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# Differential Expression of Peroxidase Isogenes During the Early Stages of Infection of the Tropical Forage Legume *Stylosanthes humilis* by *Colletotrichum gloeosporioides*

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**Infection of *Stylosanthes humilis* by the fungal phytopathogen *Colletotrichum gloeosporioides* is associated with an increase in peroxidase enzyme activity within 24 h postinoculation. Peroxidase gene expression was investigated as a first step towards understanding the regulation and functional importance of this host response to fungal attack. Four distinct cDNAs Shpx 2, 5, 6, and 12, isolated from a cDNA library of *S. humilis* contained deduced amino acid (aa) sequence motifs characteristic of peroxidases. Three of these (Shpx 2, 5, and 6) were full-length and their deduced proteins each fell into a different homology group based on comparisons with other plant peroxidases. Each cDNA appeared to hybridize to only one or two genes in *S. humilis*. mRNAs corresponding to Shpx2, Shpx6, and Shpx12 were expressed relatively abundantly in young leaves, with lesser expression of Shpx2 and Shpx6 and no expression of Shpx12 detected in roots. No expression of these genes was detected in stems or old leaves. The mRNA of Shpx5 was relatively abundant in stems and to a lesser extent in young leaves. However, infection of young leaves with *C. gloeosporioides* greatly increased expression of the mRNAs of Shpx2 and Shpx6 but not Shpx5 nor Shpx12 compared to mock-inoculated controls. The mRNA of Shpx6 was strongly induced by the pathogen 4 h postinoculation, a time which precedes fungal penetration, while Shpx2 was induced to higher levels than controls at 24 h after inoculation. The mRNAs of both Shpx2 and Shpx6 but not Shpx5 and Shpx12 were also induced by wounding. These results indicate that specific host peroxidase isogenes are induced at very early stages of the interaction of *C. gloeosporioides* with *S. humilis* and that host recognition of the pathogen appears to occur prior to physical penetration of the epidermal cell wall.**

*Additional keywords:* wounding, plant response, pathogen signals, gene family, anthracnose disease.

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The forage legumes *Stylosanthes* spp. are grown extensively in northern Australia, South-East Asia, and other tropical regions of the world (Burt et al. 1983). The fungal pathogen *Colletotrichum gloeosporioides* is the major threat to the continued expansion and usage of this legume in tropical pastures (Manners et al. 1992). *Stylosanthes* represents a genetically tractable host system for studies of legume-microbe interactions (Elliott and Manners 1993; Manners et al. 1992). Transgenic plants of the diploid annual *S. humilis* can be obtained at high frequency (Manners and Way 1989) making this species an amenable system for exploring the genetic engineering of resistant cultivars and for identifying molecular and physiological processes involved in pathogenesis.

Several investigators have shown that fungal pathogens can induce the production of new isoforms of peroxidases in their plant hosts (e.g., Svalheim and Robertson 1990; Kerby and Somerville 1989). Peroxidases (EC 1.11.1.7. donor:hydrogen-peroxide oxidoreductase) have been implicated in several plant functions of potential importance in plant-pathogen interactions including lignification (Walter 1992), cross-linking of cell wall components (Bradley et al. 1992), wound-healing (Sherf et al. 1993) and auxin oxidation (Grambow and Langenbeck-Schwich 1983). The induction of infection-related peroxidase isoforms can also be associated with systemic acquired resistance responses (Ye et al. 1990; Irving and Kuc 1990). The molecular cloning of peroxidase cDNAs and genes from various plant species (e.g. Lagrimini et al. 1987; Buffard et al. 1990; Roberts and Kolattukudy 1989) has permitted genetic analysis of the regulation and function of particular peroxidase isogenes. Although a large number of peroxidase cDNAs and genes have been cloned across the plant kingdom, there have been few comparative studies of the expression of multiple peroxidase isogenes in any particular plant–pathogen interaction. In tomato, at least two genes have been shown to be induced by *Verticillium albo-atrum* (Mohan and Kolattukudy 1990; Sherf et al. 1993), whilst another distinct gene is induced by viroid infection (Vera et al. 1993). In barley, infection with *Erysiphe graminis* f. sp. *hordei* results in the differential induction of at least two specific peroxidase isogenes (Thordal-Christensen et al. 1992). It is evident from these studies that multiple and perhaps spe-

cific infection-related isogenes of peroxidase may be induced during plant-pathogen interactions.

It has been reported that modifications of the host cell wall and induction of host peroxidase activity occur during primary infection of *S. guianensis* by *C. gloeosporioides* (Sharp et al. 1990; Ogle et al. 1990). The signaling and regulatory processes underlying the early induction of plant peroxidase activity in any plant system are not known. In the present investigation we have studied the induction of peroxidase enzyme activity at the molecular level in *S. humilis* during infection by *C. gloeosporioides*. We report the cloning of four cDNA peroxidase homologues and demonstrate that genes encoding these four distinct enzymes are differentially regulated during infection, wounding and development. Importantly, it is demonstrated that the induction of at least one peroxidase isogene occurs very early in the infection process at a time preceding primary penetration. These results provide a basis for a study of the very early recognition and signaling processes involved in peroxidase gene expression during fungal infection.

## RESULTS

### Peroxidase enzyme activity.

The molecular basis of peroxidase induction was studied in *S. humilis* cv. Paterson rather than *S. guianensis* as described previously (Sharp et al. 1990), because the efficient transformation system of the former (Manners and Way 1989) makes it more amenable to further molecular analysis. Peroxidase enzyme activity was measured over a time course of infection (Fig. 1). Significant induction of enzyme activity was detected 24 h postinoculation compared to mock-inoculated controls (Fig. 1). This result suggests that peroxidase induction occurs during the hemi-biotrophic phase of the infection process (Irwin et al. 1984; Bailey et al. 1992).

### cDNA cloning and sequence analysis of peroxidase homologues.

Four distinct cDNA clones of *S. humilis* encoding peroxidase homologues were identified and characterized by DNA sequencing to obtain deduced amino acid (aa) sequences (Fig. 2). These clones were termed Shpx2, Shpx5, Shpx6, and Shpx12 (GenBank Accession Nos. L36112, L36111, L36110, and L36231 respectively). The cDNA clones Shpx2, Shpx5, and Shpx6 appeared to be full length (see below), whilst Shpx12 was a partial cDNA clone. Significant characteristics of the DNA sequences and the deduced aa sequences of these full-length clones are summarized in Table 1. Each cDNA clone contained only one major open reading frame which commenced with the first 5′-3′ ATG codon of the sense strand in the full-length clones. These putative translation initiation codons of Shpx5 and Shpx6 were surrounded by motifs strongly homologous to the consensus translation initiation sequences reported for plants (Lutke et al. 1987), whilst that of Shpx2 had only two of the six surrounding bases in agreement with the consensus. In addition, the proposed initial methionine residues show a conserved positional relation to downstream deduced aa motifs typical of other peroxidase aa sequences reported previously (Fig. 2) further supporting the selection of these ATG codons for translation initiation.

The multiple alignment of the deduced aa sequences from other representative cDNAs encoding plant peroxidases (Fig. 2) indicated that the deduced proteins for Shpx2, 5, and 6 were complete and that these putative *S. humilis* peroxidases appeared to contain signal sequences at their N termini. Putative signal sequence cleavage sites were assigned for the deduced proteins of Shpx5 and Shpx6, according to the rules of von Heinje (1983), but no cleavage site could be determined for Shpx2 (Fig. 2). There were two, two, and nine potential N-glycosylation sites identified in the deduced aa sequences of Shpx2, Shpx6, and Shpx5, respectively (Bause 1983). The deduced proteins are cationic with probable pI values of 7.3 and 8.77 for the putative mature proteins of Shpx5 and Shpx6, respectively, with the pre-protein of Shpx2 having a pI of 8.18. The deduced amino acid sequences of the cDNA clones (Fig. 2) contain catalytic and heme binding domains characteristic of peroxidases (Henrissat et al. 1993; Kimura and Masao 1988). These two domains straddle another highly conserved intervening region of unknown function (Fig. 2, Buffard et al. 1990). These three domains are clearly present in the deduced proteins of Shpx2, Shpx5 and Shpx6, whilst the deduced aa sequence of the partial cDNA clone Shpx12 contains only the heme binding domain. Relationships among the four cDNA clones based on aa sequence homologies are shown in Figure 3, and strongly suggest that Shpx12 belongs to the same gene family as the full-length clones. Sequence homologies of the deduced aa sequences of Shpx2, Shpx5, and Shpx6 range from 38 to 64%. The three full-length cDNA clones were compared to the derived and/or deduced aa sequences of 26 previously reported plant peroxidase genes obtained from databases (Fig. 4). This analysis shows that plant peroxidases can be separated into very distinct groups based on deduced aa sequence homologies. The putative *S. humilis* peroxidases Shpx2, Shpx5, and Shpx6 each represented a different member of these diverse groups.

### Genomic organization of peroxidase genes.

The cDNA inserts did not cross-hybridize with each other under highly stringent hybridization conditions (data not shown) and therefore the cDNA inserts could be used directly as hybridization probes to investigate gene organization.

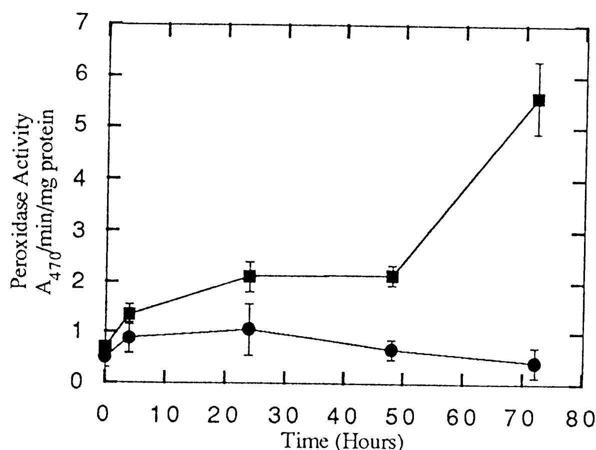


**Fig. 1.** Time course of peroxidase enzyme activity induction in *Stylosanthes humilis* cv. Paterson during infection by *Colletotrichum gloeosporioides* ( -■- ) and in mock-inoculated controls ( -●- ), the error bars indicate the range of two experiments.

Genomic DNA from *S. humilis* cv. Paterson was digested with either *Hpa*I, *Bcl*I, or *Kpn*I and resolved by 0.8% agarose gel electrophoresis. These restriction enzymes were chosen because *S. humilis* DNA appears to be highly methylated at cytosine residues and these enzymes are methylcytosine insensitive. Southern blots of agarose gels were probed with the cDNAs Shpx2, Shpx5, Shpx6, and Shpx12 (Fig. 5). Each cDNA probe gave a distinctive pattern of hybridization, indicating different genomic loci for each of the cDNA sequences. The simple banding patterns given by probes Shpx12 and Shpx2 are consistent with each being encoded by single copy genes. The more complex patterns given by Shpx6 and Shpx5 suggest that each may be encoded by at least two closely related genes. *S. humilis* is an inbreeding diploid and therefore should be homozygous for each of the Shpx genes.

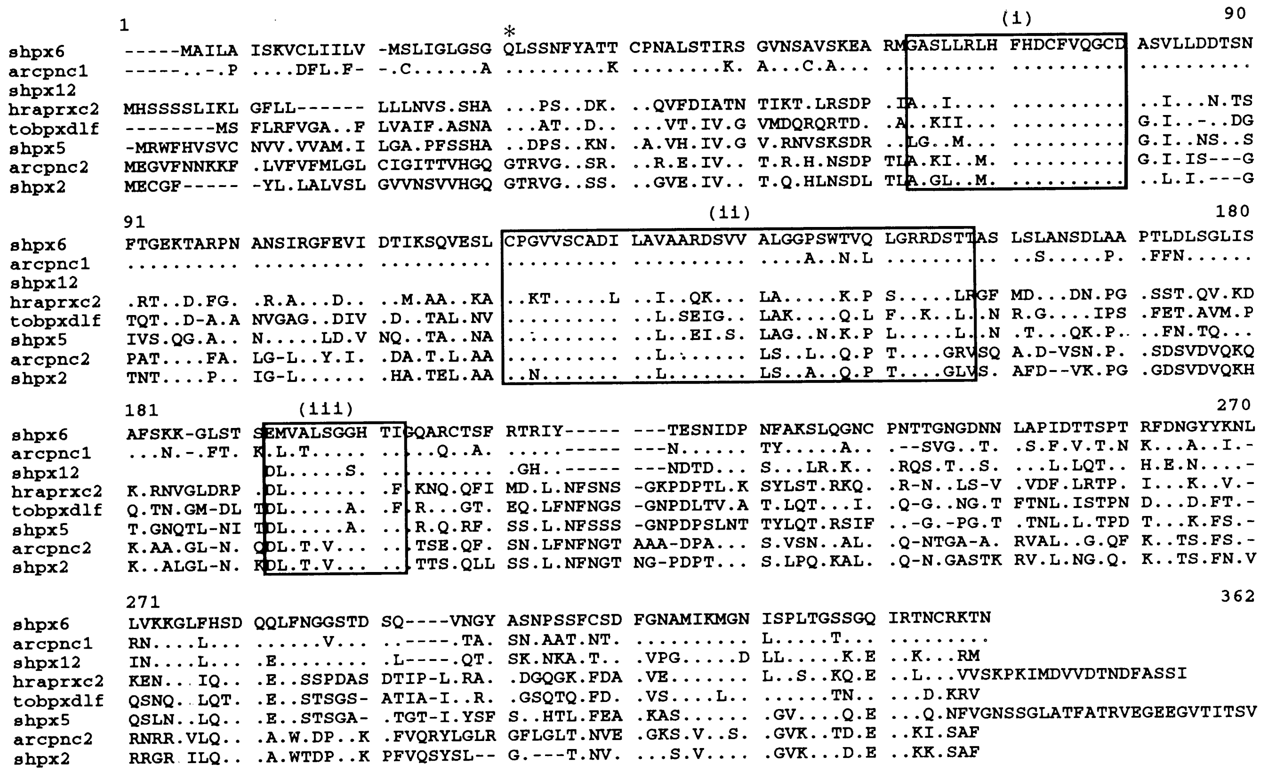


**Fig. 2.** Multiple alignment of the deduced amino acid sequences of the putative peroxidase cDNA clones of *Stylosanthes humilis* (Shpx2, Shpx5, Shpx6, and Shpx12) compared to those of other selected peroxidases (horseradish peroxidase [hraprx2], tobacco anionic peroxidase [tobpxdlf], peanut Pnc1 [arcpnc1], and Pnc2 [arcpnc2] ). The presumed cleavage sites for the signal sequences of the deduced proteins of Shpx5 and Shpx6 are indicated by *. The conserved catalytic (i), and heme-binding (iii) domains, and the conserved intervening region of unknown function (ii), are boxed. Amino acids identical to that deduced from Shpx6 are designated by a dot, and gaps (introduced to maximize alignment) are indicated by dashes.

**Table 1.** Characteristics of the nucleotide sequences and deduced amino acid sequences of the near-full length cDNAs Shpx2, Shpx5 and Shpx6 of *S. humilis*

| | Shpx2 | Shpx5 | Shpx6 |
|---|---|---|---|
| Insert length (nt[a]) | 1,175 | 1,312 | 1,144 |
| 5′ untranslated leader (nt) | 30 | 24 | 41 |
| 3′ untranslated region (nt)[a] | 185 | 213 | 159 |
| Translation start motif[b] | TTAAATGGA | AAAGATGGC | AACAATGGC |
| Possible poly A signal [c] | AATAA (21) | AATAAA (23) | AATAAA (64) |
| Deduced protein (aa[d]) | 337 | 328 | 320 |
| Deduced protein (*Mr*) | 35,980 | 34,920 | 31,250 |
| Signal sequence (aa) | ...[e] | 29 | 24 |
| Deduced preprotein pI | 8.18 | 8.33 | 9.02 |
| Deduced mature protein pI | ...[e] | 7.36 | 8.77 |
| *N*-glycosylation sites | 2 | 9 | 2 |

[a] Length in nucleotides (nt) excludes the poly $A^+$ tail.
[b] Bases in agreement with plant consensus are underlined.
[c] The distance, in nucleotides, from the poly $A^+$ tail is shown in parentheses.
[d] Amino acid residues.
[e] The probable signal sequence cleavage site in the deduced protein of Shpx2 could not be determined.

**Expression patterns of peroxidase mRNAs.**

To investigate the expression patterns of mRNAs hybridizing to the cloned peroxidase cDNAs during plant development, total RNA was isolated from young and old leaves (see Materials and Methods for sampling details), stems and roots. Northern blot analysis revealed that transcripts of all clones were expressed in young leaves, some expression of Shpx2, Shpx5, and Shpx6 was detected in roots, and Shpx5 was most abundantly expressed in stems (Fig. 6). No transcripts homologous to Shpx12 were detected in tissues other than young leaves. The size of the peroxidase mRNAs was estimated as approximately 1,300 bases by comparison with the ribosomal RNA species visualized on the gels. The expression of the peroxidase cDNAs was also examined by hybridization to total RNA isolated following the inoculation of young leaves of *S. humilis* with *C. gloeosporioides* (Fig. 7). The abundance of mRNA hybridizing to Shpx12 showed no change upon infection, whilst that of Shpx5 was slightly decreased. However the mRNAs of Shpx2 and Shpx6 showed induction following infection. The mRNA hybridizing to Shpx6 was strongly induced at 4 h following inoculation compared to controls, followed by a decrease in transcript levels at 24 h, and a large increase at 48 h after inoculation. A quite different pattern of mRNA induction was observed for transcripts hybridizing to Shpx2. First, some induction of Shpx2 mRNA was observed at 4 h after inoculation. Second, some induction was also observed for mock-inoculated controls at 4 h after treatment. Third, mRNA abundance remained elevated in the inoculated leaves at 24, 48, and 72 h following inoculation, whilst that of mock-inoculated controls declined back to 0 time levels by 48 h after treatment. Hybridization of blots to control probes for rDNA and a non-induced cDNA encoding a caffeic acid *O*-methyltransferase (McIntyre et al. 1995) demonstrated that these results were not biased by unequal loadings or blotting (Fig. 7).

For comparative purposes the effect of wounding young leaves on peroxidase gene expression was also investigated (Fig. 8). As with infection, wounding had little affect on the abundance of mRNAs corresponding to Shpx5 and Shpx12, but transcripts hybridizing to Shpx2 and Shpx6 were strongly induced within 6 h after wounding and declined in abundance at 24 h after wounding. In contrast to fungal infection, however, there was no observable difference in the pattern of expression of genes corresponding to Shpx2 and Shpx6 following wounding.

## DISCUSSION

This report describes the cloning and characteristics of four distinct cDNAs encoding peroxidase homologues from *Stylosanthes humilis*. At present these clones are assigned as peroxidases solely on the basis of compelling sequence homologies (Figs. 2 and 4) and definitive proof of function must await enzyme assays with the proteins they encode. The peroxidase cDNAs described here were so diverse that they did not cross-hybridize with each other and thus could be readily used to monitor relative mRNA abundance. The results of Northern hybridization analysis indicate the differential regulation of the genes homologous to the cloned peroxidase cDNAs. Dramatic induction of mRNAs hybridizing to Shpx2 and Shpx6 cRNAs was observed following fungal infection and wounding, whilst transcripts homologous to Shpx5 and Shpx12 were unaffected by these treatments. These results demonstrate that increased peroxidase enzymatic activity during the interaction of *S. humilis* with the phytopathogen *C. gloeosporioides* appears to be caused by the induction of expression of specific peroxidase isogenes.

A particularly interesting finding of this study was the detection of massive induction of mRNA homologous to the Shpx6 cDNA clone at very early stages of infection by *C. gloeosporioides*. This host response was detected at 4 h after

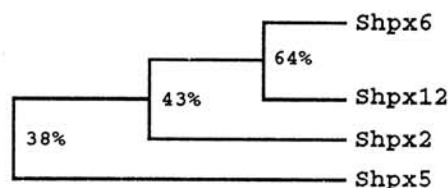


**Fig. 3.** Dendrogram showing the relatedness of the deduced aa sequences of the peroxidase cDNAs Shpx2, Shpx5, Shpx6, and Shpx12. Percentages determined using the Clustal V multiple sequence alignment program (Higgins et al. 1992).

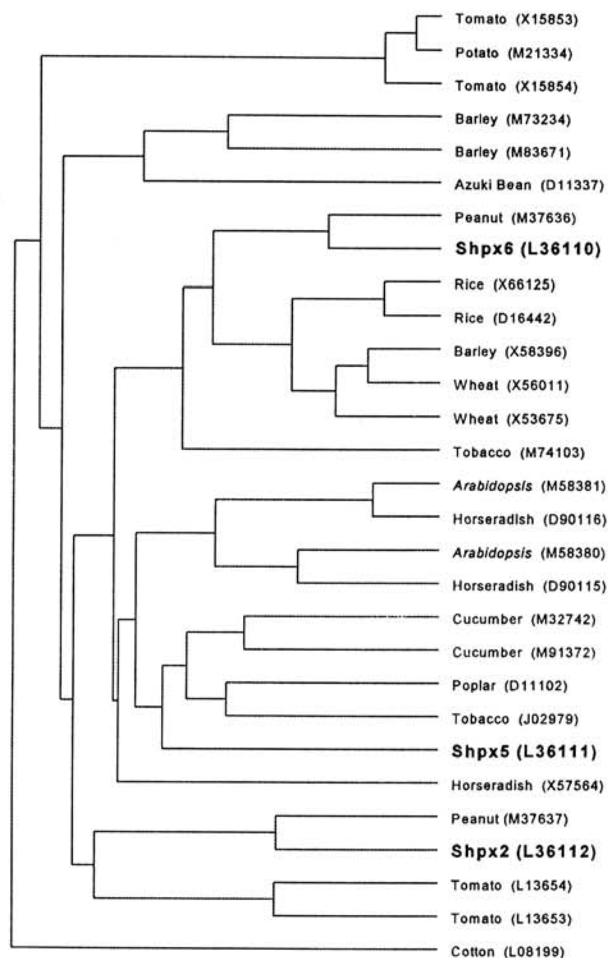


**Fig. 4.** Dendrogram showing the relationships among of the deduced aa sequences of the full-length cDNA clones Shpx2, Shpx5, and Shpx6 to those of 26 other peroxidases. The other peroxidases are indicated by the name of the plant of origin and their Genbank/EMBL accession number. The dendrogram was compiled using Pileup of the GCG package. The relative homologies between peroxidase sequences are proportional to the horizontal length of branches.

inoculation, much earlier than any response previously described for interactions between *Colletotrichum* pathogens and their hosts (e.g., Bell et al. 1986; Mahe et al. 1992). The histology of infection of biotype A of *C. gloeosporioides* has been described by Irwin et al. (1984) and Ogle et al. (1990). Spores germinate within 3 to 6 h after inoculation and then differentiate into an appressorium, which subsequently melanizes and forms a penetration peg at 6 to 12 h. Only a small percentage (5 to 10%) of melanized appressoria successfully penetrate the host cell wall and produce a vesicle in the epidermal cell. Between 12 and 48 h postinoculation secondary hyphae emerge from infected cells and infected host cells subsequently become necrotic with lesions visible at 5 to 6 days after inoculation. Substantial induction of mRNA homologous to Shpx6 was observed within 4 h of inoculation and thus must precede penetration of the host cell. This implies that signals released by either spores or germlings on the host surface are perceived by the host, but the nature of these putative signals is currently unknown. Interestingly, spores of *Colletotrichum* spp., including *C. gloeosporioides* are known to secrete an adhesion matrix prior to germination (Bailey et al. 1992; Jones et al. 1994) and it is possible that this material may be detected by the host. The identification of the signals perceived by the host at this early stage of infection is worthy of further investigation, as the early induction of peroxidase may play an important role in limiting the number of successful infections.

The biphasic induction kinetics of transcripts homologous to Shpx6 after inoculation with *C. gloeosporioides* suggests a complex range of signal exchanges and cell-cell interactions between host and pathogen during the time course of infection. Multiple phases of gene induction have been reported for other plant genes during fungal infection (Davidson et al. 1988; Clark et al. 1994). A reproducible drop in transcript abundance was observed at 24 h postinoculation following the early intense induction at 4 h noted above. Hemibiotrophic forms of *Colletotrichum* spp. such as the biotype A pathogen used in this study generally establish a transient biotrophic phase after penetration followed by host cell necrosis as the fungus ramifies through the tissue (Bailey et al. 1992). It is possible that the decline in abundance of transcripts hybridizing to Shpx6 represents a quiescent stage of host defense during this transient biotrophic phase. The cDNA clone Shpx6 appeared to be encoded on two genes in *S. humilis* and an alternative explanation for the biphasic induction observed may be that the two genes are differentially regulated during infection. The expression of each of the Shpx6 genes is currently being addressed via the isolation of genomic clones.

Transcripts homologous to Shpx2 were also induced by infection with *C. gloeosporioides*, but showed a different pattern of induction than mRNAs homologous to Shpx6. Thus, inoculated plants sustained a high level of Shpx2 mRNA throughout the experiment, and furthermore, the transient

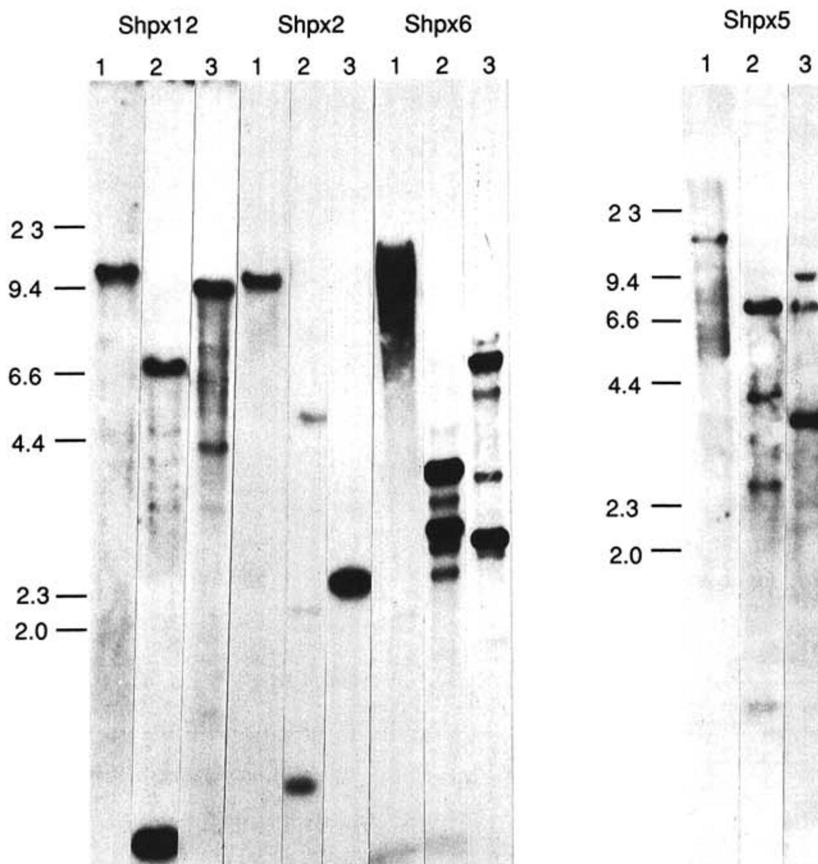


**Fig. 5.** Southern hybridization analysis of genomic DNA from *Stylosanthes humilis* cv. Paterson digested with *Bcl*I (lane 1), *Hpa*I (lane 2), and *Kpn*I (lane 3), respectively, and hybridized to the cDNA of Shpx12, Shpx2, Shpx6, and Shpx5. The sizes in kbp of *Hind*III-digested λ fragments are indicated.

induction of the Shpx2 mRNA in mock-inoculated controls was not observed for Shpx6. These differences in the induction patterns of mRNAs homologous to Shpx2 and Shpx6 indicate that the corresponding genes are regulated differentially and may respond to different signals. Gene induction of Shpx2 in mock-inoculated controls may be due either to the effects of the water treatment and/or the enclosure of the plants in a plastic bag to maintain humidity. Comparison of deduced aa sequences of these two cDNAs (Fig. 4) indicates that Shpx2 and Shpx6 show most homology to the peanut peroxidase cDNA clones Pnc2 and Pnc1, respectively (Buffard et al. 1990). The mRNAs of each of these two peanut clones were apparently absent from normal plant tissues, and were both only detected in callus and transiently in leaves following wounding; Pnc2 mRNA was also induced by ethephon, whilst Pnc1 mRNA was not (Breda et al. 1993). The possible role of ethylene in the induction of Shpx2 mRNA during infection by *C. gloeosporioides* clearly merits further investigation.

The deduced proteins of the three full-length peroxidase cDNA clones of *S. humilis*, Shpx2, Shpx5, Shpx6, and Shpx12 each fell into a different group when compared to a wide range of peroxidase aa sequences from other plant species (Fig. 4). This analysis shows that several divergent groups of homology can be recognized among plant peroxidases of known aa sequence. At present it is not known whether these aa sequence groupings have any relation to substrate specificity or in vivo function. Indeed, the in vivo function of most plant peroxidases is unknown. Northern hybridization analysis of RNA from control and inoculated leaves of *S. humilis* when probed with cDNA clones of *S. humilis* encoding phenylalanine ammonia lyase, caffeic acid

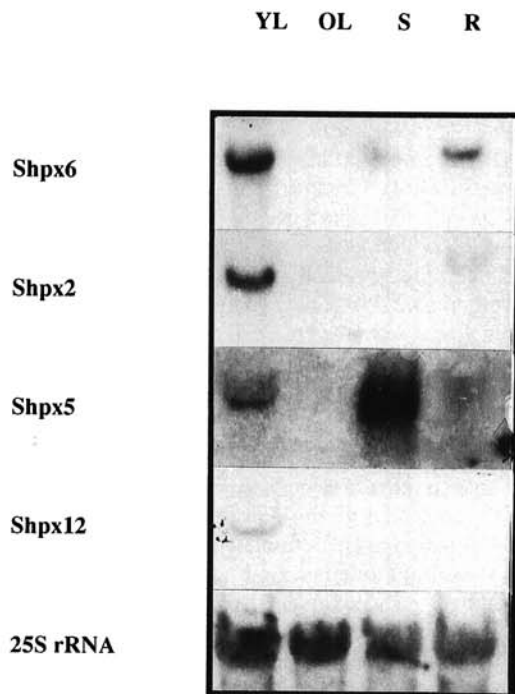


**Fig. 6.** Northern hybridization analysis showing differential expression of the mRNAs corresponding to cDNA clones Shpx2, Shpx5, Shpx6, and Shpx12 in various tissues of *Stylosanthes*. Total RNA was isolated from plant samples as described in the Materials and Methods and 20 µg aliquots of the RNA from young leaves (YL), old leaves (OL), young stem tissue (S), and root tissue (R) were hybridized to either the cDNA inserts as indicated or to a ribosomal DNA clone of wheat (pTA71, Gerlach and Bedbrook 1979) as a control on the RNA loading and blotting consistency.

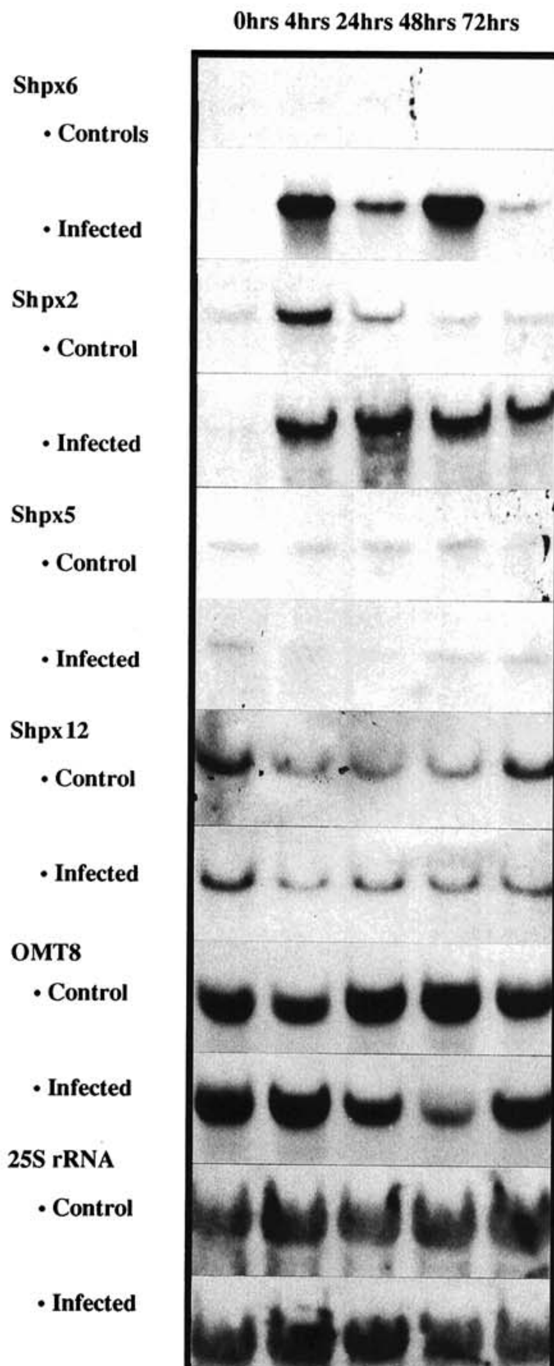


**Fig. 7.** Northern hybridization analysis showing differential expression of the mRNAs hybridizing to cDNAs Shpx2, Shpx5, Shpx6, and Shpx12 in young leaves of *S. humilis* following inoculation by *Colletotrichum gloeosporioides* or in mock-inoculated controls. Northern blots were hybridized with cDNA inserts, the rDNA probe described in Figure 6, and to a cDNA clone from *S. humilis* encoding a caffeic acid *O*-methyl transferase (OMT8, McIntyre et al. 1995) that is not induced by infection, as a control.

O-methyl transferase, and cinnamyl alcohol dehydrogenase showed that these genes were not induced over the time period studied herein (Curtis et al. 1995; McIntyre et al. 1995; Fig. 7; other data not shown). Thus induction of Shpx2 and Shpx6 mRNAs reported here does not appear to coincide with the activation of genes involved in general phenolic metabolism or lignin biosynthesis. The early induction of the putative Shpx6 peroxidase prior to penetration suggests a possible role in defense by either cross-linking host cell wall components (cf. Bradley et al. 1992) or the production of antimicrobial oxidative radicals (cf. Peng and Kuc 1992) during subsequent penetration events, and could contribute to the observation (noted previously) that most melanized appressoria of *C. gloeosporioides* fail to effect penetration and infection of *Stylosanthes* spp. (Ogle et al. 1990). Unfortunately near-isogenic lines of *S. humilis* (or any species of *Stylosanthes)* which differ in resistance and susceptibility to *C. gloeosporioides* are not available to assess the importance of peroxidase induction in resistance. However, Northern hybridization analysis of a resistant cultivar of a related species of *Stylosanthes, S. hamata* has also shown that a Shpx6 homologue is rapidly induced during infection (S. Harrison, unpublished data) suggesting that Shpx6 gene induction represents a general response. The question of whether the peroxidases encoded by Shpx2 and Shpx6 act in restricting infection can be tested by overexpression and gene suppression techniques in transgenic plants of *S. humilis*. Such experiments may provide a means for the functional dissection of individual members of this multigene family. Experiments aimed at the manipulation of Shpx2 and Shpx6 genes of *S. humilis* in transgenic plants are now in progress.

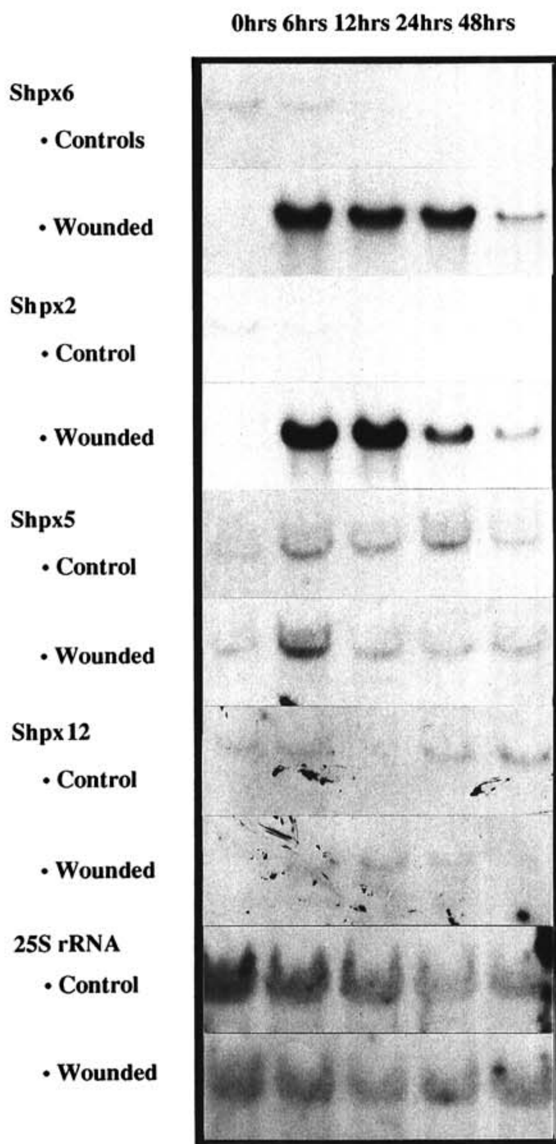


**Fig. 8.** Northern hybridization analysis showing differential expression of the mRNAs hybridizing to cDNAs Shpx2, Shpx5, Shpx6, and Shpx12 following wounding of young leaves of *Stylosanthes humilis*. Young leaves (see Materials and Methods) were wounded by squeezing between forceps and the RNA analyzed using cDNA inserts and a rDNA control as described in Figure 6.

## MATERIALS AND METHODS

### Plant and fungal material.

Mature plants of *Stylosanthes humilis* cv. Paterson were grown in controlled environment chambers with a 16 h, 28°C light period and 25°C 8 h dark period per day for approximately 2 mo after sowing. For most experiments plants were grown in soil but where root samples were to be taken plants were grown in vermiculite. A monoconidial culture of biotype A, strain SR24 of *C. gloeosporioides* was isolated by R. D. Davis, Queensland Department of Primary Industries at Southedge, North Queensland and is virulent on cv. Paterson. The fungus was cultured on oatmeal agar at 25°C under near-UV light. For inoculations, fresh spore suspensions at $10^6$ per ml were prepared in 0.1% Tween 20 and sprayed onto the plants until runoff. Control plants were sprayed with 0.1% Tween 20 only. All plants were enclosed in a plastic bag to maintain 100% humidity for 48 h at 24°C in the dark and then transferred to normal growth conditions. Disease symptoms were visible 4 to 5 days after inoculation. For sampling purposes young leaves were designated as the first three sets of unfurled and/or unexpanded leaves below the apical meristem, whilst old leaves were all fully expanded leaves below the young leaves.

### Peroxidase enzyme assays.

Freshly harvested young leaves (500 mg) were frozen in liquid $N_2$ for storage and subsequently homogenized at 4°C in a microcentrifuge tube with a custom-made tight fitting stainless steel grinder attached to an electric drill using 10 volumes per unit fresh weight of buffer, 10 mM sodium phosphate, 1% sodium metabisulphite pH 6.0. Homogenates were centrifuged at 14,000 rpm in a refrigerated microfuge at 4°C for 30 min and aliquots of the supernatant frozen at –70°C. Peroxidase assays were carried out according to Rathmell and Sequeira (1974). Reactions contained 0.28% guaiacol, 0.3% $H_2O_2$ in 50 mM sodium phosphate buffer pH 6.0. The reaction rate was monitored at 470 nm. Reaction rates were linear and proportional to the enzyme concentration added. Total protein was determined using the Bio-Rad protein assay adapted for microtiter plates.

**cDNA cloning and characterization of peroxidase homologues.**

A cDNA library of *S. humilis* cv. Paterson prepared in λgt10 (McIntyre et al. 1995) was screened by hybridization at low stringency (final filter wash conditions were 2× SSPE, 0.1% SDS at 40°C) using two peroxidase cDNA clones (Pnc1 and Pnc2) from peanut cell cultures (Buffard et al. 1990) kindly provided by R. Esnault, Institut de Science Vegetales, Gif sur Yvette, France. Filters were also probed with a degenerate 17-mer oligonucleotide matching the conserved catalytic region of plant peroxidases as described by Buffard et al. (1990). The Pnc2 probe hybridized to Shpx2 only, the Pnc1 hybridized to Shpx1, Shpx5, and Shpx12 and the oligonucleotide hybridized to Shpx1, Shpx2, and Shpx5. Positive clones were screened for cross-hybridization at high stringency and unique clones subcloned into pBluescript SK+. DNA sequencing was performed on denatured double-stranded DNA templates using either manual methods as outlined in the Sequenase Version 2.0 kit (U.S. Biochemicals) or automated methods using an Applied Biosystems (ABI) 373A instrument and the ABI PRISM DyeDeoxy Terminator Cycle Sequencing Kit. Sequence was determined on both strands with overlap. Oligonucleotide primers used for DNA sequencing were synthesized on a Beckman Oligo 1000 DNA synthesizer.

**Southern and northern blot analysis.**

Total RNA was isolated from various plant tissues and analyzed by gel electrophoresis and Northern hybridization as described previously by Higgins et al. (1985). The relative abundance of the mRNAs of Shpx1, Shpx2, Shpx5, and Shpx12 were compared directly as different autoradiographic exposure times were used for each probing, both fresh and stripped filters were used, and the specific activity of each labeled probe was not uniform. Two independent experiments were carried out for all Northern analyses with each probe giving essentially similar results. DNA isolations and Southern analysis was carried out as described by Curtis et al. (1995). Hybridizations were carried out at high stringency using cDNA inserts labeled with $^{32}$P-dCTP using the Amersham Multiprime labeling reaction.

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