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Rice Cationic Peroxidase Accumulates in Xylem Vessels during Incompatible Interactions with *Xanthomonas oryzae* pv *oryzae*[1]

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A cationic peroxidase, PO-C1 (molecular mass 42 kD, isoelectric point 8.6), which is induced in incompatible interactions between the vascular pathogen *Xanthomonas oryzae* pv *oryzae* and rice (*Oryza sativa* L.), was purified. Amino acid sequences from chemically cleaved fragments of PO-C1 exhibited a high percentage of identity with deduced sequences of peroxidases from rice, barley, and wheat. Polyclonal antibodies were raised to an 11-amino acid oligopeptide (POC1a) that was derived from a domain where the sequence of the cationic peroxidase diverged from other known peroxidases. The anti-POC1a antibodies reacted only with a protein of the same mobility as PO-C1 in extracellular and guttation fluids from plants undergoing incompatible responses collected at 24 h after infection. In the compatible responses, the antibodies did not detect PO-C1 until 48 h after infection. Immunoelectron microscopy was used to demonstrate that PO-C1 accumulated within the apoplast of mesophyll cells and within the cell walls and vessel lumen of xylem elements of plants undergoing incompatible interactions.

Peroxidases (EC 1.11.1.7, H_2O_2 oxidoreductase) have been implicated in the regulation of plant cell elongation (Goldberg et al., 1986), phenol oxidation (Schmid and Feucht, 1980), polysaccharide cross-linking (Fry, 1986), IAA oxidation (Beffa et al., 1990), cross-linking of extensin monomers (Everdeen et al., 1988), oxidation of hydroxycinnamyl alcohols into free radical intermediates (Gross, 1980), and wound healing (Espelie et al., 1986). Increases in peroxidases have been correlated with resistance in many species, including barley, cucurbits, cotton, tobacco, wheat, and rice (*Oryza sativa* L.). In these interactions the enzyme is involved in the polymerization of proteins and lignin or suberin precursors into plant cell walls (Ride, 1983; Mohan and Kolattukudy, 1990; Bradley et al., 1992), thus constructing a physical barrier that could prevent pathogen penetration of cell walls or movement through vessels. In addition, the free radical intermediates produced by peroxidase oxidative activity are toxic to pathogens (Sutherland, 1991).

In resistant or incompatible interactions between rice and the bacterial blight pathogen, *Xanthomonas oryzae* pv *oryzae*, increases in the activities of three extracellular peroxidases (two anionic [PO-A1 and PO-A2] and one cationic [PO-C1]) have been correlated with the accumulation of lignin-like compounds, a reduction in bacterial multiplication in the leaves, and the onset of the HR (Reimers and Leach, 1991; Reimers et al., 1992; Guo et al., 1993). Activity of the cationic peroxidase PO-C1 increased more dramatically with the onset of resistance when compared with the two anionic peroxidases (Reimers et al., 1992). The timing and dynamics of the events leading to the HR are dependent on the specific *X. oryzae* pv *oryzae* avirulence gene and rice resistance gene involved in the interaction. In interactions involving strains of *X. oryzae* pv *oryzae* with the avirulence gene *avrXa10* and rice cultivars with the corresponding resistance gene (*Xa-10*), an increase in PO-C1 activity is observed within 16 to 24 h, and the HR is visible at 24 h (Hopkins et al., 1992; Guo et al., 1993; Leach et al., 1993). In the incompatible interactions involving *X. oryzae* pv *oryzae* with *avrXa7* and rice with *Xa-7*, the increase in PO-C1 occurred between 24 and 48 h after inoculation and symptoms of the HR were delayed (48–72 h). PO-C1 does not accumulate until 48 to 72 h after infection in susceptible or compatible interactions. The tissues in these interactions become water soaked and the bacteria multiply in the xylem and spread rapidly through the rice leaf.

Since accumulation of PO-C1 activity is correlated with resistance in rice to *X. oryzae* pv *oryzae* infection, the isoenzyme may have a role in restricting bacterial multiplication and subsequent spread in rice plants (Reimers et al., 1992). The spatial distribution of PO-C1 in tissues undergoing the HR, however, has not yet been demonstrated. *X. oryzae* pv *oryzae* is a vascular pathogen and enters the plant through either hydathode water pores or wounds (Tabei, 1967, 1977). Upon entering a water pore, bacteria multiply in the spaces surrounding the epithem. This tissue includes modified and loosely organized mesophyll cells beneath the

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Abbreviations: HR, hypersensitive response; PLD phospholipase D; HRP, horseradish peroxidase; PVDF, polyvinylidene difluoride.

water pore and would thereby allow bacteria to gain access to the xylem. Thus, if PO-C1 plays a role in resistance, the isoenzyme would be expected to accumulate in the xylem vessels. Peroxidases have been found in xylem sap collected from stem exudates and guttation fluids of healthy *Helianthus*, strawberry, tomato, cucumber, pear, and apple (Biles and Abeles, 1991; Magwa et al., 1993). Recently, 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. In the present study, we use immunogold labeling to establish the location of the induced cationic peroxidase PO-C1 in rice leaves after infection by *X. oryzae* pv *oryzae*.

MATERIALS AND METHODS

Plant Material, Bacterial Strains, and Media

Rice (*Oryza sativa* L.) cvs Cas209 and IR-BB10 contain the *Xa-10* gene for bacterial blight resistance. IR24 is near isogenic to IR-BB10 but lacks the *Xa-10* gene and is susceptible to all strains of *Xanthomonas oryzae* pv *oryzae* used in this study. Seedlings were grown in a growth chamber as described previously (Reimers and Leach, 1991).

Strains PXO86 and PXO99^{A}(pBUavrXa10.F1) of *X. oryzae* pv *oryzae* carry the *avrXa10* gene and, when inoculated on rice cultivars containing the corresponding *Xa-10* resistance gene, elicit a resistance response (dark brown lesion). PXO99^{A}, which lacks the *avrXa10* gene, produces a susceptible response (water soaking) when inoculated onto rice cvs IR-BB10 and IR24. *X. oryzae* pv *oryzae* strains were maintained at 28°C on WF-P medium (Karganilla et al., 1973). For plant inoculations, *X. oryzae* pv *oryzae* cultures were grown in Nutrient Broth (Difco Laboratories, Detroit, MI) on a rotary shaker (250 rpm) at 28°C. When required, media were supplemented with antibiotics at the following concentrations (μg/mL): carbenicillin (100) and kanamycin (100). Inoculum (5×10^9 colony-forming units/mL) was prepared and infiltrated into multiple sites on the second leaf of rice plants as described (Reimers and Leach, 1991; Reimers et al., 1992). For guttation studies, inoculum was infiltrated at a single site two-thirds the length of the leaf from the tip.

Purification of PO-C1

Inoculated rice leaves (100 g) were harvested 48 h after infection and frozen in liquid nitrogen. Frozen leaves were ground in a coffee grinder, then homogenized with a blender (Brinkmann Instruments, Westbury, NY) in four volumes of buffer containing 20 mM Mes (pH 6.0), 1 mM DTT, 10 mM sodium ascorbate, 0.2 mM PMSF, and 0.5 μg/L leupeptin at 4°C. The homogenate was filtered through six to nine layers of sterile cheesecloth and centrifuged at 15,000*g* for 10 min. Proteins in the supernatant were precipitated with ammonium sulfate at 55 to 70% saturation, and the precipitate was dissolved in distilled water with 1 mM DTT, 0.5 mM PMSF, and 0.5 μg/mL of leupeptin. The sample was desalted by passage through a PD-10 column (Pharmacia LKB Biotechnology, Uppsala, Sweden) and concentrated in a Centricon-10 filter (Amicon, Beverly, MA).

The concentrated protein was subjected to PAGE in a 0.15 × 10.0 × 5.5 cm resolving gel (nondenaturing cathodic) (Thomas and Hodes, 1981). One lane was removed and stained with 18 mM guaiacol (Maehly, 1954), 0.01% 3-amino-9-ethylcarbazole, and 0.03% H_2O_2 in 100 mM sodium phosphate (pH 6.0) buffer (Reimers et al., 1992). The gel portion corresponding to PO-C1 was excised and cut into 7 × 10 mm pieces. Proteins were denatured, separated on SDS-polyacrylamide gels (12% PAGE), and were electrophoretically transferred to PVDF membranes (Bio-Rad) using Towbin buffer (Towbin et al., 1979), and stained with 0.025% Coomassie brilliant blue R250 in a 40% methanol and 5% acetic acid solution.

Preparation of Peptides for Sequencing

Purified PO-C1 samples for sequencing were excised from native gels and denatured. To keep Met residues in the peroxidase protein in a reduced state during separation in SDS gels, thioglycolic acid was added to the cathode buffer (final concentration 0.1 μM). After prerunning the gel for 20 min at 50 V to allow the thioglycolic acid to penetrate the gel, native gel pieces containing denatured PO-C1 were placed in sample wells. The separated proteins were electrophoretically transferred to Immobilon-P membrane (Promega) and stained with Coomassie brilliant blue. The portions of the membrane containing the PO-C1 (42 kD) were excised and the protein was cleaved with cyanogen bromide (10 mg/mL) in 70% formic acid and eluted from the membrane with a solution of 70% isopropanol, 0.2% (v/v) TFA, 0.1 mM Lys, and 0.1 mM thioglycolic acid. The peptides were concentrated by drying under vacuum and separated by SDS-PAGE in a 16.5% separating gel, a 10% spacer gel, and a 4% stacking gel (Promega, 1991). Thioglycolic acid was added to the cathode buffer (0.2 mM). Dried peptides were denatured at 95°C for 5 min in loading buffer. After separation, PO-C1 peptides were transferred to a ProBlott membrane (Promega). The membrane was stained with Coomassie brilliant blue R-250 in a 30% methanol and 5% acetic acid solution. Stained peptide bands (10 total) were excised. Four of the peptides were submitted to R. Cook at Baylor College of Medicine (Houston, TX) for sequence analysis by Edman degradation.

Peptide Synthesis

The amino acid sequence MNLAMNLAPLD, designated as POC1a, was synthesized by a manual solid-phase method as described (Takemoto et al., 1985; Henry et al., 1992). After completion of synthesis, the peptide was removed from the solid phase with anhydrous hydrogen sulfide at the Biotechnology Core Facility of Biochemistry, Kansas State University (Manhattan). Concentration of the cleaved peptide was estimated by A_{214}.

Antibodies

Anti-HRP antibodies (Sigma), which detect several rice peroxidases (Reimers et al., 1992), were used as specified

by the manufacturers. Anti-PLD antibodies were obtained from Xuemin Wang, Kansas State University (Wang et al., 1993). The antibodies were raised to purified PLD from castor bean, but they also react with rice PLD (S.A. Young, J.E. Leach, and X. Wang, unpublished data).

To prepare domain-specific antibodies for POC1a, approximately 2 mg of synthetic peptide was coupled to 3 mg of keyhole limpet hemocyanin with glutaraldehyde (Reichlin, 1980). The peptide-hemocyanin conjugate was emulsified with Freund's complete adjuvant (1:1) and injected subcutaneously into female white New Zealand rabbits. Boosters were administered subcutaneously with 125 μg of peptide-hemocyanin conjugate in Freund's incomplete adjuvant (1:1). Antibodies specific to POC1a peptide were purified either by affinity chromatography using Affi-Gel 10 (Bio-Rad) or by eluting antibodies from nitrocellulose blots according to Smith and Fisher (1984). The antibodies were used at a dilution of up to 1:1000 relative to the original antiserum.

For competitive binding studies, anti-POC1a and anti-PLD antibodies were cleaved with pepsin to isolate $F(ab)_2$ fragments as described by Harlow and Lane (1988). The $F(ab)_2$ fragments were purified from the Fc fragment by protein A column chromatography (Sigma) and the saturation titration point was determined by immunoblots.

Peroxidase Activity and Immunodetection

Extracellular peroxidases were extracted from rice leaves at 0, 12, 24, 48, and 72 h after inoculation as described (Reimers et al., 1992). Guttation fluids were collected from rice leaves at 24, 48, and 72 h after inoculation; samples were obtained 1 h before the start of the photoperiod. PO-C1 activity was measured by using nondenaturing cathodic PAGE (Thomas and Hodes, 1981) and nondenaturing anodic PAGE (Davis, 1964). Electrophoresis was performed at a constant current of 30 mA for 3 h in a minigel. Peroxidase was detected by incubating the gel in 100 mM sodium phosphate containing 18 mM guaiacol, 0.01% 3-amino-9-ethylcarbazole, and 0.03% H_2O_2 for 15 min (Reimers et al., 1992).

For immunodetection, proteins were either transferred electrophoretically to nitrocellulose membranes (Towbin et al., 1979) after separation on native gels or directly blotted onto nitrocellulose by the use of a slot blot. The nitrocellulose blots were probed with either anti-POC1a antibodies or anti-horseradish antibodies. Antigen-antibody complexes were visualized by alkaline phosphatase-conjugated antibodies.

EM and Immunocytochemistry

Tissue samples were prepared for EM as described previously (Young et al., 1994). Anti-POC1a, anti-PLD, and anti-HRP antibodies were used at a dilution of 1:50 for all labeling experiments as described (Lutkenhaus and Bi, 1991). Control reactions were treated with antibodies from preimmune serum at a dilution of 1:50. In competitive binding studies, grids containing sections were treated as described (Lutkenhaus and Bi, 1991), except that the grids were incubated first with either anti-POC1a or anti-PLD $F(ab)_2$ fragments diluted 1:50 in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% BSA (TBS buffer). Grids were then treated with anti-POC1a antibodies diluted 1:50 in TBS buffer, and finally in Protein A labeled with 10-nm colloidal gold particles (Sigma) diluted 1:50 in TBS buffer.

Quantitation of Immunogold Particles

Thin sections of infected leaf tissue were analyzed at a magnification of 30,000×. For each treatment with antibodies, two grids, two sections/grid, two fields/section, and 5 cm^2 or 5 cm per organelle per field were examined for gold particles. Each treatment consisted of two to four replicates. The gold particles in and around the different plant organelles were counted on prints with the aid of a 1-cm^2 grid. Values are reported as mean numbers ± SE. For binding studies, LSD values were calculated to compare between antibody treatments within tissue type and also between tissue types within a given antibody treatment. The experiment was a split-plot design.

RESULTS

Purification and Sequence Analysis of PO-C1

The cationic peroxidase PO-C1 (pI 8.6) was purified from rice leaves 48 h after inoculation with *X. oryzae* pv *oryzae* PXO86. After $(NH_4)_2SO_4$ fractionation, desalination, and concentration by filtration, the proteins were fractionated by a nondenaturing cathodic PAGE. The band corresponding to PO-C1 was excised, and protein in the gel fragment was denatured, separated by SDS-PAGE, and transferred to PVDF membrane. A single protein of 42 kD was detected by Coomassie brilliant blue stain (Fig. 1).

The N terminus of PO-C1 was blocked and could not be sequenced. Three of 10 fragments obtained after chemical cleavage of PO-C1 were subjected to sequence analysis by Edman degradation (Fig. 2). Alignments of the N-terminal sequences from the three PO-C1 fragments revealed simi-

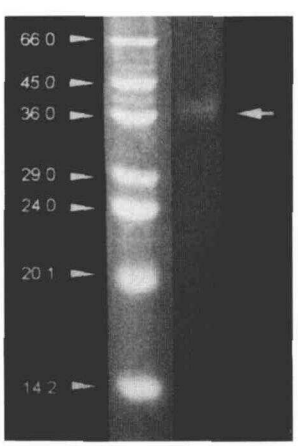

Figure 1. Partially purified PO-C1 from rice leaf extracts (negative image). Left lane, Two micrograms of low-range protein molecular mass markers (sizes are indicated on the left in kD). Right lane, Partially purified PO-C1 was separated by SDS-PAGE, transferred to PVDF membranes, and stained with Coomassie brilliant blue R-250.

Rice (Xoo)	MVALSGAHTIGQAQcSTFRSRtYt
Rice (Pss)	.....QN..D.I.N
Barley	.....A.I.G
Wheat	.....G..KD.I.N
Arabidopsis	L.....F.KN..RFIMD.L.N
Horseradish	L.....G..F.R...QF..P.L.N
Rice (Xoo)	MNLAPLDT?tADaFDrAYyg?qL
Rice (Pss)	S....V..T.PN...N...SNL.
Barley	GS..N...T..NT..N...TNLM
Wheat	GS..N...T..NT..N...TNLM
Arabidopsis	SV.VDF.LR.PLV..NK..VNLK
Horseradish	TV.VNF.VV.P....SQ..TNLR
Rice (Xoo)	MV?MGNIAPKTGTNGq
Rice (Pss)	..K....S.L...Q..
Barley	.IK.....Q..
Wheat	.IK.....Q..
Arabidopsis	.NR....T.T...Q..
Horseradish	.IR...LR.L..RQ.E

Figure 2. Alignment of amino acid sequences from fragments 1, 2, and 3 of PO-C1 to deduced amino acid sequences of other plant peroxidases. Individual peptides, cleaved from PO-C1 by cyanogen bromide, were sequenced and aligned with deduced sequences of peroxidases from rice (Reimmann et al., 1992), barley (Thordal-Christensen et al., 1992), wheat (Dudler et al., 1991), Arabidopsis (Intapruk et al., 1991), and horseradish (Fujiyama et al., 1990). Amino acids sharing identity with the PO-C1 peptides are indicated by dots. Lowercase letters indicate uncertainty and "?" indicates that the amino acid could not be identified. A peptide was synthesized based on the sequence in the shaded box.

larities to other plant peroxidase sequences from rice (Reimmann et al., 1992), barley (Thordal-Christensen et al., 1991), wheat (Dudler et al., 1991), Arabidopsis (Intapruk et al., 1991), and horseradish (Fujiyama et al., 1990). Fragments 1 and 3 from PO-C1 are more similar to the amino acid sequences of other plant peroxidases than fragment 2. The percentages of identical amino acids in fragment 1 to these peroxidases are: rice, 79%; barley, 88%; wheat, 79%; Arabidopsis, 54%; and horseradish, 63%. In fragment 2 the percentages are: rice, 61%; barley, 52%; wheat, 52%; Arabidopsis, 30%; and horseradish, 39%. In fragment 3 the percentages are: rice, 80%; barley, 87%; wheat, 87%; Arabidopsis, 69%; and horseradish, 50% (Fig. 2).

Immunological Detection of PO-C1

To aid in specific detection of PO-C1 in plant tissues or exudates, polyclonal antibodies were generated to a synthetic peptide (POC1a = MNLAMNLAPLD; see Fig. 2). The sequence of POC1a included an area of fragment 2 that was most diverged from other plant peroxidases (MNLAPLD). The sequence of the most diverged area, MNLA, was repeated in the peptide to enhance production of antibodies directed to that domain. Antibodies generated against POC1a specifically recognized PO-C1 (Fig. 3B, lane 2) in western blot analyses of extracellular fluids from rice plants undergoing an incompatible response 24 h after infection with *X. oryzae* pv *oryzae* PXO99^A(pBUavrXa10.F1). The anti-POC1a antibodies did not react with proteins in

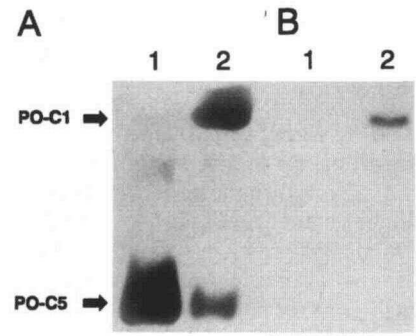

Figure 3. Anti-POC1a antibodies are specific for PO-C1. Peroxidase activity (A) and western blot analysis (B) of PO-C1 from extracellular extracts from leaves of IR-BB10 infiltrated with *X. oryzae* pv *oryzae* PXO99^A (compatible) (lanes 1) and PXO99^A(pBUavrXa10.F1) (incompatible) (lanes 2) 24 h after infiltration. Extracellular fluids were extracted as described by Reimers et al. (1992). Proteins (1.5 μg/well) were separated in a nondenaturing cathodic polyacrylamide gel and transferred electrophoretically to nitrocellulose. Anti-POC1a antibodies that were bound to PO-C1 were detected by reaction with anti-rabbit IgG-linked alkaline phosphatase.

extracellular fluids collected after 24 h from plants undergoing a compatible interaction (inoculated with the isogenic strain PXO99^A; Fig. 3B, lane 1). Extracellular fluids contain at least two cationic peroxidases that migrate faster than PO-C1 in cathodic gels (Fig. 3A, lanes 1 and 2) and at least seven anionic peroxidases (activity gels and blots not shown; Reimers et al., 1992); the anti-POC1a antibodies did not react with these peroxidases, indicating specificity to PO-C1.

In immunoblots, anti-POC1a antibodies detected PO-C1 in extracellular fluids collected from plants undergoing an incompatible response as early as 12 h and the intensity of the reaction increased up to 72 h after infection (Fig. 4). Symptoms of the HR (browning of the infiltration site) were first visible 24 h after infiltration. In the compatible interactions, the anti-POC1a antibodies did not react with extracellular fluids until 48 h after infection. The detection of PO-C1 at 48 h after inoculation in the compatible inter-

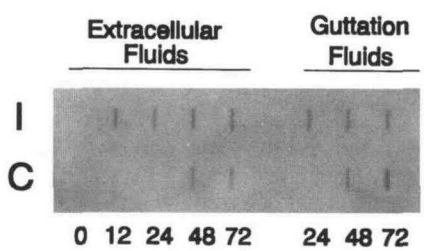

Figure 4. Anti-POC1a antibodies react with PO-C1 in extracellular and guttation fluids. Immunodetection of PO-C1 in extracellular and guttation fluids from leaves of IR-BB10 infiltrated with *X. oryzae* pv *oryzae* PXO99^A(pBUavrXa10.F1) (incompatible; I, top row) and PXO99^A (compatible; C, bottom row). Numbers at the bottom of the slots indicate the time (h) after infiltration that the proteins were extracted. Fluids (1.5 μg protein/slot) were applied to nitrocellulose, probed with anti-POC1a antibodies, and visualized using alkaline phosphatase-conjugated secondary antibodies.

actions is consistent with previous results in which activity of PO-C1 was not observed until 48 h after inoculation (Reimers et al., 1992). Guttation fluids collected from the hydathodes of infiltrated rice leaves showed a similar pattern of detection of PO-C1. In the incompatible interactions, anti-POC1a antibodies reacted to guttation fluids collected 24, 48, and 72 h after infection, but did not react with fluids from tissues undergoing compatible responses until 48 h after infection (Fig. 4).

Subcellular Localization of PO-C1

The antibodies against POC1a were used to locate PO-C1 3, 12, and 24 h after infection of rice by *X. oryzae* pv *oryzae*. Transmission electron micrographs at 24 h post-infection showed both bacterial accumulation within a xylem vessel (Fig. 5A) and the accumulation of PO-C1 (Fig. 5, B–E). More PO-C1 was detected 24 h after infection in incompatible interactions (Fig. 5, C and E) than in compatible host/pathogen interactions (Fig. 5, B and D).

The immunogold labeling of PO-C1 was quantified after EM of samples obtained 3, 12, and 24 h post-infection (Fig. 6). Labeling in the compatible interactions at 3, 12, and 24 h was relatively low in the chloroplast, cytoplasm, extracellular spaces, and membranes of mesophyll cells. More label was observed in the cell walls of mesophyll cells than in the other organelles during the compatible interaction (approximately 3.5 gold particles/cm). However, the number of particles did not vary significantly from 3 to 24 h after infection. An increase in the number of particles was observed in the cytoplasm of mesophyll cells within 12 h after infection in the incompatible interactions (Fig. 6B), but the number of particles in the chloroplasts and membranes did not increase. In the incompatible interactions, labeled particles were more abundant 24 h after infection in the apoplast than after 3 and 12 h (Fig. 6, A and B).

The number of particles in the xylem cell walls (primary and secondary walls) observed was the same (an average of 3.2 gold particles/cm^2) at all sampling times (3, 12, and 24 h after infection) during compatible interactions (Figs. 5B and 6C). However, a dramatic increase in the number of particles in xylem walls was observed in the incompatible interactions 24 h after infection (12 $\pm$ 1.1 gold particles/cm^2). Little to no label was detected in the vessel lumen during compatible interactions. However, after 24 h in the incompatible interaction, a large number of particles were observed in the vessel lumen. Particles were observed in the cytoplasm of parenchyma cells adjacent to the xylem in tissues undergoing incompatible responses (data not shown).

When tissues were first treated with $F(ab)_2$ fragments from anti-POC1a antibodies, binding of anti-POC1a antibodies to PO-C1 was blocked and little label was observed in tissues during both incompatible and compatible interactions 24 h after infection (Table I; Fig. 5, F and G). Anti-PLD $F(ab)_2$ fragments did not block the antigenic sites of PO-C1 and binding of anti-POC1a antibody to PO-C1 was detected (Table I). These experiments indicate that the binding of anti-POC1a antibodies observed in the tissue sections is specific to PO-C1 (Fig. 5, F and G).

Anti-HRP antibodies were shown to cross-react to many rice peroxidases, including PO-C1 (Reimers et al., 1992). When anti-HRP antibodies were used to label incompatible rice leaf tissue, particles were detected throughout the leaf tissue, including the cytoplasm, chloroplast, and extracellular spaces (Fig. 7B). Three-fold more gold particles were bound after detection with anti-HRP antibodies than with antibodies specific to PO-C1. Even peroxidases located within the bacteria were detected. Particles were not observed in resin surrounding the leaf tissue. Antibodies to a cytoplasmic enzyme PLD were associated primarily with the membrane and cytoplasm; little labeling occurred within the chloroplast and no labeling was detected in the apoplast or xylem lumen (Fig. 7A). Thus, gold labeling by anti-POC1a antibody was dependent on the presence of PO-C1 and was not affected by the general inability of labeled antibody to react with antigens in various cellular organelles.

DISCUSSION

In this study, we present evidence that the cationic peroxidase, PO-C1, accumulates in the apoplast of mesophyll tissue and xylem vessels of rice during resistant interactions with *X. oryzae* pv *oryzae*. Although peroxidases have been found to be associated with secondary thickenings and the middle lamella of xylem tissues from differentiating and wounded bean hypocotyl (Smith et al., 1994) and xylem sap and guttation fluids from a number of healthy plants (Biles and Abeles, 1991; Magwa et al., 1993), high accumulation of PO-C1 was dependent on infection. PO-C1 accumulated faster and to higher levels in resistant than in susceptible host/pathogen interactions. Cationic peroxidases other than PO-C1 were detected in water-treated rice plants, but the levels of these did not change.

Purification of the isoenzyme allowed us to obtain and compare amino acid sequences of three internal PO-C1 fragments with other plant peroxidases. PO-C1 sequences were highly similar to all plant peroxidases examined. Sequence identity between PO-C1 and monocot peroxidases was greater than with dicot peroxidases. The amino acid sequence of PO-C1 differs from that of the pathogen-induced peroxidase recently identified from a rice cDNA library using a wheat peroxidase clone as a probe (Fig. 2; Reimmann et al., 1992), suggesting that these two pathogen-induced peroxidases are different isoenzymes.

The domain-specific immunological probe, which recognizes the cationic peroxidase PO-C1, is specific for PO-C1. The antibodies did not react with other peroxidases, including the induced anionic peroxidases (PO-A1 and PO-A2; Reimers et al., 1992), in western blot analyses. Furthermore, anti-POC1a $F(ab)_2$ fragments inhibited the binding of anti-POC1a antibodies, but not anti-PLD antibodies.

Anti-POC1a antibodies were used to determine the location of PO-C1 during compatible and incompatible interactions in tissues after infection with *X. oryzae* pv *oryzae*. Two lines of evidence establish that PO-C1 is located in the xylem vessels. First, PO-C1 accumulates in incompatible interactions in guttation fluids within 24 h after infection. PO-C1 was not detected in guttation fluids from plants

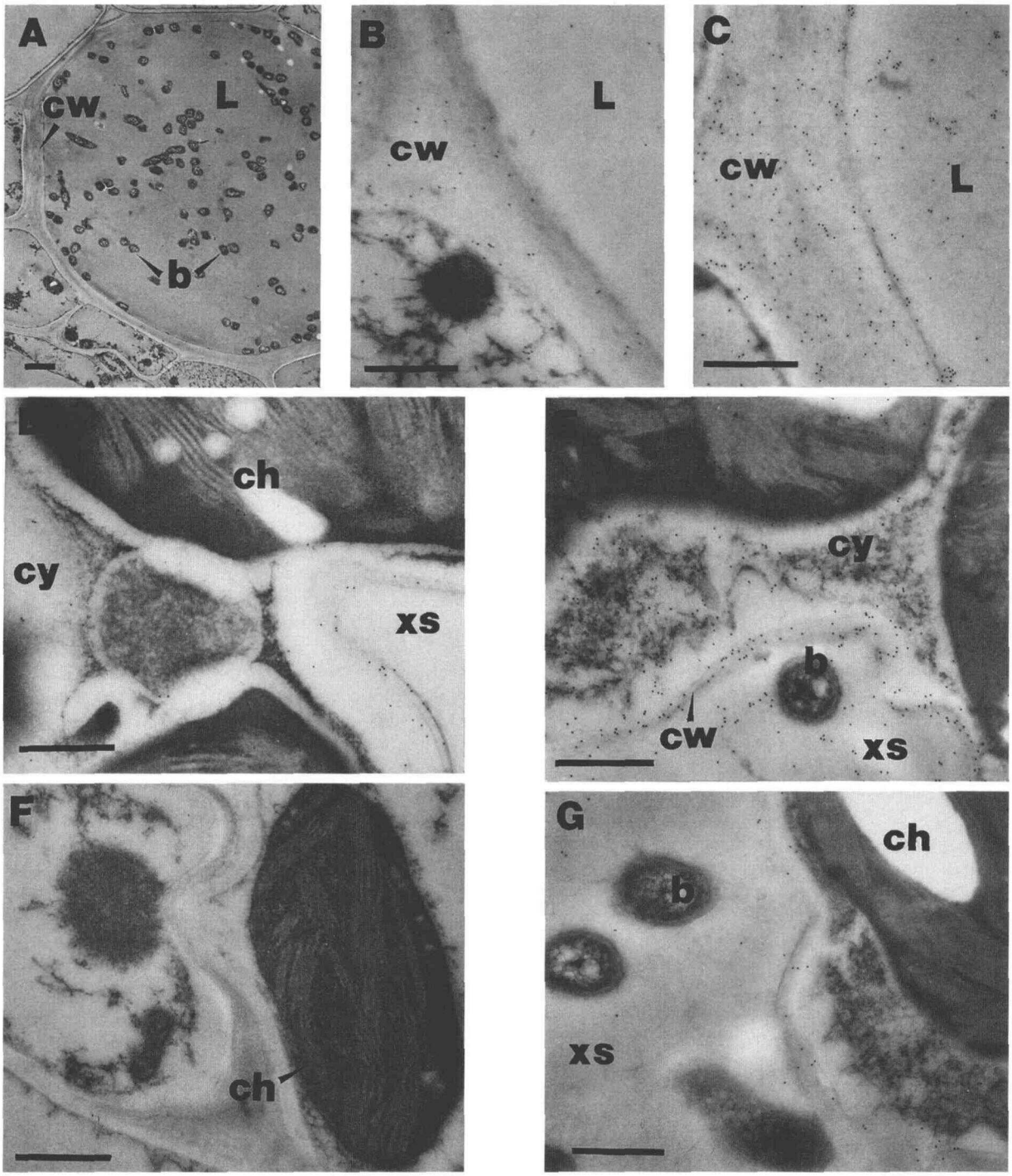

Figure 5. Transmission electron micrographs of PO-C1 immunolocalization during compatible and incompatible interactions 24 h after infiltration with *X. oryzae* pv *oryzae* PXO99^{A}(pBUavrXa10.F1). A, *X. oryzae* pv *oryzae* cells within xylem vessel in IR-BB10; B, cell wall of a xylem vessel in IR24 during a compatible interaction; C, cell wall of a xylem vessel in IR-BB10 during an incompatible interaction; D, mesophyll cell in IR24 during a compatible interaction; E, mesophyll cell and bacteria in IR-BB10 during an incompatible interaction; F, mesophyll cell in IR24 during a compatible interaction; G, mesophyll cell and bacteria in IR-BB10 during an incompatible interaction. B to E, Rice tissue was probed with anti-POC1a antibodies. F and G, Rice tissues were first treated with anti-POC1a $F(ab)_2$ antibody fragments, then treated with anti-POC1a antibodies. b, Bacteria; cw, cell wall; cy, cytoplasm; ch, chloroplast; xs, extracellular space; v, xylem vessel. Bars = 0.5 μm.

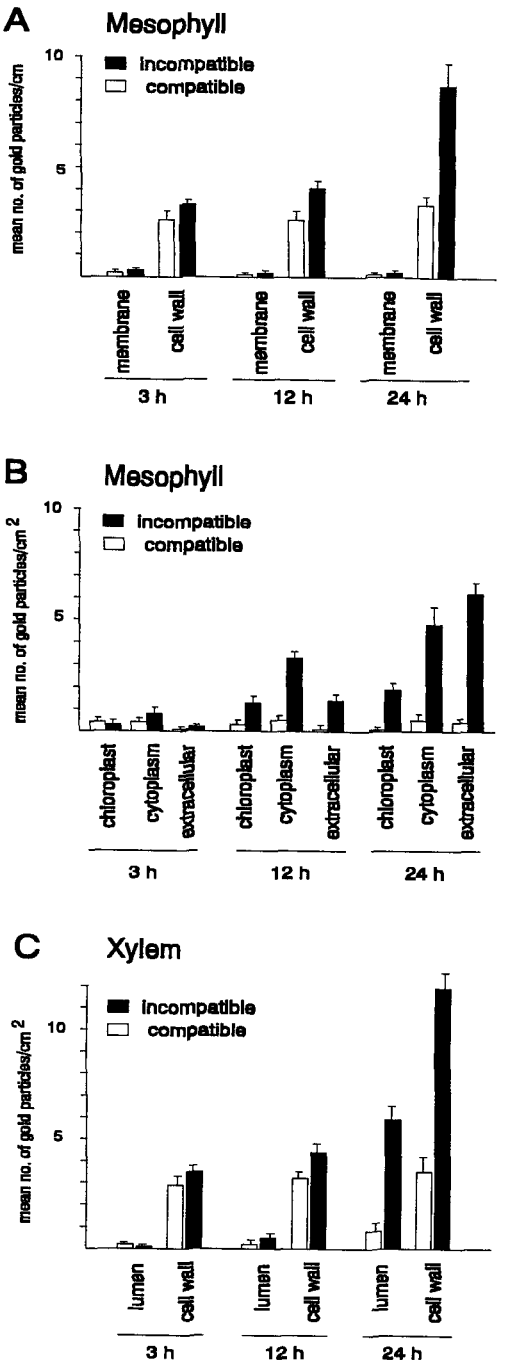

Figure 6. Quantitative analysis of immunogold particle distribution in compatible and incompatible rice tissues at 3, 12, and 24 h after inoculation with *X. oryzae* pv *oryzae* PXO99^{A}(pBUavrXa10.F1). IR24 (compatible), open bars; IR-BB10 (incompatible), solid bars. Mean number (± SE) for location indicated under bars are membrane and cell wall of mesophyll cells (A), chloroplast, cytoplasm, and extracellular space of mesophyll cells (B), and vessel and cell wall of xylem tissue (C). Sections were treated with anti-POC1a antibodies followed by gold-conjugated secondary antibodies.

undergoing compatible responses until 48 h. Second, antibodies specific for PO-C1 immuno-decorated the cell walls and lumen of xylem vessels within 24 h after infection during an incompatible response. More PO-C1 was detected in the cell walls of the xylem than in the lumen. PO-C1 also was detected in the cytoplasm of parenchyma cells adjacent to the xylem vessels. These data suggest that the isoenzyme is expressed in neighboring cells and can be distributed into the enucleated xylem vessels. PO-C1 also was found in the apoplast of mesophyll cells. Again, more PO-C1 was detected in the cell walls than in the extracellular spaces.

The accumulation of PO-C1 during the incompatible response, which occurred within the xylem vessels and the apoplast of mesophyll cells in tissues where the bacteria were found, suggests that this isoenzyme may be involved in the defense response of rice. Secretion of pathogenesis-induced peroxidases into intercellular spaces is thought to be involved in controlling the invasion of different pathogens (Nicholson and Hammerschmidt, 1992). However, the role of PO-C1 in the defense response is not clear. PO-C1 might be a lignin peroxidase. The timing of the accumulation of PO-C1 activity correlates with lignin deposition, and the isoenzyme has been shown to utilize the lignin precursor coniferyl alcohol as a substrate in vitro (Reimers and Leach, 1991). PO-C1 has not been shown to be involved in lignin synthesis in vivo. Lignin and other phenolic polymers can serve as physical barriers and, therefore, are thought to retard fungal penetration of host cells (Ride, 1983; Tiburzy and Reisener, 1990). However, bacterial plant pathogens such as X. *oryzae* pv *oryzae* do not penetrate host cells. Consequently, cell wall lignification probably would

Table I. *Specificity of anti-POC1a binding to PO-C1 at 24 h in incompatible interactions*

Tissue	Number of Gold Particles (mean/cm^2 or cm ± SE)[a] after First Treating with[b]		
	Anti-POC1a antibody	Anti-POC1a F(ab)$_2$	Anti-PLD F(ab)$_2$
Mesophyll			
Extracellular	6.3 ± 0.6	0.1 ± 0.1	6.6 ± 0.7
Wall*	8.8 ± 0.9	0.1 ± 0.15	8.5 ± 0.4
Membrane*	0.4 ± 0.1	0.4 ± 0.2	0.3 ± 0.1
Cytoplasm	4.9 ± 0.8	0.2 ± 0.15	5.2 ± 1.1
Chloroplast	2.0 ± 0.2	0.3 ± 0.15	1.8 ± 0.4
Xylem			
Lumen	5.9 ± 0.5	0.5 ± 0.2	5.7 ± 0.4
Wall	11.9 ± 0.6	1.1 ± 0.6	12.1 ± 0.7
$LSD_{0.05}$ = 1.6[c]			
$LSD_{0.05}$ = 1.3[d]			

[a] Mean number of gold particles on the wall and membrane of mesophyll cells was counted per cm. Particles on other tissue types, including the xylem walls, were counted per cm^2. [b] Tissues were treated first with anti-POC1a antibody alone, or with either anti-POC1a or anti-PLD F(ab)$_2$ antibodies. The tissues were then treated with anti-POC1a antibodies. Bound antibodies were detected by treatment with Protein A labeled with 10-nm colloidal gold particles. [c] Least significant difference for comparing antibody treatments within tissue type. [d] Least significant difference for comparing tissue types within a treatment.

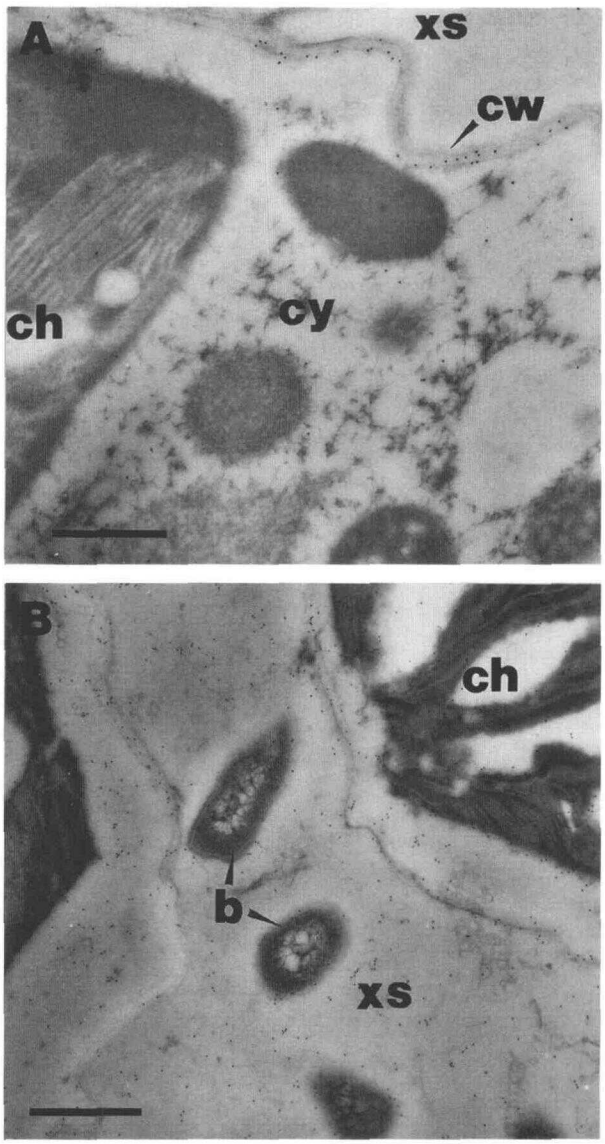

Figure 7. Transmission electron micrographs showing the location of PLD and rice peroxidases including PO-C1 during an incompatible interaction 24 h after inoculation with *X. oryzae* pv *oryzae* PXO99^{A}(pBUavrXa10.F1) (an incompatible response). A, Mesophyll cell in IR-BB10 during an incompatible interaction probed with anti-PLD antibodies. B, Mesophyll cells and bacteria during an incompatible interaction probed with anti-HRP antibodies. b, Bacteria; cw, cell wall; cy, cytoplasm; ch, chloroplast; xs, extracellular space. Bars = 0.5 μm.

not be an effective defense against these organisms unless lignified materials prevented bacterial spread by blocking movement between the epithem and xylem vessels. The lignin biosynthetic process itself could also be an important component of the defense response against bacteria, because bacteria may be inhibited by toxic phenolic compounds (Venere, 1980; Horino and Kaku, 1989), phenolic free radicals, or activated oxygen (Elstner, 1982), all of which are associated with lignification. Thus, the role of PO-C1 in resistance might be a consequence of its function in lignin biosynthesis. Further studies on the molecular characterization of the PO-C1 gene may provide valuable insights into any possible direct role of this cationic peroxidase in the defense response.

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