Activity of Class III Peroxidases in the Defense of Cotton to Bacterial Blight

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Cotton cotyledons displayed a hypersensitive reaction (HR) in the cultivar Réba B50 after infiltration with the avirulent race 18 from *Xanthomonas campestris* pv. *malvacearum*. Two sets of peroxidases were associated with the HR time course. Early but transient accumulation of peroxidase in material encapsulating the bacteria in intercellular areas was observed by immunocytochemistry at 3 h postinfection and coincided with the oxidative burst. Total guaiacol-peroxidase activity was highly increased in cells undergoing HR, from 12 h after treatment. Molecular characterization of seven cloned peroxidase genes revealed highly conserved B, D, and F domains, with similarities to plant class III peroxidases. Analysis of gene expression showed variation in transcript accumulation during both compatible (race 20) and incompatible interactions for four of these genes: *pod2*, *pod3*, *pod4*, and *pod6*. *Pod4* and *pod6* were more intensely up-regulated during resistance than during disease and in the control, while *pod3* was specifically down-regulated during the HR after the oxidative burst. *Pod2* was induced by pathogen infection and weakly stimulated in the control. These data suggest that cotton peroxidases may have various functions in the defense response to *Xanthomonas* infections.

Additional keywords: *Gossypium*, apoplasm.

Resistance of cotton (*Gossypium hirsutum*) to bacterial blight caused by *Xanthomonas campestris* pv. *malvacearum* (Hillocks 1992) is mediated by gene for gene interactions (De Feyter et al. 1993; Follin et al. 1988; Gabriel et al. 1986). The resistance phenotype is characterized by rapid localized tissue collapse, resulting in necrotization (hypersensitive response [HR]) and immobilization of the intruding pathogen at the sites of attack (Al Mousawi et al. 1982). At least 22 major resistance genes, so-called B genes, are reported as dominant resistance factors in *G. hirsutum* resistance (Hillocks 1992). The cultivar Réba B50 carries B₂B₃ bacterial blight resistance genes that confer immunity to several *X. campestris* pv. *malvacearum* races, including race 18 (Innes 1983).

Studies on the nature of cotton resistance to *X. campestris* pv. *malvacearum* have focused on a wide range of events occurring mainly within the first 24 h postinfection (pi). The oxi-

dative burst (3 h pi) is characterized by the production of active oxygen species (AOS), including superoxide anions (O₂^{•−}) and hydrogen peroxide (H₂O₂) (Martinez et al. 1998). It acts upstream of salicylic acid (SA) accumulation (6 h pi) (Martinez et al. 2000). Histopathological studies indicated that rapid membrane rupture and cell collapse were important features in the cotton HR (Cason et al. 1978; Daï et al. 1996; Hopper et al. 1975). Recently, cotton cell death during the HR was shown to be strongly associated with 9S-lipoxygenase (LOX)-dependent lipid peroxidation and correlated with water loss of infected tissues and appearance of HR lesions (Jalloul et al. 2002). Both jasmonic acid (JA), which accumulated early 2 h pi, and SA induce cotton LOX1 gene expression and activity (A. Jalloul, unpublished data). Sesquiterpene phytoalexins elicited by the pathogen (Abraham et al. 1999) accumulated in hypersensitive cells adjacent to *X. campestris* pv. *malvacearum* colonies to concentrations sufficient to account for bacteriostasis (Essenberg et al. 1990). Activity of the sesquiterpene cyclase, the first enzyme in the biosynthetic pathway of sesquiterpenoid defense compounds, was shown to increase at 24 h pi (Davila-Huerta et al. 1994). Flavonoids were detected in the cytoplasm and walls of cells reacting hypersensitively, but there was no evidence of their antibacterial activity (Daï et al. 1996).

Peroxidases (POD) (EC 1.11.1.7), a group of heme-containing glycosylated proteins, are known to be activated in response to pathogen attacks. Several roles have been attributed to plant POD in host-pathogen interactions (Chittoor et al. 1999), including xanthomonad-induced resistance (Brown et al. 1998; Chittoor et al. 1997; Do et al. 2003; Hilaire et al. 2001; Young et al. 1995). The correlation between increased POD activity during incompatible interactions and reinforcement of cell walls with phenol compounds was also reported (Reimers et al. 1992). In cotton cultivars, association of POD activity with resistance to the bacterial blight was suspected (Martinez et al. 1996; Venere 1980). Martinez and associates (1998) showed that apoplastic cationic isoforms of POD generated O₂^{•−} during the HR of cv. Réba B50 to *X. campestris* pv. *malvacearum* race 18, and they and others (Bolwell et al. 2002) suggested that POD may be involved in the oxidative burst. In addition, total POD activity was stimulated following SA or H₂O₂ treatments, both at cotyledon infection sites, and systemically in the whole plant in relation with acquired resistance (Martinez et al. 2000). Here, we investigated the possibility that the dramatic increase in total POD activity in cotton cotyledons challenged by *X. campestris* pv. *malvacearum* race 18 (Martinez et al. 1996) may be associated with variation in POD gene expression. Immunocytolocalization in planta of POD proteins was performed during the HR time course, using

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polyclonal antibodies raised against purified cotton POD. In parallel, we isolated seven class III POD genes cloned from cotton cDNA libraries, and changes in their expression were examined with emphasis on differential induction during cotton resistance and susceptibility.

RESULTS

Changes in peroxidase activity during infection.

POD activity was assessed spectrophotometrically according to time course of infection (Fig. 1). It increased significantly during the incompatible interaction between 8 and 10 h pi, as compared with the activity in the compatible interaction (Réba B50-*X. campestris* pv. *malvacearum* race 20) and the control. Tissue printing of whole *X. campestris* pv. *malvacearum* race 18-infected Réba B50 cotyledons onto nitrocellulose membranes followed by revelation of POD activity showed strong at HR sites from 10 to 18 h pi (Fig. 2A and B). A weaker reaction was noted in noninfected areas of *X. campestris* pv. *malvacearum*-infiltrated cotyledons. At the same time, *X. campestris* pv. *malvacearum* race 20-infected cotyledon tissue prints did not reveal any difference in POD activity as compared with controls (not shown).

Immunocytolocalization of peroxidase.

Ultrathin sections from *X. campestris* pv. *malvacearum*-infected resistant cotyledons were incubated on primary anti-POD antibody (1:30) followed by treatment with goat antirabbit conjugated to colloidal gold. This is a polyclonal antibody that likely reacts with several different peroxidases. At 3 h pi, ultrastructural observations of ultrathin sections from *X. campestris* pv. *malvacearum*-infected resistant cotyledons revealed dense labeling over the material encapsulating bacteria in the intercellular areas (Fig. 3A; Table 1). Gold particles were also present on the middle lamella (Fig. 3B) and secondary cell walls (Fig. 3C). Weak labeling was detected over the cytoplasm of host cells. At 6 h pi (Table 1), gold particles weakly decorated the bacteria-encapsulating material. The cytoplasm of mesophyll cells that underwent the HR displayed

irregular labeling. Gold particles were also unevenly distributed over the middle lamella. On immunotreated sections from 18-h infected cotyledons (Table 1), labeling was weak over the bacteria-encapsulating material and of variable density over host cell cytoplasm within the infected areas (Fig. 3D). An analysis of labeling density (gold particles per μm^2) showed high standard errors, indicating variation in gold decoration and suggesting local, not general, POD accumulation.

In water-infiltrated cotyledons, a weak decoration of host cell cytoplasm and walls was detected (Fig. 3E), while middle lamella (Fig. 3F) and secondary cell walls also displayed numerous gold particles. During the compatible interaction, weak or no differences in gold labeling were noted between infected cells and controls at 3, 6, and 18 h pi (Table 1). Negative results (data not shown) were obtained for controls, which included incubation of sections with i) the antibody to which the corresponding antigenic molecules was previously added (data not shown), ii) preimmune rabbit serum instead of primary antiserum (Table 1), and iii) omission of the primary antibody incubation step.

Isolation of peroxidase genes of cotton.

To isolate pathogen-inducible peroxidase genes involved in the interaction, we screened two cDNA libraries constructed with RNA isolated 2.5 and 5.5 h pi. Approximately 1.5 million phages from each library were screened, and 45 clones were

Fig. 1. Time course of total peroxidase (POD) activity during infection. A significant increase in activity is observed from 8 h postinfection in the incompatible interaction (■), as compared with the water-infiltrated plant control (▲). A weak but positive increase was also noted in the compatible Réba B50-*Xanthomonas campestris* pv. *malvacearum* race 20 interaction (●). POD activity was spectrophotometrically assessed at 470 nm, using guaiacol as hydrogen donor.

Fig. 2. Detection of peroxidase (POD) activity on cotyledon tissues from the resistant cultivar Réba B50, after blotting onto nitrocellulose membrane (0.2 μm) and incubation for 2 min in the POD substrate (0.2% guaiacol, 0.01% 3-amino-9-ethylcarbazole, 0.03% H_2O_2 in 0.05 M sodium phosphate buffer, pH 6). **A**, Cotyledon inoculated with the pathogen, 18 h postinfection. Strong activity is visible at sites of bacterial infiltration (large arrows), and weak activity is seen in portions of the noninoculated mesophyll (small arrows). **B**, Cotyledon infiltrated with water. POD activity is weakly detected in the treated areas (arrows).

Fig. 3. Immunocytochemical localization of cotton peroxidases (POD) using anti-POD polyclonal antiserum (1:30), followed by gold-labeled goat antirabbit anti-bodies (1:20). **A** through **C**, Observations of sections from Réba B50 cotyledons 3 h postinfection (pi) with *Xanthomonas campestris* pv. *malvacearum* race 18. Numerous gold particles are seen over the bacterial encapsulating material (**A**, arrows). This labeled material is closely associated with the middle lamella (**B**, arrow). Gold decoration is weak over the intercellular areas, the host cell wall (cw), the bacteria (b), and the cytoplasm. Bars : **A** = 1 μ m; **B** = 0.5 μ m. **C**, Gold particles are abundant over secondary cell walls (scw, arrow) of xylem vessels. Bar = 1 μ m. **D**, Observations of sections from Réba B50 cotyledons 18 h pi with *X. campestris* pv. *malvacearum* race 18. Numerous gold particles are seen over the cytoplasm of cells undergoing a hypersensitive reaction (cy, arrows). Bar = 0.3 μ m. **E** and **F**, Observations of sections from Réba B50 cotyledons 18 h pi with water. **E**, Over the cytoplasm (cy) and walls (cw) of mesophyll cells, only a few gold particles are seen. **F**, Labeling is regular and more intense over portions of the middle lamella (arrows). Bar = 0.3 μ m.

isolated. Sequence analysis revealed that 20 of them contained peroxidase-related sequences. Among the peroxidase-related clones, 12 were identical to *pod4* (Assigbetsé et al. 1999) (GenBank accession number AF155124), and the other clones encoded five distinct peroxidase-related sequences: *pod1* (accession number AF485266), *pod2* (accession number AY074794), *pod3* (accession number AF485265), *pod5* (accession number AF485267), and *pod6* (accession number AF485268). *Pod10* (accession number AF488305) was subsequently identified by polymerase chain reaction (PCR) from cDNA libraries.

Sequence analysis and comparison revealed that sizes of *pod1*, *pod2*, *pod3*, *pod5*, *pod6*, and *pod10* ranged from 1,125 to 1,531 bp, representing unreported full-length peroxidase coding sequences. The deduced amino-acid sequences indicated that the size of each potentially encoded protein ranged from 320 (*pod3*) to 347 (*pod10*) residues (Fig. 4), with an estimated molecular mass of 32 to 35 kDa (Table 2). As with *Arabidopsis thaliana* class III POD genes (Tognolli et al. 2002), the seven cotton genes are identified as class III POD by their three highly conserved domains B, D, and F; these domains are common to plant peroxidases and reported to be important in catalysis and protein folding (Welinder 1992). Apart from these domains, the remaining sequence of the genes is highly variable. Multiple alignment of predicted amino acid sequences of the putative peroxidase cDNA clones, *pod4*, and horseradish peroxidase C (HRPC1) showed sequence differences (Fig. 4); they displayed less than 55% identity with each other, except for *pod2* and *pod6* (79%) and *pod3* and *pod4* (62%). This high diversity may reflect the large number of roles that class III POD can have in plants. Furthermore, there are no clear homologues of these cotton genes in other plants, except for *pod2* which is 75% identical to TO7008, a tomato peroxidase induced by ethylene and during virus infections (Gadea et al. 1996). Putative signal peptide cleavage sites were identified in the coding sequence of all the clones (Fig. 4), and no clear peroxisomal targeting signal sequences were detected, thus suggesting that these POD are located extracellularly (Table 2).

Expression of peroxidase during cotton-*X. campestris* pv. *malvacearum* interactions.

The expression profile of each cDNA clone was determined by reverse transcriptase (RT)-PCR, using specific primers (Table 3) at different pi times. The mRNAs of *pod1*, *pod5*, and *pod10* were detected in healthy cotyledons and after water infiltration, showing expression from 0 to 48 h pi. These genes did not show significant modulation of their expression during either of the interactions.

Pod3 was also constitutively expressed in cotyledons. Its expression remained stable in the control but decreased during the HR; the signal was no longer detectable by 12 h pi. It also slightly decreased during the compatible interaction.

In contrast to these genes, expression of *pod4* and *pod6* was only weakly or not detected in cotyledons. However, they were rapidly and highly induced during both compatible and incompatible interactions and by water infiltration. Accumulation of mRNA, which was observed as early as 2 h pi and was still detected 36 to 48 h pi, was at much higher levels in infected resistant plants, as compared with the compatible interaction and the control.

Pod2 expression, which was weakly detected in the control, was induced by both *X. campestris* pv. *malvacearum* race 20 and race 18. Messenger RNA accumulated from 2 h pi to 36 to 48 h pi in the susceptible interaction, while a distinct pattern of expression with two phases was revealed during HR from 2 to 6 h pi and at 18 h pi.

DISCUSSION

The oxidative burst in plants infected by incompatible pathogens is a key early event in the expression of resistance (Wojtasieck 1997). It was suggested that POD of cotton participate in the defense reaction to *X. campestris* pv. *malvacearum*, including AOS generation in the HR of cotyledons (Martinez et al. 1996; Venere 1980), prompting us to further investigate cotton POD expression at cytological and molecular levels. In the present work, we demonstrated that POD activity and distribution were modified within cotyledon cells undergoing the HR. Seven peroxidase genes were cloned with similarities to plant class III POD, also referred to as classical guaiacol POD. These proteins found in the vacuole or apoplast have been implicated in a broad range of physiological processes (Hiraga et al. 2001). Variations in immunogold labeling on sections from cotton cotyledons suggest two levels of response, one occurring primarily 3 h pi, close to the encapsulated pathogen confined in the apoplast, and the second, in the cytoplasm, associated with changes in total POD activity starting around 10 h pi. Using tissue printing methods, no apparent substantial modification was detected in areas surrounding HR sites before 20 h pi. However, a subsequent systemic increase in POD activity was recorded one day after bacterial treatment, both locally in noninfected areas of cotyledons and in the whole plant (Martinez et al. 2000). A weaker increase in activity was also observed in the compatible interaction.

The highly localized accumulation of POD proteins in cotton cells 3 h after *X. campestris* pv. *malvacearum* infiltration, which was revealed by immunolabeling with semiquantitation of gold particle distribution, is consistent with diamino-benzidine cytochemistry observations showing that POD activity was con-

Table 1. Density of labeling^a

Cell compartment	Time after infection (hours)		
	3	6	18
Inoculated with the <i>X. campestris</i> pv. <i>malvacearum</i> race 18 ^b			
Plant cell wall	30 ± 26	5 ± 7	4 ± 6
Middle lamella	83 ± 30	57 ± 44	15 ± 12
Plant cytoplasm	9 ± 7	43 ± 22	32 ± 8
Bacteria	6 ± 4	3 ± 3	5 ± 4
Encapsulating material	95 ± 45	16 ± 14	2 ± 1
Background	2 ± 2	2 ± 3	2 ± 1
Infiltrated with water ^b			
Plant cell wall	3 ± 5	5 ± 5	4 ± 5
Middle lamella	17 ± 17	12 ± 7	15 ± 5
Plant cytoplasm	8 ± 5	15 ± 5	16 ± 5
Bacteria	...	...	...
Encapsulating material	...	...	...
Background	2 ± 1	3 ± 2	2 ± 1
Inoculated with the <i>X. campestris</i> pv. <i>malvacearum</i> race 20 ^b			
Plant cell wall	2 ± 3	4 ± 2	5 ± 3
Middle lamella	12 ± 6	9 ± 6	8 ± 4
Plant cytoplasm	10 ± 5	9 ± 4	21 ± 7
Bacteria	...	...	...
Encapsulating material	...	...	...
Background	1 ± 1	2 ± 2	1 ± 1
Inoculated with the <i>X. campestris</i> pv. <i>malvacearum</i> race 18 ^c			
Plant cell wall	1 ± 1	0 ± 0	0 ± 0
Middle lamella	0 ± 0	0 ± 0	1 ± 1
Plant cytoplasm	0 ± 0	0 ± 0	0 ± 0
Bacteria	0 ± 0	0 ± 0	0 ± 0
Encapsulating material	1 ± 1	0 ± 0	0 ± 0
Background	1 ± 1	0 ± 0	0 ± 0

^a Obtained with cotton antiperoxidase antiserum and gold-labeled goat antirabbit over sections from *Xanthomonas campestris* pv. *malvacearum*-infected or water-infiltrated cotton cotyledons, according to time.

^b Treated with antiperoxidase polyclonal antibodies.

^c Treated with preimmune serum.

fined in the apoplasm and close to the bacteria (Martinez et al. 1998). POD identified in the apoplastic washing fluid were shown to be i) cationic, ii) responsible for the production of superoxide anions subsequently dismutated into wall-bound H_2O_2 , iii) preformed, since they were detected in healthy cotyledons, and iv) inducible for their superoxide anion-generating NADH-oxidase activity in noninfected tissues (Martinez et al. 1998). Similarly, in other pathosystems, apoplastic POD were associated with H_2O_2 production (Blee et al. 2001; Brown et al.

1998) and oxidative cross-linking activity (Bestwick et al. 1998) with ability to reduce a wide range of substrates. POD accumulation in *X. campestris* pv. *malvacearum*-encapsulating material is a response to HR development and suggests that cotton cells challenged by the pathogen create a localized, highly toxic environment, in line with AOS production, which results in limiting bacterial growth (Nicholson and Hammerschmidt 1992). This confined apoplastic localization of active POD may explain why the change in activity 3 h pi was not observed spectrophoto-

Fig. 4. Multiple alignment of deduced amino acid sequences of putative peroxidase cDNA clones of *Gossypium hirsutum* compared with horseradish peroxidase C and *pod4*, using ClustalW 1.81 (Infobiogen, Paris). Asterisks (*) identify identical amino acids, dashes represent gaps introduced to maximize similarity. The downward arrow (↓) indicates a potential signal peptide cleavage site. The conserved cysteines (involved in the formation of disulfide bridges) are underlined, and histidines (involved in heme binding) are dashed. Horizontal lines represent the conserved catalytic and distal heme binding domain (B), proximal heme binding domain (F), and central conserved domains of unknown function (D) (Welinder 1992).

tometrically but only on isoelectric focusing (IEF) gels with reference to apoplastic cationic isoforms (Martinez et al. 1998).

Although *pod2*, *pod4*, and *pod6* genes encode cationic proteins based on their putative pI, our data showed that their transcriptional activity was not correlated with the immunological and biochemical observations. In contrast, the expression of *pod3*, which also putatively encodes an apoplastic cationic protein, was constitutive but restricted to photosynthetic tissues (data not shown) and was inhibited by 10 to 12 h pi during the cotton HR. This *pod3* profile was associated with peaks in accumulation of immunolabeled POD and IEF activity and coincided with the oxidative burst. Investigations are currently under way to determine whether *pod11* might correspond to apoplastic POD with superoxide anion-generating NADH-oxidase activity (Martinez et al. 1998) and whether it might be cationic POD associated with plant defense to xanthomonads. In *Capsicum annuum* infected with *X. campestris* pv. *vesicatoria* (Do et al. 2003), the drastic increase in H₂O₂ production was correlated with extinction of ascorbate and thioredoxin POD-like gene expression.

Immunocytological observations in cotton showed localization of POD within the middle lamella bordering intercellular areas and the matrix surrounding the pathogen, suggesting close physical interactions between POD and pectin. Recent cell biological analysis revealed the presence of pectinlike molecules in material encapsulating the bacteria (E. Delannoy and M. Nicole, *unpublished data*). Binding of plant POD with oligogalacturonides in the presence of calcium was reported,

thus suggesting that pectin may influence the distribution of POD within the apoplast (Carpin et al. 2001; Penel and Greppin 1996; Penel et al. 1999, 2000). The possibility that calcium may have a crucial dual role during HR of the cotton genotype Réba B50 to *X. campestris* pv. *malvacearum* should be investigated in light of regulation of the oxidative burst by optimizing apoplastic POD activity (Bolwell 1999) and formation of pectic gels that trap the pathogen.

The increase in total POD activity from 12 to 18 h pi (Fig. 1), dramatic during resistance and slight during disease, was associated with cytoplasmic localization of POD and an increase in transcriptional activity of *pod4* and *pod29* genes, mainly during HR. The detection of these gene activities in water-infiltrated tissues suggests that these POD are stress-related rather than resistance-related proteins. Conversely, *pod1*, *pod5*, and *pod10* encoding putatively anionic isoforms did not display significant transcriptional activity related to either of these interactions. Preferential stimulation of transcriptional activity of the putative cationic protein-encoding *pod2* gene in both interactions but not in the control (Fig. 5) indicates that this gene may play a role in the cotton response to *X. campestris* pv. *malvacearum* infection. The expression profile and high sequence identity of *pod2* with a virus- and ethylene-induced POD gene (Gadea et al. 1996) suggests that *pod2* might encode a pathogen-induced defense protein that is differentially regulated during resistance versus disease.

The physiological function that proteins encoded by *pod2*, *pod4*, and *pod6* genes may have in local expression of cotton defense to *X. campestris* pv. *malvacearum* still remains ob-

Table 2. Relevant features of peroxidase sequences from cotton

Sequence features	Peroxidase clones						
	<i>pod1</i>	<i>pod2</i>	<i>pod3</i>	<i>pod4</i>	<i>pod5</i>	<i>pod6</i>	<i>pod10</i>
Length (bp)	1,125	1,330	1,335	1,369	1,531	1,389	1,250
Amino acid sequence length of the putative protein	329	328	320	317	323	328	347
Potential signal peptide cleavage sites ^a	19 to 20	27 to 28	23 to 24	19 to 20	21 to 22	26 to 27	26 to 27
Estimated molecular mass of mature protein (kDa) ^b	34.3	32.8	34.4	31.7	32.5	32.4	34.6
Estimated pI for mature protein ^b	5.29	8.87	8.46	8.72	5.66	8.34	5.15
Potential N-glycosylation sites ^c	3	1	3	2	0	1	10
Probable cellular location ^d	E	E	E	E	E	E	E

^a Sequence analysis performed with SignalP (Center for Biological Sequence Analysis, Lyngby, Denmark).

^b Sequence analysis performed with Mwcalc (Infobiogen, Paris).

^c Sequence analysis performed with Proscan (Pole Bio-Informatique Lyonnais, Lyon, France).

^d Sequence analysis performed with PSORT (GenomeNet, Kyoto, Japan); E = extracellular.

Table 3. List of primers used for polymerase chain reaction (PCR)

Primer name	Primer sequence (5' to 3')	Amplified gene	PCR product expected size (bp)
P18a	TTCCATGACTGCTTTGTTAATGGTTGTGATGG		
Pk1	CAG CTT CTG CTT TCG TTC TC	<i>pod4</i>	1,002
Pk2	ACC GAT AAT TGG ATA TGG GC		
Pod1for	GCC CGT CAA CTG TTT CTT GT	<i>pod1</i>	254
pod1rev	GCT TTG CCT ATG GAG TGT GA		
pod2for	CAG TTC AGT CCC ATT TCC GTT C	<i>pod2</i>	727
pod2rev	GAG CCT CTC TCA CCC AAG AA		
pod3for	AAG GCC AAG GTG GAG AAA GT	<i>pod3</i>	503
pod3rev	GAT CCG CCA TTG AAG AGT TC		
pod5for	GGC CAA CTA AGA GTG GGG TTC	<i>pod5</i>	725
pod5rev	CCA ACA CTG CAA ACC CAT TCC T		
pod6for	CGC TGC TCG TGA TTC TGT AG	<i>pod6</i>	362
pod6rev	CCT GTG TCC AAT CCA ATC CT		
5'pod10	CGA AGC AAG CCT CGA TTA AC	5'pod10	
3'pod10	CAC AAC ACC TGA TGG TTT CG	3'pod10	
pod10for	ACC CCG ACA TTT TAC GAT GA	<i>pod10</i>	725
pod10rev	CGA AGC AAG CCT CGA TTA AC		
ACT3	TAY CCN ATH gAR CAYgg	actin	300
ACT4	RTA DAT Ngg NAC NgT RTg		
RACT	GTA GAT GGG GAC GGT GTG AG	actin	275
LACT	ATT GTG AGC AAC TGG GAT GA		

scure. In rice infected by *X. oryzae*, the activity of the peroxidase gene PO-C1 was shown to be correlated with thickening of vessel secondary cell walls and probably associated with an increase in lignification (Hilaire et al. 2001; Young et al. 1995). However, the lignification response that is sometimes observed in plant HR cells was not detected in cotton cells challenged by the avirulent *X. campestris* pv. *malvacearum* race 18 (Dai et al. 1996). Recently, an extensive study on the expression of *A. thaliana* class III POD (Welinder et al. 2002) suggested that even similar POD (>70% identity) do not fulfill similar biological roles. Hence, it is hard to predict the role of a particular POD on the basis of its similarity to another known POD. One response of cotton to *X. campestris* pv. *malvacearum* was found to be a drastic accumulation of flavonoids in walls and cytoplasm of cells undergoing hypersensitive collapse (Dai et al. 1996), suggesting a possible relation between increased POD activity and phenol oxidation. Since POD were shown to be responsible for peroxidatic degradation of flavonones (Patzlaff and Barz 1978) and to catalyze H₂O₂-dependent oxidation of flavonols (Yamasaki et al. 1997), it has been suggested that the flavonoid-POD reaction can function as an H₂O₂-scavenging mechanism. Consistent with observations that H₂O₂ is synthesized apoplastically and flavonoids produced abundantly in cells at the edge of lesions during cotton HR to *X. campestris* pv. *malvacearum*, the role of POD as an oxidative damage protectant must be examined in light of the fact that flavonoids are elec-

tron donors. Accordingly, increase in anionic isoform activity was observed in noninfected areas of the inoculated cotyledons between 18 and 20 h pi (Martinez et al. 2000). Investigations are currently in process to elucidate the function of *pod2*, *pod3*, *pod4*, and *pod6* genes using transformed *Arabidopsis* mutants and to understand the underlying mechanisms involved in regulation of POD genes by the signaling hormones produced in cotton HR to *X. campestris* pv. *malvacearum*.

MATERIALS AND METHODS

Bacterial strains.

Races 18 and 20 of *X. campestris* pv. *malvacearum* were maintained at 30°C on YPG agar medium (0.5% wt/vol yeast extract, 0.5% wt/vol bacteriological peptone, and 0.5% wt/vol glucose as a carbon source, solidified with 1.5% wt/vol agar [Difco, Detroit]) in distilled water (pH 7.2). Bacteria for inoculation were grown in 150 ml of YPG medium in a shaking incubator at 150 rpm/min at 30°C. After approximately 18 h of growth, cultures were washed once with water by centrifugation at 10,000 × g for 10 min, to remove nutrients and exopolysaccharides. The bacterial pellet was then resuspended in water and was adjusted to an A₆₀₀ = 10⁷ CFU/ml.

Plant material.

The cotton cultivar (*G. hirsutum*) Réba-B50 (Allen × Stoneville 2B) was used in this study. This cultivar, similar to the 101-102B line, contains B₂B₃ blight resistance genes, which confer immunity to all *X. campestris* pv. *malvacearum* races, including race 18, except race 20 (Hillocks 1992; Innes 1983). Ten-days-old cotyledons were inoculated by infiltration of the bacterial suspension (Dai et al. 1996). Two interactions were investigated: the incompatible Réba-B50/*X. campestris* pv. *malvacearum* race 18, and the compatible Réba-B50/*X. campestris* pv. *malvacearum* race 20. In control plants, cotyledons were infiltrated with sterile water. These interactions were carried out in a growth chamber under a 30/24°C and 80/90% relative humidity/light/dark cycle with 12 h of light.

Peroxidase assay.

POD were extracted in sodium acetate buffer (0.05 M, pH 5, plus 25 mM β-mercapto-ethanol). POD activity of crude extracts was spectrophotometrically assessed at 470 nm, using guaiacol as hydrogen donor without 3-amino-9-ethylcarbazole. The molar extinction coefficient is 26.6 × 10³ M⁻¹ cm⁻¹. Total activity is expressed in nKat per mg of proteins. Protein determination is performed by the method of Bradford (1976) with serum albumin bovine as a standard. POD activity on gels was revealed using 0.2% guaiacol, 0.01% 3-amino-9-ethylcarbazole, and 0.03% H₂O₂ in 0.05 M sodium phosphate buffer, pH 6.

Tissue printing.

Tissue printing was performed on whole *X. campestris* pv. *malvacearum*-infected or water-infiltrated fresh cotyledons collected 3, 6, 10, and 18 h pi. Cotyledons were immersed for 30 s in 70% ethanol and were gently dried on filter paper before being carefully blotted onto nitrocellulose membrane (0.2 μm). Both cotyledons and membrane were placed between combined layers of No. 1 Whatman filter paper and aluminum foil. A 20 kg/cm² pressure (hydraulic press) was applied for 1 min. The nitrocellulose membrane was then incubated for 2 min in the POD substrate (0.2% guaiacol, 0.01% 3-amino-9-ethylcarbazole, and 0.03% H₂O₂ in 0.05 M sodium phosphate buffer, pH 6), was rinsed with distilled water, and then, was air-dried. Areas that stained red exhibited POD activity. Controls were carried out in the absence of H₂O₂.

Fig. 5. Changes in peroxidase gene expression within inoculated cotton cotyledons. Total RNA was extracted from water-treated (control), *Xanthomonas campestris* pv. *malvacearum* race 18 (avirulent)- or *X. campestris* pv. *malvacearum* race 20 (virulent)-infected cotyledons at different times after infiltration. Using specific primer pairs, the expression profile of each cDNA clone was determined by reverse transcriptase-polymerase chain reaction (PCR). PCR products were run on 1.5% agarose gels in Tris-acetate-EDTA buffer, and the band intensities within each experiment were compared after ethidium bromide staining.

Preparation of antiperoxidase polyclonal antibodies.

New Zealand white rabbits were immunized by injection of cotton-purified nondeglycosylated anionic POD isoforms (100 mg/ml), three times at 2-week intervals and one time 70 days following the first inoculation. Freund's incomplete adjuvant (200 µl) was added for the first injection, and 250 µl of Freund's incomplete adjuvant was added for the other injections. The rabbit serum titer was determined by enzyme-linked immunosorbent assay after each injection. The final serum was diluted (1:2) in 1 M phosphate buffer saline (PBS) (pH 7.4) at 4°C and was partially purified with 40% ammonium sulfate. After centrifugation, the pellet was resuspended in PBS and dialyzed using Amicon P10 membrane (Amicon, Beverly, MA, U.S.A.). Preimmune serum was also collected and treated similarly.

Immunocytochemical localization of peroxidase.

Fragments of inoculated or healthy leaf tissues (about 2.0 mm²) were fixed for 4 h in 1% glutaraldehyde, 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2, at 4°C. After dehydration in graded series of ethanol at 4°C until 70% ethanol and -20°C until 100% ethanol, samples were embedded in LR-White Medium Grade (TAAB, Aldermaston, England) in a Reichert AFS (Automatic Freeze substitution System), according to a progressive low-temperature protocol at -19°C. Resin supplemented with 0.5% benzoin methyl ether (Sigma, St. Louis) was polymerized for 48 h at -19°C, and then for 3 h at 10°C under UV light. Samples were thin (90 nm) sectioned with a diamond knife on a Reichert Ultracut E microtome (Leica, Paris) and were examined under a transmission electron microscope operating at 80 kv (Jeol 100EX), after treatment. Sections cut from 3-, 6-, and 18-h-pi cotyledons were monitored. The peak of superoxide anion generation and the end of the oxidative burst in the incompatible interaction corresponded to 3 and 6 h pi, respectively. The increasing phase of POD activity, as seen in Figure 1, coincided with 18 h pi. From 2 days pi onward, fixation and embedding of infected tissues were difficult, due to dryness of HR lesions.

For immunolocalization, all solutions were prepared in 20 mM PBS (pH 7.2) with 0.5% bovine serum albumin (BSA) and 0.05% Tween 20 (TBST). After blocking in 2.5% BSA (20 min at room temperature), sections were incubated on a drop of anti-cotton peroxidase polyclonal antibody diluted in TBST (1:30) for 60 min at 37°C. After three washes in TBST (10 min each under gentle shaking), sections were incubated for 30 min at 37°C on gold-labeled goat antirabbit antibodies (GAR-15, Biocell Research Laboratories, Cardiff, U.K.) diluted 1:20 in TBST.

Specificity of labeling was assessed through the following control experiments performed on sections from healthy and infected cotyledons: i) incubation with antibody previously adsorbed with the corresponding antigen, and ii) incubation with preimmune rabbit serum instead of the primary antiserum. Quantitation of labeling (D) over sections was compared by determining the number of gold particles per square µm ± standard error. The density of labeling was calculated as follows: D = number of gold particles (Ns) divided by surface area (Sa). Densities were determined by counting the number of gold particles over specified cell areas on 8 to 15 micrographs taken randomly from serial sections made on each of three blocks from *X. campestris* pv. *malvacearum*-infected or water-infiltrated cotyledons.

Construction and screening of two cDNA libraries.

Two *X. campestris* pv. *malvacearum* 18-induced cDNA libraries were constructed on the basis of the observed dual-phase POD activity during the incompatible interaction. Total RNA was isolated 2.5 and 5.5 h pi, using the RNeasy plant

mini kit (Qiagen, Courtaboeuf, France). Both libraries were constructed using the SMART PCR cDNA library construction kit (Clontech, Palo Alto, CA, U.S.A.) and packaged with the Gigapack III gold packaging extract (Stratagene, Amsterdam), according to the supplier's instructions.

The libraries were screened using the P18a oligonucleotide (Table 3) as probe. P18a is derived from the catalytic site of *pod4* (accession number AF155124), which is highly conserved among guaiacol peroxidases. P18a was labeled with γ -³²P dATP, using the 5' end labeling kit (Amersham, Bucks, UK), and was purified on mini Quick Spin Oligo columns (Boehringer Mannheim, Indianapolis, IN, U.S.A.). The plaques were transferred to nylon Hybond N+ filters (Amersham Pharmacia, Varsay, France). The membranes were prehybridized 3 h at 65°C in a buffer containing 6 × NET (0.9M NaCl, 0.09M Tris, pH 8.3, 6 mM EDTA), 0.05% SDS, 0.05% NaPyrophosphate, 5× Denhardt's reagent, and 100 µg of sheared salmon sperm DNA per ml and were hybridized overnight at 42°C in fresh buffer without DNA. The membranes were then washed 5 min at 42°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS, 30 min at 32°C, and 5 min at 42°C, before exposure to autoradiogram films.

Plaques that hybridized to P18a were purified by three rounds of replating and screening. Plasmids were excised from the phage according to the manufacturer's instructions (Clontech).

The 5' and 3' ends of a peroxidase-related expressed sequence tag (EST) provided by M. Patil and M. Essenberg (Oklahoma State University, Tulsa, U.S.A.) were cloned by PCR from the cDNA libraries, using the 5' *pod10* and 3' *pod10* primers (Table 3). An actin EST was also isolated from the cDNA libraries by PCR, using the degenerated primers ACT3 and ACT4, provided by J. Tregor (IRD, Montpellier, France) (Table 3). A 300-bp band was gel-purified and cloned into pGEM-T (Promega, Charbonnières, France). The POD cDNA sequences are available in the GenBank database.

The deduced proteins were aligned using the ClustalW (1.81) multiple sequence alignment program (Infobiogen, Paris).

RT-PCR.

Total RNA was extracted from water-treated and *X. campestris* pv. *malvacearum*-infected cotyledons by the guanidium thiocyanate/CsCl method described by Maliyakal (1992), at different times after infiltration. Using specific primer pairs (Table 1), the expression profile of each cDNA clone was determined by RT-PCR. Total RNA (7.5 µg) was reverse-transcribed with StrataScript Reverse Transcriptase (Stratagene) using oligo-(dT)₁₈ as primer. PCR products were run on 1.5% agarose gels in Tris-acetate-EDTA buffer, and band intensities within each experiment were compared after ethidium bromide staining. For each primer pair, bands from three independent PCR reactions were cloned and were sequenced to confirm their identities. The entire experiment was repeated twice.

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LITERATURE CITED

Abraham, K. J., Pierce, M. L., and Essenberg, M. 1999. The phytoalexins desoxyhemigossypol and hemigossypol are elicited by *Xanthomonas* in *Gossypium* cotyledons. *Phytochemistry* 52:829-836.

- Al-Mousawi, A. H., Richardson, P. E., Essenberg, M., and Johnson, W. M. 1982. Ultrastructural studies of a compatible interaction between *Xanthomonas campestris* pv. *malvacearum* and cotton. *Phytopathology* 72:1222-1230.
- Assigbetsé, K., Cuny, G., Valette, V., Delannoy, E., Bresson, E., Jalloul, A., Daniel, J. F., Geiger, J. P. and Nicole, M. 1999. Cloning and characterization of a bacterial-induced peroxidase encoding cDNA from cotton (GenBank accession No. AF155124). *Plant Physiol.* 121:312.
- Bestwick, C. S., Brown, I. R., and Mansfield, J. W. 1998. Localized changes in peroxidase activity accompany hydrogen peroxide generation during the development of a non-host hypersensitive reaction in *Lactuca sativa* L. *Plant Physiol.* 118:1067-1078.
- Blee, K. A., Jupe, S. C., Richard, G., Zimmerlin, A., Davies, D. R., and Bolwell, G. P. 2001. Molecular identification and expression of the peroxidase responsible for the oxidative burst in French bean (*Phaseolus vulgaris* L.) and related members of the gene family. *Plant Mol. Biol.* 47:607-620.
- Bolwell, G. P. 1999. Role of active oxygen species and NO in plant defense responses. *Curr. Opin. Plant Biol.* 2:287-294.
- Bolwell, G. P., Bindschedler, L. V., Blee, K. A., Butt, V. S., Davies, D. R., and Gardner, S. L., Gerrish, C., Minibayeva, F. 2002. The apoplastic oxidative burst in response to biotic stress in plants: A three-component system. *J. Exp. Bot.* 53:1367-1376.
- Bradford, M. M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Brown, I., Trethowan, J., Kerry, M., Mansfield, J., and Bolwell, G. P. 1998. Localization of components of the oxidative cross-linking of glycoproteins and of callose synthesis in papillae formed during the interaction between non-pathogenic strains of *Xanthomonas campestris* and french bean mesophyll cells. *Plant J.* 15:333-343.
- Carpin, S., Crevecoeur, M., de Meyer, M., Simon, P., Greppin, H., Penel, C. 2001. Identification of a Ca²⁺-pectate binding site on an apoplastic peroxidase. *Plant Cell* 3:511-20.
- Cason, E. T., Jr., Richardson, P. E., Essenberg, M. K., Brinkerhoff, L. A., Johnson, W. M., and Venere, R. J. 1978. Ultrastructural cell wall alterations in immune cotton leaves inoculated with *Xanthomonas malvacearum*. *Phytopathology* 68:1015-121.
- Chittoor, J. M., Leach, J. E., and White, E. F. 1997. Differential induction of a peroxidase gene family during infection of rice by *Xanthomonas oryzae* pv. *oryzae*. *Mol. Plant-Microbe Interact.* 10:861-871.
- Chittoor, J. M., Leach, J. E., and White, E. F. 1999. Induction of peroxidase during defense against pathogens. Pages 171-193 in *Molecular Biology Intelligence Unit: Pathogenesis-Related Proteins in Plants*. S. K. Datta and S. K. Muthukrishnan, eds. CRC Press, New York.
- Daï, G. H., Nicole, M., Martinez, C., Bresson, E., Daniel, J. F., Andary, C., and Geiger, J. P. 1996. Flavonoids accumulate in cell walls, middle lamellae and callose-rich papillae during an incompatible interaction between *Xanthomonas campestris* pv. *malvacearum* (Race 18) and cotton. *Physiol. Mol. Plant Pathol.* 49:285-306.
- Davila-Huerta, G., Hamada, H., Davis, G. D., Stipanovic, R. D., Adams, C., and Essenberg, M. 1994. Cadinane-type sesquiterpenes induced in *Gossypium* cotyledons by bacterial inoculation. *Phytochemistry* 39:531-536.
- De Feyter, R., Yang, Y., and Gabriel, D. W. 1993. Gene-for-gene interactions between cotton R genes and *Xanthomonas campestris* pv. *malvacearum*. *Mol. Plant-Microbe Interact.* 6:225-237.
- Do, H. M., Hong, J. K., Jung, H. W., Kim, S. H., Ham, J. H., and Hwang, B. K. 2003. Expression of peroxidase-like genes, H₂O₂ production, and peroxidase activity during the hypersensitive responses to *Xanthomonas campestris* pv. *vesicatoria* in *Capsicum annuum*. *Mol. Plant-Microbe Interact.* 16:196-205.
- Essenberg, M., Grover, P. B., Jr., and Cover, E. C. 1990. Accumulation of antibacterial sesquiterpenoids in bacterially inoculated *Gossypium* leaves and cotyledons. *Phytochemistry* 29:3107-3113.
- Follin, J. C., Girardot, B., Mangano, V., and Benitez, R. 1988. Nouveaux résultats sur le déterminisme génétique de la résistance foliaire totale du cotonnier (*Gossypium hirsutum* L.) à la bactériose (*Xanthomonas campestris* pv. *malvacearum* (Smith)) Dye, race 18 et 20. *Cot. Fib. Trop.* 3:167-175.
- Gabriel, D., Burges, A., and Lazo, G. R. 1986. Gene for gene interaction of five cloned avirulence genes from *Xanthomonas campestris* pv. *malvacearum*. *Proc. Natl. Acad. Sci. U.S.A.* 83:6415-6419.
- Gradea, J., Mayda, M. E., Conejero, V., and Vera, P. 1996. Characterization of defense-related genes ectopically expressed in viroid-infected tomato plants. *Mol. Plant-Microbe Interact.* 9:409-415.
- Hilaire, E., Young, S. A., Willard, L. H., McGee, J. D., Sweat, T., Chittoor, J. M., Guikema, J. A., and Leach, J. E. 2001. Vascular defense responses in rice: Peroxidase accumulation in xylem parenchyma cells and xylem wall thickening. *Mol. Plant Microbe Interact.* 14:1411-9.
- Hillocks, R. J. 1992. *Cotton Diseases*. CAB International. Redwood Press. Melksham, U.K.
- Hiraga, S., Sasaki, K., Ito, H., Ohashi, Y., and Matsui, H. 2001. A large family of class III plant peroxidases. *Plant Cell Physiol.* 42:462-468.
- Hopper, D. G., Venere, R. J., Brinkerhoff, L. A., and Gholson, R. K. 1975. Necrosis induction in cotton. *Phytopathology* 65:206-213.
- Innes, N. 1983. Bacterial blight of cotton. *Biol. Rev.* 58:157-176.
- Jalloul, A., Montillet, J. L., Assigbetsé, K., Agnel, J. P., Delannoy, E., Daniel, J. F., Marmey, P., Geiger, J. P., and Nicole, M. 2002. Lipid peroxidation in cotton—*Xanthomonas* interactions. Role of lipoxygenases during the hypersensitive reaction and leaf blight. *Plant J.* 32:1-12.
- Maliyakal, E. J. 1992. An efficient method for isolation of RNA and DNA from plants containing polyphenolics. *Nucleic Acids Res.* 20:2381.
- Martinez, C., Geiger, J. P., Bresson, E., Daniel, J. F., Dai, G. H., Andary, C., and Nicole, M. 1996. Isoperoxidase are associated with resistance of cotton to *Xanthomonas campestris* pv. *malvacearum* (race 18). Pages 327-332 in: *Plant Peroxidases: Biochemistry and Physiology* O. Obinger, U. Burner, R. Ebermann, C. Penel, and H. Greppin, eds. University of Geneva, Switzerland and University of Vienna, Austria.
- Martinez, C., Montillet, J. L., Bresson, E., Agnel, J. P., Daniel, J. F., Geiger, J. P., and Nicole, M. 1998. Apoplastic peroxidase generates superoxide anions in cells of cotton cotyledons undergoing the hypersensitive reaction to *Xanthomonas campestris* pv. *malvacearum* race 18. *Mol. Plant-Microbe Interact.* 11:1038-1047.
- Martinez, M., Baccou, J. C., Bresson, E., Baissac, Y., Daniel, J., Jalloul, A., Montillet, J. L., Geiger, J. P., Assigbetsé, K., and Nicole, M. 2000. Salicylic acid mediated by the oxidative burst is a key molecule in the local and systemic resistance of cotton challenged by an avirulent race of *Xanthomonas campestris* pv. *malvacearum* race 18. *Plant Physiol.* 122:757-766.
- Nicholson, R., and Hammerschmidt, R. 1992. Phenolic compounds and their roles in disease resistance. *Ann. Rev. Phytopathol.* 30:369-389.
- Patzlaff, M., and Barz, W. 1978. Peroxidatic degradation of flavanones. *Zeit Naturfor.* 33c:675-684.
- Penel, C., and Greppin, H. 1996. Pectin binding proteins: Characterization of the binding and comparison with heparin. *Plant Physiol. Biochem.* 34:479-488.
- Penel, C., Van Cutsem, P., and Greppin, H. 1999. Interactions of a plant peroxidase with oligogalacturonides in the presence of calcium ions. *Phytochemistry* 51:193-198.
- Penel, C., Carpin, S., Crèvecoeur, M., Simon, P., and Greppin, H. 2000. Binding of peroxidase to Ca²⁺ pectate: Possible significance for peroxidase function in cell wall. *Plant Peroxidase Newsl.* 14:33-40.
- Tognolli, M., Penel, C., Greppin, H., and Simon, P. 2002. Analysis and expression of the class III peroxidase large gene family in *Arabidopsis thaliana*. *Gene* 288:129-38.
- Reimers, P. J., Guo, A., and Leach, J. E. 1992. Increased activity of a cationic peroxidase associated with an incompatible interaction between *Xanthomonas oryzae* pv. *oryzae* and rice (*Oryza sativa*). *Plant Physiol.* 99:1044-1050.
- Venere, R. J. 1980. Role of peroxidase in cotton resistant to bacterial blight. *Plant Sci. Lett.* 20:47-54.
- Welinder, K. G. 1992. Superfamily of plant, fungal and bacterial peroxidases. *Curr. Opin. Struct. Biol.* 2:388-393.
- Welinder, K. G., Justesen, A. F., Kjaergard, I. V. H., Jensen, R. B., Rasmussen, S. K., Jespersen, H. M., and Duroux, L. 2002. Structural diversity and transcription of class III peroxidases from *Arabidopsis thaliana*. *Eur. J. Biochem.* 269:6063-6081.
- Wojtaszek, P. 1997. Oxidative burst, an early plant response to pathogen infection. *Biochem. J.* 322:681-692.
- Yamasaki, H., Sakihama, Y., and Ikehara, N. 1997. Flavonoid-peroxidase reaction as a detoxification mechanism of plant cells against H₂O₂. *Plant Physiol.* 115:1405-1412.
- Young, S. A., Guo, A., Guikema, J. A., White, F. F., and Leach, J. E. 1995. Rice cationic peroxidase accumulates in xylem vessels during incompatible interaction with *Xanthomonas oryzae* pv. *oryzae*. *Plant Physiol.* 107:1333-1341.