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The location of (1→3)-β-glucans in the walls of pollen tubes of *Nicotiana alata* using a (1→3)-β-glucan-specific monoclonal antibody

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Abstract. The location of the (1→3)-β-glucan, callose, in the walls of pollen tubes in the style of *Nicotiana alata* Link et Otto was studied using specific monoclonal antibodies. The antibodies were raised against a laminarin-haemocyanin conjugate. One antibody selected for further characterization was specific for (1→3)-β-glucans and showed no binding activity against either a cellopentaose-bovine serum albumin (BSA) conjugate or a (1→3, 1→4)-β-glucan-BSA conjugate. Binding was inhibited by (1→3)-β-oligoglucosides (DP, 3–6) with maximum competition being shown by laminaripentaose and laminarihexaose, indicating that the epitope included at least five (1→3)-β-linked glucopyranose residues. The monoclonal antibody was determined to have an affinity constant for laminarihexaose of 2.7. 10^4 M^{-1}. When used with a second-stage gold-labelled, rabbit anti-mouse antibody, the monoclonal antibody probe specifically located the (1→3)-β-glucan in the inner wall layer of thin sections of the *N. alata* pollen tubes.

Key words: Callose – Cell wall – (1→3)-β-Glucan – *Nicotiana alata* – Pollen tube – Monoclonal antibody

Introduction

The pollen-tube wall in *Nicotiana alata* Link et Otto is composed of a (1→3)-β-glucan, an arabinan and probably some cellulose (Rae et al. 1985). The fluorochrome, Calcofluor, stains the pollen-tube wall intensely, indicating the presence of a β-glycan; the aniline-blue fluorochrome, Sirofluor, induces strong fluorescence indicative of the presence of a (1→3)-β-glucan (Stone et al. 1984; Rae et al. 1985). At the electron-microscope level *N. alata* pollen-tube walls show two distinct layers, an outer fibrillar layer and an inner layer with a non-fibrillar, electron-lucent appearance (Anderson et al. 1987). This is characteristic of callose in other tissues. However, the location of the (1→3)-β-glucan in the inner wall layer is not proven by these observations.

Immunocytochemistry has been used successfully to localize (1→3)-β-glucans in cell walls. Horisberger and coworkers, (Horisberger and Rouvet-Vauthey 1985; Horisberger et al. 1985; Latge et al. 1986) used an anti-laminaribiose serum, produced in rabbits against a laminaribiose-edestin conjugate, with gold-labelled protein A to localize callose in the cell walls of the yeasts *Saccharomyces rouxii* and *Schizosaccharomyces pombe* and in the ballistospore wall of the fungus *Conidiobolus obscurus*. Northcote et al. (1989) also used serum from rabbits, immunized with a (1→3)-β-oligoglucoside-BSA conjugate, to locate callose in the cell plate and primary cell walls in dividing and differentiating cells of bean (*Phaseolus vulgaris*) roots. Kishida et al. (1989) have reported the preparation and characterization of polyclonal antibodies to the branched (1→3)-β-D-glucan of *Volvariella volvacea* and its use in studies of antitumor action. In this paper we report the generation and characterization of a (1→3)-β-glucan-specific monoclonal antibody, and observations on the immunostaining of the walls of *N. alata* pollen tubes using the antibody and a second-stage, gold-labelled, rabbit anti-mouse antibody.

Materials and methods

Polysaccharides and proteins. Laminarin from *Laminaria digitata* was obtained from Sigma Chemical Co. (St. Louis, Mo. USA), water-soluble barley *(Hordeum vulgare)*, (1→3, 1→4)-β-glucan from Biosupplies (Melbourne, Australia) and laminaribiose, laminaritriose, laminaritetraose, laminaripentaose and laminarihexaose were from Seikagaku Kogyo Co. (Tokyo, Japan). Cellopentaose was a gift from Dr. D.R. Whitaker (NRC, Ottawa, Canada). Bovine serum albumin (BSA) Fraction V was from Boehringer Mannheim (Germany), haemocyanin Type VIII was from Sigma.

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Abbreviations: BSA = bovine serum albumin; PBS = phosphate-buffered saline; ELISA = enzyme linked immunosorbent assay; DP = degree of polymerization; PVC = polyvinyl chloride

Preparation of oligosaccharide- and polysaccharide-protein conjugates. Oligosaccharides and polysaccharides were conjugated to BSA and haemocyanin by reductive amination (Roy et al. 1984). Laminarin (50 mg), a (1→3)-β-D-glucan with an average degree of polymerization (DP) of 23, was oxidized in 0.25 M $NaIO_4$ (5 ml) at 20° C for 60 min. The reaction was stopped with 250 µl of ethylene glycol and the oxidized laminarin desalted on a column of Sephadex G–25 (Pharmacia LKB Biotechnology, Uppsala, Sweden), then freeze-dried. (1→3, 1→4)-β-Glucan (60 mg) was oxidized in 10 mM $NaIO_4$ (6 ml) at 20° C for 75 min. Oxidation of the (1→3, 1→4)-β-glucan was monitored by measuring the conversion of periodate to iodate at 223 nm. The reaction was stopped by the addition of 40 µl ethylene glycol when approx. 4% of the glucose residues had been oxidized. The oxidized (1→3, 1→4)-β-glucan solution was made up to 0.5 M HCl and heated at 40° C for 3 h. It was neutralized with 5 M NaOH and desalted on a column of Sephadex G–25. The oxidized, hydrolysed (1→3, 1→4)-β-glucan, which eluted as a broad peak near V_0, was freeze-dried. Oxidized laminarin (22 mg) with either BSA (10 mg) or haemocyanin 10 mg was dissolved in 1.0 ml of 0.2 M sodium-borate buffer (pH 9.0) containing 20 mg/ml sodium cyanoborohydride. The reaction mixture was stirred at 40° C for 5 d. The laminarin-protein conjugates were then separated from unconjugated polysaccharide on a column of Sepharose CL–6B (43 cm long, 1.6 cm i. d.) (Pharmacia LKB Biotechnology) by eluting with 20 mM phosphate-buffered saline (PBS, pH 7.4). The eluted conjugates were dialysed against water and freeze-dried.

Oxidized, hydrolysed (1→3, 1→4)-β-glucan (15 mg) and cellopentaose (20 mg) were each conjugated to BSA (10 mg) by the same procedure. The relative proportion of protein to carbohydrate in the conjugates was determined using the phenol-sulphuric acid reagent (Dubois et al. 1956) for carbohydrate determination and the Lowry procedure (Lowry et al. 1951) for protein determination using glucose and BSA, respectively, as standards.

Production of monoclonal antibodies. Female Balb/c mice (eight weeks old) were immunized intraperitoneally with the laminarin-haemocyanin conjugate according to the following schedule: three injections of 200 µl, containing 50 µg conjugate in PBS: complete Freunds adjuvant (1:1, v/v) (injection 1), conjugate in PBS: incomplete Freunds adjuvant (1:1, v/v) (injection 2), or conjugate in PBS (injection 3) were given at three-week intervals.

Three days after the last injection, the spleens of two mice were used for the fusion of plasma cells with P3–X63 Ag8 myeloma cells (Horibata and Harris 1970) by the procedure of Galfré et al. (1977). Hybridoma supernatants were screened for the presence of antibodies against laminarin by indirect enzyme-linked immunosorbent assay (ELISA) using laminarin conjugated to a different protein from that used to immunize the mice. Polyvinyl-chloride (PVC) microtitre plates (96-well) were coated with laminarin-BSA conjugate (10 µg/ml) in PBS for 3 h at 20° C, washed three times with PBS then incubated for 2 h at 20° C with 100 µl of hybridoma supernatant. The plate was again washed three times with PBS, then incubated for 1 h at 20° C with 100 µl of 1:500 dilution of goat anti-mouse IgG (immunoglobulin G)-alkaline-phosphatase conjugate (Sigma) in PBS containing 1% BSA. After washing three times with PBS, 100 µl of substrate solution (1 mg/ml *p*-nitrophenyl phosphate in 50 mM sodium-hydrogen-carbonate buffer pH 9.8, 1 mM $MgCl_2$) was added and the *p*-nitrophenol measured at 405 nm after 60 min.

Hybridomas were cloned by limiting dilution (Oi and Herzenberg 1980). Monoclonal antibodies were purified from hybridoma supernatant on a protein A-Sepharose CL–4B column (Pharmacia LKB Biotechnology) at 4° C, eluting with 0.1 M sodium-citrate buffer, pH 4.0. Monoclonal antibodies obtained in this way were dialysed against 20 mM PBS, pH 7.4 and stored at 4° C with 0.02% sodium azide. The isotype of the antibodies was established using a kit for isotyping mouse monoclonal antibodies (Amersham International plc, Amersham, UK).

Characterization of a monoclonal antibody specific for (1→3)-β-glucan. The binding specificity of a (1→3)-β-glucan-specific antibody (LAMP2HI2H7) was determined using a combination of indirect ELISA against various β-glucan-protein conjugates and indirect competitive ELISA, using a series of highly purified (1→3)-β-oligoglucosides to inhibit the antibody binding.

For the indirect ELISA, 96-well PVC plates were coated with either laminarin-BSA, (1→3, 1→4)-β-glucan-BSA or cellopentaose-BSA conjugates (100 µl of 10 µg/ml in 20 mM PBS pH 7.4). The plates were washed three times with PBS, then incubated with serial dilutions of protein-A-purified antibody diluted in PBS containing 1% BSA, for 2 h at 20° C. The plate was again washed three times with PBS and developed with a goat anti-mouse IgG-alkaline-phosphatase conjugate as described earlier.

Indirect competitive ELISA was also performed in 96-well PVC plates coated with 10 µg/ml laminarin-BSA conjugate. Each well was incubated overnight with 50 µl of a 0.5 µg/ml solution of purified antibody in PBS containing 1% BSA together with 50 µl of the same buffer containing various concentrations of (1→3)-β-oligoglucoside inhibitor. The plate was washed three times with PBS and developed with the goat-anti-mouse IgG-alkaline-phosphatase conjugate as described.

Determination of affinity constants. The affinity constant of the monoclonal antibody LAMP2H12H7 for laminarihexaose was determined by the ELISA method of Friguet et al. (1985). A 96-well PVC plate was coated with 10 µg/ml laminarin-BSA conjugate in PBS. Solutions of antibody (2 µg/ml) in PBS, containing laminarihexaose at concentrations from 1 to 1000 µM and BSA (1%) were incubated overnight at 20° C. The solutions (3 × 100 µl) were then applied to the coated microtitre wells and incubated at 20° C for 30 min. The wells were washed and developed with goat anti-mouse IgG-alkaline-phosphatase conjugate as described. The concentration of free antibody in each solution was determined by reference to a calibration curve (0–2.0 µg/ml) prepared using the same incubation conditions. For an accurate determination of the affinity constant by this method, it is essential that a limiting fraction of antibody is bound to the laminarin-BSA-coated plates in the 30-min incubation. This was determined by incubating known concentrations of the antibody in coated wells for 30 min. The content of each well was then transferred into another coated well and incubated for a further 30 min. The antibody bound in each of the two series of wells was then detected with a goat anti-mouse IgG–alkaline-phosphatase conjugate. The fraction of antibody which had been retained in the first series of wells was calculated by

$$f = \frac{A_1 - A_2}{A_1}$$

where A_1 = absorbance in the first well and A_2 = absorbance in the second well. With the concentrations used in the calibration curve the values found for f ranged from 0.05 to 0.20.

Plant material. Nicotiana alata seeds, genotype S_2S_2 and S_3S_3, were a gift from the late Dr. K.K. Pandey, DSIR Grasslands Division, Palmerston North, New Zealand. Plants were maintained in a glasshouse at 21°–25°C, 14 h daylight/10 h darkness. Compatible pollinations were made and after 12 and 48 h material was collected for fixation. The upper 5–7 mm of the pistil was collected for the 12-h sample and the upper 10–15 mm for the 48-h sample. In each case the material was excised under the fixation medium.

Electron microscopy. The fixation medium was a mixture of 2% formaldehyde, freshly prepared from paraformaldehyde powder, and 2% glutaraldehyde in (piperazine-N,N′-bis[2-ethanesulfonic acid]; 1,4-piperazinediethanesulfonic acid) buffer 0.09 M, pH 7.2 (Salema and Brandao 1973). After 4 h fixation at room temperature, the material was washed in buffer and post-fixed in 1% OsO_4 followed by several washes in buffer and dehydration in an ethanol–H_2O series from 10% to 100%, and embedded in Spurr's resin (Spurr 1969).

Immunolabelling at the electron-microscope level. The anti-(1→3)-β-glucan monoclonal antibody was used to probe sections of the style

containing pollen tubes as follows. Sections (80–90 nm) were immunostained on gold grids, blocked in PBS (0.50 M NaCl) containing 1% BSA (Sigma A; No. 4503) or gelatine (Biorad Laboratories, Richmond, Cal., USA) for 30 min, and incubated with monoclonal antibody (8.2 μg/ml) for 60 min followed by a secondary rabbit anti-mouse antibody conjugated to 15-nm gold particles. Sections were counterstained with aqueous uranyl acetate and lead citrate and examined by electron microscopy (model 1200; Joel, Tokyo, Japan).

Controls were performed in which the first antibody was omitted or in which the monoclonal antibody was preincubated with laminarin (100 μg/ml).

Results

Preparation of glycoconjugates. Laminarin from *Laminaria digitata* has a substantial proportion of reducing termini glycosidically linked to mannitol (Peat et al. 1958). These residues lack the free aldehyde needed for reductive amination to protein. However, aldehyde groups can be introduced into laminarin by periodate oxidation. The (1→3)-linkages between glucose residues in laminarin render them resistant to periodate oxidation so that only terminal residues will be oxidized. Conjugates of the oxidized laminarin with BSA and haemocyanin contained approx. 52% carbohydrate (Table 1).

A similar (1→3, 1→4)-β-glucan-BSA conjugate was prepared by controlled periodate oxidation of the β-glucan, to oxidize approx. 1 in 25 glucose residues, followed by a mild acid hydrolysis of the polysaccharide to cleave acetal linkages at the oxidation sites. This material was used to produce a BSA conjugate with a carbohydrate-to-protein ratio similar to the laminarin-BSA conjugate (Table 1).

Cellopentaose required no modification prior to conjugation to BSA and produced a conjugate containing 32% carbohydrate (Table 1).

Table 1. Carbohydrate-protein conjugates

Conjugate	Carbohydrate (%)	Protein (%)
Laminarin-BSA	52	48
Laminarin-haemocyanin	52	48
(1→3, 1→4)-β-glucan-BSA	61	39
Cellopentaose-BSA	32	68

Table 2. Monoclonal antibody (LAMP2H12H7) binding to BSA glycoconjugates

Glycoconjugate	Titre[a]
Laminarin-BSA	30 ng/ml
(1→3, 1→4)-β-glucan-BSA	>100,000 ng/ml
Cellopentose-BSA	>100,000 ng/ml

[a] Antibody level required to produce an OD=0.2 after 60 min incubation with substrate

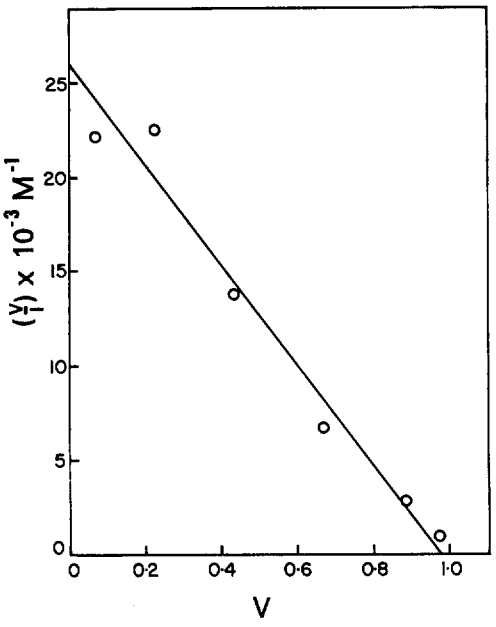

Fig. 2. Scatchard plot of the binding of monoclonal antibody LAMP2H12H7 to laminarihexaose. *V* is the fraction of bound antibody and *I* is the concentration of free laminarihexaose

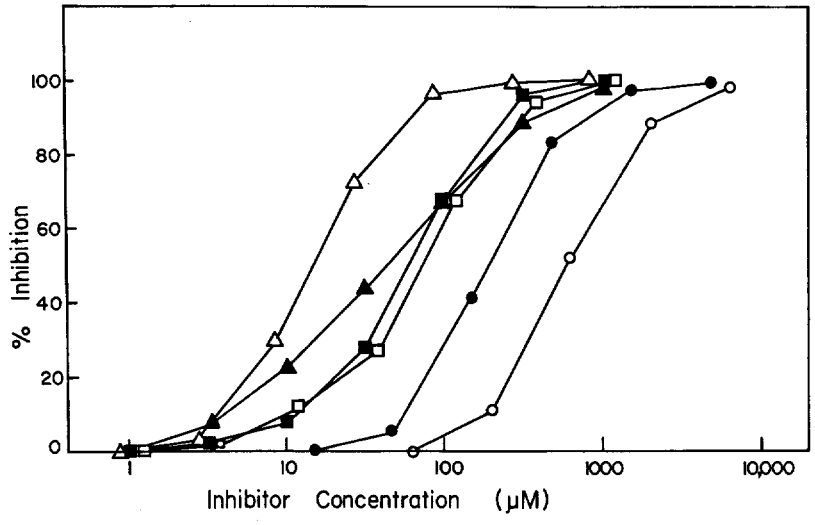

Fig. 1. Competitive inhibition of the binding of monoclonal antibody LAMP2H12H7 to laminarin-BSA conjugate with laminaritriose (○), laminaritetraose (●), laminaripentaose (□), laminarihexaose (■) and laminarin (△). The method of Friguet et al. (1985) was used for the non-competitive inhibition with laminarihexaose (▲)

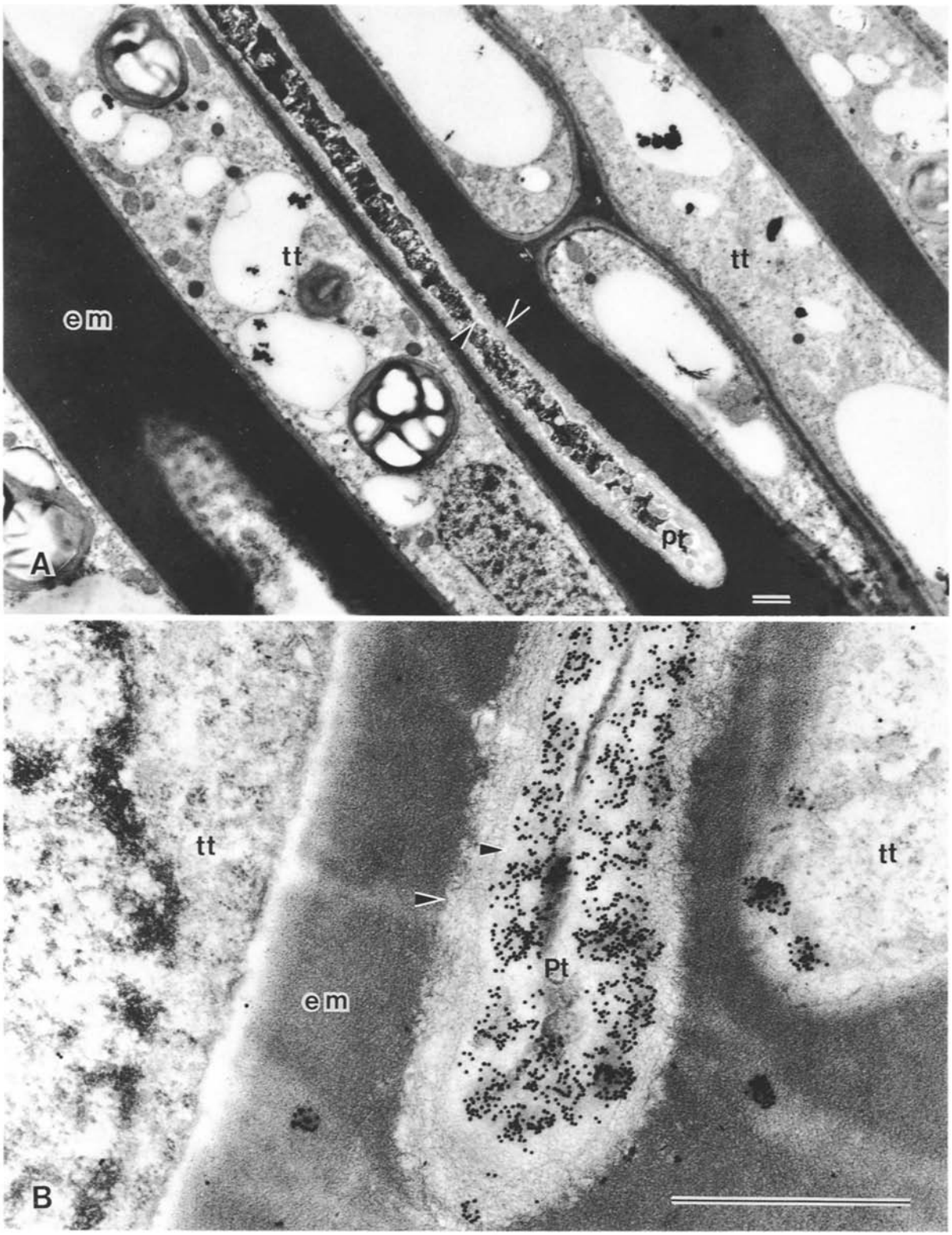

Fig. 3A–C. Longitudinal sections through a style of *Nicotiana alata*, 12 h after pollination. This section is from material about 4 mm beneath the stigma. The profiles of the pollen tubes (*pt*), show that they grow through the extracellular matrix (*em*) of the transmitting tissue (*tt*). **A** The growing pollen tube has a bilayered wall *(arrowheads)*. Bar = 1 μm. **B** Section similar to that in **A**, treated with monoclonal antibody to the (1→3)-β-glucan and the gold-labelled secondary antibody (rabbit anti-mouse). Labelling is intense over the inner, electron-lucent layer of the pollen-tube wall. *Arrowheads* indicate the inner, labelled wall and the outer wall layer which is fibrillar but is not labelled by the antibody. Accumulations of gold label are apparent in the transmitting tissue cell which could correspond to plasmodesmata. Bar = 1 μm. **C** Section showing gold label at the interface of the inner layer and the outer fibrillar layer of the pollen tube (*pt*) wall. The extracellular matrix (*em*) is not labelled. Bar = 0.1 μm

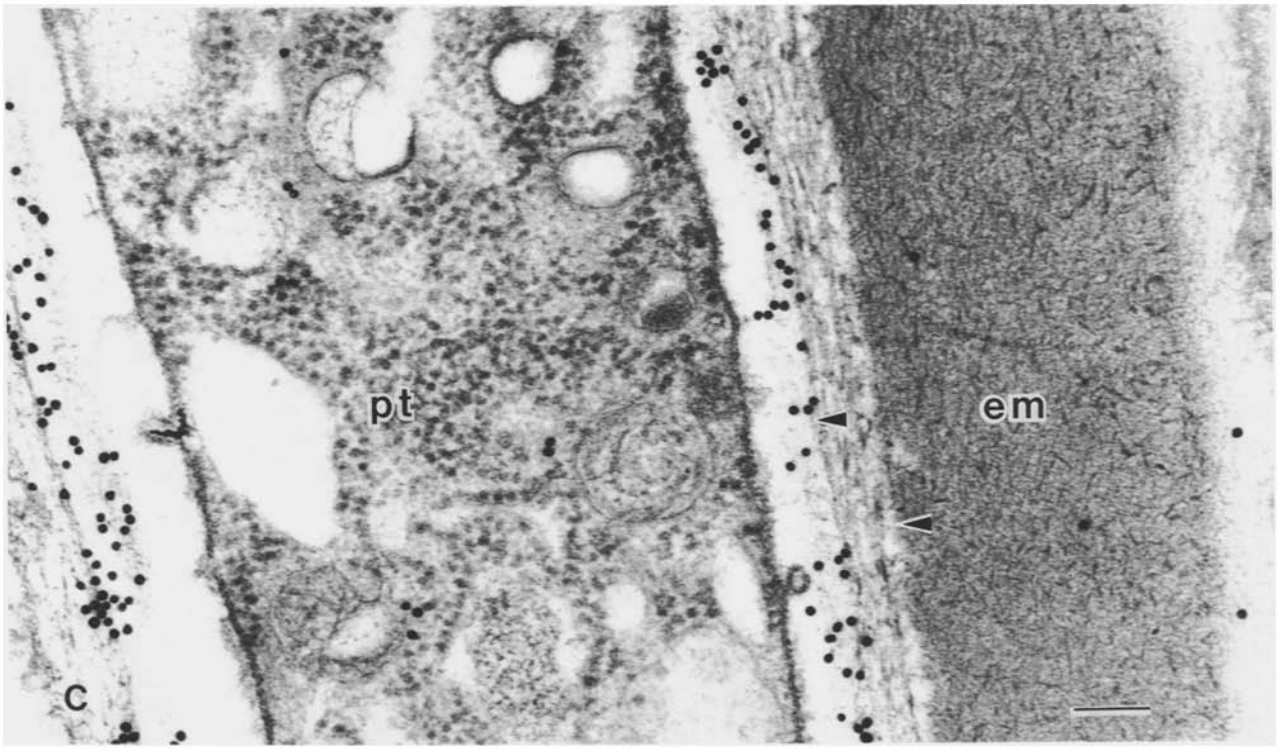

Fig. 3C

Table 3. Oligosaccharide inhibition of monoclonal antibody LAMP2H12H7 by indirect competitive ELISA

Oligosaccharide	Concn. of oligosaccharide ($\times 10^{-6}$ M) required for 50% inhibition
Laminaribiose	>9240
Laminaritriose	593
Laminaritetraose	192
Laminaripentaose	74
Laminarihexaose	60
Laminarin	15

Production and characterization of monoclonal antibodies. Immunization of mice with the laminarin-haemocyanin conjugate produced a strong humoral response. From a single fusion 180 cell lines were obtained, and of these 20 screened positive against the laminarin-BSA conjugate. Three of these cell lines were cloned by limiting dilution to produce three monoclonal antibodies, LAMP2H12H7 (IgG_1, kappa light chain), LAMP2G10H5 (IgG_1, kappa light chain) and LMAP2F7A10 (IgG_{2B}, kappa light chain). The binding specificity of the monoclonal antibody LAMP2H12H7 was further characterized by indirect ELISA against the three BSA glycoconjugates. In this assay the antibody showed no reactivity against (1→3, 1→4)-β-glucan or cellopentaose at antibody levels up to 100 μg/ml but bound to the laminarin-coated wells at levels as low as 30 ng/ml (Table 2). Indirect competitive ELISA with the (1→3)-β-oligoglucosides (Fig. 1) showed increasing inhibition from DP 3–5 levelling off at DP 5–6. The polysaccharide, laminarin, inhibited significantly better than laminarihexaose, requiring only 25% of the concentration to produce the same inhibition of binding (Table 3). The anti-(1→3)-β-glucan monoclonal antibody is quite specific for (1→3)-β-glucans and appears to recognize a polymer epitope at least five glucose residues long.

The inhibition data obtained from laminarihexaose using the method of Friguet et al. (1985) differed only slightly from the competitive-inhibition data (Fig. 1) but produced an inhibition curve which more closely approximates the theoretical sigmoidal curve expected for antibody inhibition. When these results are represented on a Scatchard plot (Fig. 2) a close fit to a straight line is obtained and the intercept on the x-axis is close to one. From linear regression analysis the K_a obtained is $2.7.\ 10^4\ M^{-1}$.

Immunochemical staining of pollen tubes. The longitudinal section of a pollen tube shown growing intercellularly in the transmitting tissue of the style 12 h after pollination (Fig. 3A) has a bilayered wall. In the immunolabelled glancing section of a pollen tube from the same preparation (Fig. 3B), the outer fibrillar wall layer is unlabelled but the inner, translucent wall layer binds the (1→3)-β-glucan-specific monoclonal antibody (LAMP2H12H7). This binding is demonstrated by the gold-labelling technique. In the pollen-wall sections shown in Fig. 3C, the label appears to be confined to a zone in the inner wall layer just beneath the fibrous layer.

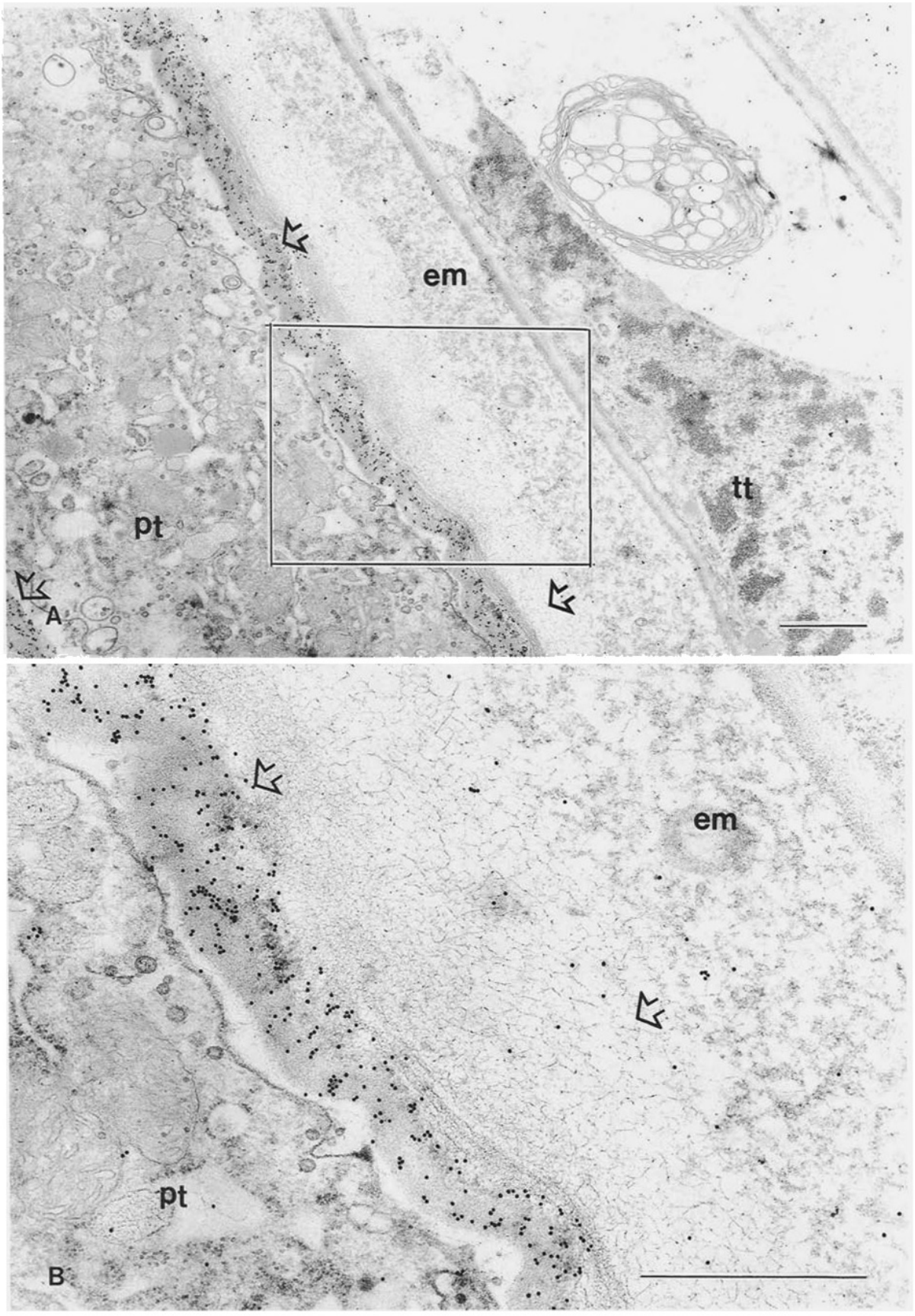

Fig. 4A, B

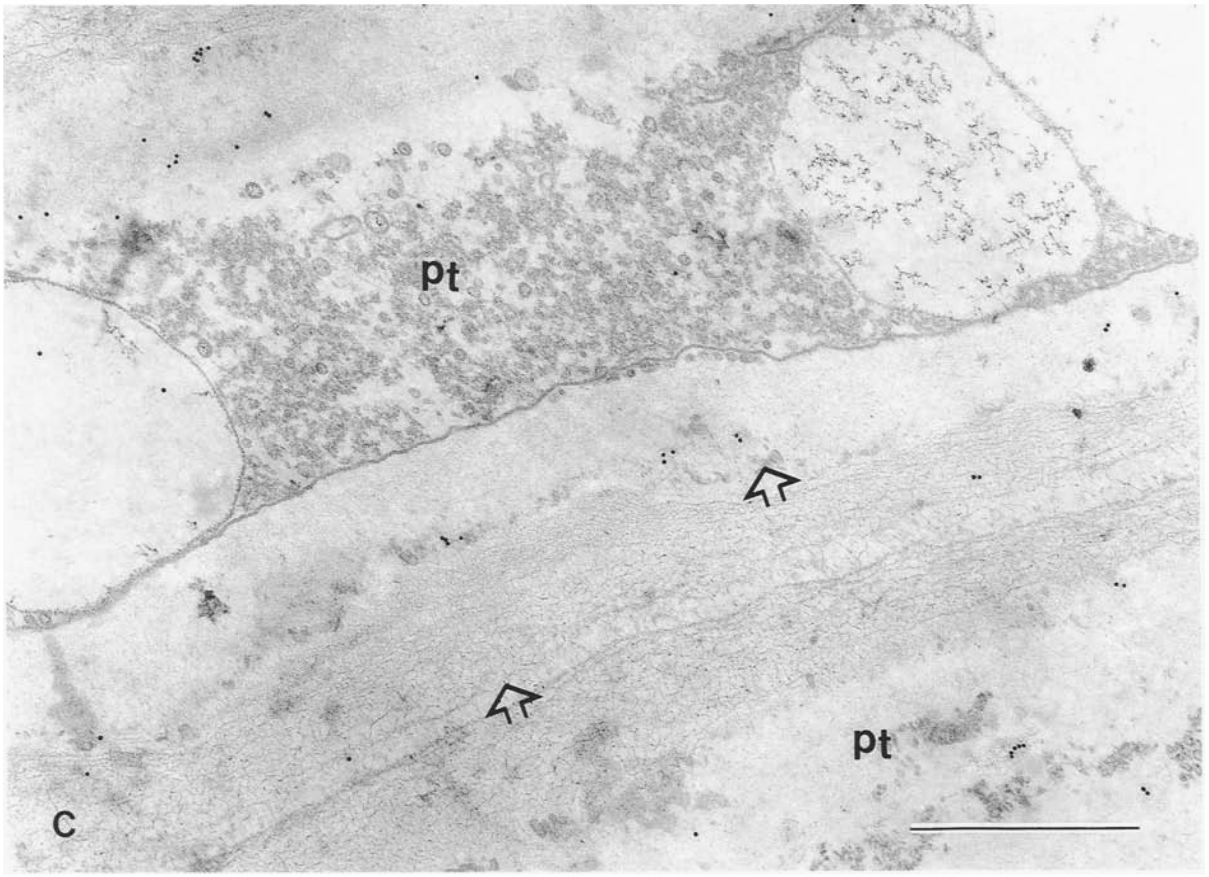

Fig. 4A–C. Longitudinal sections through a style of *Nicotiana alata*, 48 h after pollination. This section is from material about 8 mm beneath the stigma. The pollen tube (*pt*) is growing in the extracellular matrix (*em*) secreted by cells of the transmitting tract (*tt*). *Arrows* indicate the fibrillar layer and the electron-lucent layer of the pollen tube wall. **A** Section treated with monoclonal antibody to the (1→3)-β-glucan and the gold-labelled secondary antibody (rabbit anti-mouse). Labelling is more evenly distributed across the wall than is seen in tubes 12 h after pollination (Fig. 3B). Bar = 1 μm. **B** Higher magnification of *boxed area* of **A** showing even distribution of gold label. Bar = 1 μm. **C** Section treated with monoclonal antibody pre-incubated with laminarin (100 μg/ml) before application to the section. This section was subsequently treated with gold-labelled secondary antibody (rabbit anti-mouse). Few gold particles are present on the section. Bar = 1 μm

In pollen tubes which have grown for 48 h within the extracellular matrix of the transmitting tissue, the distribution of gold-label is more even within the inner layer (Fig. 4A, B) than in the tubes which had grown for 12 h (Fig. 3C). In a control experiment (Fig. 4C), the anti-(1→3)-β-glucan monoclonal antibody was blocked with 100 μg/ml laminarin prior to application to the sections. This effectively abolished labelling, confirming the specificity of the immunostaining procedure. In a further control experiment, in which primary antibody was omitted, there was no detectable labelling of the section. (Not shown)

Discussion

The monoclonal antibody generated against the laminarin-haemocyanin conjugate (LAMP2H12H7) is quite specific for (1→3)-β-glucans with no detectable cross-reactivity with either (1→4)-β-glucan or (1→3,1→4)-β-glucan in an indirect ELISA. Indirect competitive ELISA with the (1→3)-β-oligoglucosides has identified the optimum binding epitope as being a penta- to hexasaccharide with a K_a of approx. $2.7.10^4$ M^{-1}. The large epitope size explains why no cross-reaction is observed with the barley (1→3,1→4)-β-glucan which contains only single (1→3)-linkages flanked by (1→4)-linkages (Woodward et al. 1983). This explanation is further supported by the failure of laminaribiose to inhibit binding. The higher inhibitory power of laminarin compared with the (1→3)-β-oligoglucosides is likely to result from the multiple binding sites present along the length of the polysaccharide. The specificity of this anti-(1→3)-β-glucan antibody makes it an outstanding reagent for the immunostaining of (1→3)-β-glucans in tissue sections.

The anti-(1→3)-β-glucan antibody binds specifically to the inner wall layer of pollen tubes growing within the style of *N. alata*. As already pointed out in the *Introduction* the pollen-tube walls of *N. alata* contain two distinct layers, an inner electron-lucent layer and an outer fibrillar layer. Results of methylation analysis of pollen-tube wall preparations are consistent with the presence of two major polysaccharides, a (1→5)-arabinan and a (1→3)-glucan (Rae et al. 1985). A monoclonal antibody with a specificity for terminal arabinosyl residues binds primarily to the outer fibrillar layer of the pollen tube (Anderson

et al. 1987), indicating the association of the arabinan with this fibrillar layer. The current immunoelectron-microscopical observations confirm the presence of a (1→3)-β-glucan component in the inner wall layer. The characteristic electron-lucent nature of this region of the wall is also consistent with the presence of a (1→3)-β-glucan component.

The labelling pattern in the inner wall changes with time after initiation of pollination. Twelve hours after pollination the label is restricted to the outer zone of the inner wall, while after 48 h the inner wall is more evenly labelled. This presumably relates to the assembly and deposition of the (1→3)-β-glucan within the wall and may indicate the presence of a higher proportion of the longer (1→3)-β-glucan chains, and hence of more antibody-binding sites in the more mature wall. The lack of binding of the antibody to walls of the transmitting-tissue cells and intracellular sites is consistent with the absence of (1→3)-β-glucan in these sites. Patches of labelling associated with discrete regions of the transmitting tissue walls could correspond to plasmodesmata which connect adjacent transmitting-tissue cells. Similar binding of a polyclonal anti-(1→3)-β-glucan to sections of root tips of *Phaseolus vulgaris* has been reported (Northcote et al. 1989).

The experiments reported here demonstrate the utility of a monoclonal antibody specific for (1→3)-β-oligoglucosides to detect (1→3)-β-glucan ("callose") within plant tissues. In particular they confirm the presence of (1→3)-β-glucan in the inner walls of growing pollen tubes.

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