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Immunocytochemical Location of Pathogenesis-related b_1 Protein Induced in Tobacco Mosaic Virus-infected or Polyacrylic Acid-treated Tobacco Plants

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SUMMARY

Pathogenesis-related b_1 (PR-b_1) protein and other serologically related PR-b proteins, known to be associated with both tobacco mosaic virus infection and chemical treatments, such as with polyacrylic acid (PAA), were detected by immunofluorescence microscopy around epidermal, palisade and lacunar leaf cells in hypersensitive tobacco plants (PAA^+ line). After gentle fixation and embedding in Lowicryl K4M, PR-b proteins revealed by immunogold labelling were located in the cytoplasm and apoplast. After fixation and embedding in Epon, the PR-b proteins were found to accumulate mainly in the intercellular spaces. These observations support results obtained from biochemical tests.

The hypersensitive reaction of tobacco plants to viral, fungal and bacterial infection is accompanied by the appearance of abundant amounts of pathogenesis-related b proteins (PR-b proteins) (Gianinazzi *et al.*, 1970, 1980; Van Loon & Van Kammen, 1970; Ahl *et al.*, 1981; Van Loon, 1983). These host-coded proteins are first produced near the point of entry of the pathogen, where a necrotic lesion later appears, and reach their highest concentrations in a zone immediately outside the lesion (Antoniw & White, 1986). They subsequently appear throughout the shoot (Gianinazzi, 1982), which is then resistant to further infection (Ross, 1961*a*, *b*). It has been suggested that the PR-b proteins may be involved in active defence mechanism(s) of plants (Gianinazzi, 1982, 1984; Van Loon, 1985), especially as acetylsalicylic acid and polyacrylic acid (PAA) induce both the appearance of PR-b proteins and resistance to pathogens (Gianinazzi & Kassanis, 1974; White, 1979; Ahl *et al.*, 1981). However, tobacco cultivars differ in their response to these chemicals (Ahl & Gianinazzi, 1985), and recently the genetics of PAA-induced resistance to tobacco mosaic virus (TMV) and tobacco necrosis virus, and PR-b_1 protein production have been determined (Dumas *et al.*, 1985, 1987).

The functions of the PR-b_1 protein and other PR-b proteins serologically related to it (Ahl *et al.*, 1985) are still unknown. Nevertheless, although their role in TMV localization and in induced non-specific resistance has been questioned (Fraser, 1982; Dumas & Gianinazzi, 1986), the possibility remains that they are involved in such mechanisms against pathogens. This is particularly likely because other PR-b proteins not serologically related to the PR-b_1 group were recently identified as chitinases (Legrand *et al.*, 1987) and β-1,3-glucanases (Kauffman *et al.*, 1987), for which strong evidence exists that they are involved in plant defence mechanisms (Boller, 1987). There is indirect biochemical evidence from TMV-infected (Parent & Asselin, 1984; Ohashi & Matsuoka, 1987) and chemically treated leaves (Hooft van Huijsduijnen *et al.*, 1986; Dumas *et al.*, 1987; Ohashi & Matsuoka, 1987), as well as direct immunological evidence,

that PR-b proteins accumulate in extracellular spaces between mesophyll cells (Carr *et al.*, 1987). In this paper, we present new data on the location in tissues and cells of tobacco leaves of PR-b_1 protein and other serologically related PR-b proteins.

A tobacco line that reacts hypersensitively to infection by TMV and which reacts to PAA induction (PAA^+) (Dumas *et al.*, 1985, 1987) was propagated by cuttings and used for our experiments. This line possesses the PR-b_1 protein of *Nicotiana tabacum*, as well as $b_{1'}$, b_2 and b_3 of the same species that react, but to a lesser extent than homologous antigen, with a polyclonal antiserum to the PR-b_1 protein (Van Loon *et al.*, 1983; Ahl *et al.*, 1985). This same polyclonal antibody (prepared by Antoniw *et al.*, 1985) was used in our experiments. Samples were taken 1·5 mm from the edge of the necrotic lesion (mean diameter of lesions 2·5 mm) on leaves infected 7 days previously with TMV (common strain). Control samples were taken from untreated leaves of healthy plants. Infected samples contained 250 μg/g fresh weight of PR-b_1 protein, measured by ELISA as described by Antoniw & White (1986).

Samples were fixed by vacuum infiltration for at least 4 h at 4 °C in 4% formaldehyde/0·1% glutaraldehyde in 100 mM-sodium cacodylate buffer pH 7·2. Free aldehydes were removed by treatment for 1 h with 50 mM-ammonium chloride in 100 mM-phosphate buffered saline (PBS) pH 7·2. Tissues were then dehydrated through a graded ethanol series and embedded in LKB Historesin (LKB Bromma) or in Lowicryl K4M (Polysciences). Embedding in Lowicryl K4M was performed according to the method of Altman *et al.* (1985), modified by increasing infiltration times to 1 h in 1 : 2 (v/v) Lowicryl K4M/dimethylformamide, 3 h in a 1 : 1 mixture and overnight in 100% Lowicryl K4M. Polymerization was done by direct u.v. irradiation in Beem capsules in a cooled (4 °C) box for 2 h, followed by irradiation for 2 h at room temperature. Before sectioning, the blocks were stored as suggested by Fryer & Wells (1983). Thick sections, produced on a Reichert Ultracut E microtome, of Historesin-embedded (4 μm) or Lowicryl-embedded (0·5 μm) tissues were collected on microprint slides. Indirect immunofluorescent staining was done as suggested by Johnson *et al.* (1978) and Franklin (1984) in a moist chamber by sequential incubations in (i) 10% normal goat serum (Biosys, France) plus 1% ovalbumin in 100 mM-PBS–0·02% thimerosal (PBS–T) for 30 min at 20 °C, followed by 1% ovalbumin in PBS–T and PBS–T rinses for 30 min, (ii) rabbit PR-b_1 antibody diluted 1 : 50 in PBS–T for 1 h at 37 °C followed by PBS–T rinses for 30 min and (iii) fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (FITC–GAR from Biosys, France) diluted 1 : 800 in PBS–T for 1 h at 37 °C followed by rinses in PBS–T for at least 1 h at 20 °C. Sections were then mounted in PBS–T plus fluorescence-free glycerol (10% v/v).

Fluorescence microscopy [Leitz Laborlux K fluorescence microscope equipped with an epi-illumination system and filter combination I2 (Leitz)] of samples embedded in both Lowicryl K4M (Fig. 1*e*) and Historesin (Fig. 1*f*) revealed detectable amounts of PR-b proteins (Fig. 1*e*, *f*). No specific immunofluorescence was obtained when PR-b_1 antibody was omitted (Fig. 1*a*, *b*) or replaced with preimmune rabbit IgG (a gift from E. Boudon, INRA) (Fig. 1*c*, *d*). PR-b proteins were present around epidermal, palisade and lacunar cells and sometimes also in large amounts apparently in the intercellular spaces (arrowheads in Fig. 1*e*, *f*).

Carr *et al.* (1987) also observed faint immunostaining in the extracellular spaces of the mesophyll cells, but not around the epidermal cells. However, we have not confirmed their further observation, in infected samples, of a predominant immunofluorescent labelling in xylem. We consider that the fluorescence detected may have been due to non-specific labelling as well as to autofluorescence of lignin-like compounds (Perrot-Rechenmann & Gadal, 1986). As the resolution in immunofluorescent light microscopy was insufficient to demonstrate unequivocally where the PR-b proteins were located, immunolabelling was done at the ultrastructural level.

Samples were taken from leaves 7 days after inoculation with TMV or 3 days after treatment with PAA (0·02% M_r 2000, pH 6·5; Aldrich) or from untreated leaves. They were then fixed as previously described and embedded in Lowicryl K4M or fixed in a 2% formaldehyde/3% glutaraldehyde mixture in 100 mM-cacodylate buffer, followed by post-fixation in 1% osmium tetroxide in the same buffer for 1 h at 4 °C and then treated with ammonium chloride. Tissues were dehydrated with ethanol and embedded in Epon 812. Thin sections (85 nm) of Epon-

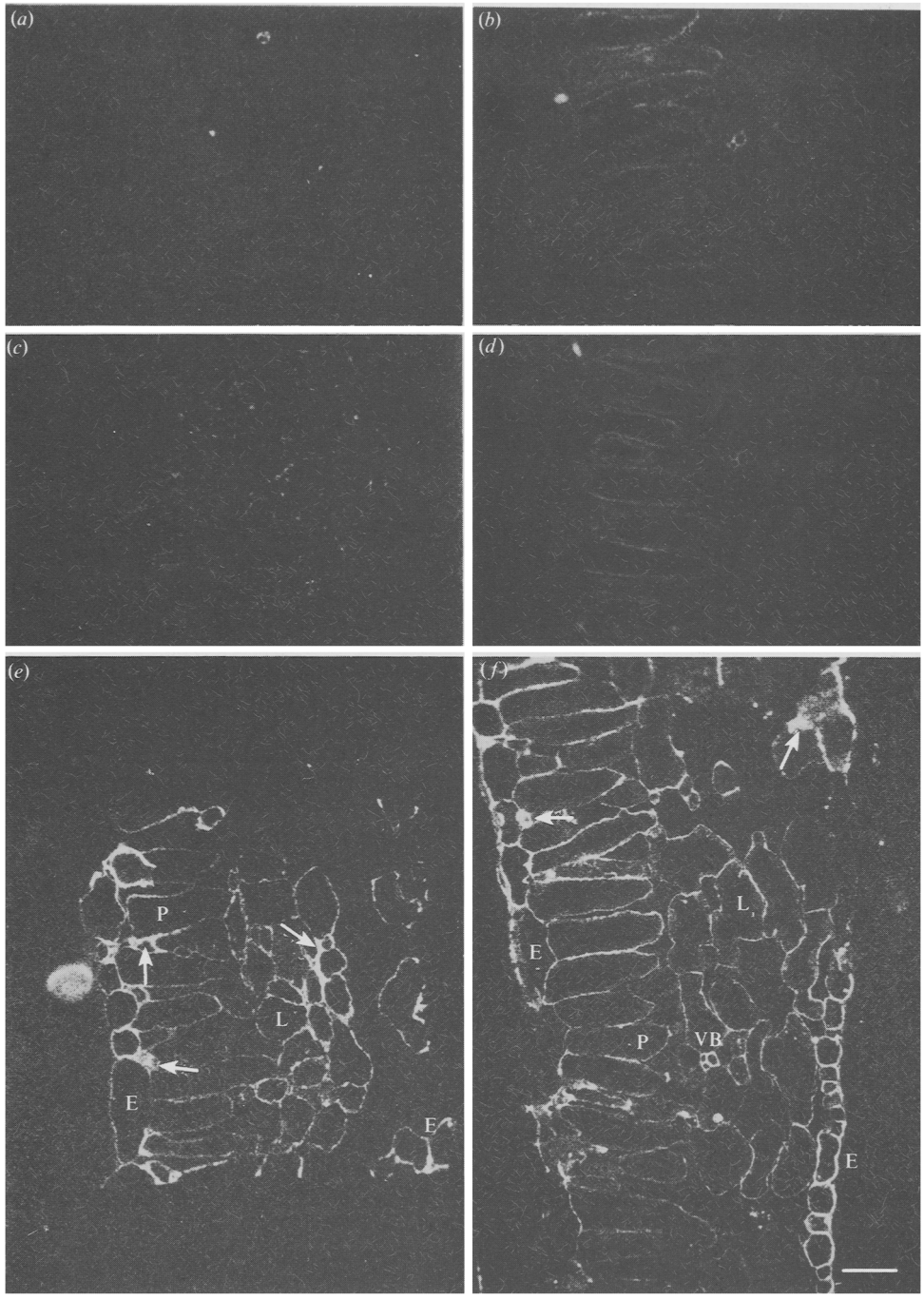

Fig. 1. Tissue location by immunofluorescence of the PR-b_1 protein in a TMV-infected PAA$^+$ line of tobacco. Successive sections of (*a*, *c*, *e*) Lowicryl K4M and (*b*, *d*, *f*) Historesin-embedded material. (*a*, *b*) Control sections omitting the first antibody, in (*c*) and (*d*) the sections were treated with preimmune rabbit IgG (1 : 100) and in (*e*) and (*f*) with PR-b_1 antibody (1 : 50); all sections were then incubated with FITC–GAR (1 : 800). E, epidermal cells; P, palisade cells; L, lacunar cells; VB, vascular bundle. Bar marker represents 50 μm.

embedded tissue were mounted on nickel grids, while those embedded in Lowicryl were mounted on Formvar-covered, carbon-coated nickel grids.

Indirect immunogold labelling was carried out in duplicate by floating the grids at 20 °C on 1% bovine serum albumin (BSA) plus 10% normal goat serum in TBS-T (10 mM-Tris-buffered saline pH 7·2, 0·02% thimerosal) for 30 min, followed by incubation with rabbit antibody to PR-b_1 diluted 1 : 100, 1 : 500 or 1 : 1000 in 100 mM-TBS–T overnight at 4 °C followed by rinsing for 1 h with 1% BSA in TBS–T. Grids were then incubated with 10 nm gold-conjugated goat anti-rabbit IgG (Gold–GAR from Janssen Pharmaceutica, Beerse, Belgium) diluted 1 : 10 in TBS–T for 1 h, rinsed five times with 1% BSA in TBS–T (total 30 min), then for 30 min with TBS–T and finally with distilled water. Some experiments were done wih monospecific PR-b_1 antibody obtained from the polyclonal antiserum, using the method of Rybicki (1986). After incubation of 100 µl of polyclonal antiserum with 100 µg of purified PR-b_1 blotted on nitrocellulose, bound antibodies were eluted from the nitrocellulose. Lyophilized antibodies were dissolved in 2 ml of TBS–T and used at a 1 : 50 dilution. Controls for all staining included substitution of PR-b_1 antibody with TBS–T, or with purified IgG from preimmune rabbit, or with anti-PR-b_1 serum (1 : 100 and 1 : 1000) preadsorbed with 10 µg/ml purified PR-b_1 (Berna *et al.*, 1986). All sections were counterstained with 2·5% aqueous uranyl acetate for 10 min and lead citrate for 10 min and examined with a Hitachi H600 electron microscope at 75 kV.

Samples embedded in Lowicryl K4M and treated with the polyclonal PR-b_1 antibody revealed immunogold labelling in the cytoplasm (Fig. 2*a*) and sometimes in the intercellular spaces (apoplast) of the leaves infected with TMV (Fig. 3*a*). The immunolabelling was particularly intense on the endoplasmic reticulum (Fig. 2*a*). Similar results were obtained in leaves treated with PAA. No labelling occurred in the control plants (results not shown) nor when the anti-PR-b_1 serum has been preadsorbed with purified PR-b_1 (Fig. 2*b*). Moreover, using monospecific antiserum (Fig. 3*c*, *d*) or monoclonal antibody (3-3G 1 line kindly provided by J. P. Carr) we also observed labelling in the cytoplasm and sometimes in the intercellular spaces.

After fixation and embedding in Epon, no specific labelling was found on TMV-infected or PAA-treated tissues unless the sections were etched. After different etching treatments, adapted from Bendayan & Zollinger (1983), Smart & Millard (1983) and Craig & Goodchild (1984), satisfactory immunolabelling was obtained on thin sections pretreated with 1% NaOH/ethanol for 30 s. However, the labelling was very weak on the cytoplasm and was found mainly in the intercellular spaces (Fig. 3*b*) and sometimes in the middle lamella (results not shown); this could be due to the two fixation steps used preventing the loss of small immunoreactive peptides during sample preparation (Bendayan & Zollinger, 1983), but decreasing the antigenicity of the proteins (Roth *et al.*, 1981; Craig & Goodchild, 1982; Bendayan & Zollinger, 1983).

Immunolabelling by different methods demonstrated that the PR-b_1 and other serologically related proteins were clearly localized both in the cytoplasm and in the intercellular spaces. This observation is in agreement with the results of biochemical studies in which PR protein was extracted from TMV-infected or PAA-treated tobacco plants by vacuum infiltration of the intercellular spaces (Carr *et al.*, 1985; Hooft van Huijsduijnen *et al.*, 1985; Dumas *et al.*, 1987; Bol, 1988).

The location of the PR-b_1 protein appears, therefore, to be different from other serologically unrelated PR-b proteins (β-1,3-glucanases and chitinases) which accumulate mainly in the vacuole (Mauch & Staehelin, 1988), where we never observed immunolabelling after treatment with polyclonal, monospecific or monoclonal antibody to PR-b_1. However, although it seems highly unlikely that vacuolar contents were lost during processing of glutaraldehyde- and osmium-fixed tissues, this possibility cannot be completely excluded.

The role of the PR-b_1 protein group in plant resistance remains an open question. Their accumulation in the intercellular spaces together with the fact that PR-b_1 protein is synthesized as a precursor with a signal sequence of 30 amino acids, which is probably cleaved off during maturation (Bol, 1988), support the hypothesis that they may act as intercellular 'messengers' (Hooft van Huijsduijnen *et al.*, 1985; Matsuoka *et al.*, 1987) in induced resistance phenomena (Gianinazzi, 1982).

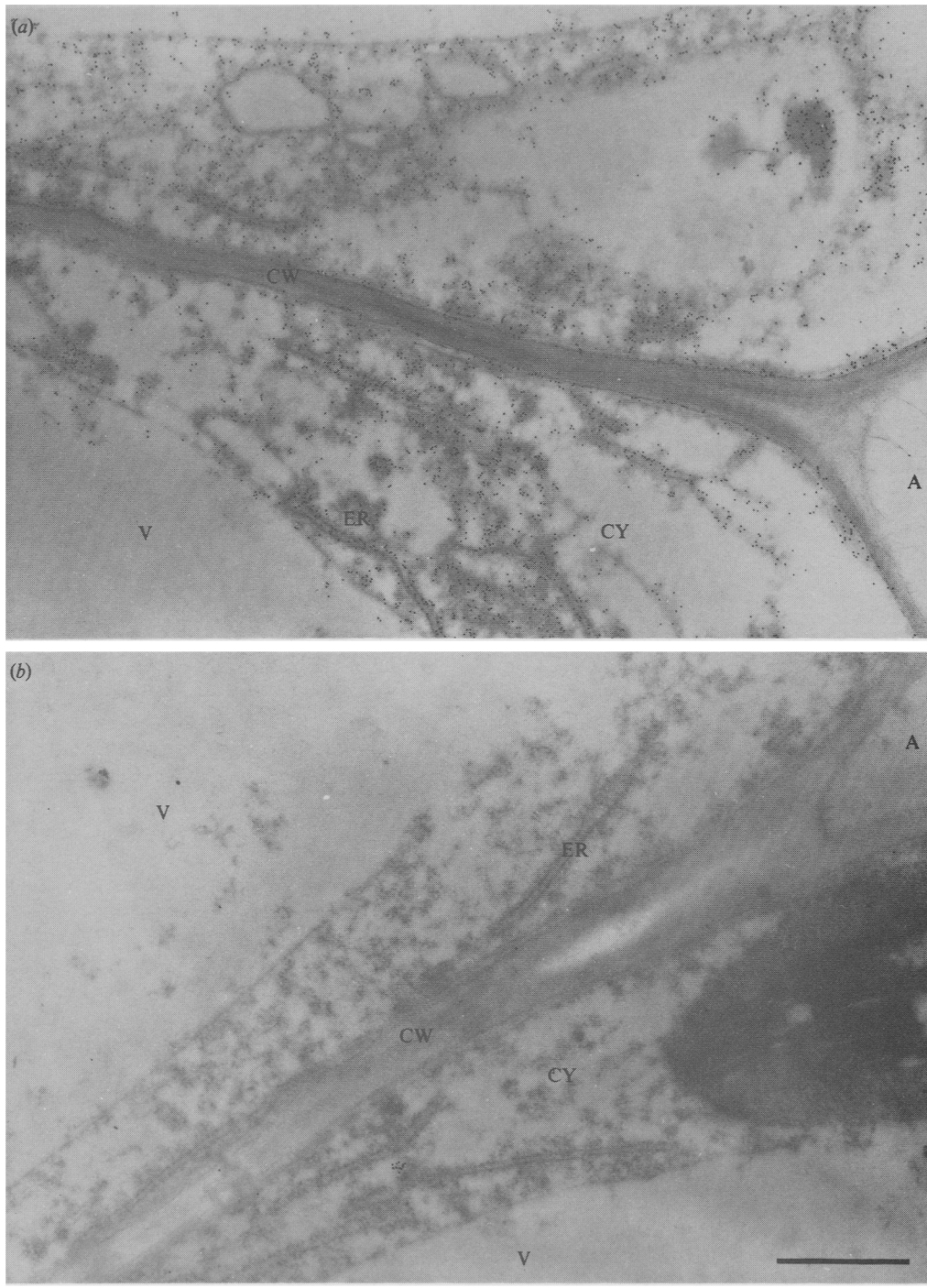

Fig. 2. Gold immunolabelling of the PR-b_1 protein in leaves 7 days after inoculation with TMV after Lowicryl K4M embedding. (*a*) PR-b_1 antibody (1:1000); (*b*) anti-PR-b_1 preadsorbed serum (1:1000). CY, cytoplasm; CW, cell wall; A, apoplast; ER, endoplasmic reticulum; V, vacuole. Bar marker represents 0·5 μm.

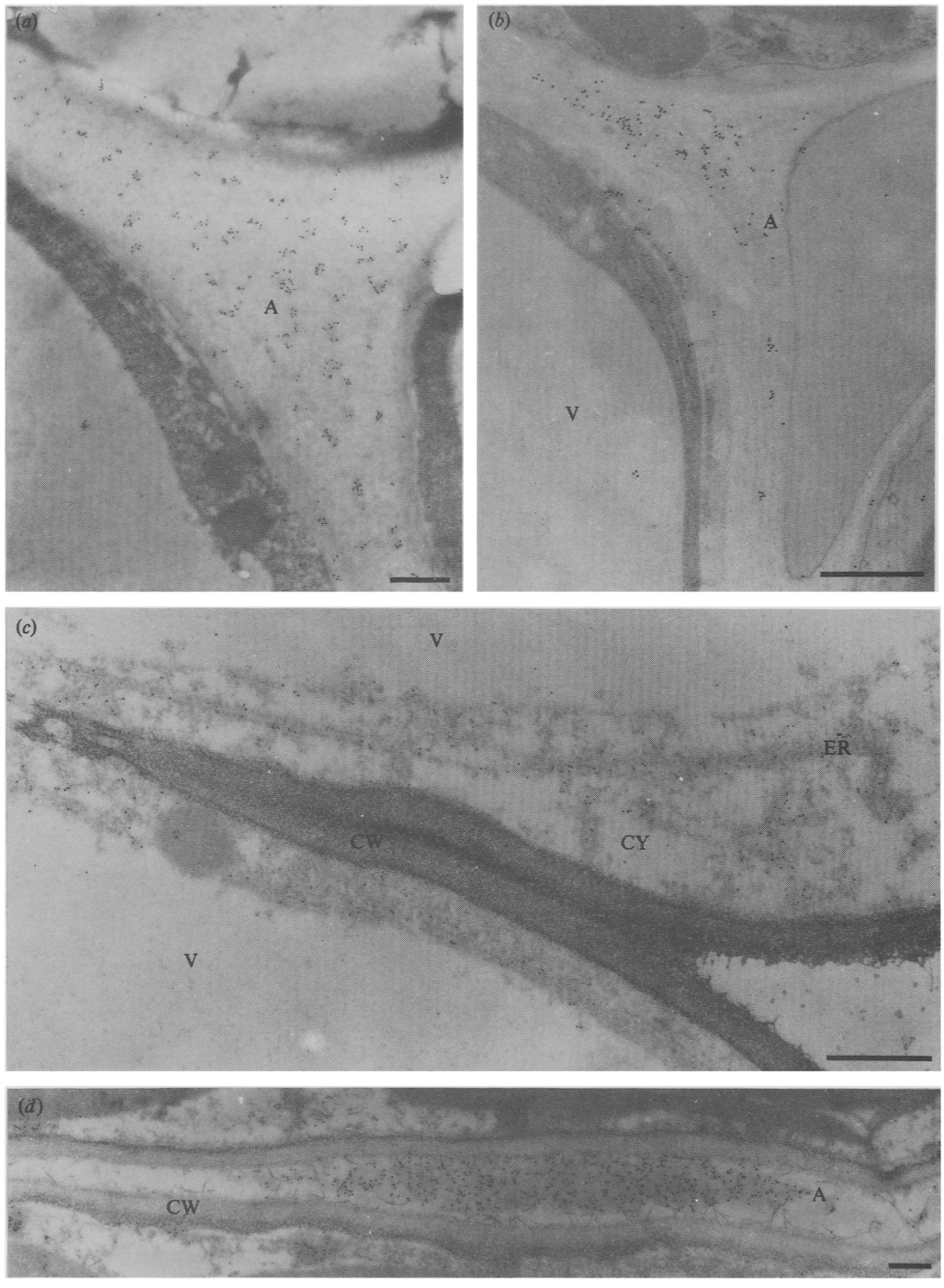

Fig. 3. Gold immunolabelling of PR-b_1 protein in TMV-infected or PAA-treated leaves after Lowicryl K4M and Epon embedding. (*a*, *c*, *d*) Embedding in Lowicryl K4M of TMV-infected (7 days) leaves; (*b*) embedding in Epon of PAA-treated (3 days) leaves; (*a*, *b*) PR-b_1 antibody diluted 1:100; (*c*, *d*) PR-b_1 monospecific antibody. CY, cytoplasm; CW, cell wall; A, apoplast; ER, endoplasmic reticulum; V, vacuole. Bar marker represents 0·5 μm.

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