Vascular Defense Responses in Rice: Peroxidase Accumulation in Xylem Parenchyma Cells and Xylem Wall Thickening

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The rice bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* is a vascular pathogen that elicits a defensive response through interaction with metabolically active rice cells. In leaves of 12-day-old rice seedlings, the exposed pit membrane separating the xylem lumen from the associated parenchyma cells allows contact with bacterial cells. During resistant responses, the xylem secondary walls thicken within 48 h and the pit diameter decreases, effectively reducing the area of pit membrane exposed for access by bacteria. In susceptible interactions and mock-inoculated controls, the xylem walls do not thicken within 48 h. Xylem secondary wall thickening is developmental and, in untreated 65-day-old rice plants, the size of the pit also is reduced. Activity and accumulation of a secreted cationic peroxidase, PO-C1, were previously shown to increase in xylem vessel walls and lumen. Peptide-specific antibodies and immunogold-labeling were used to demonstrate that PO-C1 is produced in the xylem parenchyma and secreted to the xylem lumen and walls. The timing of the accumulation is consistent with vessel secondary wall thickening. The *PO-C1* gene is distinct but shares a high level of similarity with previously cloned pathogen-induced peroxidases in rice. *PO-C1* gene expression was induced as early as 12 h during resistant interactions and peaked between 18 and 24 h after inoculation. Expression during susceptible interactions was lower than that observed in resistant interactions and was undetectable after infiltration with water, after mechanical wounding, or in mature leaves. These data are consistent with a role for vessel secondary wall thickening and peroxidase PO-C1 accumulation in the defense response in rice to *X. oryzae* pv. *oryzae*.

Additional keywords: lignification, pit aperture.

Considerable progress has been made in understanding events involved in the activation of plant defense responses by bacterial pathogens (Galan and Collmer 1999). Based on the best characterized of these interactions, contact between the bacterium and a metabolically active leaf mesophyll cell is predicted to be required for transfer of avirulence signals from the pathogen to the host cell and the induction of resistance. The xylem vessels of plants offer a particularly hospitable environment for plant pathogens. In fact, several important bacterial pathogens, including the rice bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae*, remain within xylem vessels throughout the disease interaction (Goodman et al. 1986; Tabei 1967). During its existence in the xylem vessel, the pathogen's contact with living cells, i.e., the xylem parenchyma, occurs at the pit membranes. The pit membranes, consisting of the primary wall of the parenchyma cell, the pectinaceous middle lamella, and the primary cell wall of the tracheary cell, are more complex than the walls of mesophyll cells.

Xylem parenchyma cells adjacent to fungal-infected xylem vessels have been reported to undergo drastic metabolic changes, including the production of callose, chitinase, and ligninlike compounds (Beckman et al. 1991; Benhamou 1995; Mueller et al. 1994; Shi et al. 1992). Only limited information, however, is available on the changes occurring in the xylem and xylem parenchyma during interactions with bacterial pathogens (Goodman et al. 1986; Horino 1976, 1981; Horino and Kaku 1989; Sequeira et al. 1977).

Ultrastructural analyses of interactions between rice (*Oryza sativa*) and *X. oryzae* pv. *oryzae* show bacterial multiplication in the vessels in both compatible and incompatible interactions (Horino 1976, 1981; Horino and Kaku 1989). In the incompatible interactions, *X. oryzae* pv. *oryzae* cells within rice vessels were irregular in shape and were enveloped by abundant fibrillar material after 3 days. In contrast, during the compatible interactions, bacterial cells appeared normal and were not surrounded by the fibrillar material until after 20 days (Horino 1976, 1981). Using an inoculation technique that distributed bacteria predominantly into the leaf mesophyll, Reimers and coworkers (Guo et al. 1993; Reimers and Leach 1991; Reimers et al. 1992) demonstrated that incompatible interactions were characterized by the accumulation of lignin polymers in the cell walls and the increased activity of three

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isozymes of peroxidase (two anionic and one cationic) in the apoplast of leaf parenchyma. The pattern of accumulation and cellular location of PO-C1, a cationic pathogen-induced peroxidase, was determined using domain-specific antibodies (Young et al. 1995). PO-C1 was detected in the cell walls and extracellular spaces in leaf mesophyll tissues and in the vessel lumen and walls of xylem elements within 12 h after inoculation with avirulent strains of *X. oryzae* pv. *oryzae* (Young et al. 1995). Based on the temporal and spatial patterns of activity and accumulation and on the substrate utilization pattern of the isoenzyme, PO-C1 was predicted to contribute to the rice defense response (Reimers et al. 1992; Young et al. 1995).

Induction of peroxidase genes, enzyme activity, or both, has been linked with disease resistance in many plant species

(Chittoor et al. 1999; Kristensen et al. 1999). However, precisely how the peroxidases participate in the defense response is not clear, but a variety of roles have been proposed (Bradley et al. 1992; Moerschbacher 1992; Mohan and Kolattukudy 1990; Ride 1983). One possible role for peroxidases is the formation of structural barriers such as cell wall enhancement and deposition of cell wall appositions, both of which can involve the polymerization of lignin or suberin (Hammerschmidt and Kuc 1982; Espelie et al. 1986), the cross-linking of wall glycoproteins or polysaccharides (Bradley et al. 1992; Fry 1986; Iiyama et al. 1994), and the dimerization of antimicrobial phenols (Ikegawa et al. 1996; Stoessl 1967). Another possibility is that the free radical intermediates produced by peroxidase-oxidative activity are toxic to the pathogens (Martinez et al. 2000; Sutherland 1991).

Fig. 1. A, Transmission electron microscopy (TEM) images and, (B to F) scanning electron microscopy (SEM) images of rice leaf tissue infiltrated with avirulent and virulent strains of *Xanthomonas oryzae* pv. *oryzae*. A, *X. oryzae* pv. *oryzae* strain PXO99^A(pBUavrXa10.F1) cells within a xylem vessel in 'IRBB10' (incompatible interaction); inset, a bacterial cell with associated fibrillar material. B, Xylem vessel and associated parenchyma with connecting pits in an uninfected 'IRBB10' 12-day-old rice leaf. C, *X. oryzae* pv. *oryzae* strain PXO99^A(pBUavrXa10.F1) cells in the apertures of pits of a xylem vessel of 'IRBB10' (incompatible interaction). D and E, PO-C1 location (arrows) during incompatible ('IRBB10') interactions 24 h after infiltration with *X. oryzae* pv. *oryzae* strain PXO99^A(pBUavrXa10.F1). F, PO-C1 location (arrows) during a compatible interaction 24 h after infiltration with *X. oryzae* pv. *oryzae* strain PXO99^A(pHM1). Bar = 3 μ m; b, bacteria; f, fibrillar material; p, pit aperture; pw, primary wall; and sw, secondary wall.

In this study, we focused on two resistance-associated events during the interactions of rice with *X. oryzae* pv. *oryzae*: the ultrastructural changes in xylem secondary walls and xylem parenchyma cells, and the correlated changes in *PO-C1* gene induction and isoenzyme location. We show that xylem secondary walls thicken during incompatible interactions and that this thickening effectively reduces the exposure of the pit membrane to the bacterial pathogen. These changes correlate with the accumulation of cationic peroxidase PO-C1 in xylem parenchyma and in the vessel walls and lumen. The *PO-C1* gene was cloned and characterized and shown to be transiently induced between 12 and 48 h postinoculation during the incompatible interactions.

RESULTS

Ultrastructural changes in xylem and xylem parenchyma during incompatible interactions.

Transmission electron microscopy (TEM) images revealed that xylem vessels at the inoculation site were filled with bacteria in both incompatible (Fig. 1A) and compatible interactions (data not shown) by 24 h postinoculation. In incompatible, but not compatible, interactions, fibrillar material was observed around the bacteria within the vessel at 24 h (Fig. 1A, inset), and the presence of organellar remains in the cytoplasm (data not shown) suggests xylem parenchyma cells were degraded. In contrast, during the compatible interactions and in the water-infiltrated control, vascular parenchyma cells displayed a well-preserved ultrastructure with intact organelles (data not shown). Bacteria were never observed within the xylem parenchyma in any treatment at any time.

Scanning electron microscopy (SEM) of 12-day-old rice seedlings was used to study the consequences of interactions occurring within the xylem vessels. In 12-day-old rice seedlings, pits separating the xylem parenchyma and the xylem element are the prominent feature within the vessels (Fig. 1B). In both incompatible and compatible interactions (Fig. 1C to F), bacteria were frequently associated with the pit membranes.

Measurements of the diameter of the pit and the width of the secondary xylem walls from the SEM study were used to quantify xylem secondary wall thickening in rice seedlings during interactions with *X. oryzae* pv. *oryzae* and in mock-inoculated controls (Table 1). At 3 h postinoculation, no differences were observed in the diameter of pits after treatment with either the virulent or avirulent *X. oryzae* pv. *oryzae* strains or the mock-inoculated rice control. However, by 48 h

postinoculation, the secondary walls of the xylem vessels in incompatible interactions were thickened relative to the 3 h time point, the compatible interaction, and the mock-inocu-

Fig. 2. A, Longitudinal section of a xylem vessel from a noninoculated 65-day-old 'IRBB10' rice leaf viewed by scanning electron microscopy. B, Higher magnification showing the aperture of the pits (average diameter of short axis = $2.1 \mu\text{m} \pm 0.5$) and the thickened secondary walls (average distance between aperture short axis = $7.4 \mu\text{m} \pm 0.5$). Bar = $3 \mu\text{m}$; p, pit; and sw, secondary wall.

Table 1. Mean size ($\mu\text{m} \pm$ standard error) of rice xylem vessel secondary wall and pit aperture changes during incompatible, but not compatible, interactions with *Xanthomonas oryzae* pv. *oryzae*^y

Rice cultivar	<i>X. oryzae</i> pv. <i>oryzae</i> strain or treatment	Interaction	Mean size of changes			
			Secondary wall		Pit aperture	
			3 h	48 h	3 h	48 h
IRBB10	Mock inoculation	...	$4.7 \pm 0.1 \text{ a}^z$	$4.8 \pm 0.1 \text{ a}$	$4.6 \pm 0.1 \text{ a}$	$4.7 \pm 0.2 \text{ a}$
	PXO99 ^A (pHMI)	Compatible	$4.8 \pm 0.1 \text{ a}$	$4.8 \pm 0.1 \text{ a}$	$4.7 \pm 0.1 \text{ a}$	$4.7 \pm 0.2 \text{ a}$
	PXO99 ^A (pBUavrXa10.F1)	Incompatible	$4.8 \pm 0.2 \text{ a}$	$5.8 \pm 0.1 \text{ b}$	$4.7 \pm 0.1 \text{ a}$	$3.8 \pm 0.1 \text{ b}$
IR24	Mock inoculation	...	$5.2 \pm 0.2 \text{ a}$	$5.4 \pm 0.2 \text{ a}$	$5.6 \pm 0.4 \text{ a}$	$5.4 \pm 0.3 \text{ a}$
	PXO99 ^A (pBUavrXa10.F1)	Compatible	$5.3 \pm 0.1 \text{ a}$	$5.3 \pm 0.3 \text{ a}$	$5.7 \pm 0.4 \text{ a}$	$5.7 \pm 0.3 \text{ a}$

^y Secondary wall thickness and pit aperture size were measured on the short axis of the ellipse in scanning electron microscopy photos as described in text. Experiments with 'IRBB10' and 'IR24' were performed at two different times. Each experiment was repeated, with similar results.

^z Mean thickness or size within a cell structure with the same letter are not different in that particular cultivar according to least squares means at $P = 0.01$.

lated control (Table 1). As a consequence of secondary wall thickening, the diameter of the pit was reduced in incompatible interactions relative to the other treatments.

To determine if cell wall thickening could be induced by AvrXa10 in the absence of the *Xa10* gene product in rice, cultivar IR24, which does not contain *Xa10*, was inoculated with *X. oryzae* pv. *oryzae* containing *avrXa10*. No wall thickening was observed in the treated versus control tissues, suggesting that AvrXa10 does not cause thickening in the absence of recognition by the *Xa10* product (Table 1).

Since mature rice plants are more resistant to bacterial blight than young seedlings are, we wondered if pits of mature (65-day-old) plants could accommodate bacterial cells. We observed that secondary xylem wall thickening and the reduction in diameter of the pit are also consequences of development in rice (Fig. 2A and B). The pit diameters in mature rice vessels were even smaller than those observed in incompatible interactions, averaging $2.1 \mu\text{m} \pm 0.5$ ($\pm$ standard error [SE]).

Fig. 3. Immunolocalization of PO-C1 in rice xylem parenchyma cells infiltrated with *Xanthomonas oryzae* pv. *oryzae* observed by transmission electron microscopy. Images show PO-C1 location during compatible ('IR24') and incompatible ('IRBB10') interactions at 24 h after infiltration with PXO99^A(pBUavrXa10.F1). **A**, Xylem parenchyma cell in 'IRBB10' during an incompatible interaction. **B**, Xylem parenchyma cell in 'IR24' during a compatible interaction. **A and B**, Rice tissue was probed with peptide-specific anti-PO-C1 antibodies and detected with gold-labeled Protein-A. The arrows indicate PO-C1-associated gold particles. Bar = $2 \mu\text{m}$; l, lumen; and xpc, xylem parenchyma cell.

The secondary wall thickness in mature vessels was $7.4 \mu\text{m} + 0.6$ ($\pm$ SE).

Localization of PO-C1 in xylem and xylem parenchyma cells.

Domain-specific antibodies were previously generated to a synthetic peptide (MNLAMNLAFLD) that included a region (MNLAPLD) of the cationic peroxidase PO-C1 that diverged from other plant peroxidases (Young et al. 1995). Immunogold-labeling analysis using affinity-purified anti-PO-C1 antibody showed many PO-C1-associated gold particles were present on the pit membranes and secondary walls of the xylem in incompatible interactions (Figs. 1D and E and 3A) at 24 h postinoculation, whereas few particles were observed in xylem during compatible interactions (Figs. 1F and 3B).

The anti-PO-C1 antibodies were used to determine if PO-C1 accumulated in xylem parenchyma (Fig. 3A and B) and to quantitate the subcellular localization of PO-C1 at 3, 12, and 24 h after infection in rice undergoing incompatible or compatible interactions with *X. oryzae* pv. *oryzae* (Fig. 4). More gold particles associated with PO-C1 were detected 24 h after infection during incompatible interactions (Figs. 1D and E and 3A and 4) than during compatible interactions (Figs. 1F, 3B, and 4). The gold particles associated with PO-C1 were found in the xylem walls and lumen (Young et al. 1995) (Figs. 3A and 4) and, in addition, in the walls of the xylem parenchyma (Figs. 3A and 4). These gold particles were distributed along both secondary and primary walls of the xylem parenchyma and, of relevance to this study, were clearly observed in the thinned primary walls of the pit membranes.

Quantitation of the gold particles over time showed a gradual increase within the cytoplasm and the primary and secondary walls of xylem parenchyma cells during the incompatible interactions (Fig. 4). At 3 h, very few gold particles were associated with the cell walls in either kind of interaction. No differences in the number of gold particles were detected in the plasma membrane and organelles (data not shown). In contrast, no accumulation of gold particles was observed in the xylem parenchyma cells or walls during compatible interactions at any of the three time points (Fig. 4). In this treatment,

Fig. 4. Location of PO-C1 in xylem parenchyma and xylem vessels of rice tissues during incompatible ('IRBB10', dark bars) and compatible ('IR24', white bars) interactions at 3, 12, and 24 h postinoculation with *Xanthomonas oryzae* pv. *oryzae* PXO99^A(pBUavrXa10.F1). The mean number of PO-C1-associated particles per square centimeter are reported for each cellular location. Cellular locations indicated are cy, cytoplasm; w1, primary cell wall; and w2, secondary cell wall.

little labeling was detected in the cytoplasm, vacuoles, plasma membrane, or walls of xylem parenchyma cells.

PO-C1 gene and amino acid sequence analysis.

The genomic copy of *PO-C1* was cloned by inverse polymerase chain reaction, and the *PO-C1* cDNA was identified from a cDNA library constructed from rice undergoing a resistant interaction with *X. oryzae* pv. *oryzae* at 12 h postinoculation. Both were sequenced and used to deduce the *PO-C1* gene and protein primary structure. The 1.3-kb *PO-C1* gene (GenBank accession no. AF247700) contains three introns and a 297-bp 3' untranslated region (UTR). The putative PO-C1 protein contains 313 amino acids and has an estimated molecular weight of 32.6 kDa before glycosylation (Peptidesort; Genetic Computer Group, Madison, WI, U.S.A.). A predicted-signal peptide cleavage site is located between amino acids 23 and 24 (SignalP; Center for Biological Sequence Analysis, Lyngby, Denmark) (Fig. 5). The PO-C1 amino acid sequence is distinct but is highly similar to two previously cloned (Chittoor et al. 1997) rice peroxidases (70% similar to POX8.1 and 75% similar to POX22.3) (Fig. 5).

The PO-C1 sequence contains MNAPLD, the peptide that was previously chosen, based on partial protein sequencing, for the production of the PO-C1-specific antibody (Young et al. 1995). This peptide sequence was not found in POX8.1, POX22.3 (Fig. 5), or any other reported rice or plant peroxidase.

PO-C1	1	MAKAT----	CISLLAVVALATAAQAQLSATFYDTSCPRAMSIKSAVTAA
Pox22.3	1	MASATNSSLSMLLVAAAMASVASAQLSATFYDTSCPNALSTIKSVITAA	
Pox8.1	1	MAS-----	SLSVAVLLCLAAAAAQLSPFFYDTSCPRALATTKSAVTAA
			
			1
PO-C1	47	VNSEPRMGATLLRLHFRDCFVQGCDAVLLSG----	NEQDAPPNKDSLRL
Pox22.3	51	VNSEARMGASLLRLHFDHCFVQGCDAVLLSG----	QEONAGPNVGSRL
Pox8.1	45	VNSEPRMGASLLRLHFDHCFVQGCDAVLLADTATFTGEQNALPNKNSLR	
			
			2
PO-C1	92	GYGVDSIKAQIEAVCNQTVSCADILTVAAARDS--	VLGGPTWTVPLGRRD
Pox22.3	96	GFSVIDNAKARVEAICNQTVSCADILAVAARDSVVALGGPSWTVLLGRRD	
Pox8.1	95	GFNVVDSIKTQLEGICSQTVSCADILAVAARDSVVALGGPSWTVGLGRRD	
		..	
			3
PO-C1	140	STGSAALAIISDLPPPTASLQELVDAFAKGLSVTDMVALSGISGAHTIG	
Pox22.3	146	STTASEALANTDLPAPSSSLAELIGNFSRKLDTMDVALS--	GAHTIG
Pox8.1	145	STTASMSDANNDLPPFPDLENLIKAFGDKGFSVTDMVALS--	GAHTIG
			
PO-C1	190	QAQST-FRGRIYNETNIDSAFATQRQANCPTSG--	<u>D</u> <u>M</u> <u>N</u> <u>L</u> <u>A</u> <u>P</u> <u>L</u> <u>D</u> <u>T</u> <u>T</u> <u>T</u> <u>N</u> <u>A</u>
Pox22.3	193	QAQCQNFRDRIYNETNIDSAFATQRQANCPTSGSDSNLAALDITPTTNA	
Pox8.1	192	QAQCTNFRGRIYNETNIDAGYAASLRANCPPTAGTGDNLAAALDITPTTYS	
			
PO-C1	237	FDNAYYTNLLSNKGLLHSDQVLFNNGSTDNTVRNFASNAAAFSSAFATAM	
Pox22.3	243	FDNAYYSNLLSNKGLLHSDQVLFNNGSADNTVRNFASNAAAFSSAFATTAM	
Pox8.1	242	FDYAYYSNLLSNKGLLHSDQVLFNNGSTDNTVRNFASNRAAFSSAFSSAM	
			
PO-C1	287	VNMGNTPAKTGITNGQIRLSCSKVNS	
Pox22.3	293	VKMGNISPLTGTGQIRLSCSKVNS	
Pox8.1	292	VKMANLGPLIGSQGQIRLSCSKVN-	
			

Fig. 5. Comparison of the deduced PO-C1 amino acid sequence with those of two pathogen-inducible rice peroxidases (POX8.1 and POX22.3). Bold and underline indicates the amino acids used to generate peptide-specific antibodies (MNAPLD). The arrow shows a predicted-signal peptide cleavage site between amino acids 23 and 24. Numerals 1, 2, and 3 refer to the location of introns, “.” indicates all three amino acids are the same, and “.” indicates two of the three peroxidases contain the same amino acid.

DNA blot analysis of rice DNA, digested with different restriction enzymes and probed with either the first intron region or the 3' UTR of *PO-C1*, was used to estimate the copy number of *PO-C1* in the rice genome. The *PO-C1* intron-specific probe hybridized to a single fragment after digestion with *Hind*III and *Xmn*I, *Hind*III and *Nhe*I, *Hind*III and *Bam*HI, *Bam*HI and *Xmn*I, and *Bam*HI and *Nhe*I (Fig. 6). Similarly, the 3' UTR of *PO-C1* hybridized to only a single fragment in rice DNA digested with *Eco*RI, *Eco*RV, *Hind*III, *Xba*I, and *Dra*I. These results suggest that the *PO-C1* gene exists as a single copy in the rice genome.

PO-C1 gene induction.

RNA blot analyses, using the 3' UTR of *PO-C1* as a probe, demonstrated that induction of *PO-C1* occurred as early as 12 h postinoculation during the incompatible interactions, with maximum expression at 18 h postinoculation (Fig. 7). Little to no *PO-C1* transcript was detected at 48 h (Fig. 7), and no tran-

Fig. 6. DNA blot analysis of genomic DNA from rice cultivar IRBB10 hybridized with a gene-specific probe for *PO-C1*. The DNA was digested with *Nhe*I and *Xmn*I (lane 1), *Hind*III and *Xmn*I (lane 2), *Hind*III and *Nhe*I (lane 3), *Hind*III and *Bam*HI (lane 4), *Bam*HI and *Xmn*I (lane 5), and *Bam*HI and *Nhe*I (lane 6).

Fig. 7. RNA blot analysis of *PO-C1* gene induction pattern after inoculation of 'IRBB10' leaves with the virulent *Xanthomonas oryzae* pv. *oryzae* strain PXO99^A(pBU) (compatible) or the avirulent strain PXO99^A(pBUavrXa10) (incompatible). RNA was extracted from rice leaves harvested at 0.5, 3, 6, 12, 18, and 24 h (upper blot) and 0.5, 24, 36, and 48 h (lower blot) after infiltration with *X. oryzae* pv. *oryzae*. Control leaves were either untreated (U) or infiltrated with water (Water). Equal loading was assessed using 18S or 28S rRNA probes.

script was detected at 72 h (data not shown). During the compatible interactions, *PO-C1* expression was barely detected after 18 h, weak expression was observed to 48 h (Fig. 7), and no transcript was detected at 72 h postinoculation (data not shown). No *PO-C1* transcript was detected in water controls. *PO-C1* expression was only detected in pathogen-treated leaves; expression was not detected in mechanically wounded leaves or in roots or leaves of 1- or 3-month-old plants (data not shown).

DISCUSSION

The rice bacterial blight pathogen *X. oryzae* pv. *oryzae* is primarily a vascular pathogen that elicits a defensive response through interaction with metabolically active rice cells. Using ultrastructural analyses, we show that the pathogen contacts the xylem parenchyma cells bordering the vessel, and that this contact has several consequences that are specific to the incompatible interactions. Within 12 h of inoculation, expression of a defense-specific peroxidase gene *PO-C1* is induced, and the enzyme accumulates in the xylem parenchyma cells and in the xylem vessel lumen and walls. The xylem secondary walls thicken, reducing the pit diameter and effectively reducing access of bacterial cells to the living parenchyma cells.

A number of biochemical and ultrastructural responses of xylem parenchyma cells proximal to infected xylem vessels have been previously reported (Beckman et al. 1991; Benhamou 1995; Mueller et al. 1994; Shi et al. 1991). For example, Benhamou (1995) reported a cytoplasmic reorganization in paratracheal parenchyma cells adjacent to infected xylem vessels, and Shi and associates (1992) described an increase in cytoplasmic content and activity in cells in contact with infected vessels. In our study, significant ultrastructural changes observed during the incompatible interactions included cytoplasmic degradation in the xylem parenchyma cells within 24 h of inoculation and thickening of the xylem secondary walls with a reduction in pit diameter by 48 h postinoculation.

Concomitant with the ultrastructural changes, we observed that cationic peroxidase PO-C1 accumulated in the cytoplasm, the primary and secondary walls of the xylem parenchyma, and the secondary walls and lumen of the xylem cells as early as 12 h after infection during incompatible interactions. Significant levels of PO-C1 were not detected in the cytoplasm or walls of xylem parenchyma cells undergoing compatible interactions. In addition, immunogold-labeling analysis revealed that PO-C1 accumulated in the cytoplasm and cell walls of xylem parenchyma cells surrounding infected xylem vessels during incompatible interactions with *X. oryzae* pv. *oryzae*. These data are consistent with an induced expression of the isoenzyme in the xylem parenchyma cells and the subsequent distribution into the enucleated xylem vessels.

Both the genomic DNA and cDNA copies of *PO-C1* were cloned and sequenced. As demonstrated previously for four other rice peroxidases (Chittoor et al. 1997), DNA blot analyses with two *PO-C1*-specific probes indicate that the *PO-C1* gene exists as a single copy in the rice cultivar IRBB10. The PO-C1 protein shares a high level of similarity with other pathogen-induced rice peroxidases (Chittoor et al. 1997) and other monocotyledons such as wheat and barley (Rebmann et al. 1991), suggesting that they share a common ancestor gene.

PO-C1 was not detected in a previous study designed to investigate pathogen-inducible peroxidases from a rice cDNA library constructed at 12 h postinoculation with an avirulent strain of *X. oryzae* pv. *oryzae* (Chittoor et al. 1997). However, two other pathogen-induced peroxidases, POX8.1 and POX22.3, were identified, and enhanced expression of these enzymes was first detected at 6 h postinoculation during the incompatible interactions (Chittoor et al. 1997). Expression of these peroxidases during compatible interactions was reduced or delayed, and only POX8.1 showed brief enhanced expression after wounding. Initial induction of *PO-C1* is delayed comparable to POX8.1 and POX22.3 (12 to 18 h versus 6 to 12 h), but it is induced only during pathogen interactions. The detection of the *PO-C1* transcript during incompatible interactions is consistent with the detection of PO-C1 protein by immunogold labeling (12 h) and with the thickening of the xylem vessel walls (48 h). Thus, our data are consistent with a role for PO-C1 in the defense response of rice against *X. oryzae* pv. *oryzae*. Whether or not PO-C1 acts alone or in concert with other peroxidases, particularly those that are induced earlier in incompatible interactions, to cause the wall thickening is not known.

Bestwick and associates (1997, 1998) reported peroxidase activity and the deposition of cerium perhydroxide precipitates at the site of adhesion of *Pseudomonas syringae* pv. *phaseolicola* to lettuce cell walls. H₂O₂ is known to accumulate in lignifying cell walls (Czaninski et al. 1993) and to potentially have antimicrobial activity (Lamb and Dixon 1997). We have also observed deposition of cerium perhydroxide precipitates in *X. oryzae* pv. *oryzae* interactions (E. Hilaire and J. E. Leach, unpublished data). However, as it is induced relatively late in the defense interaction, PO-C1 is unlikely to be involved in the very early accumulation of hydrogen peroxide.

Nicholson and Hammerschmidt (1992) have proposed that secretion of pathogenesis-induced peroxidases into intercellular spaces is involved in controlling the invasion of pathogens, either by the production of reactive oxygen species that are toxic to pathogens or by the construction of physical barriers that would impede a pathogen's ability to penetrate the plant cell (Benhamou 1995; Ride 1983; Tiburzy and Reisener 1990). Because PO-C1 utilizes the lignin precursor coniferyl alcohol as a substrate (Reimers et al. 1992), accumulates in cell walls during lignification (Reimers and Leach 1991), and is associated with secondary xylem wall thickening (Table 1), it is a good candidate for a lignin peroxidase. Lignin and other phenolic polymers are polymerized into plant cell walls forming physical barriers (Benhamou 1995; Ride 1983; Tiburzy and Reisener 1990). Since plant bacterial pathogens such as *X. oryzae* pv. *oryzae* do not penetrate host cells, we had previously proposed that cell wall lignification would not be an effective way of preventing bacterial spread unless lignified materials would block movement between the epithem and xylem vessels (Reimers et al. 1992; Young et al. 1995). In this study, we show that thickening of the xylem secondary wall decreases the extent to which the pit membrane is exposed to bacterial cells, reducing their access to the xylem parenchyma cells. Thus, lignified walls in xylem vessels may provide a barrier to the transfer of materials essential to virulence or avirulence from bacterial pathogens to plant cells.

Mature rice plants, which are less susceptible to *X. oryzae* pv. *oryzae* (Mew 1987), also exhibit thickened xylem secondary walls with small pit diameters. In addition to activation of the defense response, pathogenic colonization of the xylem vessel may require access by the bacteria to living xylem parenchyma cells; perhaps contact is required to initiate nutrient leakage into the xylem lumen. Some *X. oryzae* pv. *oryzae* avirulence genes are known to contribute to pathogen virulence (Bai et al. 2000), and the products of these genes are thought to function inside the plant cell (Zhu et al. 1999). Thus, reduction of access to living cells by thickened secondary walls could be one reason for enhanced resistance in mature rice plants. Because expression of the *PO-C1* gene was not observed in untreated mature leaves and was only observed during interactions with the bacteria, *PO-C1* may not be involved in the developmental thickening of xylem parenchyma secondary walls. However, our assays may not have detected a very low level of *PO-C1* expression that could contribute to wall thickening in aging plants.

Finally, the lignin biosynthetic process, which is enhanced during the rapid xylem secondary wall deposition that occurs during the defense response, could also be an important component of the defense response against bacteria. Toxic phenolic compounds (Horino and Kaku 1989; Venere 1980), phenolic free radicals, and activated oxygen (Elstner 1982), all of which are associated with lignification, may inhibit or kill bacteria. Since peroxidase catalyzes the polymerization of lignin, *PO-C1* may contribute to the defense response as a consequence of its function in lignin biosynthesis.

MATERIALS AND METHODS

Plant and bacterial culture.

Rice (*Oryza sativa* L.) cultivar IR24 is susceptible to the *X. oryzae* pv. *oryzae* strains used in this study. Cultivar IRBB10 contains the *Xa10* gene for bacterial blight resistance, is near-isogenic to 'IR24', and is resistant to *X. oryzae* pv. *oryzae* PXO99^A(pBUavrXa10) and PXO99^A(pBUavrXa10.F1), which contain avirulence gene *avrXa10* (Young et al. 1995). 'IRBB10' is susceptible to strains PXO99^A(pHMI) or PXO99^A(pBU), which contain the cloning vectors pHMI and pBU (a pUFR027/pBlueScript SK⁺ fusion) but lack the *avrXa10* gene (Young et al. 1995). Seedlings were grown in growth chambers or greenhouses as described previously (Reimers and Leach 1991).

X. oryzae pv. *oryzae* strains were maintained at 28°C on modified Wakimoto's medium (Karganilla et al. 1973) amended with kanamycin (50 µg/ml) as required. For plant inoculations, bacteria were cultured in nutrient broth (Difco Laboratories, Detroit, MI, U.S.A.) on a rotary shaker (250 rpm) at 28°C and the inoculum concentration was adjusted to 5×10^9 CFU/ml (Reimers and Leach 1991). The first fully expanded leaf of 12-day-old rice plants was inoculated by infiltration into multiple sites (Reimers and Leach 1991; Reimers et al. 1992) for use in RNA extraction or TEM. Bacteria were inoculated by the leaf-clipping technique (Kauffman et al. 1973) for SEM studies. Infiltrated leaves were frozen for RNA extraction or fixed for TEM at 0, 6, 12, 18, 24, 36, and 48 h postinoculation. For SEM, a 2-cm section of leaf immediately below the clipped site was excised and frozen at 3 and 48 h after clip inoculation. Samples for SEM were also taken from leaves of 65-day-old untreated plants.

Tissue preparation for electron microscopy.

For SEM of freeze-fractured samples, rice tissue samples were immersed in liquid nitrogen, fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2), and dehydrated in 100% ethanol (Boyde 1974). After a second rinse in 100% ethanol, the samples were freeze-fractured in liquid nitrogen (Humphreys 1975). For immunolabeling of tissue for SEM, samples were probed with anti-*PO-C1* antibodies, fixed as above, dehydrated in an ethanol series, and critical point-dried. The samples were mounted on specimen stubs and sputter coated with gold to a thickness of 200 Å. Specimens were observed with a Hitachi H-300/3010 scanning electron microscope (Hitachi Instruments Inc., San Jose, CA, U.S.A.).

For TEM, rice leaf material was fixed overnight in freshly prepared 4% (vol/vol) paraformaldehyde and 0.05% (vol/vol) glutaraldehyde in cacodylate buffer at 4°C. Fixed leaf tissues were dehydrated through a graded ethanol series and infiltrated in several changes of London resin White resin as described by the manufacturer (Polysciences, Inc., Warrington, PA, U.S.A.). The tissue was embedded in BEEM capsules (Taab Laboratories, Aldermaston, Berks, U.K.) by polymerization of the resin for 24 to 48 h at 60°C. Ultrathin sections on parlodion-covered nickel grids were immunogold-labeled and stained as described by Lutkenhaus and Bi (1991). The sections were examined with a Hitachi H-300 transmission electron microscope (Hitachi Instruments Inc.).

Anti-*PO-C1* antibodies against the synthetic peptide MNLAMNLAFLD (Young et al. 1995) were used to detect the rice cationic peroxidase *PO-C1* in immunogold-staining studies. Antibodies were purified either by affinity chromatography using peptide bovine serum albumin (BSA) conjugates linked to Affi-gel 10 (Bio-Rad Laboratories, Hercules, CA, U.S.A.) or by eluting antibodies from peptide-BSA conjugates bound to nitrocellulose according to Smith and Fisher (1984). The antibodies were used at a dilution of the same concentration for all labeling experiments. Specificity of labeling was assessed in TEM experiments by the following controls: incubation with preimmune serum instead of primary antiserum, and competitive binding studies using anti-*PO-C1* and anti-PLD F(ab)₂ fragments as described by Young and associates (1995, 1996).

Quantitation of immunogold particles in TEM and secondary cell wall thickening in SEM experiments.

Thin sections of infected leaf tissue were analyzed at a magnification of $\times 30,000$ by TEM. For each treatment with antibodies, two grids with two sections per grid, two fields per section, and 5 cm² or 5 cm per organelle per field were examined for gold particles. Each treatment consisted of two replicates. The gold particles in and around the different plant organelles were counted on prints with the aid of a 1-cm² grid. Values are reported as mean numbers $\pm$ the standard error of the mean.

Cross sections of five rice leaves were analyzed by SEM for each treatment and for each time point. At least 10 pictures for each time point and treatment were used to obtain 30 measurements each of the short axis of the ellipse of the xylem secondary wall width (relative to what is exposed in the xylem vessel) and the pit length. Values are reported as mean numbers $\pm$ the standard error of the mean.

PO-C1 cloning.

'IRBB10' DNA (10 µg) was digested with *Pst*I or *Eco*RI and then ligated in a final volume of 250 µl. Two sets of primers were designed according to a partial amino acid sequence of the purified PO-C1 protein (Young et al. 1995). These were 5'CGGGCGCCAGGTTTCATGTCGCC3' combined with either 5'TGCTGTCTGAACAAGGGCT3' or 5'TGAGGAACTTCGCGTCCAA3'. For inverse polymerase chain reactions (I-PCR), samples contained 200 ng of ligated 'IRBB10' DNA; 25 pmol of each primer; 50 pmol of dATP, dCTP, dGTP, and dTTP (Promega, Madison, WI, U.S.A.); 2 units of *Taq* DNA polymerase (Promega); and 0.1 M MgCl₂. The I-PCR products were subcloned into the TOPOII vector (Invitrogen, Carlsbad, CA, U.S.A.) and sequenced at the Iowa State University DNA Sequencing Facility (Ames, IA, U.S.A.).

The 3' UTR of *PO-C1* was missing in the original I-PCR-derived clone. To obtain this sequence, total RNA was extracted from 'IRBB10' leaves undergoing resistant interactions for 24 h (RNeasy Plant Mini Kit; Qiagen, Santa Clarita, CA, U.S.A.), and polyA mRNA was purified by means of an oligo(dT) column (Stratagene, La Jolla, CA, U.S.A.). The 3' UTR of *PO-C1* was amplified using the 3' Rapid Amplification cDNA Ends Kit (Life Technologies, Gaithersburg, MD, U.S.A.), and the fragment was cloned and sequenced.

The *PO-C1* cDNA was identified from a pathogen-induced cDNA library constructed from RNA isolated 12 h after infiltration of 'IRBB10' leaves with *X. oryzae* pv. *oryzae* strain PXO99A(pBUavrXa10). The cDNA was synthesized from poly(A)⁺-enriched RNA and ligated into hybridzap vector according to the manufacturer's instructions (Stratagene). DNA was packaged using the MaxPlax Lambda packaging kit (Epicentre Technologies Corporation, Madison, WI, U.S.A.). The primary library with a titer of 10⁶ phage per µl was amplified. Plaques were transferred to nylon membrane and screened for hybridization with the 3' UTR of *PO-C1*. Plaques that hybridized to *PO-C1* were purified by three rounds of re-plating and screening.

DNA and RNA blot analyses.

'IRBB10' DNA was isolated as described by Saghai-Maroo and associates (1984). DNA (10 µg per lane) was digested by restriction enzymes individually (*Eco*RI, *Eco*RV, *Hind*III, *Xba*I, and *Dra*I) or in combination with others (*Nhe*I + *Xmn*I, *Bam*HI + *Nhe*I, *Bam*HI + *Xmn*I, *Hind*III + *Bam*HI, *Hind*III + *Nhe*I, and *Hind*III + *Xmn*I). After separation in agarose gels, the fragments were transferred to nylon membranes. The blots were prehybridized at 42°C with ULTRAHyb buffer (Ambion, Austin, TX, U.S.A.) and hybridized using a ³²P-labeled probe amplified from the first intron in *PO-C1* or the 5' UTR of *PO-C1*. After overnight hybridization, the membrane was washed twice (15 min per wash) at 42°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and twice in 1× SSC.

Total RNA was extracted from frozen leaves using the RNeasy Plant Mini Kit (Qiagen). RNA (5 µg per lane) was separated on a formaldehyde gel and then transferred to nylon membranes (Sambrook et al. 1989). The membranes were prehybridized at 65°C with the ULTRAHyb buffer (Ambion), hybridized overnight with the ³²P-labeled 3' UTR of *PO-C1*, and washed twice in 2× SSC and twice in 1× SSC (15 min at 65°C per wash).

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