Three differentially expressed basic peroxidases from wound-lignifying Asparagus officinalis

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Abstract
The activity of ionically bound peroxidases from an asparagus spear increased from 5–24 h post-harvest. Isoelectric focusing showed that the post-harvest increase of the total peroxidase activity was due to the increase of several distinct isoperoxidases. Concomitantly, a decrease in the activity of two anionic peroxidases was observed. Peroxidases with pl 5.9, 6.4 and 9.2 were detected only at 24 h post-harvest, whereas four peroxidases, with 8.7, 8.1, 7.4, and 6.7, detected throughout the time-course, increased in their activity. Histochemical staining demonstrated that lignin and peroxidase activity were located in the vascular bundles throughout the period of measurement. Lignin was detected in the cell walls of the protoxylem in the vascular bundles of the asparagus stem. A cDNA library of mRNA isolated from asparagus spears 24 h post-harvest was screened for peroxidases using homologous and heterologous probes. Three clones were isolated and the corresponding mature asparagus peroxidases displayed 70%, 76% and 81% amino acid sequence identity to each other. These new asparagus peroxidases are typical class III plant peroxidases in terms of conserved regions with a calculated pl >9.2, which is consistent with most basic peroxidases. One of the genes was shown to be a constitutively expressed single-copy gene, whereas the others showed an increased expression at post-harvest. The highest similarity in the amino acid sequence (71–77%) was found in peroxidases from roots of winter grown turnip TP7, to Arabidopsis AtP49, to an EST sequence from cotton fibres and to TMV-infected tobacco.

Key words: Asparagus, DNA sequence, histochemical staining, lignin, peroxidase, suberin.

Introduction
Lignin is a characteristic chemical and morphological component of the tissue in higher plants. Next to cellulose, lignin is the most abundant organic substance in the plant world and makes up 20–30% of the global plant biomass (Lewis, 1999). Lignins occur in great quantity in the secondary cell walls of fibres, xylem vessels and tracheids. Several functions are recognized for lignin in the extracellular matrix of plants; it supplies the cell wall with mechanical support (Monties, 1989) and acts as a water-impermeable seal for the xylem vessels (Northcote, 1989). Agro-industrial uses of plant material are affected by lignin composition, quantity and distribution. Digestibility and dietary conversion of herbaceous crops are affected negatively by lignin content and composition (Akin et al., 1991). It is also an undesirable component in the conversion of pulp to paper and removal of lignin is a major step in the paper-making process from wood chips. Peroxidase (EC 1.11.1.7) has been proposed as a candidate enzyme catalysing the last step in the polymerization of monolignols like p-coumaroyl alcohol, coniferyl alcohol, 5-hydroxyconiferyl alcohol and sinapyl alcohol (Campbell and Sederoff, 1996; Freudenberg, 1965; Hatfield and Vermerris, 2001). However, conclusive evidence for the enzymatic role(s) of lignification is still missing.

The Arabidopsis genome contains 73 plant peroxidase-encoding genes (Tognolli et al., 2002; Welinder et al., 2002) of which at least 58 are expressed during the plant life cycle. The remaining genes may be expressed
following biotic or abiotic stress. Peroxidases are haem-containing enzymes which mediate oxidation of a variety of molecules using hydrogen peroxide as an electron acceptor (Dunford, 1999). They also play a role in cell suberization by catalysing the deposition of the aromatic residues of suberin on the cell wall (Bernards et al., 1999), some peroxidases are expressed as a response to wounding (Egea et al., 2001) and others are involved in the metabolism of auxin (Lagrimini, 1999).

Asparagus (Asparagus officinalis L.) spears are known to increase lignin production during post-harvest storage (Hennion et al., 1992) and, for this reason, asparagus has been chosen as a model for tissues expressing lignin-related peroxidases. The expression of peroxidase iso-enzymes during storage of asparagus spear tissue, cloning of their cDNA and their potential involvement in the synthesis of phenol-containing polymers are reported here.

Materials and methods

All reagents used were of chemical grade from Sigma or Merck, unless otherwise stated.

Plant material

Green shoots of 22–44 cm length were harvested at ground level from sister plants of a tetraploid variety of green asparagus (var. Aarslev nr. 270), developed at the Research Centre Aarslev, Danish Institute of Agricultural Sciences (Sørensen and Thuesen, 1991), and immediately processed as described by Goldstein et al. (1972). The basal, stringer portions of the spears (5–20 cm) and the tips (6–15 cm) were cut off and discarded. The remaining central portion of each spear (diameter 5–12 mm) was divided into four segments and each segment was cut into discs 3 mm thick. The discs were kept on filter paper saturated with 0.1 M potassium phosphate buffer, pH 7.0, in loosely closed Petri dishes at room temperature under illuminating light for up to 24 h. Discs were sampled randomly at 0, 0.5, 1, 2, 5, and 24 h post-harvest, snap-frozen in liquid nitrogen, and stored at −80 °C for up to one year.

Peroxidase extraction and assay

For each post-harvest sampling time, four discs, one from each segment, were removed from storage, pooled and ground to a fine powder in a mortar under liquid nitrogen. Ionically-bound peroxidases were extracted in a native extraction buffer adjusted with salt (50 mM potassium acetate at pH 5.2, 0.5 M NaCl, 1 mM CaCl₂, 1 mM ascorbic acid, 0.1% Triton X-100). The ratio of ground plant material to extraction buffer was 1:2 (w/v) and the extraction was carried out on ice for 15 min. Extracts were cleared by centrifugation (15 000 g at 4 °C for 20 min), dialysed overnight at 4 °C against 5 mM sodium acetate pH 5.5, 0.5 mM CaCl₂ and subsequently concentrated by lyophilization.

Peroxidases were quantified in duplicate with Coomassie Plus protein assay reagent (Pierce) using bovine serum albumin (BSA) as a standard. The peroxidase activity of each sample was determined in duplicate using 2, 2'-azinobis(3-ethylbenzothiazoline-6-sulphonate) (ABTS) (0.36 mM ABTS, 6 mM H₂O₂, 100 mM sodium acetate pH 5.5) as substrate. Peroxidase activity (λ=405 nm) was measured continuously at 30 °C for 1.5 min using a PowerWave, spectrophotometer (BIO-TEK Instruments Inc.).

Isoelectric focusing

Proteins (37 μg) were focused at 11 °C for a total of 2 kVh in a wide range, non-denaturing isoelectric-focusing (IEF) polyacrylamide gel (Ampholine PAGplate pH 3.5–9.5, Amersham Biosciences). An IEF standard calibration kit (Amersham Biosciences) was used to determine the pH-gradient across the gel. The IEF markers were visualized by Coomassie staining according to the manufacturer’s recommendation. Peroxidase activity was visualized with 3-amino-9-ethylcarbazole as substrate (Kerby and Sommerville, 1989).

Histochemical stain for lignin and peroxidase activity

All the tissue stem sections were cut free hand, transverse to the asparagus stem, and incubated for 10 min at room temperature in an appropriate medium. Lignin was visualized using phloroglucinol/HCl (Weisner reaction; Gahan, 1984), where phloroglucinol in acidic conditions gives a red-pink product primarily by reaction with the lignin cinnamaldehyde groups. Fresh transverse sections were incubated for 3 min in 10% phloroglucinol (w/v) in 100% EtOH solution followed by 3 min incubation in HCl conc and mounted in 50% glycerol. The plant sections were examined directly under a light microscope (Zeiss). The intensity of the reaction product was visually estimated and photographs were taken (MC80, Zeiss; Kodak Ektachrome 64T). Autofluorescence was detected on sections incubated in water. The sections were observed in a fluorescence microscope (Axioplan, Zeiss) in the excitation range 410–460 nm (filter set 9, Zeiss) and photographs were taken (Cool Snap, Rs Photomatics). Peroxidase activities were visualized using 2.02 mM 3-3′-diaminobenzidine tetrahydrochloride (DAB) dissolved in 50 mM TRIS-HCI buffer pH 6.8 containing 22.5 mM H₂O₂ or 0.1% alcoholic syringaldazine (Goldberg et al., 1983). Hydrogen peroxide was detected using the same substrate and buffer combination, but excluding H₂O₂ from the medium.

Construction of cDNA library

A unidirectional cDNA library enriched for lignin-related sequences was prepared using a Lambda ZAP® II XR Library Construction Kit (Stratagene). The template poly(A)⁺RNA was isolated from the discs of asparagus spears stored for 24 h as described above.

Peroxidase probe generation

DNA from the cDNA library was prepared using QIAGEN® mini Lambda phage DNA Kit (Qiagen). A peroxidase probe was generated by PCR amplification using two degenerated primers, [5′-CGS-CTSCACCTCCACGACTGC] and [5′-GTGSGCGCGSAG-SGC], encoding the conserved sequences (Tyson and Dhindsa, 1995) at the proximal and distal histidine, respectively. PCR was performed in a final volume of 50 μl containing 100 ng cDNA, 6 pmol of each primer, 2.5 U Taq-polymerase (Perkin-Elmer), 0.2 mM dNTP (Amersham Biosciences), PCR-buffer (Perkin-Elmer) and 1.5 mM MgCl₂. Amplification was achieved by denaturing at 92 °C for 5 min followed by 40 cycles of 30 s at 50 °C, 1 min at 72 °C, and 15 s at 94 °C. A 390 bp fragment was cloned into the pCR4-TOPO vector using TOPO TA cloning kit (Invitrogen). Plasmid DNA was purified using the Wizard Miniprep System (Promega), and the peroxidase identity of the PCR product was subsequently confirmed by DNA sequencing (see below).

Library screening with peroxidase probes

An aliquot of the cDNA library (approximately 200 000 pfu) was screened using a 32P-labelled probe of the histidine-spanning region (see above) followed by plaque purification (Sambrook et al., 1989). Hybridization and washes were carried out under low stringency conditions.
The full length clones Aoprx1 and Aoprx2 were retrieved by excision in vivo into pBluescript II SK(-) according to the manufacturer’s protocol (Stratagene).

The sequencing of the cloned DNA was performed on an Applied Biosystems ABI PRISM™ 377 DNA sequencer using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit, according to the manufacturer’s protocol (PE Applied Biosystems). Sequence traces were assessed and contigs assembled using Sequencer 3.1.1 (Genecode). Sequences and contigs were translated and analysed by MacVector 7.0 (Oxford Molecular Software). Similarity searches were performed using TBLASTN 2.2.5 with a BLOSUM62 matrix and expect value of 10 with low complexity filter at the NCBI website (http://www.ncbi.nlm.nih.gov/).

Some 200,000 pfu of the asparagus cDNA library were screened with a 32P-labelled fragment of barley peroxidase clone Prx7 (Kristensen et al., 1999). Isolated clones were characterized and the partial sequence of Aoprx3 was extended at the 5’ end by PCR and sequenced using primers from the sequenced 3’ segment: [5’-TTCACCTTTGACTGGCGCTC], [5’-GACAAGGGCAACCATTTCCG], and [5’-AGAATCCACCACCTTATGG]. All cloning and DNA-blotting procedures were performed according to Sambrook et al. (1989).

Southern blot analysis
DNA was isolated from asparagus spears, using the protocol developed for green barley leaves (Sharp et al., 1988). Subsequently, the genomic asparagus DNA (20 μg) was digested with BamHI, HindIII and EcoRI (10 U μg⁻¹ DNA) for 3 h, fractionated by size on a 0.7% agarose gel, and transferred to a Hybond N+ nylon membrane (Amersham Biosciences) by alkali capillary blotting (Ausubel et al., 1999). Blots were probed either with thehistidine spanning probe or a specific 32P-labelled Aoprx1 probe made by PCR using the genome-specific primers [5’-AGACCTCTCAACTCCGACAACC] and [5’-GCCACCTACTAATTCTTC]. Hybridizations were performed in Rapid-hyb buffer (Amersham Biosciences) as described by the manufacturer, and washes were performed at moderate stringency in 0.1× SSC at 65 °C and at high stringency in 0.1× SS at 65 °C.

Isolation of RNA
Total RNA was isolated from pools of 3 mm discs of asparagus spears after homogenization with an Ultra-Turrax T25 (Janke and Kunkel, Germany) in a buffer containing 4 M guanidiniumthiocyanate (GTC), and [5

RT-PCR of post-harvest expression
Reverse transcription of 1 μg total RNA was carried out using Ready to go RT-PCR-beads (Amersham Biosciences) as described by the manufacturer. Positive and negative controls were primed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (GAPDH1: [5’-CAAGGACTGAGRGGTGGG] and GAPDH2: [5’-CCCACCTGTGTCTCRTACC]). The negative control was heated at 95 °C prior to reverse transcription for 10 min to denature the reverse transcriptase. RT-PCR was started by reverse transcription at 42 °C for 15 min, followed by 5 min denaturation at 92 °C. The reverse transcription was followed by 20, 30 or 40 cycles of PCR, each consisting of a 15 s denaturation step at 92 °C, a 30 s annealing step at 55 °C, and a 1 min elongation step at 72 °C. This was followed by a 4 min extension at 72 °C. Two primer combinations, which were specific for Aoprx1 and Aoprx2, were used. The gene-specific primers for Aopnx1 [5’-AGACCTCTCAACTCCGACAACC] and [5’-GCCACCTACTAATTCTTC] produce a 227 bp product while the Aoprx2 gene-specific primers [5’-AAGAGACTCGGTCGTAATCC] and [5’-AAGGAAGCAGCAAGCGTC] produce a 277 bp product. The expected size of the product of the GAPDH primer in the control reactions is 380 bp.

Differential display
Differential display was performed as described by Jørgensen et al. (1999) and detailed at http://biobase.dk/~ddbase/. Briefly, total RNA was reverse transcribed using three different primers (AAGCT11A, AAGCT11G, AAGCT11C). Each of the three resulting cDNA populations was used as template for PCR reactions with suitable primers as described in the ‘Results’ section. Finally, the resulting DNA fragments were analysed by denaturing polyacrylamide gel-electrophoresis and autoradiography.

Results
Changes in peroxidase activity and lignin content during storage
To study the increase in peroxidase activity, H2O2 and lignin accumulation and localization in asparagus for 24 h of post-harvest storage, peroxidase activity was measured and histochemically localized as were H2O2 and lignin.

The activity of the ionically bound peroxidases decreased between 0 h and 1 h post-harvest, levelled out at 1–5 h and increased between 5 h and 24 h of storage to twice the amount at the time of harvesting (Fig. 1). Phenylalanine-ammonia-lyase activity (data not shown) showed an activity profile similar to that of peroxidases in agreement with Hennion et al. (1992), indicating that the observed changes were related to lignin or suberin synthesis.

Cross-sections of the asparagus stem (Fig. 2) revealed a primary rind and the stele, the vascular tissue had a scattered bundle system, with closed collateral bundles. The primary rind consisted of an epidermis with assimilating tissue underneath. The stele was separated from the primary rind by the sclerenchyma belt. In the inner part of the sclerenchyma belt there were small vascular bundles, those in the stele had a larger diameter. There were no vascular bundles in the middle of the stem. The xylem formed a V-shaped figure in the vascular bundles, with the phloem enclosed between the two arms of the V in agreement with Esau (1965). The protoxylem was located at the bottom of the V and the xylem vessels at the top of the arms of the V.

Peroxidase staining showed different patterns depending on the substrate used. Syringaldazine is commonly used to discriminate lignin-specific peroxidases and this substrate specifically stained the xylem cells of the asparagus spear at T0 and at T24. The staining was slightly more intense at T24 as compared to T0 (Fig. 2a, b). This was more obvious using the general peroxidase substrate 3-3’-diaminobenzidine (DAB). DAB staining in the xylem cells significantly increased from T0 to T24 (Fig. 2c, d), showing the accumulation of peroxidases over time. DAB staining of the phloem was also highest at the late time point, showing...
the accumulation of peroxidases in the phloem cells in response to harvest.

Endogenous hydrogen peroxide was detected by the peroxidase-dependent oxidation of DAB. The capacity of vascular bundles to produce hydrogen peroxide was also enhanced during the time-course (Fig. 2e, f) and correlated with the accumulation of lignin in the proto- and metaxylem.

To localize lignin and visually detect changes of its abundance during storage, hand cut asparagus sections were stained in phloroglucinol/HCl immediately and after 24 h of storage. At time 0 (T0) the phloroglucinol stained area was limited to the protoxylem (Fig. 2i), while at time 24 (T24) the area had increased and included the metaxylem (Fig. 2j). The same was seen for cell wall autofluorescence (Fig. 2g, h), indicating that the autofluorescence originated from polyphenols.

These differences in lignification and peroxidase staining were unambiguous in the sections from the tip of the asparagus spear, but became less and less apparent in the sections towards the base of the spear. This suggests that the younger, uppermost parts of the spear were not lignified at harvest, but had the capacity to lignify in response to harvest by cutting (wounding).

The changes in peroxidase activity described in Fig. 1 could indicate either a general increase in the amount of peroxidase isoenzymes or the response to harvesting by a specific peroxidase isoenzyme. Therefore, peroxidases were extracted in a native extraction buffer with a high salt concentration and analysed by isoelectric focusing (IEF) from pH 3.5–9.5 (Fig. 3). The protein extracts contained a number of distinct isoperoxidases, which were detected at all times. The peroxidases at pI 3.8 and 8.7 appeared as double bands. A neutral and a cationic isoenzyme were detected only at 24 h post-harvest (T24) (pI: 6.4, 9.2). Two anionic peroxidase (pI: 4.4, 4.7) activities decreased over the time. The rest of the detected isoperoxidase activities were present at all times and increased 24 h post-harvest. Activity-stained bands corresponding to pI 5.3 and ~9.6 were detected 24 h post-harvest, but only when higher amounts of total protein were loaded (data not shown).

cDNA library screening for lignin related peroxidases

An asparagus cDNA library from poly(A)+ RNA isolated from spears 24 h post-harvest was screened using a partial asparagus peroxidase cDNA encoding the sequence spanning the highly conserved distal and proximal
histidine domains as a probe. Sequencing of the cDNA, followed by Blast analysis, showed that the probe had the highest similarity to the corresponding region of mRNA for a *Nicotiana tabacum* peroxidase (AB027752, included in Fig. 6).

Approximately 200 000 pfu of the 24 h post-harvest cDNA library were screened with this probe and several independent clones were isolated. Two clones harbouring all of the coding regions for two different peroxidases (hereafter named Aoprx1 (AJ544514) and Aoprx2 (AJ544515), respectively) were subcloned into pBluescript II KS-, sequenced completely, and further characterized. Aoprx1 and the probe sequence were identical. The peroxidase Aoprx3 (AJ544516) was obtained by screening the same library using the biotic stress-related barley Prx7 as a probe.

The Aoprx1 cDNA sequence had a length of 945 base pairs encoding a 315 amino acid precursor protein containing at the N-terminal a signal peptide of 19 amino acid residues as predicted using the sequence analysis program of Nielsen *et al.* (1997). The deduced mature protein had a calculated pI of 9.26 and a MW of 31.9 kDa. The deduced mature Aoprx2 protein contained 296 amino acid residues, had an estimated pI of 9.55, and a calculated MW of 31.7 kDa. The Aoprx3 sequence encodes a 320 amino acid precursor protein. The precursor protein is predicted to contain a signal peptide of 25 amino acid residues and the mature protein of 295 amino acid residues with a calculated pI of 9.35 and a deduced molecular weight of 31.5 kDa. However, these calculations do not take into account any post-translational modifications. Based on empirical observations (Rasmussen *et al.*, 1991; Kristensen *et al.*, 1999) the actual pIs of the native peroxidases may be higher than the calculated values.

To determine the gene copy numbers of peroxidases in general and, in particular, of the Aoprx1 gene, Southern transfer analysis was performed (Fig. 4). Genomic DNA was restricted with *Bam*H1, *Hind*III and *Eco*RI and the membranes were exposed to the histidine domain-spanning probe from asparagus and to a probe specific for the Aoprx1 C-terminal. Several restriction fragments were detected using the homologous probe at low stringency, where most peroxidases are expected to cross hybridize, but only a subfamily of a large number of peroxidase genes was detected. Typically, three restriction fragments were observed in the *A. officinalis* genome by hybridization with the histidine domain-spanning probe at high stringency and the gene-specific probe at low stringency. Only one restriction fragment was observed using the gene-specific probe at high stringency. Since *A. officinalis* is naturally a diploid species, but has been made tetraploid by cholchicine treatment during the 1950s (Braak and Zelinga, 1957), it seems reasonable that each
restriction band represents two identical genes, due to the duplication of its genome.

RT-PCR was used to determine if transcription of the Aoprx1 and Aoprx2 genes was induced during storage. The RT-PCR was performed on total RNA using specific primers, resulting in a 227 bp product for Aoprx1 and a 277 bp product for Aoprx2 after 20 and 30 cycles and showing saturation at 40 cycles. The analysis showed that both Aoprx1 and Aoprx2 were expressed at all times during the time-course (Fig. 5), but while Aoprx1 decreased, Aoprx2 increased, reaching the highest level at 24 h.

The time-course of expression of the three cloned A. officinalis peroxidases was also analysed by ‘gene-specific differential display’. In this kind of analysis, differential display is performed with a gene-specific sense primer designed from a known cDNA sequence (Jørgensen et al., 1999). The specificity of the primer ensures that a band corresponding to the mRNA under investigation will be present in the gel-electrophoretic analysis of the RT-PCR products, and the size of this band can be predicted from the sequence of the cDNA. At the same time, the low annealing temperature used for the PCR in the differential display protocol ensures that a number of unrelated bands will also be present in the gel image, and these bands are ideal internal standards for the analysis of expression patterns. Figure 5 shows the temporal expression profile of Aoprx3 determined by gene-specific differential display on two independent series of RNA preparations. Evidently, Aoprx3 was induced during storage, especially after 24 h, as would be expected for a peroxidase related to lignin synthesis.

The expression profiles of Aoprx1 and Aoprx2 were both investigated by gene-specific differential display (results not shown). These analyses confirmed the suggestions from Fig. 5 that the expression of Aoprx1 was reduced during storage, and the expression of Aoprx2 increased during storage.

The sequence identity shared between Aoprx1 and Aoprx2 was 81%, Aoprx3 and Aoprx1 was 70%, and Aoprx2 and Aoprx3 was 76% identity at the amino acid level. The identity between Aoprx1, Aoprx2, and Aoprx3 and the tobacco peroxidase (AB027752) was 71%, 73% and 77%, respectively, for the deduced mature protein sequence. A previously identified asparagus peroxidase sequence (Takeda et al., 2003) was found to have 50% sequence identity with the three peroxidases identified in this study (Fig. 6). The aligned sequences are listed by degree of similarity to the Aoprx sequences presented in this paper. In total, 101 amino acid residues were conserved between the peroxidases compared in Fig. 6 and 28 of those were found in all 73 peroxidases in Arabidopsis (Welinder et al., 2002) as well as in the classic horseradish peroxidase HRPC.

The cleavage site for the signal peptide was located in the amino acid sequence motif [AQLS] (Nielsen et al., 1997; Fig. 6). The Aoprx1 sequence contained a [QLSTTFY] motif, Aoprx2 had a [HLSTNPY] motif, and the Aoprx3 contained the sequence [QLSPNPY] near the N-terminus. This motif was found in most known plant peroxidase sequences (Tyson and Dhindsa, 1995). Highly conserved regions, typical for plant peroxidases, were found in the three deduced protein sequences for Aoprx1, 2, 3, containing the distal histidine (His42) at the catalytic
site, the proximal histidine (His170) at the heme-binding site and a conserved cysteine motif [VSCADILA] at Cys97 (Tyson and Dhindsa, 1995). The [VSCXD] is present in all Arabidopsis peroxidases. Eight conserved cysteines are found in the same relative positions as in other class III peroxidases forming four disulphide bridges by cysteine residues 11–91, 44–49, 97–301, and 177–209 (Fig. 6).

A phylogenetic tree was constructed from the multiple alignments of the cloned Aoprx sequences and related peroxidases with the highest similarity. The NCBI accession numbers are as follows: N. tabacum: gi 5381253; A. thaliana: AtP6 gi 15239370, AtP44 gi 15237187, AtP49 gi 15239075; B. rapa: TP7 gi 464365; G. hirsutum1 gi 1969846; G. hirsutum2 gi 543379; A. officinalis gi 763147. The conserved regions (bottom) show invariant residues and active site residues, which are indicated by bold face type (such as the 8 cysteines). Residues also invariant in 77 Arabidopsis peroxidases are indicated in italics and underlined (bottom).

The functions of the peroxidases shown in the phylogenetic tree (Fig. 7) have not been determined. The first cluster consists of the three Aoprx sequences and is linked to a cluster of tobacco (Higara et al., 1999) and turnip root TP7 peroxidases, which is 90% identical to Arabidopsis AtP49. The tobacco peroxidase (AB027752) was isolated from tobacco leaves infected with Tobacco Mosaic Virus and shows the closest identity to Aoprx3 (77%). Two cotton peroxidases, one isolated from bacterial infected tissue, are 63–70% identical to Aoprx1-3. The previously characterized asparagus sequence was an apoplastic peroxidase secreted from suspension-cultured cells of a male somatic embryo (AB042103), and its branching away from the other asparagus peroxidases suggests a different function. It is 73% identical to Arabidopsis AtP6 expressed in 35-d-old roots. The three Arabidopsis peroxidases in Fig. 7 are

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Fig. 6. ClustalW alignments of the three Aoprx mature protein sequences and related peroxidases with the highest similarity. The NCBI accession numbers are as follows: N. tabacum: gi 5381253; A. thaliana: AtP6 gi 15239370, AtP44 gi 15237187, AtP49 gi 15239075; B. rapa: TP7 gi 464365; G. hirsutum1 gi 1969846; G. hirsutum2 gi 543379; A. officinalis gi 763147. The conserved regions (bottom) show invariant residues and active site residues, which are indicated by bold face type (such as the 8 cysteines). Residues also invariant in 77 Arabidopsis peroxidases are indicated in italics and underlined (bottom).

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Hennion et al. (1992) have shown that this plant continues lignification during post-harvest storage (Hennion et al., 1992), and it could supply plant tissues expressing lignin-related peroxidases.

The plant peroxidase activity was found to increase between 5 and 24 h post-harvest. The increase in peroxidase activity was shown by isoelectric focusing to be due to the accumulation of several peroxidases, suggesting the involvement of a set of peroxidases in polymerization of phenolic compounds. The results are in agreement with the peroxidase activity measured by Hennion et al. (1992). The histochemical stain for lignin showed an increase in the lignified area in the differentiating cells of the xylem during the time of measurement. Peroxidase activity and hydrogen peroxide were located in the same cells as the lignification front. These results, which show co-localization of lignification zones, peroxidase activity, and hydrogen peroxide, are correlative, suggesting that hydrogen peroxide and peroxidases are required for polymerization. However, although quantitative relationships between peroxidase activity and lignification have frequently been reported in the literature (McDougall et al., 1994; Barceló, 1995; Christensen et al., 1998), in no case has a specific peroxidase been shown to be exclusively involved in lignification. It seems likely that Freudenberg’s hypothesis (Freudenberg, 1965) where both laccases and peroxidases co-operate in the lignin polymerization will prove to be correct. Since laccases operate without the production of toxic hydrogen peroxide, Stejiades et al. (1993) suggested that laccases could be involved in the early stages of lignification around living cells. However, recent data show peroxidase transcript accumulation in the early stages and laccase transcript accumulation in the later stages of lignification in Z. elegans xylemogenesis (Milioni et al., 2002). In the asparagus system at 24 h post-harvest, both xylem and surrounding cells showed accumulation of peroxidase activity, suggesting that peroxidases participate both in polymerization of phenolics other than monolignols and in xylem-specific lignification.

Activity-stained IEF gels showed predominant expression of basic peroxidases of pI 8.1 and 8.7 and, after 24 h, also 9.2 (Fig. 3), and the three cloned peroxidases were also basic although with a higher calculated pI (>9.2). Unfortunately, there is no simple connection between the calculated and experimentally determined pI for class III peroxidases because of Ca²⁺ binding and the haem prosthetic group. Using two screening methods of the cDNA library, it is possible that all the major plant peroxidases have been identified and, therefore, it is tempting to claim that these three are among those basic peroxidases extracted from the same tissue detected by IEF.

The deduced amino acid sequence of the three new asparagus peroxidase genes reported here are typical plant peroxidases in terms of the conserved regions, including the two regions involved with the heme group and positions and numbers of disulphide bridges, as shown in Fig. 6. The similarity in these regions approaches 100% identity between the various peroxidase isoenzymes and they are critical for peroxidase activity. The deduced amino acid sequences of Aoprx1 and Aoprx2 are clustered together with a tobacco peroxidase in the dendrogram (Fig. 7) and they share 71% and 73% sequence identity, respectively, for the mature protein; Aoprx3 shares 77% identity. The tobacco gene encoded a pathogen-induced peroxidase, and lignification of the infected cells is often seen as a response to pathogens in plants (Egea et al., 2001). The asparagus peroxidases presented here have been extracted from wound-lignifying tissue and they are differentially expressed in the stem post-harvest. These properties suggest that the Aoprx2 and Aoprx3 sequences are lignin- or polyphenol-related peroxidases. Whereas the down-regulation of Aoprx1 may suggest that this peroxidase is involved in the early steps of the wound response or not related to the formation of lignin. Their differential expression probably reflects different roles or sites of action. In this context, it is interesting that a peroxidase in the Z. elegans cell system (Milioni et al., 2002) also shows...
a transient expression profile early in the xylem differentiation process. It will, however, be very difficult to obtain direct conclusive evidence for the involvement of the three characterized asparagus peroxidases in the lignin biosynthesis because it is possible that the whole set of oxidases are involved in the polymerization of monolignols.

None of the three peroxidases showed high similarity to putative lignin-peroxidases isolated from poplar (Christensen et al., 1998). In fact, they were more similar to peroxidases isolated from suberizing tissue, for example, TP7 from winter turnip roots, from which suberin has been isolated (Kolattukudy et al., 1975). TBLASTN searches using Aoppx3 also showed high similarity to potato root EST (gi 17075760), well known for its content of suberin in the periderm. High similarity was also found with a tree cotton EST (gi 18100104), which had been isolated from a fibre library. Although cotton fibres are not suberized, suberin has been detected at the fibre base, where it may serve in the transport of assimilates (Ryser, 1992).

Confirmation that the asparagus peroxidases have a functional role in lignin biosynthesis depends on multiple lines of evidence, including biochemical, molecular and histochemical techniques. Gene knock-out experiments are not necessarily the key to identifying involvement in lignin polymerization unambiguously. This is because downregulation of a specific lignin peroxidase isoform may be compensated by other peroxidases or oxidases. Experiments may, however, reveal more about the enzymes involved in the polymerization process, making it possible to engineer plants with altered lignin contents. However, it seems very unlikely that classification based on sequence similarity alone will lead to the correct function assignment for individual peroxidases. Of the 73 peroxidases present in the Arabidopsis genome, two showed 90% sequence identity to peroxidase from a distinct related species (turnips and horse radish), the remaining 71 were less than 80% identical compared with the large number of cloned peroxidases now available. Thus, having the entire peroxidase complement of a single plant genome cannot support a functional classification based on amino acid sequence alone.

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