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Identification of glycoproteins specific to biotrophic intracellular hyphae formed in the *Colletotrichum lindemuthianum*-bean interaction

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SUMMARY

Monoclonal antibodies (MAbs) specific for intracellular hyphae (IH, i.e. infection vesicles and primary hyphae), appressoria/germ tubes and conidia of *Colletotrichum lindemuthianum* (Sacc. & Magn.) Briosi & Cav. isolated from infected leaves of *Phaseolus vulgaris* L. were obtained using a co-immunization procedure. One of the MAbs, UB25, bound specifically to IH in immunofluorescence, immunogold and Western blot assays: it showed *no* affinity for conidia, conidial germ tubes, appressoria or appressorial germ tubes growing *in vitro*, or for any plant components. Immunogold labelling of infected tissue prepared by high pressure freezing, freeze-substitution and low temperature embedding showed that the UB25 antigen was present in the interfacial matrix surrounding IH and in the fungal wall. The antigen was confined to infection vesicles and primary hyphae in contact with host protoplasts and could not be detected in primary hyphae growing in intercellular spaces. UB25 recognizes a protein epitope present in a set of *N*-linked glycoproteins. These glycoproteins are expressed at an early stage of intracellular development, suggesting a possible role in biotrophy or recognition.

Key words: Biotrophic pathogen, *Colletotrichum lindemuthianum*, high pressure freezing, intracellular hyphae, monoclonal antibody.

INTRODUCTION

Infection of bean tissues by the facultative biotrophic anthracnose fungus *Colletotrichum lindemuthianum* (Sacc. & Magn.) Briosi & Cav. involves several specialized infection structures. Conidial germ-tubes form appressoria and, following penetration of the host cuticle and epidermal cell wall, the fungus forms infection vesicles within host epidermal cells which give rise to one or more primary hyphae. During the initial biotrophic phase of infection the intracellular hyphae (IH, i.e. infection vesicles and primary hyphae) invaginate the host plasma membrane, and are separated from this membrane by a carbohydrate-rich matrix layer (O'Connell, Bailey & Richmond, 1985).

The composition of the cell surface of *C. lindemuthianum* has been studied using lectin cytochemistry to detect and locate specific sugars. This revealed differences in cell wall composition between cell types and species of *Colletotrichum* (O'Connell, 1991; O'Connell, Nash & Bailey, 1992). Recently, MAbs were raised to the cell surface of germlings of *C. lindemuthianum*, race γ (Pain *et al.*, 1992). Two MAbs, UB20 and UB22, bound to carbohydrate epitopes on two distinct sets of glycoproteins present on the surface of different infection structures. UB20 labelled conidia, whereas UB22 primarily labelled fibrillar material around germ tubes. Both MAbs also labelled the wall and matrix around the IH (Pain *et al.*, 1992).

The aim of the present work was to obtain MAbs specific to the IH of *C. lindemuthianum*, since the interface between these structures and living host

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cells is likely to be the site of molecular traffic between plant and fungus, including for example nutrient transport and recognition signals involved in the induction or suppression of host cell death (Bailey, 1991). A similar approach has been used to study molecular differentiation in the pea powdery mildew (*Erysiphe pisi*) system, and MAbs which bind to specific components of the haustorial complex have been obtained (Green *et al.*, 1992). For example, MAbs UB8 and UB10 recognized 62 kDa and 45 kDa glycoproteins located in the haustorial plasma membrane (Mackie *et al.*, 1991; 1993), whereas UB11 recognized a 250 kDa glycoprotein specific to the extrahaustorial membrane (Roberts *et al.*, 1993).

Haustorial complexes of *E. pisi* can be isolated from infected pea leaves to approx. 50% purity (Gil & Gay, 1977; Mackie *et al.*, 1991), so it was not unreasonable to expect to generate haustorial complex-specific antibodies. In contrast, the IH of *C. lindemuthianum* only constituted approx. 3% of a preparation isolated by similar methods from infected bean leaves: contaminants included spores, appressoria, chloroplasts and cell wall fragments (Pain *et al.*, 1994). It is therefore far less likely that IH-specific MAbs would be generated. In other systems, approaches used to generate MAbs specific to particular cell types have involved modifications to the immunization protocol, such as tolerization (Golumbeski & Dimond, 1986), immunosuppression (Matthew & Sancrock, 1987) and co-immunization (Barclay & Smith, 1986). In the latter technique, a polyclonal antiserum is prepared in mice against antigens or cells which are not of interest (or to common antigens). This antiserum is used to co-immunize more mice along with a sample of the cells/antigens of interest. Hybridomas producing MAbs are then produced by conventional methods. It is generally considered that the antibodies present in the polyclonal antiserum may mask the immune response of the second mouse to unwanted cells or common antigens, and therefore produce an increased response to antigens of interest. This paper describes the use of co-immunization procedures in the generation of MAbs specific to the IH of *C. lindemuthianum*, and the characterization and localization of antigens recognized by one of these MAbs.

MATERIALS AND METHODS

Fungal and plant material

Colletotrichum lindemuthianum, race γ (ATCC 56987) was cultured as described previously (Pain *et al.*, 1992). Seedlings of *Phaseolus vulgaris* L. cv. Kievitsboon Koekoek were grown as described in Pain *et al.* (1994).

Preparation of fungal and plant homogenates

Mycelial and germling homogenates were prepared as described by Pain *et al.* (1992). To prepare plant homogenates, leaves from 10-day-old seedlings of *P. vulgaris* were homogenized with a mortar and pestle at 4 °C in buffer (20 mM Tris, 1 mM $MgCl_2$, 1 mM PMSF, pH 7·5). This crude homogenate was transferred to an Atomix blender and homogenized at half speed for 1 min. The homogenate was filtered twice through a 45 μm nylon mesh, prior to centrifuging at 1080 ***g*** for 10 min at 4 °C. The pellet, containing mainly wall material, was resuspended in 5 ml buffer and the supernatant was centrifuged at 50000 ***g*** for 65 min at 4 °C. The final supernatant containing the cytosol fraction was removed and the pellet containing membranes and organelles was discarded. The protein content of each sample was determined using the Bio-Rad Protein Assay Reagent. Samples were stored at −20 °C.

Isolation of infection structures

Leaves from 10 day-old seedlings of *P. vulgaris* were inoculated with a suspension of spores from 7 day cultures of *C. lindemuthianum* race γ (Pain *et al.*, 1994). Inoculated leaf tissue was incubated for either 40 h or 4 days at 17 ± 1 °C before infection structures were isolated using an isopycnic centrifugation (IPC) procedure (Pain *et al.*, 1994). The preparation of infection structures isolated 40 h after inoculation contained spores, appressoria, chloroplasts and cell wall fragments, but no IH, whereas the preparation isolated 4 days after inoculation also included IH. In the case of samples to be used for immunizations and ELISA, the protein content of each sample was determined using the Bio-Rad Protein Assay Reagent. Samples to be used for immunization were used immediately or stored at −70 °C.

Immunization and production of MAbs

Female Balb/c mice (6–8 week-old) were given two intra-peritoneal injections, three weeks apart, of the IPC preparation isolated 40 h after inoculation (approx. 500 μg protein in 250 μl of phosphate buffered saline, PBS). Eleven days after the final immunization, the mice were bled and serum was prepared (Johnstone & Thorpe, 1987). The serum was tested in an ELISA (Pain *et al.*, 1992) using microtitre plates coated with the IPC preparation (isolated 40 h after inoculation). A serum obtained in a similar manner from a non-immune mouse was titrated alongside the polyclonal serum under test for comparison.

In a second series of immunizations, Balb/c mice of the same sex and age as above were given intraperitoneal injections of IPC preparations isolated 4 days after inoculation, which had been incubated with polyclonal antiserum (at 1:200 dilution in PBS) for 30 min. Three injections, each containing approx.

5×10^5 IH (representing approx. 3 % of total cells/organelles present in the sample) in 250 μl PBS, were made at two week intervals. A final injection of a sample which had not been incubated with the polyclonal antiserum was made into the tail vein, and 200 μl of polyclonal antiserum was injected intraperitoneally. Four days later the spleen was removed and fused with NS1 myeloma cells (Galfrè & Milstein, 1981). The tissue culture supernatants (TCS) from growing hybridomas were screened for MAb production by indirect immunofluorescence (IIF) labelling of an IPC preparation from a 4-day infection dried onto multiwell microscope slides as described below. Selected cell lines were cloned by limiting dilution and re-screened by IIF 10–14 days later. The immunoglobulin class and subclass of the selected MAbs were determined using a mouse Monoclonal Antibody Typing Kit (Amersham plc, Aylesbury, Bucks, U.K.). Other MAbs used included UB20 and UB22, which bind to *C. lindemuthianum* (Pain *et al.*, 1992), UB40, which was raised to pea membranes (Mitchell *et al.*, unpublished results) and UBIM 22, which was raised to rat bone cells and was used as a negative control (Perry *et al.*, 1990).

Indirect immunofluorescence

Slides coated with infection structures were prepared as follows. A suspension of cells (10 μl) isolated by IPC from 4-day infections containing 3×10^5 IH ml^{-1} was placed in the wells of multiwell microscope slides (ICN Biomedicals Ltd, High Wycombe, Bucks, UK), pre-coated with 0·5 % (w/v) gelatin. The slides were air-dried and 10 μl 4 % (v/v) *p*-formaldehyde in PBS was added to each well. Slides were incubated with fixative in trays lined with damp filter paper for up to 16 h before being washed in PBS and stored at −20 °C. To obtain germ-tubes and appressoria *in vitro*, conidia were germinated on uncoated slides (Pain *et al.*, 1992). Appressorial germ-tubes were obtained by allowing appressoria to penetrate Formvar plastic membranes supported over deionized water (O'Connell, 1991). MAb binding was localized by IIF (Pain *et al.*, 1992), and slides were viewed by epi-fluorescence, using either a Zeiss Axiophot microscope, or a Zeiss Axioplan attached to a confocal laser scanning microscope (MRC 600, Bio-Rad, UK).

Preparation of infected tissue for electron microscopy

Hypocotyls excised from 6 day-old seedlings of *P. vulgaris* cv. Kievitsboon Koekoek were inoculated with droplets (7 μl) of a conidial suspension (5×10^5 spores ml^{-1}). Four days after inoculation, segments of hypocotyl tissue (*c.* 1 cm × 5 mm × 5 mm) were infiltrated with 8 % (v/v) methanol in distilled water under vacuum (20 mmHg) for 3 min to remove air from intercellular spaces (Mendgen *et al.*, 1991). Strips of tissue (*c.* 0·5 mm thick) were cut parallel to the hypocotyl surface with a razor blade and infected areas were then excised using a 2 mm cork borer. Samples were mounted in 8 % (v/v) methanol between two aluminium holders (0·3 mm deep) and immediately fixed by ultra rapid freezing using the Balzers HPM 010 high pressure freezing apparatus (Müller & Moor, 1984). Samples were exposed to the methanol solution for no longer than 15 min prior to freezing. Tissues were freeze-substituted with acetone containing 2 % (w/v) osmium tetroxide at −90 °C for 10 h, −88 °C for 10 h, −80 °C for 14 h and −25 °C for 8 h using the Balzers FSU 010 freeze-substitution apparatus. Samples were then rinsed with absolute ethanol (3 × 1 h), infiltrated with LR White resin containing 0·5 % (w/v) benzoin methyl ether for 3 days at −20 °C and polymerized using UV light at −20 °C (27 h), then −10 °C (14 h) and finally at room temperature (18 h). Ultrathin sections were mounted on uncoated 400 mesh nickel grids prior to immunogold labelling.

Immunogold labelling

PBS containing 1 % (w/v) bovine serum albumin and 0·1 % (v/v) cold water fish skin gelatin (Sigma) was used for all rinsing steps and for dilution of reagents. Sections were immunolabelled by immersing grids in drops (20 μl) of reagent in the following sequence: (1) 10 % (v/v) normal goat serum in buffer, 30 min; (2) undiluted TCS, 18 h at 4 °C; (3) five changes of buffer, 3 min each; (4) goat anti-mouse IgG antibody conjugated with 5 nm colloidal gold (BioCell Research Laboratories, Cardiff, UK) diluted 1:50 in buffer, 2 h; (5) five changes of buffer, 2 min each; (6) five changes of deionized water, 3 min each; (7) silver enhancement with Aurion R-GENT (Aurion, Wageningen, NL), 5 min at 22 °C; (8) five changes of deionized water, 3 min each. Sections were then air-dried onto a Formvar film (Moran & Rowley, 1987), stained with uranyl acetate and lead citrate and viewed with an Hitachi H7000 TEM.

Western blotting

Fungal and plant homogenates to be used for SDS-PAGE and Western blotting were diluted so that samples had similar protein contents. An equal volume of double strength sample buffer (0·125 M Tris-HCl pH 6·8, 20 % (v/v) glycerol, 4 % (w/v) SDS and 0·0025 % (w/v) bromophenol blue), either with or without 10 % (v/v) 2-mercaptoethanol (i.e. reducing or non-reducing sample buffer) was added to each sample homogenate. Samples of the IPC preparation of infection structures were centrifuged

at 11600 ***g*** for 5 min and resuspended in single strength sample buffer. All samples were heated at 70 °C for 15 min and centrifuged at 11600 ***g*** for 10 min before being loaded onto 7·5 % (w/v) acrylamide gels for SDS-PAGE (Laemmli, 1970) and transferring to nitrocellulose (Pain *et al.*, 1992). The nitrocellulose sheets were blocked and incubated with MAbs, alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulins (AP-RAMIG) and the appropriate substrate as described by Stafford, Green & Callow (1992), except that 0·05 % Tween 20 in PBS was used for dilution of reagents and all washing steps.

Antigen modification

Periodate oxidation Carbohydrate side-chains of glycoproteins were oxidized with periodate as follows. Nitrocellulose strips with separated, blotted proteins were washed in 50 mM sodium acetate buffer (pH 4·5) and incubated in 20 mM sodium metaperiodate in acetate buffer for 2–4 h. The strips were washed three times in acetate buffer and once in PBS before blocking and incubating with antibodies and substrate. Control strips were incubated in acetate buffer alone.

Protease digestion Trypsin digestion was performed on proteins separated by SDS-PAGE and blotted onto a nitrocellulose membrane using a method adapted from that of Jones, Callow & Green (1990). Strips of nitrocellulose were incubated in 1 mg ml^{-1} trypsin (Sigma) in PBS for 1 h at 37 °C, washed twice in PBS and incubated in 0·1 mg ml^{-1} trypsin inhibitor (Sigma) for 10 min, washed twice and finally blocked and developed as above. Pronase (Protease XIV) digestion was adapted from Mackie *et al.* (1991). Nitrocellulose strips were incubated in the presence of 100 units of enzyme per strip in PBS for 1 h at 37 °C, and washed four times in PBS. Strips were blocked and developed as above. Control strips were incubated in PBS instead of enzyme, and otherwise treated identically.

Peptide-N-glycosidase digestion Samples (50 μg) in 20 μl of 20 mM sodium phosphate pH 7·5 containing 50 mM EDTA, 0·5 % SDS, 50 % (v/v) glycerol, with or without 5 % (v/v) 2-mercaptoethanol were heated at 100 °C for 2 min. After cooling on ice, 5 μl of 10 % (v/v) octylglucoside and two units of peptide-*N*-glycosidase F (Oxford GlycoSystems Limited, Abingdon, Oxon, UK) were added to each sample and incubated at 37 °C for 16 h. After adding an equal volume of double strength sample buffer to each sample, they were heated at 70 °C for 15 min, centrifuged at 11600 ***g*** for 10 min and finally loaded onto 7·5 % (w/v) acrylamide gels for SDS-PAGE as above.

RESULTS

Co-immunization and the production of MAbs

After the co-immunization procedure and fusion of the immune spleen cells from one mouse with

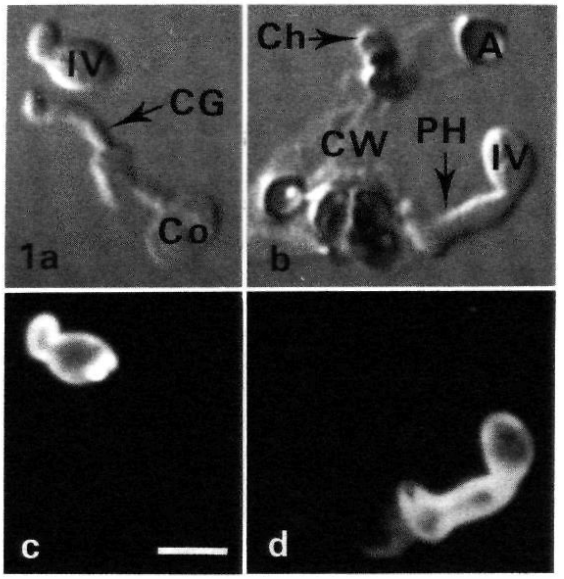


Figure 1. Immunofluorescence labelling of *Colletotrichum lindemuthianum* infection structures with MAb UB25. Infection structures were isolated from bean leaves by IPC, dried onto slides and fixed with 4 % formaldehyde. Bar = 10 μm. (**a, b**) Differential interference contrast microscopy showing infection vesicles (IV), primary hyphae (PH), conidium (Co), conidial germ-tube (CG), appressoria (A), chloroplasts (Ch) and plant cell wall fragments (CW). (**c, d**) Fluorescence images acquired using a confocal laser scanning microscope. Note only infection vesicles and primary hyphae are labelled.

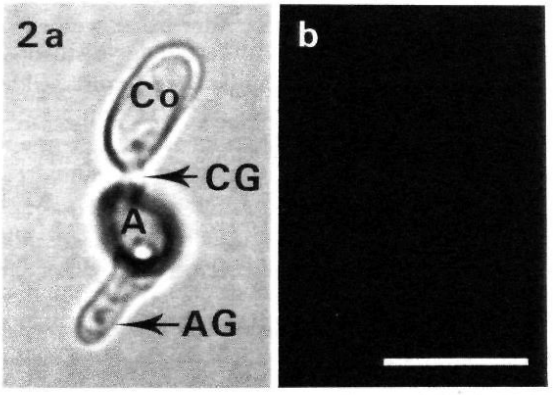


Figure 2. Immunofluorescence labelling with MAb UB25 of *Colletotrichum lindemuthianum* germling penetrating a Formvar plastic membrane (100 nm thick). Infection structures were not fixed and the membrane was immersed in the antibody solutions. Bar = 10 μm. (**a**) Bright field microscopy showing conidium (Co), conidial germ-tube (CG) and appressorium (A) above, and appressorial germ-tube (AG) below, Formvar membrane. (**b**) Epi-fluorescence microscopy showing complete absence of labelling from all fungal structures.

myeloma cells, 69 hybridomas were generated in a total of 382 wells. The TCS were screened by IIF on a crude preparation of infection structures which contained IH, appressoria, spores, chloroplasts and cell wall fragments dried onto slides. Ten hybridomas produced antibodies which reacted specifically with chloroplasts; three antibodies (UB23, UB24, UB25; all IgG1) were specific to the IH; one was specific to spores (UB28; IgM); one to appressoria (UB27; IgG1) and one labelled appressoria and germ tubes (UB26; IgG1). Of the three MAbs that were reactive with IH, UB25 proved to be the most useful in subsequent tests (Western blotting and immunogold labelling procedures) and is described below.

Localization of antigens recognized by MAb UB25

IIF of infection structures isolated from leaves by IPC and dried onto slides showed that UB25 bound only to the IH. It did not label fungal spores and appressoria, or plant chloroplasts and cell wall fragments which were also present in the preparation (Fig. 1*a–d*). Similarly, UB25 did not label conidia, conidial germ-tubes, appressoria or appressorial germ-tubes growing on glass slides and Formvar membranes (Fig. 2*a*, *b*). No structures were labelled by UBIM 22 in IIF assays.

EM immunogold labelling was performed on sections of infected bean hypocotyls prepared by high pressure freezing, freeze-substitution in osmium-acetone and low temperature embedding in LR White resin. The method gave excellent preservation of host and fungal cytoplasm, but membrane contrast was low (Figs 3–7; see also Mendgen & Deising, 1993). All membranes were smooth in profile and organelles had a turgid appearance. The host plasma membrane invaginated around young infection vesicles contained many coated pits (Fig. 3), similar to those observed by O'Connell (1987) in samples prepared by chemical fixation.

Freeze-substitution also retained high levels of antigenicity: preliminary experiments had shown that antigens recognized by UB25 were not well preserved by aldehyde fixation, dehydration by the progressive lowering of temperature method and low temperature embedding (Pain *et al.*, 1992). The efficiency of immunogold labelling was increased by immersing grids in the reagents, which allowed labelling of both sides of the section, and by the use of a small (5 nm) colloidal gold probe (Slot & Geuze, 1981). Silver enhancement facilitated detection of the gold probe at low magnifications.

UB25 labelled young infection vesicles formed soon after initial penetration of epidermal cells (Fig. 3). The walls of infection vesicles and primary hyphae and the surrounding matrix, which separates the fungal wall from the host plasma membrane, were both labelled (Figs 4–6). Fungal cytoplasm and septa were not labelled (Fig. 5). The primary hyphae of *C. lindemuthianum* ramify through bean hypocotyl tissue intracellularly, but sometimes enter small intercellular spaces when penetrating from cell to cell (see Fig. 4*b* in O'Connell *et al.*, 1985). In contrast to primary hyphae inside host cells, those present in intercellular spaces were not labelled by UB25 (Fig. 6). Infection pegs were sparsely labelled (not illustrated) but UB25 did not label the walls or extracellular mucilage of appressoria (Fig. 7), or any other fungal or plant structures (e.g. plant cytoplasm, Fig. 4; plant cell wall, Fig. 6).

Western blotting and antigen modification

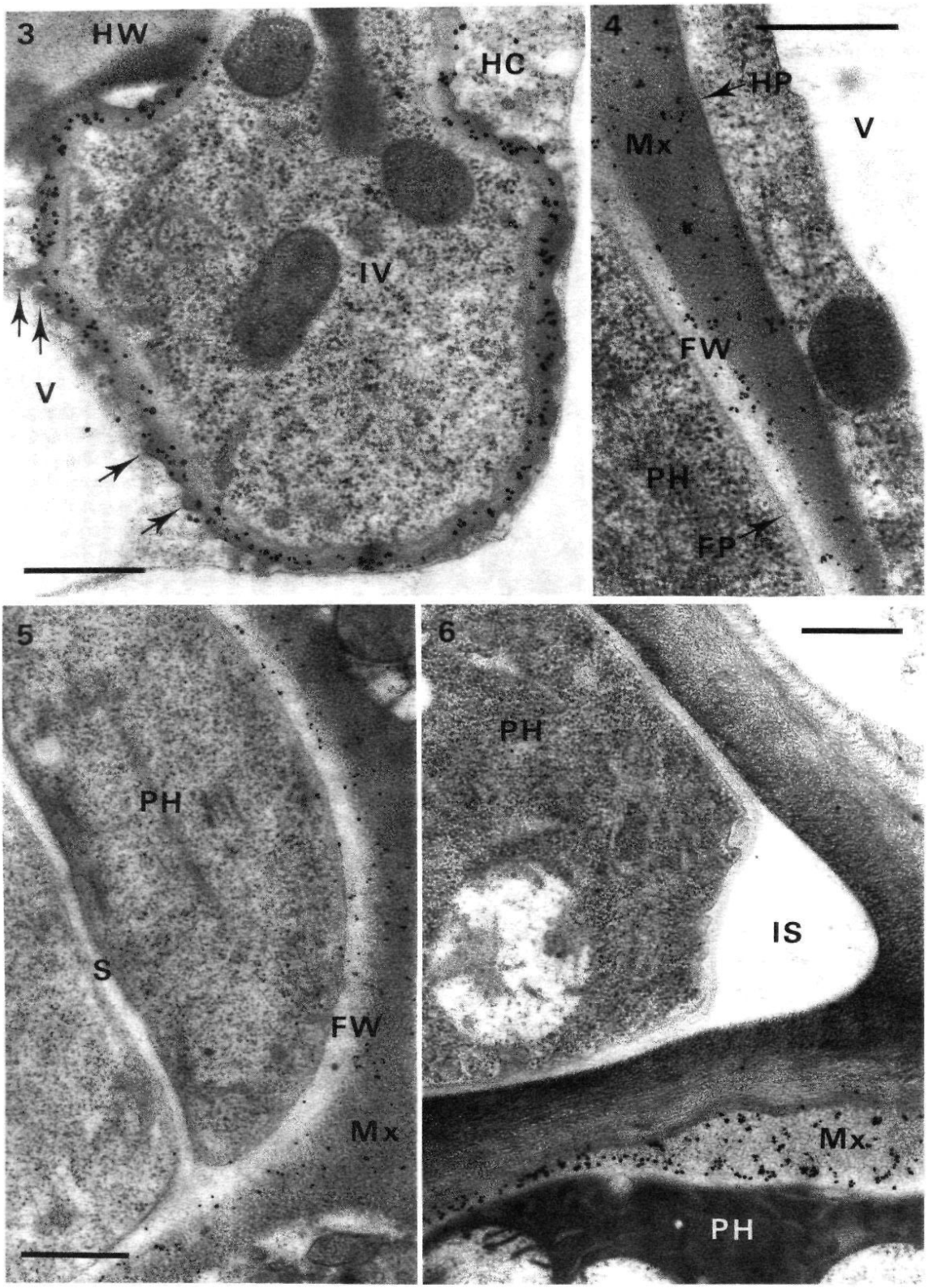
Proteins and glycoproteins of infection structures prepared by IPC were separated by SDS-PAGE in reducing and non-reducing conditions. After transfer to nitrocellulose, blotted proteins were incubated with antibodies. UB25 recognized a ladder of bands in both reduced and non-reduced samples (M.Wt. 100–180 kDa non-reduced; major bands at 40·5, 83, 150, 190, 217, 245 kDa reduced; Fig. 8), but did not bind to fungal mycelium, germ-tube or bean proteins (Fig. 8). MAbs UB20 and UB40 (that recognize fungal and plant glycoproteins respectively) were used as positive controls in these experiments.

Blotted proteins were pre-treated with periodate, trypsin or pronase and incubated with UB25 to determine the nature of the epitopes recognized (Fig. 9). Periodate increased UB25 binding to blotted proteins, however it abolished binding of UB22, which recognizes antigens known to be susceptible to periodate oxidation (Fig. 9; Pain *et al.*, 1992). Pretreatment of antigens with pronase and trypsin (Fig. 9) abolished binding of UB25. After treatment of the infection structure preparation with peptide-*N*-glycosidase, which removes *N*-linked carbohydrate side chains from glycoproteins, the bands labelled by UB25 were reduced in M.Wt. by approx. 5 kDa compared to the untreated controls (Fig. 9). Blots incubated with MAb UBIM 22 showed no labelling (Figs 8 and 9).

DISCUSSION

Co-immunization results in the production of MAbs recognizing specific infection structures in C. lindemuthianum

A co-immunization procedure was used in this study in an attempt to generate MAbs specific for the IH produced by *C. lindemuthianum* on bean tissues. As well as obtaining MAbs that recognized IH (UB23, UB24, UB25), other antibodies recognizing germ-tubes and appressoria (UB26, UB27) and conidia (UB28) were obtained. The nature and location of



Figures 3–6. Electron micrographs showing sections of bean hypocotyl tissue infected by *Colletotrichum lindemuthianum*, immunogold labelled with MAb UB25. HW, host cell wall; V, vacuole. Bars = 0·5 μm.

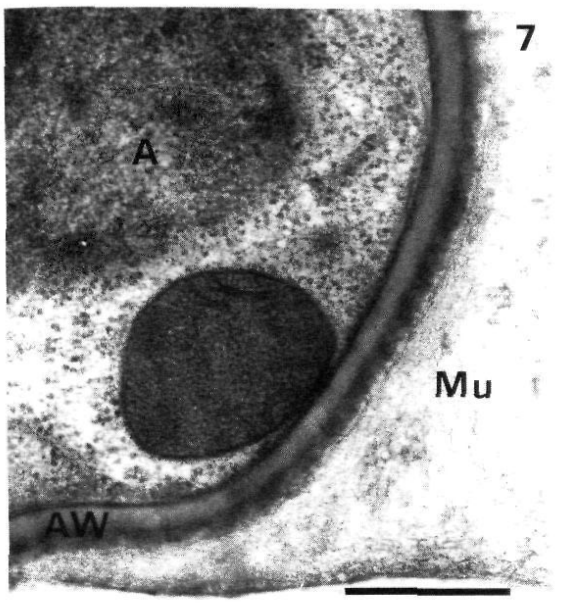


Figure 7. Part of appressorium (A) of *Colletotrichum lindemuthianum* immunogold labelled with UB25. Note absence of labelling from appressorial wall (AW) and extracellular mucilage (Mu). Bar = 0·5 μm.

the antigens recognized by UB25 are described here, while the use of this MAb to purify IH using an immunomagnetic separation technique is reported in an accompanying paper (Pain *et al.*, 1994). MAbs UB26 and UB27 will be described elsewhere.

A co-immunization approach has previously been used in the preparation of MAbs specific to slime mold differentiation antigens (Barclay & Smith, 1986), human myeloma cells (Fisher *et al.*, 1982), rat bone cells (Perry *et al.*, 1990) and higher plant arabinogalactan proteins (Pennell *et al.*, 1991). In the present study, fusion with immune spleen cells yielded very few hybridomas, but many of these produced MAbs with cell-type specificity. In particular, although the IH only constituted approx. 3% of the immunogen (Pain *et al.*, 1994), three out of the sixteen MAbs obtained (i.e. 18%) were specific for this structure suggesting that co-immunization was important in increasing the number of MAbs raised to IH. It is difficult to perform a control experiment to show the efficiency of the co-immunization approach. However, in a previous conventional immunization, a fusion using a mouse immunized with conidia and germ-tubes produced a large number of hybridomas which were not cell specific (Pain *et al.*, 1992). Similar results obtained with *Dictyostelium discoideum* indicated that co-immunization reduced the number of hybridomas produced, compared to a conventional immunization protocol, but raised the number of specific antibodies obtained (Barclay & Smith, 1986).

UB25 recognizes N-linked glycoproteins specific to IH

Western blotting and antigen modification procedures have shown that UB25 recognizes a set of *N*-linked glycoproteins. Treatment with trypsin or pronase abolished antibody binding, indicating the proteinaceous nature of the antigens recognized by UB25. Furthermore, periodate treatment did not inhibit antibody binding (in fact binding was slightly increased, possibly due to enhanced access of the antibody to the epitope recognized), which suggests that carbohydrate epitopes (at least those containing vicinal hydroxyl groups) were not being recognized by this antibody. After peptide-*N*-glycosidase treatment, which removes the entire *N*-linked carbohydrate side chains from asparagine moieties of glycoproteins, the M.Wt. of the glycoproteins recognized by UB25 were reduced by approx. 5 kDa suggesting the presence of *N*-linked side chains. Since UB25 recognized a protein epitope yet bound to multiple bands in Western blots, it is possible that subunits (monomers, dimers, trimers etc.) of a polymeric structure are being recognized. Since these are observed in reducing conditions, disulphide bonds are probably not involved in their overall structure.

The evidence from IIF of infection structures, Western blotting of infection structure preparations and plant and fungal extracts, and EM immunogold labelling of infected bean leaves, shows that UB25 binds specifically to the IH produced by *C. lindemuthianum*. The results provide further evidence for molecular differentiation in specialized infection structures formed in fungal-plant interactions and can be compared with findings on pea powdery mildew haustorial complexes (Mackie *et al.*, 1991; 1993; Roberts *et al.*, 1993), fungal zoospores and

Figure 3. Young infection vesicle (IV) in epidermal cell, sectioned close to site of initial penetration of host wall. IV is surrounded by a very thin layer of host cytoplasm (HC) and the invaginated host plasma membrane contains numerous coated pits (arrows). Note intense labelling of fungal wall and matrix (these structures are too thin to be distinguished in this micrograph). **Figure 4.** Part of primary hypha (PH) in epidermal cell. Fungal wall (FW) and matrix (Mx) between wall and invaginated host plasma membrane are strongly labelled. Host cytoplasm (HC) is not labelled. Plasma membranes of host (HP) and fungus (FP) are smooth in profile. **Figure 5.** Part of primary hypha (PH) in cortical cell. Note matrix (Mx) and fungal wall (FW) are strongly labelled, but the septum (S) is not labelled. **Figure 6.** Matrix (Mx) around primary hypha (PH) inside cortical cell is strongly labelled whereas PH in adjoining intercellular space (IS) is not labelled. The host wall is also not labelled.

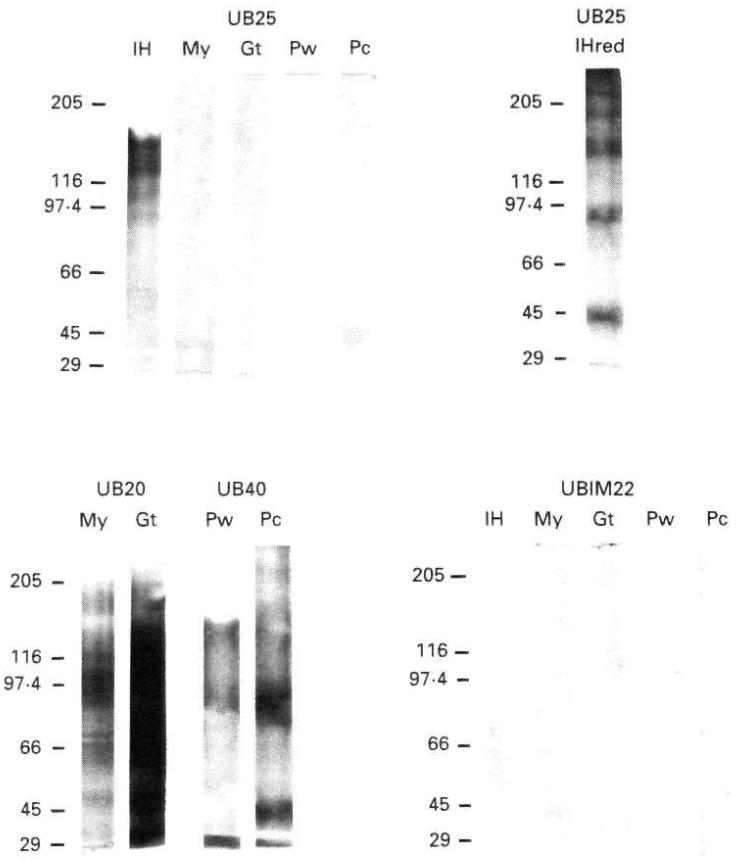


Figure 8. Western blotting. Samples were subjected to SDS-PAGE with subsequent blotting to nitrocellulose and probing with MAbs UB25, UB20, UB40 or UBIM22. MAb binding was detected using AP-RAMIG followed by the appropriate substrate. IH: intracellular hyphae (IPC preparation) in non-reducing sample buffer. IHred: intracellular hyphae (IPC preparation) in reducing sample buffer. My: mycelium; Gt: germ-tubes and spores; Pw: plant wall; Pc: plant cytoplasm (all in non-reducing sample buffer). The positions of molecular mass markers (kDa) are indicated on the left of each set of blots.

cysts (Estrada-Garcia *et al.*, 1989; Hardham, 1992) and mycorrhizae (Hilbert & Martin, 1988).

EM immunogold labelling was used to localize binding of UB25. High pressure freezing is the only method which can be used to cryo-fix thicker specimens, e.g. hyphae inside epidermal and cortical cells (Knauf *et al.*, 1989) and the combination of this technique with freeze-substitution and low temperature embedding in LR White resin yielded good structural preservation of infected tissue and retained antigenicity of protein epitopes. EM-immunogold labelling showed that UB25 bound to the walls of the IH and the matrix material present between the fungal wall and the host plasma membrane. Previous immunocytochemical studies with polyclonal and monoclonal antibodies suggested that antigens present in cell walls of *C. lindemuthianum* also occur in the matrix surrounding the IH (O'Connell *et al.*, 1986; Pain *et al.*, 1992). MAbs UB20 and UB22 which recognize carbohydrate epitopes on two distinct sets of glycoproteins, also labelled walls of mycelia growing *in vitro*, suggesting a fungal origin for the matrix components (Pain *et al.*, 1992). In contrast, UB25 recognizes glycoproteins specific to the IH that are not present *in vitro*. The presence of these glycoproteins in the walls of the IH would infer that they are synthesized by the fungus. Furthermore, proteins from uninfected bean tissue were not labelled by UB25 in Western blots. However, at present the possibility that the glycoproteins recognized by UB25 are produced by plant cells in response to infection cannot be excluded.

UB25 recognizes glycoproteins expressed at the biotrophic stage of infection in the Colletotrichum-*bean interaction*

The expression of specific glycoproteins in the matrix surrounding the IH suggests that this is a key region in the plant-fungal interaction. This is in contrast to the plasma membrane surrounding IH, which does not appear to become structurally or physiologically specialized (O'Connell, 1987). In addition, EM immunogold labelling of infected leaves has shown that the glycoproteins recognized by UB25 are expressed at a very early stage of IH

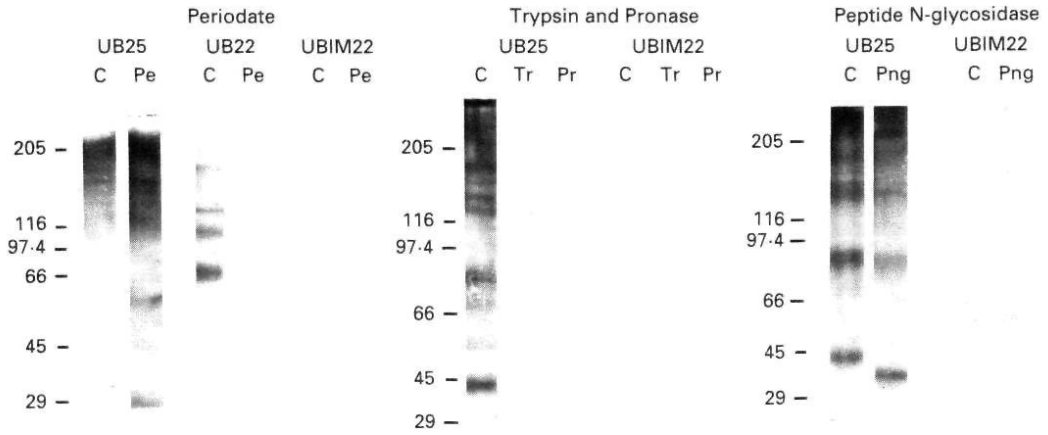


Figure 9. Effects of periodate, trypsin, pronase and peptide-*N*-glycosidase F on MAb binding as assessed by Western blotting. All samples (infection structure proteins from IPC preparations) were in reducing sample buffer, apart from those used for the periodate procedure. C: Controls. Blotted proteins were treated with periodate (Pe), trypsin (Tr) or pronase (Pr) before incubation with MAbs, AP-RAMIG and substrate. Infection structure proteins were treated with peptide-*N*-glycosidase F (Png) before separation on SDS-gels and Western blotting. The positions of molecular mass markers (kDa) are indicated on the left of each set of blots.

formation, when the fungus first contacts the host protoplasts. These glycoproteins are not expressed by hyphae occupying intercellular spaces, and IIF has shown that UB25 does not bind to appressorial germ-tubes growing *in vitro*. Thus, these glycoproteins are only produced during *intra*cellular development of hyphae in contact with living host protoplasts. They may therefore be involved in functions specific to IH, e.g. plant-pathogen recognition or biotrophic nutrition. The results reported would support the notion that a set of 'biotrophy genes' which function to promote pathogenic success are switched on early in the development of the IH. Similar conclusions have been made for the pea powdery mildew system, in which two fungal glycoproteins specific to the haustorial plasma membrane have been identified with MAbs (Mackie *et al.*, 1991; 1993; Callow *et al.*, 1992). These glycoproteins, like those recognized by UB25 in *C. lindemuthianum*, are expressed very early in haustorial development.

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