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Immunocytolocalization of Cell Wall Peroxidase and Other Wall Antigens from Maize Seedlings

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Immunocytochemistry utilizes the specificity of the antigen-antibody reaction to localize specific antigens in cells or cellular organelles. Here we report the use of monoclonal antibodies, in conjunction with gold-labeled second antibodies to study the ultrastructural localization and tissue distribution of the *Mr* 98,000 anionic peroxidase and other wall antigens. The antibody specific for this wall peroxidase, mWP3, labeled mainly the cell wall area. At the tissue level, the *Mr* 98,000 peroxidase is located predominantly in the leaf mesophyll, internal coleoptile and sieve elements, but not in the root, as assayed with these procedures. The coleoptile walls were less heavily stained than the walls of leaf mesophyll cells. At the subcellular level, it is localized mainly in intercellular regions of the cell walls. A similar staining pattern was revealed by mWP19, one of anti-β glucosidase antibody, though it looked less heavily stained than one with mWP3. In order to serve as a control wall staining using IgM monoclonal antibodies, mWP18 was used. Most of the label is localized over wall regions of cells of the young leaf mesophyll and coleoptile.

Keywords: immunocytolocalization, cell wall, peroxidase, wall antigens, maize

Plants contain several peroxidase isozymes whose pattern of expression is tissue specific, developmentally regulated, and under control by many environmental stimuli (Lagrimini and Rothstein, 1987). Plant peroxidases are of importance in a variety of cellular functions such as IAA oxidation (Nakajima and Yamazaki, 1979; Smith *et al.*, 1982), lignin biosynthesis (Mäder and Amberg-F., 1982), NADH-dependent hydrogen peroxide formation (Mäder *et al.*, 1980), suberization (Espelie and Kolattukudy, 1985), metabolism of aromatic compounds (Stafford, 1974), and ethylene production (Yang, 1967). A number of wall peroxidase isoenzymes have been identified and partially purified from different plant species and tissues. Extracellular anionic isoperoxidases can be classified on the basis of their interaction with the plant cell wall. These peroxidases are either bound to the cell wall or freely soluble in the intercellular spaces (Mäder, 1976).

Peroxidase is a widespread enzyme in plants. Histochemical and cytochemical investigations have contributed to an understanding of the ultrastructural compartmentation of peroxidase activity in different plant tissues (Hall and Sexton, 1972; Hall and Sexton, 1974; Goff, 1975). They reveal considerable variations in the localization of peroxidase activity. Some peroxidases are preferentially secreted into the cell wall where they occur ionically or covalently bound (Zaar, 1979).

Localization studies have utilized a variety of different techniques, including cytochemical staining (Griffing and Fowke, 1985), radioactive labeling (Zaar, 1979), and immunofluorescence assays with polyclonal antibodies (Espelie *et al.*, 1986). Various colorimetric stains and histochemical methods are helpful but are not specific enough (Northcote *et al.*, 1989). Immunocytochemistry utilizes the specificity of the antigen-antibody reaction to localize specific antigens in cells or cellular organelles. Among the applicable techniques is the use of a gold-labeled second antibody to visualize the presence and location of specific antigenic sites at the electron microscopy level (Cohn *et al.*, 1989). Young *et al.* (1995) used immunogold labeling to establish the location of the induced cationic peroxidase PO-C1 in

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rice leaves after infection by *Xanthomonas oryzae* cv. *oryzae*.

Here we report the use of mWP3, in conjunction with gold-labeled second antibodies to study the ultrastructural localization and tissue distribution of the *M*r 98,000 anionic peroxidase. The study also employs, as positive controls, other monoclonal antibodies in our library, designated mWP19 and mWP18, which are raised against *M*r 58,000 β-glucosidase and other cell wall protein, respectively, to localize these cell wall molecules in thin sections of maize (Kim *et al.*, 1988).

MATERIALS AND METHODS

Plant material

Maize seedlings were grown on vermiculite in the dark for 4-5 days at 23°C as previously described in Kim *et al.* (1988). For fixation, samples were taken from the coleoptile with enclosed young leaves and from root tips.

Sample processing

Both maize coleoptiles with enclosed primary leaves and root tips were fixed in 2% formaldehyde and 0.17% glutaraldehyde in 0.1 M Na phosphate buffer (pH 7.5) for 1 hr and then fixed in 2% formaldehyde for 4 additional hours at room temperature as described in Kim *et al.* (1988). Samples were washed in cold Na phosphate buffer, dehydrated through an ethanol series, and then embedded in Lowicryl K4M (Polysciences, Inc.) and polymerized by UV irradiation at 4°C overnight. Silver to gold sections were cut on a microtome (LKB Ultratome), and picked up on Parlodion-coated nickel grids.

Staining procedure

Some sections for etching were treated on grids with saturated Na metaperiodate for 10 min in order to determine whether staining was specific for the protein. They were then washed in 0.1 N HCl for 10 min followed by a wash in distilled H_2O for 5 min. They were blocked in 5% nonfat dry milk in PBS (pH 7.5) for 30 min, incubated for 1 hr in partially purified mWP3 or mWP19 or mWP18 from CM-Affi gel Blue diluted in 0.05% PBST (final antibody concentration ranged from 150 to 200 ng/μL), washed two times with 0.5% PBST followed by TBS (20 mM Tris, 150 mM NaCl, pH 8.2) containing 0.5% Tween-20 (0.5% TBST), and incubated with gold (15 nm or 10 nm)-labeled goat anti-mouse antibody (Jansen) diluted 1 : 20 in 0.05% TBST for 30 min. In most cases, anti-mouse IgM was used as second antibody, unless otherwise mentioned.

Control sections were treated with control ascites or preabsorbed antibody and were processed in parallel with antibody-treated sections. Control ascites used were NS-1 or M75 ascites. Preabsorption of antibodies took place in the presence of a fivefold excess of crude wall protein preparation overnight before use in immunogold staining, and the resultant antibody solution was used. After the sections were washed in 0.5% TBST three times and once in distilled H_2O, they were dried and poststained with 5% saturated uranyl acetate for 30 min. They were then washed five times in distilled H_2O and dried.

All steps were carried out at room temperature. Stained samples were viewed with an Hitachi HU 11E (or Hitachi HS8) electron microscope. Kodak electron microscope film (Eastman-Kodak) was used for photography.

Western Blots

Discontinuous SDS-PAGE was performed according to Laemmli (1970). Antibody preparation was electrophoretically separated on a 9% SDS-polyacrylamide gel in a Hoefer minislab gel apparatus, and then transferred by electrophoresis to a nitrocellulose membrane (0.45 μm pore size) by the method of Towbin *et al.* (1979) in a Hoefer Transfer apparatus at 80V for 1.5 h at 4°C. All the following procedure were done at room temperature.

RESULTS

The preservation of maize coleoptile cells, embedded in Lowicryl K4M, was generally good. The antibody specific for *M*r 98,000 wall peroxidase, mWP3, labeled mainly the cell wall area of both coleoptiles and enclosed young leaves of maize, as shown in Fig. 1 (Kim *et al.*, 1988). Because there was so little cytoplasm present in coleoptile cells, the mesophyll cells of leaf sections were also chosen for most of the localization study. The most consistent staining patterns were seen in leaf mesophyll cells. In general the coleoptile walls were less heavily stained than the walls of leaf mesophyll cells. Except for some non-specific binding in starch grains, little staining was seen in most cytoplasmic regions.

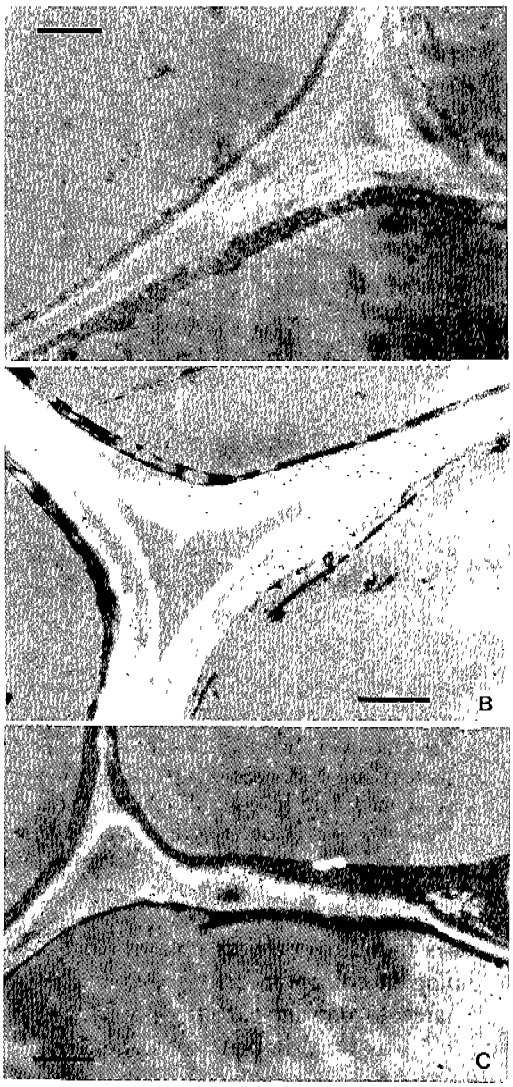

Fig. 1. Immunostaining of coleoptile cells and leaf mesophyll cells reacted with mWP3. Walls of the internal cells of coleoptile (A, etched; B, non-etched) were stained. In general, they were less heavily stained than the walls of leaf mesophyll cells (C) in the same sample. Figures are with 15 nm gold (A, C) and 10 nm gold (B). A, X18,100; B, X20,100; C, X16,800; bar=1 μm.

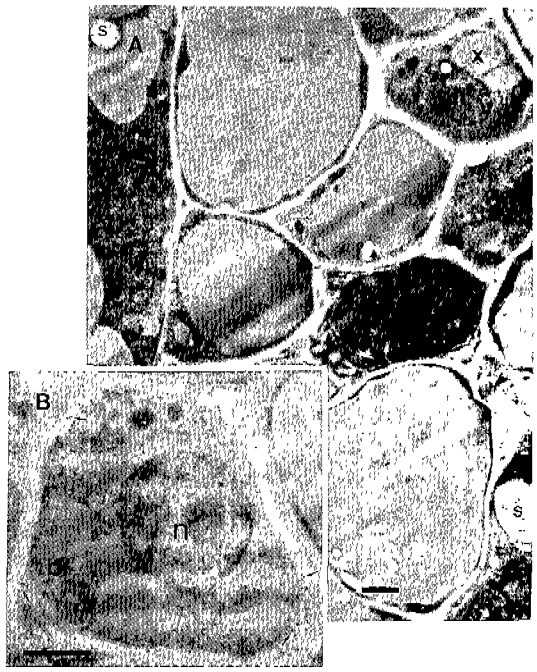

Fig. 2. Immunostaining of the vascular bundles reacted with mWP3. The walls of most vascular cells were not stained. Only walls of young sieve elements (x) showed some specific staining (A). A sieve element is shown at higher magnification in (B). Figures are with 15 nm gold. A, X9,300; B, X17,400; bar=1 μm. n, nucleus; s, starch grains

Preabsorption of mWP3 with a partially purified wall peroxidase preparation significantly reduced its staining of wall antigens (data not shown).

While most vascular cells were not stained, only walls of young sieve elements showed consistent staining (Fig. 2). There was no staining in the cell walls of any apical root cells (Fig. 3). There was neither staining of the cell walls nor of wall associated materials in any root cap cells. Epidermal cells are shown in Fig. 3A, and cells from columella region are shown in Fig. 3B. The sparse staining in the cytoplasm is not above the background staining seen in the controls.

A similar staining pattern was revealed by mWP 19, as shown in the Fig. 4, though it looked less heavily stained than one with mWP3. *Mr* 58,000 polypeptide recognized by mWP19 was turned out to be a wall β-glucosidase (Nematollahi, 1996). Most of the labeling with mWP19 was located in the wall area.

There was no labeling of cell wall with the NS-1 control ascites as shown in Fig. 5A. Other controls tested were no primary antibody (Fig. 5B). None of these showed any specific staining in the wall, though a small amount of staining was visible in the wall in Fig. 5A. This staining is much less and in a different pattern than those shown in other mWP3- or mWP19-specific staining. The minimum background staining was removed by the treatment of the sections with Na metaperiodate (etching, data not shown). This confirmed the specificity of the immunocytological procedure.

The purpose of the experiments whose results are shown in Fig. 6 was to determine whether the as-

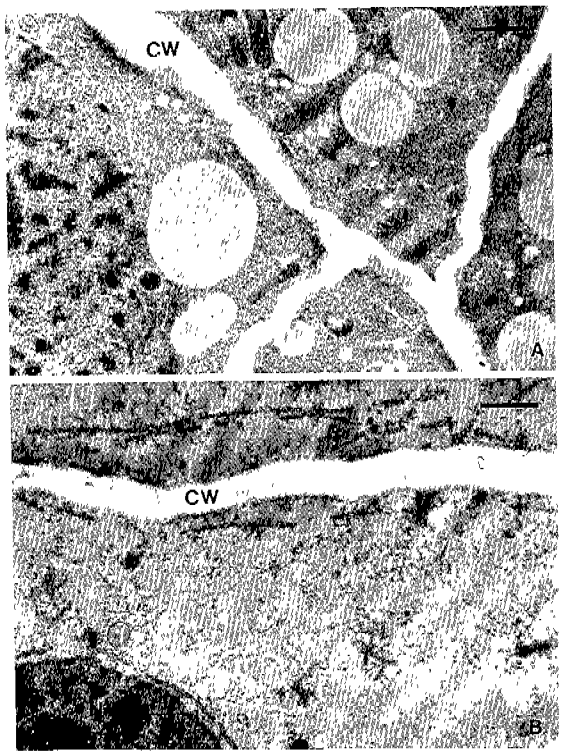

Fig. 3. Immunostaining of cells in the root apex and the rootcap reacted with mWP3. There was no staining in the cell walls of any apical cells of the root. Epidermal cells are shown in (A). Cells from columella region are shown in (B). Figures are with 15 nm gold. A and B, X17,400; bar=1 μm.

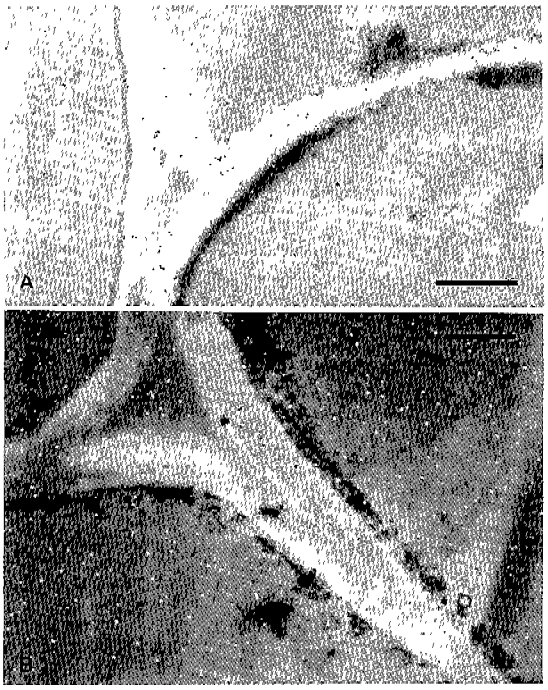

Fig. 4. Immunostaining of coleoptile cells and leaf mesophyll cells reacted with mWP19. Walls of the coleoptile internal cells (A) and leaf mesophyll cell wall (B) of the sample were stained, but, in general, they were less heavily stained than the ones stained with mWP3. Figures are with 15 nm gold. A, X23,200; B, X20,000; bar=1 μm.

cites fluids of mWP3 and mWP19 had both IgG and IgM in its antibody population. When only protein A was used to bind and precipitate antibody from either mWP3 or mWP19, it selectively brought down only IgG (Lanes 2 and 3 in Fig. 6). However, when rabbit anti-mouse IgM absorbed to protein A was used, it brought down IgM as well as a small amount of IgG (Lanes 4, 5, and 6 in Fig. 6). Since IgGs of both antibodies were not very active when they were used for immunogold staining, most studies were done with IgMs of each antibody by use of anti-mouse IgM second antibody.

In order to serve as a control wall staining using IgM monoclonal antibodies, mWP18 was used. Previously, we had shown that mWP18 reacted with only one protein band which had been immunoprecipitated with polyclonal anti-9.5 cellulase antibodies (Kim *et al.*, 1987). Most of the label is localized over wall regions of cells of the young leaf mesophyll (Fig. 7A) and coleoptile (Fig. 7B). There was some non-specific binding of starch grains, but little gold is found over the cytoplasm (Fig. 7A). Immunostaining in coleoptile cells shows a similar pattern compared to one in leaf mesophyll cells. When the antibody was preabsorbed with a crude wall protein preparation, the staining in the wall of leaf mesophyll cells and of coleoptile was significantly diminished (data not shown).

DISCUSSION

At the tissue level the *M*r 98,000 peroxidase is located predominantly in the leaf mesophyll, internal coleoptile and sieve elements, but not in the root, as assayed with these procedures. At the subcellular level it is found mainly in intercellular regions of the cell walls. When unpurified crude ascites was used, there was always prominent non-specific staining throughout the cell including in the cell wall area (data not shown). This indicated that a false positive in immunogold staining could result unless the antibody preparation was purified.

Our results indicate that both mWP3 and mWP19 contain both IgG and IgM, even though they are

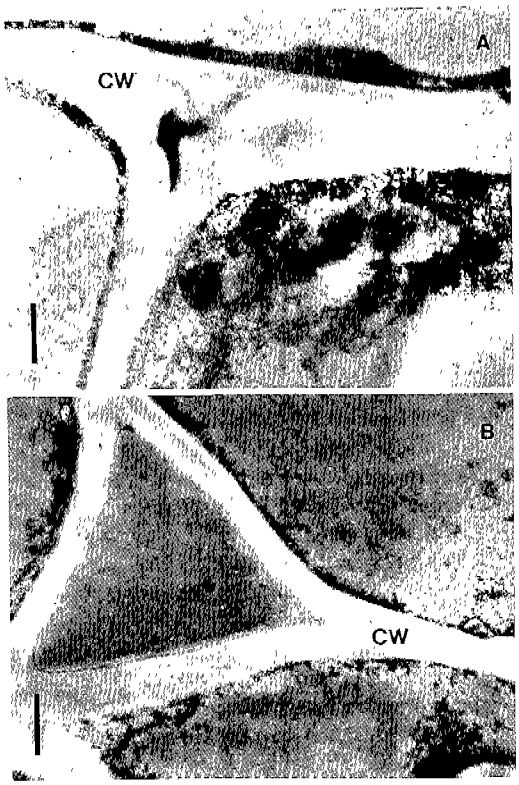

Fig. 5. Immunostaining of leaf mesophyll cells reacted with control ascites. Samples were treated with NS-1 ascites (A) or no primary antibody (B). There is a small amount of staining in the wall in A, but this is much less and in a different pattern than those shown in other Figures. Figures are with 15 nm gold. A, X16,800; B, X17, 400; bar=1 μm.

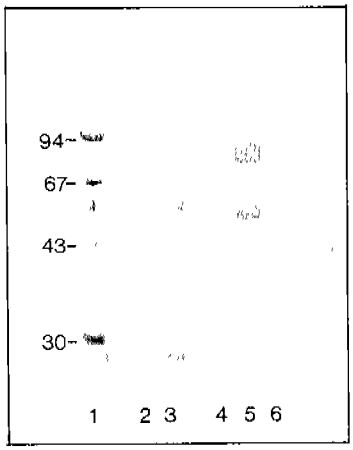

Fig. 6. Identification of IgG and IgM subclasses both in mWP3 and mWP19 by Western blot analysis. The lanes were as follows: (1) molecular weight markers (values in kD) stained with India ink; (2) immunosorbent of mWP3 coupled to protein A only was eluted, electroblotted and visualized using phosphatase-labeled goat anti-mouse IgG (heavy chain specific); (3) same as lane (2) except mWP19 was used; (4) immunosorbent of mWP3 coupled to rabbit anti-mouse IgM absorbed protein A was eluted, electroblotted and visualized using phosphatase-labeled goat anti-mouse IgM (heavy chain specific); (5) same as lane (4) except mWP19 was used; and (6) same as lane (4) except M75 ascites was used.

considered to be monoclonal antibodies. One possible explanation is that both of these hybridoma clones could be heavy chain class switch variants that secret both IgG and IgM against the same epitope of the antigen. During B-cell differentiation, different heavy chain classes can be expressed sequentially by the same cell [e.g., the μ to γ switch] (Nossal *et al.*, 1964) or simultaneously [e.g., μ and γ chains] (Radbruch et al., 1980).

According to De Jong (1967), who used cytochemical staining, peroxidase localization is cytoplasmic in juvenile onion root tissues and associated with the walls in mature cells. The peroxidase activity is strongest in tissues which do not lignify, but is wholly absent in the lignifying xylem throughout all stages of development. These results did not support the theory that plant peroxidase is involved in cell wall synthesis. Espelie *et al.* (1986) studied the time course of appearance of the immunochemically detected peroxidase. Their results were consistent with both the changes in the level of peroxidase activity measured in the periderm layer of potato during wound-healing and with the changes in the anionic peroxidase protein level measured by the immunoblot assays. Their results, which were derived from fluorescent antibody staining, also showed that the anionic peroxidase is localized in the inner side of suberizing walls.

Smith *et al.* (1994) reported that after wounding of French bean hypocotyl tissue, a cationic peroxidase accumulated in the xylem at sites of secondary thickening and in the middle lamella, based on their immunolocalization studies. Other immunocytolocalization studies (Griffing and Fowke, 1985; Chibbar and van Huystee, 1986) have shown that some peroxidases are associated with Golgi vesicles and small membranous vesicular structures in the cytoplasm. Our immunogold localization study shows that mWP3 binds to antigens present in the cell walls of both coleoptiles and enclosed young leaves. The ultrastructural localization of the peroxidases re-

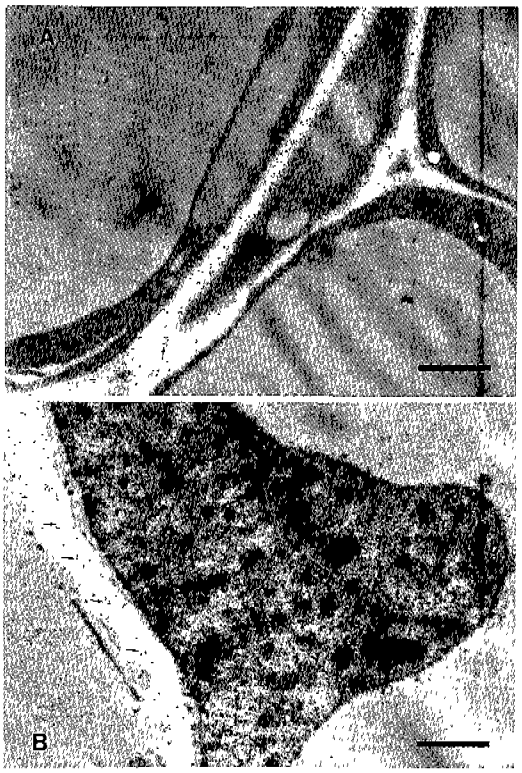

Fig. 7. Characteristic wall immunostaining of the cell wall of young leaf mesophyll cells and coleoptile cells reacted with mWP18. Most of the label is localized over wall regions both of young leaf mesophyll cells (A) and coleoptile cells (B). Little gold is found over the cytoplasm. Immunostaining in coleoptile cells shows a similar pattern compared to one in leaf mesophyll cells. A and B, X 18,100; bar=μm.

cognized by mWP3 is predominately in the cell walls of both developing and differentiated tissues of maize. These results clarify questions relating to the potential functions of these peroxidases.

In conclusion, the use of characterized monoclonal antibodies has led us to localize various cell wall antigens. These antibodies will be useful for detecting with precision dynamic changes in the wall antigens they recognize during plant growth and differentiation.

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LITERATURE CITED

Chibbar, R.N. and R.B. van Huystee. 1986. Immunochemical localization of peroxidase in cultured peanut cells. *J. Plant Physiol.* **123**: 477-486.

Cohn, N.S., J.P. Mitchell and Z. Zhou. 1989. Ultrastructural immunolocalization of developmental proteins in phloem cell wall regions of *Pisum sativum* L. shoot tissue. *Plant Sci.* **60**: 137-143.

De Jong, D.W. 1967. An investigation of the role of plant peroxidase in cell wall development by the histochemical method. *J. Histochem. Cytochem.* **15**: 335-346.

Espelie, K.E., V.R. Franceschi and P.E. Kolattukudy. 1986. Immunocytochemical localization and time course of appearance of an anionic peroxidase associated with suberization in wound-healing potato tuber tissue. *Plant Physiol.* **81**: 487-492.

Espelie, K.E. and P.E. Kolattukudy. 1985. Purification and characterization of an abscisic acid-inducible anionic peroxidase associated with suberization in Potato (*Solanum tuberosum*). *Arch. Biochem. Biophys.* **240**: 539-545.

Goff, C.W. 1975. A light and electron microscopic study of peroxidase localization in the onion root tip. *Amer. J. Bot.* **62**: 280-291.

Griffing, L.R. and L.C. Fowke. 1985. Cytochemical localization of peroxidase in soybean suspension culture cells and protoplasts: Intracellular vacuole differentiation and presence of peroxidase in coated vesicles and multivesicular bodies. *Protoplasma* **128**: 22-30.

Hall, J.L. and R. Sexton. 1972. Cytochemical localization of peroxidase activity in root cells. *Planta* **108**: 103-120.

Hall, J.L. and R. Sexton. 1974. Fine structure and cytochemistry of the abscission zone cells of Phaseolus leaves. II. Localization of peroxidase and acid phosphatase in the separation zone cells. *Ann. Bot.* **38**: 855-858.

Kim, S.-H., M.E. Terry and S.J. Roux. 1987. Production of monoclonal antibodies to cell wall peroxidase and cellulase. *In* D.J. Cosgrove, D.P. Knievel, eds, Physiology of cell expansion during plant growth. American society of plant physiologists, Rockville, pp. 289-291.

Kim, S.-H., M.E. Terry, P. Hoops, M. Dauwalder and S.J. Roux. 1988. Production and characterization of monoclonal antibodies to wall-localized peroxidases from corn seedlings. *Plant Physiol.* **88**: 1446-1453.

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

Lagrimini, L.M. and S. Rothstein. 1987. Tissue specificity of tobacco peroxidase isozymes and their induction by wounding and tobacco mosaic virus infection. *Plant Physiol.* **84**: 438-442.

Mäder, M. 1976. Die Lokalization des Peroxidase-Isoenzymgruppe G1 in der Zellwand von Tabak-Geweben. *Planta* **131**: 11-15.

Mäder, M. and V. Amberg-F. 1982. Role of peroxidase in lignification of tobacco cells. I. Oxidation of nicotinamide adenine dinucleotide and formation of hy-

drogen peroxide by cell wall peroxidases. *Plant Physiol.* **70**: 1128-1131.

Mäder, M., J. Ungemach and P. Schlob. 1980. The role of peroxidase isozyme groups of *Nicotiana tabacum* in hydrogen peroxide formation. *Planta* **147**: 467-470.

Nakajima, R. and I. Yamazaki. 1979. The mechanism of indole-3-acetic acid oxidation by horseradish peroxidases. *J. Biol. Chem.* **254**: 872-878.

Nematollahi, W.P.S. 1996. Studies on a β-glucosidase from the cell wall of maize (*Zea mays* L.). Ph.D. thesis. Univ. of Texas at Austin, Austin.

Northcote, D.H., R. Davey and J. Lay. 1989. Use of antisera to localize callose, xylan and arabinogalactan in the cell-plate, primary and secondary walls of plant cells. *Planta* **178**: 353-366.

Nossal, G.J. V., A. Szenberg, G.L. Ada and C.M. Austin. 1964. Single cell studies on 19S antibody production. *J. Exp. Med.* **119**: 485-502.

Radbruch, A., B. Liesegang and K. Rajewsky. 1980. Isolation of variants of mouse myeloma X63 that express changed immunoglobulin class. *Proc. Natl. Acad. Sci. USA* **77**: 2909-2913.

Smith, A.M., W.L. Morrison and P.J. Milham. 1982. Oxidation of indole-3-acetic acid by peroxidase: Involvement of reduced peroxidase and compound III with superoxide as a product. *Biochem.* **21**: 4414-4419.

Smith, C.G., M.W. Rodgers, A. Zimmerlin, D. Ferdinando and G.P. Bolwell. 1994. Tissue and subcellular immunolocalisation of enzymes of lignin synthesis in differentiating and wounded hypocotyl tissue of French bean (*Phaseolus vulgaris* L.). *Planta* **192**: 155-164.

Stafford, H.A. 1974. The metabolism of aromatic compounds. *Ann. Rev. Plant Physiol.* **25**: 459-486.

Towbin, H., T. Staehelin and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**: 4350-4354.

Yang, S.F. 1967. Biosynthesis of ethylene. Ethylene formation from methional by horseradish peroxidase. *Arch. Bioch. Biophy.* **122**: 481-487.

Young, S.A., A. Guo, J.A. Guikema, F.F. White and J. E. Leach. 1995. Rice cationic peroxidase accumulates in xylem vessels during incompatible interactions with *Xanthomonas oryzae* cv. *oryzae. Plant Physiol.* **107**: 1333-1341.

Zaar, K. 1979. Peroxidase activity in root hairs of Cress. Cytochemical localization and radioactive labelling of wall bound peroxidase. *Protoplasma* **99**: 263-274

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