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MOLECULAR BIOLOGY AND PHYSIOLOGY

Molecular Analysis of Class III Peroxidases from Cotton

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ABSTRACT

A molecular analysis of class III peroxidases from cotton (*Gossypium hirsutum* L.) was undertaken using the sequence of 12 genes available in databanks. Sequence comparison, phylogenetical analysis, and investigation of expression in organs were performed to characterize this group of peroxidases in cotton. All 12 genes possess the characteristics of class III peroxidases, including three conserved domains with the catalytic histidines involved in haem-binding and catalysis, four disulfide bridges, a salt bridge, a signal peptide that targets the protein to the secretory pathway, and putative N-glycosylation sites. Possible functions for these peroxidases according to their phylogenetical position and the existence of close orthologs in other plant families are suggested. Two class III peroxidases from cotton play a role in the oxidative burst response of cotton to bacterial blight.

Peroxidases are iron haem-containing oxidoreductases (EC 1.11.1.7) (Dunford, 2000) that reduce peroxides, mainly hydrogen peroxide, to water and subsequently oxidize small molecules, often aromatic oxygen donors (Penel et al., 1992). Based on their primary sequence, they fall into two superfamilies, the animal peroxidases and the plant peroxidases (Welinder, 1992). The plant peroxidase superfamily can be further divided into three classes: (a) class I contains intracellular peroxidases related to bacterial peroxidases, such as ascorbate peroxidases or bacterial catalase-peroxidases, or the cytochrome c peroxidase of *Saccharomyces cerevisiae* Meyen ex E.C. Hansen, (b) class II contains fungal-secreted peroxidases, i.e. lignin peroxidases or manganese peroxidases, and (c) class III peroxidases are plant peroxidases that target the secretory pathway by their signal peptide. Class III peroxidases form a large multigenic family in plants (Hiraga et al., 2001; Delannoy et al., 2004). Seventy-three genes were identified in the genome of *Arabidopsis thaliana* (L.) Heynh (Tognolli et al., 2002), and 138 were identified in *Oryza sativa* (L.) cv. Japonica (Passardi et al., 2004). At least 31 ESTs were found in *Glycine max* (L.) Merr. (TIGR Soybean Gene Index, release 12.0), and 27 in *Solanum tuberosum* L. (TIGR Potato Gene Index, release 9.0). These findings reinforce the importance of class III peroxidases in plant metabolism, since they have been associated with various processes, such as cell wall organization (Blee et al., 2003; Carpin et al., 2001), germination, hormonal, and/or stress responses (Chittoor et al., 1998; Dong et al., 1999).

In recent studies on the hypersensitive response (HR) of *G. hirsutum* cv. Réba B50 to the causal agent of bacterial blight, *Xanthomonas campestris* pathovar *malvacearum* (Smith) Dye *(Xcm)*, nine class III peroxidases were cloned that showed differential expression in response to the pathogen (Delannoy et al., 2003). In this research, the sequences of 12 cotton peroxidase genes now available in databanks were analysed and compared with other class III plant peroxidase genes.

MATERIALS AND METHODS

Sequence retrieval and alignments. Genes encoding class III peroxidases were extracted from the non–redundant section of GenBank (August 2004; National Center for Biotechnological Information; http://www.ncbi.nlm.nih.gov/GeneBank/index/html) and from the TIGR Cotton Gene index (release 6.0; Institute for Genomic Research; http://www.tigr.org) by keyword searches. In the TIGR database, EST sequences were assembled in overlapping contigs to form tentative consensus sequences (TC). Full-length cDNAs were aligned using ClustalW (European Bioinformatics Institute; http://www.ebi.ac.uk/clustalw/index.html) with the parameters as follows: gap open penalty = 15, gap extension penalty = 6,66,

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and delay divergent sequence = 60%. Default values were used for the other parameters.

Deduced, full-length amino-acid sequences were aligned using ClustalX (Bioinformatics Platform of Strasbourg; http://bips.u-strasbg.fr/en/Documentation/ClustalX/) as described (Duroux and Welinder, 2003). Briefly, the parameters used were as follows: gonnet protein weight matrix, gap open penalty = 10, gap extension penalty = 1, and delay divergent sequence = 70%. Default values were used for the other parameters.

Identity and similarity matrix were calculated using Vector NTI alignX software (InforMax, Inc.; North Bethesda, MD).

Phylogenetic construction. A phylogenetic analysis of cotton peroxidases was performed using 98 selected plant peroxidases. The peroxidase sequence of *Marchantia polymorpha* L. (Genbank AB97197), a liverwort, was used to root the trees as described (Passardi et al., 2004). Distance and maximum likelihood analyses were done with the help of the Phylogeny Inference Package (PHYLIP 3.63a; http://evolution.genetics.washington.edu/phylip.html) (Felsenstein, 2001). Pair-wise distances were calculated from the alignment with PROTDIST using the PAM substitution model and 250 bootstrap replicates were carried out for this method with the SEQBOOT option. The maximum likelihood tree was inferred using PROML with the PAM substitution model, global optimization, and multiple jumble (10 times) options selected.

Expression profiles. Cotton peroxidase expression profiles were studied by RT-PCR in *G. hirsutum* cv. Réba B50 as previously described (Delannoy et al., 2003). Briefly, samples were ground in an extraction buffer containing 4M guanidine isothiocyanate, 200 mM Tris-acetate (pH 8.5), 1% polyvinyl pyrrolidone (MW 40kD), 0.62% sodium lauroyl sarcosine, and 0,7% β-mercaptoethanol. After centrifugation, RNA was purified from the clear supernatant by ultracentrifugation on a cesium chloride cushion. Total RNA was extracted from cotyledonary leaves, root tips, and stems of 10-d-old seedlings and from 6-wk-old flower buds and mature leaves. Experiments were repeated three times.

RESULTS

Genes retrieved from databases and used for the analysis. Eleven full-length cDNAs of class III peroxidases (*pods*) from *G. hirsutum* were found in the databases. Nine of them, *pod1* (AF485266), *pod2* (AY074794), *pod3* (AF485265), *pod4* (AF155124), *pod5* (AF485267), *pod6* (AF485268), *pod7* (AY311597), *pod9* (AY366083), and *pod10* (AF488305), were identified during previous work on cotton interaction with *Xcm* (Delannoy et al., 2003). Three others peroxidases were identified: *pod8* (Cotproxds; L08199) was highly induced in cotyledons during cotton embryo development and germination, *pod11* (AF311351) was isolated from aphid infected leaves, and *pod12* (TC20645) was an EST found in the TIGR database and corresponded to a virtual partial cDNA. Since it was only a partial sequence, *pod12* was not used in DNA sequence analysis, but produced information about the putative mature protein. Several other cotton peroxidase genes in Genbank, such as AI725603 and AI728054, or in the TIGR Cotton Gene index were not taken into account in the present molecular analysis because of their incomplete nucleotide sequence and the absence of putative protein sequence.

Comparisons of sequences of cotton peroxidases. Nucleotide sequences of cotton peroxidases cDNAs range from 1125 to 1531bp (Table 1). The percentage of similarity between sequences is highly variable, ranging from 29 to 71% between *pod5/pod11* and between *pod2/pod6*, respectively (Table 2). The alignment of the putative amino-acid sequences also confirmed the variability of cotton peroxidase proteins (PODs) with the sequence similarity ranging from 17% between POD10 and POD11 to 82% between POD2 and POD6 for the putative mature proteins (Table 3). POD11 had the lowest sequence similarity with all other PODs. POD7, POD3, POD4 and POD6, POD2, POD5 form two groups that are confirmed by a similar analysis on cDNA sequences (data not shown).

Analysis of cotton peroxidase proteins. Based on bioinformatic predictions, these genes encode proteins of 316 to 347 amino acids with a signal peptide (Table 1). After maturation, their molecular weights range from 31.8 to 34.9 kD, and they are potentially secreted extracellularly. The molecular weights of these PODs in planta are likely different due to the carbohydrate moiety that increases their weight. POD1, POD5, POD9, POD11, and POD12 are predicted to be cationic proteins, whereas POD2, POD3, POD4, POD6, POD7, POD8, and POD10 are predicted to be anionic.

Table 1. Molecular characteristics of twelve cotton peroxidase genes and their corresponding deduced proteins

	cDNA length (bp)	AA sequence length of the putative mature protein	Potential signal peptide cleavage sites[z]	Molecular weight (kD)[y]	Estimated pI for mature protein[y]	Potential N-glycosylation sites[x]	Probable cellular localization[w]
pod1	1125	329	19 to 20	34.3	5.29	3	EC
pod2	1330	328	27 to 28	32.8	8.87	1	EC
pod3	1335	320	23 to 24	34.4	8.46	3	EC
pod4	1369	317	19 to 20	31.7	8.72	2	EC
pod5	1531	323	21 to 22	32.5	5.66	0	EC
pod6	1389	328	26 to 27	32.4	8.34	1	EC
pod7	1282	330	34 to 35	31.8	9.29	2	EC
pod8	1175	322	23 to 24	32.4	7.89	5	EC
pod9	1250	347	26 to 27	34.6	5.15	10	EC
pod10	1333	332	25 to 26	34.9	8.10	1	EC
pod11	1143	328	24 to 25	33.0	4.94	8	EC
pod12	N/A	332	26 to 27	33.0	5.23	2	EC

[z] Sequence analysis performed with SignalP ; Center for Biological Sequence Analysis Lyngby, Denmark

[y] Sequence analysis performed with Mwcalc; Infobiogen, Paris, France

[x] Sequence analysis performed with Proscan; Pole Bio-Informatique Lyonnais, Lyon, France

[w] Sequence analysis performed with PSORT; GenomeNet, Kyoto, Japan; EC = Extracellular

Table 2. Similarity matrix of cDNAs

pod2	48									
pod3	50	51								
pod4	49	50	58							
pod5	37	50	36	36						
pod6	47	71	50	50	48					
pod7	52	48	64	67	36	49				
pod8	46	46	48	49	34	48	52			
pod9	57	50	52	50	38	48	54	48		
pod10	51	52	54	52	39	51	53	50	62	
pod11	46	42	50	48	29	42	51	42	51	45
	pod1	*pod2*	*pod3*	*pod4*	*pod5*	*pod6*	*pod7*	*pod8*	*pod9*	*pod10*

Table 3 . Similarity matrix of putative mature proteins

POD2	41									
POD3	44	44								
POD4	46	48	67							
POD5	40	56	42	41						
POD6	40	82	46	47	56					
POD7	43	46	75	74	42	46				
POD8	40	50	51	53	42	50	50			
POD9	38	45	45	48	39	45	48	55		
POD10	32	38	40	37	36	36	40	34	32	
POD11	20	23	28	29	22	22	28	31	24	17
POD12	34	41	43	42	43	44	41	43	37	36	22
	POD1	POD2	POD3	POD4	POD5	POD6	POD7	POD8	POD9	POD10	POD11

Figure 1. Sequence alignment of the putative class III peroxidases cloned from *G. hirsutum* L. Predicted cleavage site of the signal peptide:/; amino acids C in green: cysteines involved in disulfide bridges; amino acids in blue background: residues involved in a salt bridge; amino acids in brown background: putative N-glycosylation sites; amino acids in yellow background: residues involved in calcium binding: amino acids in red: catalytic amino acids. The three conserved domains (A, D, and F) of class III peroxidaese are indicated by red boxes.

The characteristics of class III peroxidases were provided by aligning all amino acid sequences (Fig. 1). The three conserved domains of class III peroxidases, B, D and F, are present in all cotton POD, except POD11 that seems to lack domain D. This domain has no known function in peroxidase activity, whereas B and F domains are the distal and proximal haem-binding domains, respectively, and each contains one catalytic histidine (Gajhede, 2001). The alignment also showed eight conserved cysteines potentially forming four disulfide bridges, four amino acids involved in a buried salt bridge, and the residues forming two calcium-binding sites. Other conserved amino acids are the catalytic residues, such as two histidines in the B and F domains involved in electron transfer and haem-binding, respectively. Except POD5, these proteins are putatively glycosylated to varying degrees. In addition to their signal peptide, POD1 and POD9 display a carboxy-terminal extension.

Expression profile of cotton class III peroxidase genes in various tissues. The expression profile of 10 cotton *pod* genes in several tissues was studied by RT-PCR (Fig. 2). *Pod1, pod5, pod9,* and *pod10* were expressed in all tested organs. *Pod5* was expressed less in leaves than the other *pods*.

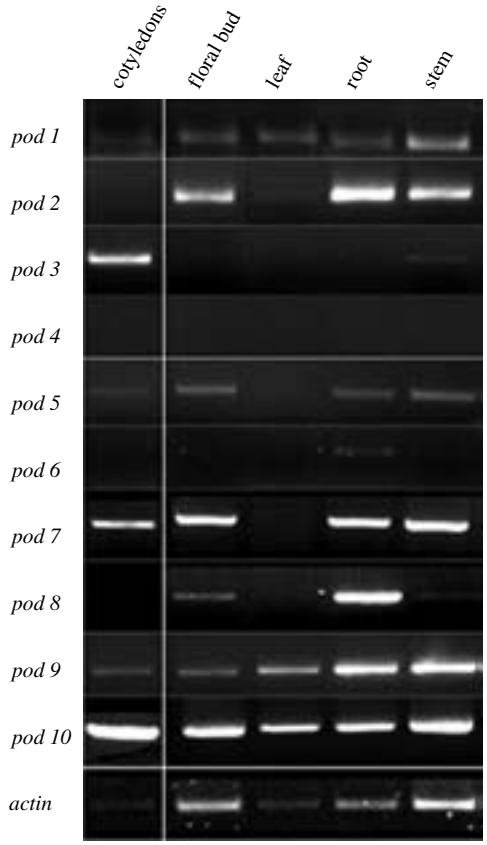

Figure 2: Expression profile by RT-PCR of 10 cotton peroxidase genes in various organs of *G. hirsutum* cv. Réba B50.

Pod2 and *pod6* were not detected in cotyledons, while *pod7* was not observed in leaves. *Pod3* was expressed in cotyledons and stems. *Pod8* was not detected in foliar organs. *Pod4* was not detected in any of the tested organs. None of these genes was organ specific, but in *A. thaliana* (Valerio et al., 2004) and *O. sativa* (Hiraga et al., 2001), root, stem, and floral bud tissues seem to be a preferential place for *pod* expression.

Phylogenetical analysis. One hundred and ten full-length protein sequences from class III PODs were selected for the phylogenetic analysis, covering 28 genera of both monocotyledons and dicotyledons. Among the analyzed sequences were 15 from *O. sativa*, 14 from *A. thaliana*, and 12 from *G. hirsutum*. A tree was constructed that discriminates all PODs (Fig. 3). PODs from mono and dicotyledons are interspersed, as it is also the case with PODs from the same genus, with the exception of PODs from *Populus*. Cotton PODs are also dispersed in the tree. The position of POD11 in this tree is not supported by bootstrap analysis probably because this peroxidase is only distantly related to other peroxidases, which reinforces the peculiar status of POD11. POD10 grouped with P42 (*A. thaliana*) and GM2 (*G. max*) at the base of the tree, forming a small group of closely related peroxidases (between 79 and 87% identity) that are distinct from other peroxidases.

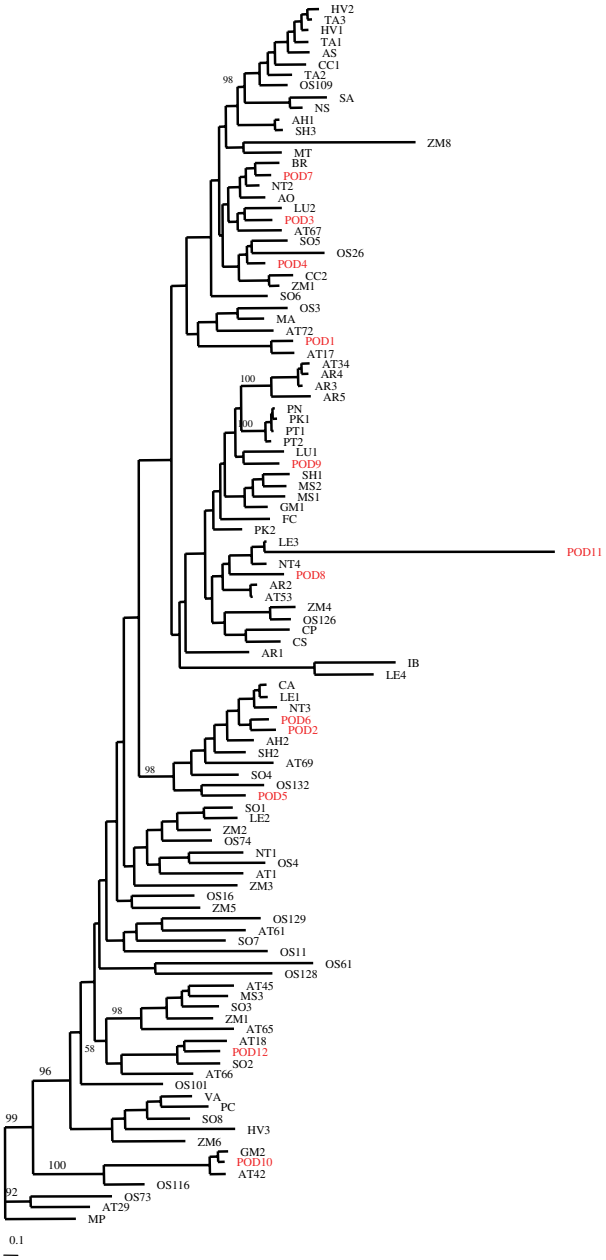

Figure 3: Phylogenetic comparison of cotton peroxidases based on predicted sequence of the protein. The tree was contructed using the maximum likelihood method and rooted with the peroxidase sequence of *Marchantia polymorpha*. Bootstrap values over 50% are indicated. All branches are drawn to scale and the scale represents 0.1 substitution per site. Cotton peroxidases are indicated in red. AH: *Arachis hypogea* L. 1: M37636, 2: M37637. AO: *Asparagus officinalis* L. CAD67479. AR: *Armoracia rusticana* G. Gaertner, B. Meyer & Scherb.. 1: Q42517, 2: P80679, 3: P15233, 4: P00433, 5: P17180. AS: *Avena sativa* L. AAC31551. AT: *Arabidopsis thaliana* L. Peroxidases named according to Tognolli et al. (2002). BR: *Brassica rapa* L. P00434. CA: *Capsicum annuum* L. AF442386. CC: *Cenchrus ciliaris* L. 1: U12315, 2: U12314. CS: *Cucumis sativus* L. AAA33121. CP: *Cucurbita pepo* L. Y17192. FC: *Ficus carica* L. AAL85344. GM: *Glycine max* (L.) Merr. 1: pdb|1FHF|, 2: AAC83463. HV: *Hordeum vulgare* L. 1: L36093, 2: CAB99487, 3: CAA80667. IB: *Ipomea batatas* Lam. AAF00094. LE: *Lycopersicon esculentum* Mill. 1: X94943, 2: S51584, 3: S32768, 4: P15003. LU: *Linum usitatissimum* L. 1: L07554, 2: L24120. MA: *Medicago annua* L. X91232. MP: *Marchantia polymorpha* L. BAB97197. MS: *Medicago sativa* L. 1: L36156, 2: L36157, 3: X90695. MT: *Medicago truncatula* L. AAB48986. NS: *Nicotiana sylvestris* L. M74103. NT: *Nicotiana tabacum* L. 1: T02960, 2: AB027752, 3: AB027753, 4: P11965. OS: *Oryza sativa* L. Peroxidases named according to Passardi et al. (2004). PC: *Petroselinum crispum* (Mill.) Nyman ex AW Hill L36981. PK: *Populus kitakamiensis* L. 1: BAA06334, 2: S60054. PN: *Populus nigra* L. D83224. PT: *Populus trichocarpa* Torr. & Gray. 1: X97349, 2: X97350. SA: *Striga asiatica* (L.) Kuntze AF043234. SH: *Stylosanthes humilis* Kunth. 1: L36111, 2: L36112, 3: AAB02554. SO: *Spinacia oleracea* L. 1: CAA76374, 2: CAA71496, 3: CAA71495, 4: CAA71494, 5: CAA71493, 6: CAA71492, 7: CAA71489, 8: CAA71488. TA: *Triticum aestivum* L. 1: AAM76682, 2: S61405, 3: S61407. TR: *Trifolium repens* L. CAA09881. VA: *Vigna angularis* (Willd.) Ohwi & Ohashi JQ2252. ZM: *Zea mays* L. 1: CAD57868, 2: CAD57867, 3: CAD57866, 4: CAD57861, 5: CAD57856, 6: CAD57853, 7: CAD57852, 8: AAC79953.

DISCUSSION

The recent isolation of several class III *pods* in cotton permitted molecular analysis of this gene family in *G. hirsutum* and comparison of them with other plant species. Eleven of the 12 cotton peroxidase genes available for the analyses possess the following characteristics of class III peroxidases: three conserved domains with the catalytic histidines involved in haem-binding and catalysis, four disulfide bridges and a salt bridge that constrain the tertiary structure of the enzyme, a signal peptide that targets the protein to the secretory pathway, and N-glycosylation sites that may influence peroxidase stability and activity (Lige et al., 2001; Duarte-Vazquez et al., 2003). The carboxy-terminal extension present in POD1 and POD9 is probably removed during maturation of the protein (Johansson et al., 1992; Theilade and Rasmussen, 1992). Although its function is unknown, this peptide may be involved in targeting the peroxidase to the vacuole (Huh et al., 1997).

Pod11 seems to encode a peculiar peroxidase lacking several characteristics of the other class III enzymes. This putative peroxidase lacks the conserved D domain, and its B domain is shifted toward the amino-end of the protein. The B domain is the distal haem-binding site and contains several catalytic amino-acids, one of the two calcium binding pockets, and one disulfide bridge. Furthermore, POD11 lacks four cysteines that potentially correspond to the lack of three of the four disulfide bridges existing in all class III peroxidases. This suggests that the tertiary structure of POD11 is different from classical class III peroxidases. Because folding of class III peroxidases is highly conserved, POD11 may not be functional, or may not function as a classical class III peroxidase. It is interesting to note that no EST corresponding to this gene cloned from aphid-infected leaves is available in the TIGR Cotton Gene Index (release 6.0).

The phylogenetic analysis confirmed the high diversity of cotton peroxidases since the 12 cotton peroxidases are not clustered, but are part of several groups of peroxidases. Even if there are several other class III peroxidases to be identified in the cotton genome, the analysis of these 12 genes suggests that cotton class III peroxidases cannot be distinguished from other plant peroxidases when considering their sequence or their phylogenetic position. This analysis may provide some hints about the possible function of cotton peroxidases, such as *pod9* and *pod10.* A cotton peroxidase gene, *pod10,* and *P42*, a closely related gene from Arabidopsis (Valerio et al. 2004), were constitutively expressed in all tested organs of their respective plants. The basal position in the tree, the existence of close orthologs in other plant families, and its expression profile suggest an important but yet unknown function for *pod10.*

A phylogenetic analysis of plant peroxidases suggests that class III PODs appeared as plants emerged from water in correlation with the appearance of cell wall structure adapted to terrestrial life (Passardi et al., 2004). Interestingly, the only coherent group of PODs in the phylogenetic tree is mainly composed of PODs from woody plants (*Populus* or *Ficus*), and this group is strongly supported by distance analysis (data not shown). Furthermore, PXP3-4 (PT2: CAA66036) oxidizes syringaldazine and is involved in lignin biosynthesis (Christensen et al., 2001), so this cluster may correspond to PODs specifically involved in the formation of wood. It would be interesting to test whether class III PODs from other trees are included in this cluster. Interestingly, POD9 is part of this cluster and is 71% identical to PXP3-4, which indicates it may also be involved in lignification.

Other cotton *pod* genes have orthologs identified in a functional study of plant-pathogen interactions. POD2 is 77% identical to CAPO1 (CA: AF442386) from *Capsicum annuum* L. whose expression is inhibited during a hypersensitive response to *X. c.* pathovar *vesicatoria* (Do et al., 2003). POD6 is 78% identical to CEVI16 (LE1: X94943) from *Lycopersicon esculentum* Mill that is induced upon infection by a viroid (Gadea et al., 1996). POD3 and POD7 show 72 and 78% identity to TPOXC1 (NT2 : AB027752) from tobacco that is induced by tobacco mosaic virus infection (Hiraga et al., 1999).

Although these orthologs may give hints about cotton peroxidase functions, inferring the putative role of a peroxidase based on its sequence or phylogenetic position remains tentative for several reasons. First, the expression profile of peroxidase genes does not correlate with their phylogenetic position. *Pod1, pod5, pod9,* and *pod10* are expressed in the five organs tested, but they are dispersed in the phylogenetical tree. In addition, very orthologous peroxidase genes from *A. thaliana* have different expression profiles in selected organs (Valerio et al., 2004 ; Welinder et al., 2002). These observations indicate that even closely related peroxidase genes may not have the same physiological function. Second, most peroxidases do not have a known function, despite the fact that their activity

has been correlated with a wide range of physiological responses (Dunford, 1999). Very few peroxidase isoforms have been demonstrated to have a specific function. The expression profiles only yield correlations with organs or tissues. Because peroxidases genes may be subjected to translational regulation, the determination of precise roles remains elusive, which illustrates the observation that very homologous genes exhibited different expression profiles (Valerio et al., 2004; Dunand et al., 2003). Biochemical and reverse genetic studies appear necessary to unravel precise functions of the class III peroxidases.

In cotton, physiological studies on resistance to *Xcm* (Delannoy et al., 2005), identified key roles for peroxidase isoforms in the hypersensitive response of the cv Réba B50 to the avirulent race 18 (Delannoy et al., 2003; Venere, 1980). Several lines of evidence strongly indicated that a wall-bound peroxidase was involved in the production of superoxide anions ($O_2^{\cdot-}$) at the onset of the oxidative burst 3 h post-inoculation (Martinez et al., 1998). Increase in the activity of cationic peroxidase isoforms (pI 9.0 - 9.4), positive effects of peroxidase inhibitors on $O_2^{\cdot-}$ generation, immunolocalization of peroxidase proteins close to bacteria, and differential transcriptional activity of the *pod4* and *pod2* candidate genes reinforced the idea of a strong role of cotton peroxidase in the oxidative burst response to *Xcm* (Delannoy et al., 2003). The important and local accumulation of flavonoids in hypersensitive response areas at 10 h post-inoculation (Daï et al., 1996) was shown to be associated with an increase in the activity of peroxidase anionic isoforms (pI 4.1 – 4.6), indicating that they may act as a protectant from oxidative damage caused by phenols (Yamasaki et al., 1997).

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