	surface	surface	+	+	wide range; many bands
	Pn15G8	surface	surface	+	+	wide range; many bands
	Pn16D4	surface	surface	+	+	wide range; many bands
	Pn16D7	surface	surface	+	+	wide range; many bands
	Pn18E2	surface	surface	−	+	−
	Pn18G5	surface	surface	−	+	−
	Pn19B10	surface	surface	+	+	wide range; many bands
	Pn19C3	surface	surface	+	+	wide range; many bands
6 (PnCpa)	Pn10F6	few spots	surface	−	+	~100 kDa; two bands
	Pn10F8	few spots	surface	−	+	−
	Pn12F4	few spots	surface	−	+	~100 kDa; two bands
	Pn13F6	few spots	surface	−	−	~100 kDa; two bands
	Pn14G2	few spots	surface	−	+	−
7 (PnLpv)	Pn9F6	large spots	−	−	+	>>200 kDa; two bands
	Pn19B3	large spots	−	−	+	>>200 kDa; two bands
8 (PnVsv)	Pn2G2	ventral spots	surface	−	+	−
	Pn3F4	ventral spots	surface	−	+	230 kDa; one band
	Pn5G6	ventral spots	surface	−	+	230 kDa; one band
	Pn6E7	ventral spots	surface	−	+	230 kDa; one band
	Pn7B10	ventral spots	surface	−	+	230 kDa; one band
	Pn8G8	ventral spots	surface	−	+	230 kDa; one band
	Pn11B9	ventral spots	surface	−	+	230 kDa; one band
	Pn17E7	ventral spots	surface	−	+	230 kDa; one band
	Pn18F10	ventral spots	surface	−	+	230 kDa; one band
	Pn19F2	ventral spots	surface	−	+	230 kDa; one band

[a] −, absence of labelling; +, presence of labelling
[b] −, no reaction on immunoblots. Indicated are the ranges or approximate values of relative molecular masses at which *P. nicotianae* polypeptides were detected and the number of bands
[c] VH, vegetative hyphae
[d] SH, sporulating hyphae
[e] WEV, water expulsion vacuole

these antibodies also reacted with the surface of sporulating hyphae and sporangia, and 10 labelled the surface of vegetative hyphae. Nine of these 10 antibodies produced high-background fluorescence on the microscope slide.

Labelling with antibodies Pn10F6, Pn10F8, Pn12F4, Pn13F6, and Pn14G2 gave a variable number of brightly fluorescent spots in the periphery of formaldehyde-fixed *P. nicotianae* zoospores (Figs. 8 and 9). Many cells contained only two or three spots (Fig. 8), while others contained 10–20 spots (Fig. 9). The addition of glutaraldehyde to the fixative abolished zoospore labelling (Fig. 10). These antibodies gave strong surface labelling of cysts in both fixa-



Figs. 4–7. Immunofluorescence micrographs of *P. nicotianae* zoospores and sporangia. Bars: Figs. 4, 6, and 7, 10 μm; Fig. 5, 25 μm. Pn17B3 labels the water expulsion vacuole complex in zoospores fixed with formaldehyde (Fig. 4) and in a cryosection of sporangia (Fig. 5). The surface of zoospores fixed in glutaraldehyde-formaldehyde is labelled with Pn10E7 (Fig. 6) and Pn7D9 (Fig. 7)

tion protocols (Figs. 10 and 11). None of these antibodies reacted with *P. cinnamomi* zoospores or cysts. The labelling pattern of this group of antibodies is similar to that of the labelling of dorsal vesicles in *P. cinnamomi* zoospores by antibodies in the Cpa group (Hardham 1995), although the number of labelled vesicles in the *P. nicotianae* zoospores is less than that observed with Cpa antibodies in *P. cinnamomi* cells. The antibodies also labelled spots in sporulating hyphae (Table 1).

Two antibodies, Pn9F6 and Pn19B3, displayed the same labelling pattern as that observed with monoclonal antibodies targetting the large peripheral vesicles of *P. cinnamomi* zoospores (Gubler and Hardham 1988), the Lpv group. In *P. nicotianae* zoospores fixed with formaldehyde, labelling with Pn9F6 or Pn19B3 (Fig. 12) took the form of numerous large spots in the cell periphery. Many fewer spots were seen in cells fixed with formaldehyde-glutaraldehyde (Fig. 13). Similar patterns of labelling were observed in *P. cinnamomi* zoospores (Figs. 14 and 15). The antibodies also labelled vesicles in sporulating hyphae (Table 1).

Ten of the 40 antibodies gave rise to a pattern of small spots that occurred predominantly along the ridges of the groove on the ventral surface of *P. nicotianae* zoospores fixed in formaldehyde (Fig. 16). They labelled the surface of cysts (Fig. 17). This labelling pattern is the same as that of an antibody, Vsv-1, that labels the contents of ventral vesicles in *P. cinnamomi* zoospores and cysts (Hardham and Gubler 1990). On young cysts with the groove still visible and the cells not yet fully spherical, fluorescence in the form of patches on the ventral side of the cell could be detected (Fig. 17). On fully encysted cells, the entire surface was brightly labeled (data not shown). When tested on sporulating hyphae, vesicles in *P. nicotianae* hyphae and sporangia were labeled (data not shown). Nine of the 10 antibodies cross-reacted with ventral vesicles in zoospores of *P. cinnamomi* (Fig. 18) and with the surface of *P. cinnamomi* cysts (Fig. 19). Labelling of infected *E. sieberi* roots showed a patch of bright fluorescence between host root and pathogen (Fig. 19).

Ultrastructural localisation of vesicle antigens in P. nicotianae and P. cinnamomi zoospores

The monoclonal antibodies that labelled the putative dorsal vesicles in *P. nicotianae* zoospores (the PnCpa group: Pn10F6, Pn10F8, Pn12F4, Pn13F6, and Pn14G2) failed to recognise their antigen in *P. nicotianae* zoospores embedded in Lowicryl K4M for immunogold labelling. However, postembedding immunogold labelling of sections of *P. nicotianae* zoospores and cysts with Pn9F6 or Pn19B3 (PnLpv group) gave strong labelling of the contents of the large peripheral vesicles (approximately 400 μm in diameter) in the cell cortex of zoospores (Fig. 20) and of vesicles distributed throughout the cytosol in cysts (Fig. 21). Preembedding labelling with Pn9F6 of *P. nicotianae* zoospores fixed in formaldehyde showed reaction of the antibody with the outer layer of material in the large peripheral vesicles (Fig. 22). Postembedding immunogold labelling of sections of *P. cinnamomi* zoospores with Pn9F6 or Pn19B3 also led to labelling of the contents of the large peripheral vesicles (data not shown).

Postembedding immunogold labelling of sections of *P. nicotianae* zoospores with monoclonal antibodies in the PnVsv group (Table 1) led to labelling of the small vesicles (approximately 200 μm in diameter) in the cell periphery (Fig. 23). These vesicles were less electron dense than the large peripheral vesicles and typically showed an outer shell that was less electron dense than the core. In many cases, gold particles were confined to this outer region. Consistent with the results of the immunofluorescence assay, the population of small vesicles labelled by these antibodies was mainly present along the ridges of the ventral groove.

The PnVsv antibodies were also tested in postembedding immunogold labelling on sections of *P. cinnamomi* and *Pythium aphanidermatum* zoospores. The antibodies labelled small vesicles occurring predominantly under the ridges of the groove in *P. cinnamomi* (Fig. 24) and *Pythium aphanidermatum* zoospores (data not shown), as previously demonstrated by the Vsv-1 raised against *P. cinnamomi* (Cope et al. 1996). Pn19F2 labelled small vesicles

in the microsomal fraction from *P. nicotianae* zoospores embedded in Lowicryl K4M (Fig. 25).

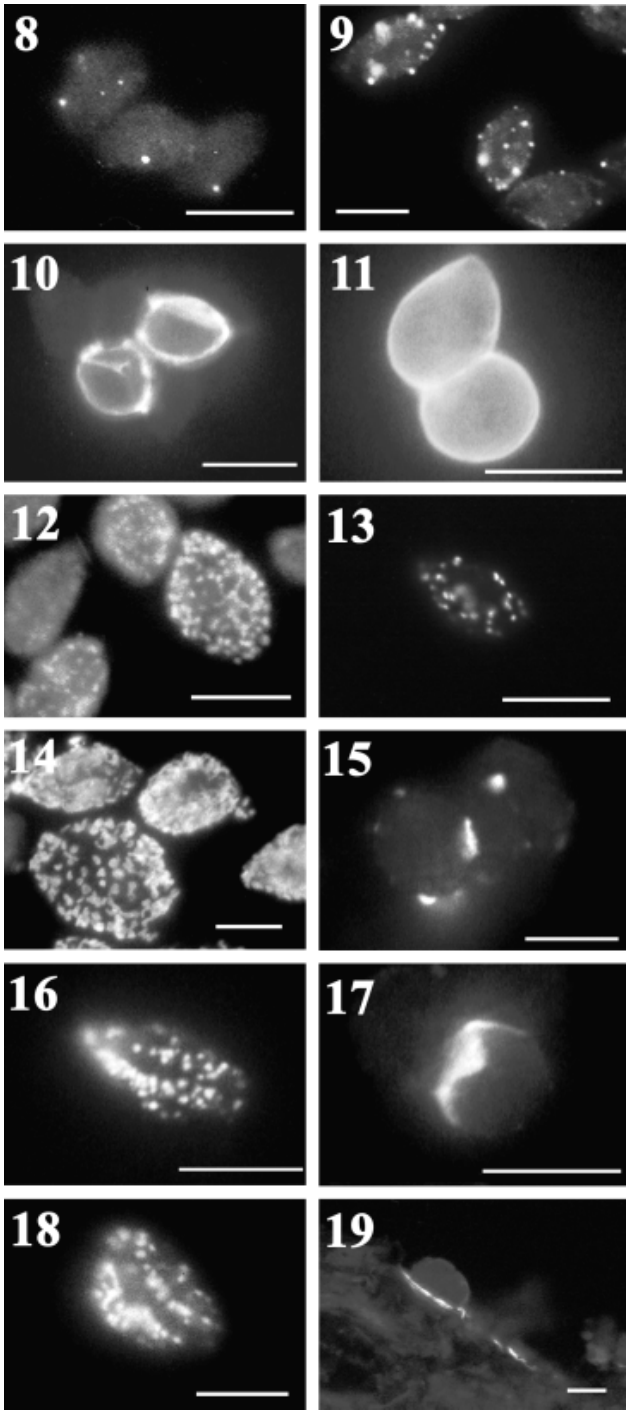
Immunoblot characterisation of zoospore antigens

The reaction of the 40 antibodies was tested on immunoblots of proteins extracted from zoospores, vegetative hyphae, or sporulating hyphae of *P. nicotianae* or *P. cinnamomi*. None of the monoclonal antibodies directed against the cytosolic reticulum, the anterior flagellum, the groove, or the water expulsion vacuole reacted on the immunoblots. Twelve of the 15 monoclonal antibodies directed towards the zoospore and cyst surface (PnZCp group) reacted positively with *P. nicotianae* extracts (Table 1). Ten of these gave strong labelling of multiple bands with a range of molecular weights in extracts from zoospores, vegetative hyphae, and sporulating hyphae (Fig. 26A and Table 1). Pn7D9 (Fig. 26A) and Pn11B2 labelled a single band with a relative molecular mass greater than 200 kDa in blots of proteins from zoospores and sporulating hyphae.

Three of the five antibodies, Pn10F6, Pn12F4 and Pn13F6, in the PnCpa group (Table 1) and reacting with the putative dorsal vesicles labelled a double band at about 100 kDa in zoospores (Fig. 26B) and sporulating-hyphae extracts. The other two antibodies in this group were negative in all developmental stages that were tested.

Pn9F6 and Pn19B3 (PnLpv group) reacted with two polypeptides with relative molecular masses greater than 200 kDa (Fig. 26C). The intensity of labelling was stronger with Pn9F6 than that with Pn19B3, and many polypeptides of lower molecular mass were also labelled. Pn19B3 reacted only with zoospore proteins, but Pn9F6 also interacted with polypeptides on immunoblots of sporulating hyphae.

Of the 10 PnVsv antibodies, nine reacted with a single polypeptide with an approximate molecular mass of 230 kDa on immunoblots of proteins extracted from *P. nicotianae* zoospores (Fig. 26D, lane 2) or sporulating hyphae (data not shown). There was no labeling of blots from vegetative hyphae. Only Pn2G2 failed to recognise its epitope on

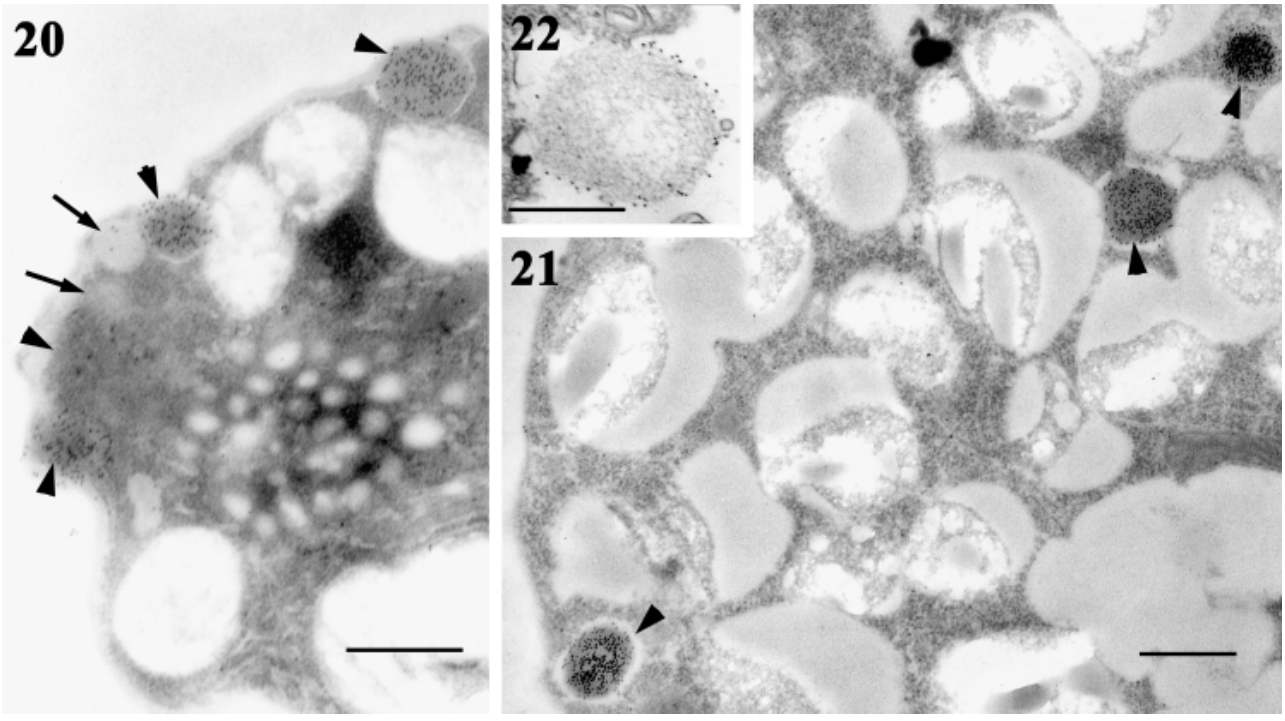


Figs. 8–19. Immunofluorescence micrographs of *P. nicotianae* or *P. cinnamomi* zoospores and cysts labelled with monoclonal antibodies in PnCpa (Figs. 8–11), PnLpv (Figs. 12–15), and PnVsv (Figs. 16–19) groups. Bars: 10 μm

Figs. 8–11. *P. nicotianae* zoospores labelled with Pn10F6 (Fig. 8) or Pn13F6 (Fig. 9). Young *P. nicotianae* cysts labelled with Pn10F6 (Fig. 10). Zoospores surrounding the cysts are not labelled and are not visible under fluorescence optics; mature *P. nicotianae* cysts labelled with Pn13F6 (Fig. 11)

Figs. 12–15. *P. nicotianae* zoospores fixed in formaldehyde and labelled with Pn19B3 (Fig. 12). *P. nicotianae* zoospores fixed in glutaraldehyde-formaldehyde and labelled with Pn9F6 (Fig. 13). *P. cinnamomi* zoospores fixed in formaldehyde and labelled with Pn19B3 (Fig. 14). *P. cinnamomi* zoospores fixed in glutaraldehyde-formaldehyde and labelled with Pn9F6 (Fig. 15)

Figs. 16–19. *P. nicotianae* zoospore fixed in formaldehyde and labelled with Pn3F4 (Fig. 16). Young *P. nicotianae* cyst labelled with Pn17E7 (Fig. 17). *P. cinnamomi* zoospore fixed in formaldehyde and labelled with Pn17E7 (Fig. 18). *P. cinnamomi* cysts adhering to the surface of an *E. sieberi* root and labelled with Pn17E7 (Fig. 19)



Figs. 20–22. Transmission electron micrographs of *P. nicotianae* spores labelled with PnLpv antibodies. Large peripheral vesicles (arrowheads) are heavily labelled. Ventral vesicles (arrows) are not labelled by the PnLpv antibodies. Bars: 0.5 μm

Fig. 20. Zoospore after postembedding immunogold labelling with Pn9F6

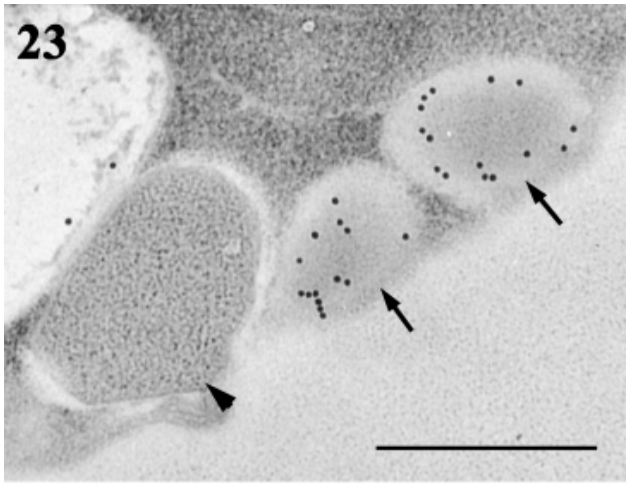
Fig. 21. Cyst after postembedding labelling with Pn9F6

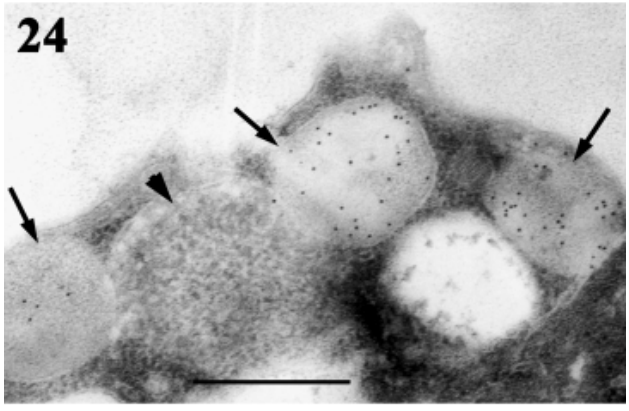
Fig. 22. Zoospore after preembedding labelling with Pn9F6

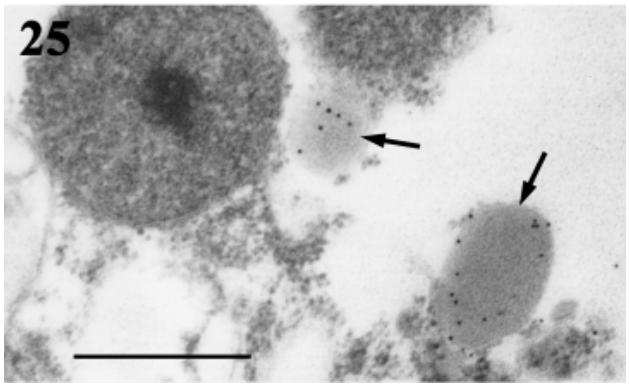
immunoblots. With *P. cinnamomi* protein extracts, Pn3F4, Pn8G8, Pn17E7, Pn19F2, and Pn7B10 labelled a polypeptide with a molecular mass of approximately 220 kDa (Fig. 26 D, lane 1). A polypeptide with the same molecular mass is recognised by antibody Vsv-1 in *P. cinnamomi* (Fig. 26 D, lane 3) but not in *P. nicotianae* extracts (Fig. 26 D, lane 4). Immunodot blots were carried out to determine the chemical nature of the epitopes of the PnVsv antibodies (Fig. 27). All but Pn2G2 recognised an epitope that was sensitive to treatment with pronase, indicating that the epitope consisted of protein only. Antibody Pn2G2 did not recognise its epitope in immunodot blots.

Discussion

Phytophthora nicotianae is a favourable model system for studies of sporulation and zoospore biology in the members of genus *Phytophthora*. Like *P. cinnamomi*, it is a highly destructive plant pathogen with a broad host range (Erwin and Ribeiro 1996). This contrasts with the species that have been the focus of most genomic studies to date, namely, *P. infestans* and *P. sojae*, which have narrow host ranges (Kamoun 2003). In previous work, three sets of monoclonal antibodies have been raised against *P. nicotianae* spores, principally with the aim to produce species-specific antibodies that could be used in diagnostic assays (Gautam et al. 1999, Robold and Hardham 1998). In these earlier fusions, as in the present study, antibodies that target components associated with the surface of both zoospores and cysts (PnZCp group) dominated the immune response, and antibodies directed towards the anterior flagellum were also frequently produced. In contrast to the results of similar experiments with *P. cinnamomi* (Hardham et al. 1986), there has been a complete absence of antibodies that react with the contents of zoospore peripheral vesicles in any of the earlier fusions using *P. nicotianae* antigens. As the main goal of the present study was the production of antibodies directed towards *P. nicotianae* zoospore peripheral vesicles or other zoospore-specific components, the coimmunisation strategy developed by Barclay and Smith (1986) was followed. This proved to be a successful approach. Amongst the 40 monoclonal antibodies that were isolated, 70% are specific for components in sporulating hyphae, zoospores, or cysts but not in vegetative hyphae, and 42% react with proteins in the zoospore peripheral vesicles.







Figs. 23–25. Transmission electron micrographs of zoospores after postembedding immunogold labelling with PnVsv antibodies (arrows). Ventral vesicles are labelled but large peripheral vesicles (arrowheads) are not labelled by the PnVsv antibodies. Bars: 0.5 μm

Fig. 23. *P. nicotianae* zoospore labelled with Pn3F4

Fig. 24. *P. cinnamomi* zoospore labelled with Pn17E7

Fig. 25. Microsomal fraction from *P. nicotianae* zoospores labelled with Pn19F2

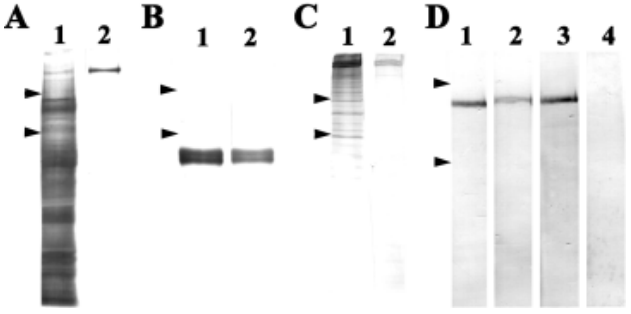


Fig. 26. Immunoblots of *P. nicotianae* (**A–C**) and *P. nicotianae* and *P. cinnamomi* (**D**) zoospore proteins labelled with PnZCp (**A**), PnCpa (**B**), PnLpv (**C**), and PnVsv antibodies (**D**). **A** *1*, Pn1B4; *2*, Pn7D9. **B** *1*, Pn10F6; *2*, Pn12F4. **C** *1*, Pn9F6; *2*, Pn19B3. **D** *1* and *3*, *P. cinnamomi* proteins; *2* and *4*, *P. nicotianae* proteins; *1* and *2*, Pn3F4; *3* and *4*, Vsv-1. Molecular-mass markers indicate 216 and 132 kDa in panels A–C and 250 and 148 kDa in panel D

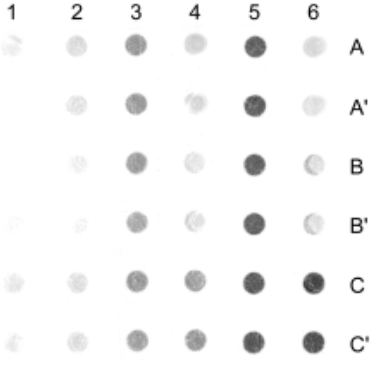


Fig. 27. Immunodot blot showing antibody labelling after pronase or periodate treatment of *P. nicotianae* zoospore protein extracts. In the columns results are shown after incubation without primary antibody (*1* and *2*), after labelling with Pn8G8 (*3* and *4*), and after labelling with Pn17E7 (*5* and *6*). Columns 1, 3, and 5 have been treated with periodate or acetate buffer; columns 2, 4, and 6, with pronase or PBS. Rows A and A′ are duplicates treated with either 20 mM periodate (*1*, *3*, and *5*) or 1 mg of pronase per ml (*2*, *4*, and *6*). Rows B and B′ are duplicates treated with 10 mM periodate (*1*, *3*, and *5*) or 100 μg of pronase per ml (2, 4, and 6). Rows C and C′ are duplicates treated with acetate buffer (*1*, *3*, and *5*) or PBS (*2*, *4*, and *6*). Antibody labelling is abolished by pronase digestion of the antigen (columns *4* and *6*)

A major problem that may arise in the production of antibodies when mixtures of antigens are used in the immunisation is that of immunodominant antigens. This problem can be circumvented by using various strategies, including the depletion of unwanted antigens (Springer 1980), neonatal immunotolerisation (Golumbeski and Dimond 1986, Hardham et al. 1991, Quintáns and Quan 1983, Weigle 1973), the use of immunosuppressant agents like cyclophosphamide (Matthew and Patterson 1983), or coimmunisation (Barclay and Smith 1986, Fisher et al. 1982). Coimmunisation has aided the generation of antibodies to components that have a low abundance or a weak antigenicity in situations in which it is not possible to purify the target molecules away from other contaminating components in the immunogen. In studies of plant–pathogen interactions, the procedure has enhanced the production of monoclonal antibodies specific for conidia, in-

fection hyphae, or appressoria of *Colletotrichum lindemuthianum* (Pain et al. 1994, 1995), for haustoria of *Melampsora lini* (Murdoch et al. 1998), and for the species *P. nicotianae* (Robold and Hardham 1998). In the present study, this approach was combined with the use of microsomal fractions instead of whole-cell extracts in order to enrich for vesicular components in the immunogen.

Antibodies directed towards proteins on the surface of zoospores and cysts, Group ZCp as designated in the earlier study of *P. cinnamomi* (Hardham et al. 1986), dominate the immune response to the *P. nicotianae* spore components in all fusions conducted to date, with 37.5% and 26% showing this labelling pattern in the present and earlier studies (Gautam et al. 1999, Mitchell et al. 2002, Robold and Hardham 1998), respectively. This pattern of labelling was also displayed by antibodies PA7 and PA8 raised against *Pythium aphanidermatum* (Estrada-Garcia et al. 1989). Further characterization of the *P. nicotianae* PnZCp antibodies showed that they could be divided into four groups (Mitchell et al. 2002).

In the present study, one third (5 out of 15) of the PnZCp antibodies are specific for asexual sporulation. The antigen is present in sporulating hyphae, zoospores, and cysts but absent from vegetative hyphae. Two of the five sporulation-specific monoclonal antibodies (Pn7D9 and Pn11B2) react with a polypeptide with a relative molecular mass greater than 200 kDa. Their labelling characteristics in both immunofluorescence and immunoblot assays are similar to those of antibodies Pn3B3 and Pn3C3, which were also specific for cells involved in asexual sporulation (Mitchell et al. 2002). The other 10 PnZCp antibodies reacted with components in vegetative hyphae and are thus not specific for sporulation. Nine of these 10 antibodies gave rise to high-background fluorescence on the microscope slide, a phenomenon seen previously with the ZCp monoclonal antibodies raised against *P. cinnamomi* zoospores (Hardham et al. 1986). All 10 of these antibodies recognised a large number of polypeptides on immunoblots, a feature similar to that shown by the Pn6G9 antibody (Mitchell et al. 2002).

Five (12.5%) of the 40 antibodies isolated in the present study reacted with material in small vesicles that occurred predominantly on the dorsal surface of formaldehyde-fixed zoospores and with material on the surface of cysts. This labelling pattern is similar to that reported for the Cpa category of antibodies, which label dorsal vesicles in *P. cinnamomi* (Hardham et al. 1986), and for antibodies PA3 to PA6 on *Pythium aphanidermatum* cells (Estrada-Garcia et al. 1989). This labelling pattern has been interpreted to indicate that material stored in the dorsal vesicles in the zoospores is released onto the cyst surface during encystment. The main difference between the results in *P. cinnamomi* and *Pythium aphanidermatum* and those in *P. nicotianae* is the number of fluorescent spots observed in the formaldehyde-fixed zoospores. In *P. nicotianae* often only three or four brightly fluorescent spots were observed, although other zoospores contained larger numbers of spots. By contrast, in *P. cinnamomi* and *Pythium aphanidermatum*, all zoospores contained numerous spots. A possible explanation for these observations is that the dorsal vesicles in *P. nicotianae* zoospores are less likely to rupture when the cells are fixed in formaldehyde than are those of *P. cinnamomi* or *Pythium aphanidermatum*. This would mean that the contents of fewer vesicles in the *P. nicotianae* zoospores were accessible to the antibody.

In *P. cinnamomi* and *Pythium aphanidermatum* the dorsal-vesicle antigen dominates the immune response in conventional immunisations and also after neonatal tolerisation with cysts (Estrada-Garcia et al. 1989, Hardham et al. 1991), but in the two previous studies of *P. nicotianae* not a single antibody towards the dorsal vesicles was obtained. The three Cpa-like antibodies that gave a positive reaction on immunoblots of *P. nicotianae* proteins reacted with two polypeptides with a molecular mass of approximately 100 kDa in zoospore and sporulating-hyphae protein extracts. In *P. cinnamomi*, the Cpa antibodies label three polypeptides with relative molecular masses well above 200 kDa (Gubler and Hardham 1988). Without further information on the nature of these molecules, it is not possible to know what relationship the 100 kDa *P. nicotianae* proteins might have to the $\gg$200 kDa *P. cinnamomi* proteins. They could be variants of the same polypeptide or could be completely different proteins stored in the dorsal vesicles. Cloning of the genes encoding the dorsal-vesicle proteins would clarify this situation.

Two monoclonal antibodies that react with the contents of the large peripheral vesicles in *P. nicotianae* and *P. cinnamomi* in immunofluorescence and immunogold assays were generated. Both monoclonal antibodies labelled two high-molecular-weight polypeptides in *P. nicotianae* zoospores. The Lpv antibodies that label the contents of the large peripheral vesicles in *P. cinnamomi* recognise three high-molecular-weight bands in extracts of *P. cinnamomi* (Gubler and Hardham 1988, Marshall et al. 2001). The fact that two bands are recognised in *P. nicotianae* and three bands in *P. cinnamomi* zoospores might be due to the presence of only two instead of three genes encoding the Lpv polypeptides in *P. nicotianae*.

Ten monoclonal antibodies (25%) are directed towards an antigen present in the ventral vesicles of *P. nicotianae* and

P. cinnamomi zoospores and sporulating hyphae and on the surface of cysts. Previously, only two monoclonal antibodies against the ventral vesicles of Phytophthora zoospores had been generated and this was following an immunisation protocol employing neonatal tolerisation with *P. cinnamomi* cysts (fusion III; Hardham et al. 1991). The *P. nicotianae* anti-Vsv antibodies did not react with vegetative hyphae of *P. nicotianae* indicating that the recognised antigen is produced during sporulation as has been demonstrated for the Vsv-1 antigen in *P. cinnamomi* (Dearnaley et al. 1996). All but one of the PnVsv antibodies labelled proteins on immunoblots of *P. nicotianae* zoospores and sporulating hyphae, labelling a polypeptide with a relative molecular mass of 230 kDa. Several of these antibodies cross-react with the protein recognised by antibody Vsv-1 in *P. cinnamomi* zoospore extracts. These results indicate that the PnVsv antibodies produced in the present study recognise at least three different epitopes within the Vsv antigen. All three epitopes are recognised in both species in immunofluorescence assays. However, one epitope is recognised on immunoblots of *P. nicotianae* protein extracts only, one is recognised on immunoblots of protein extracts from both species, and one is not recognised on immunoblots from either species. The latter could be part of a second antigen in the ventral vesicles of *P. nicotianae*.

The Vsv protein is thought to be important for the adhesion of Phytophthora cysts to their potential host (Hardham and Gubler 1990) and adhesion is likely to play a crucial role in the early infection process of many pathogens to their hosts. As the epitopes recognised by the PnVsv antibodies are proteinaceous, these antibodies could be used to screen cDNA expression libraries in order to clone the gene encoding the Vsv protein.

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