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Molecular cloning of complementary DNA encoding the lignin-forming peroxidase from tobacco: Molecular analysis and tissue-specific expression

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ABSTRACT **Plant peroxidases play a major role in lignin formation and wound healing and are believed to be involved in auxin catabolism and defense to pathogen attack. The function of the anionic peroxidase isozymes is best understood in tobacco. These isozymes catalyze the formation of the lignin polymer and form rigid cross-links between lignin, cellulose, and extensin in the secondary plant cell wall. We report the purification of the anionic peroxidase isozymes from tobacco and their partial amino acid sequence. An oligonucleotide probe deduced from the amino acid sequence was used to screen a tobacco leaf cDNA library and a 1200-base-pair cDNA clone was isolated and sequenced in its entirety. The predicted amino acid sequence revealed a 22-amino acid signal peptide and a 302-amino acid mature protein (M_r, 32,311). The amino acid sequence was compared to that of the cationic peroxidases from horseradish and turnip and was found to be 52% and 46% homologous, respectively. By RNA blot analysis, the messenger for the tobacco isozyme was found to be abundant in stem tissue while expressed at very low levels in leaf and root tissue. Four distinguishable copies of the gene were found on genomic DNA blots. The gene copy number may reflect the allotetraploid nature of *Nicotiana tabacum*.**

The peroxidases (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7) have been studied extensively in a wide variety of higher plants [for a review, see Gaspar *et al.* (1)]. They play an integral role in secondary cell wall biosynthesis by the polymerization of cinnamyl alcohols into lignin (2) and by forming rigid cross-links between cellulose, pectin, hydroxyproline-rich glycoproteins, and lignin (2). Peroxidases are also critical in wound healing, in which they in part form a water-tight barrier over the wound by the deposition of polymeric aliphatic and aromatic compounds (3). Peroxidases may also have a role in auxin catabolism (4) and defense of the plant against pathogen attack (5, 6).

Most higher plants possess a number of different peroxidase isozymes whose pattern of expression is tissue specific, developmentally regulated, and influenced by environmental factors (6). In the tobacco plant there are at least 12 distinguishable isozymes, which fall into three subgroups; the anionic, the moderately anionic, and the cationic. Each group is thought to serve a different function in the cell.

The cationic peroxidase isozymes (pI, 8.1–11) efficiently catalyze the synthesis of H_2O_2 from NADH and H_2O (7), and have recently been localized to the central vacuole (8). These isozymes have also been shown to possess an indoleacetic acid oxidase activity in the absence of H_2O_2 (9). There has been speculation that these isozymes regulate auxin levels, form ethylene from 1-aminocyclopropane-1-carboxylic acid (10), and provide H_2O_2 for other peroxidase isozymes (7). Their actual function in the plant is as yet unclear.

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The moderately anionic peroxidase isozymes (pI, 4.5–6.5) from tobacco are highly expressed in wounded stem tissue (6). These isozymes have previously been localized to the cell walls, and they have a moderate activity toward lignin precursors. They may serve a function in wound healing or suberization by forming a water-tight barrier over the wound (3).

The function of the anionic peroxidase isozymes (pI, 3.5–4.0) is understood best. This isozyme group is also cell wall associated and has a high activity for the polymerization of cinnamyl alcohols *in vitro* (11). They function in lignification and the cross-linking of extensin monomers and feruloylated polysaccharides (12). Since the formation of the secondary cell wall prohibits cell expansion, the activity and perhaps the *de novo* synthesis of the anionic isozymes are likely regulated by auxin levels (13).

We have begun to apply the tools of molecular biology to better understand how peroxidases function in growth and development and what role they may play in the plant's ability to recover from environmental stress, wounding, and infection. Our approach entails the isolation of molecular probes to monitor the expression of the various peroxidase isozymes. In this paper, we report the cloning and sequence of a cDNA encoding one of the two anionic peroxidases from tobacco. A cDNA probe was used to observe the levels of expression of the anionic peroxidase genes in several plant tissues. The tobacco genome was also probed to determine the gene copy number and to characterize the nucleotide homology between peroxidase isozyme classes. In addition, the predicted amino acid sequence of the anionic peroxidase of tobacco was compared to that of other plant peroxidases.‡

MATERIALS AND METHODS

Plant Materials and Growth Conditions. *Nicotiana tabacum* (Coker 176), *Nicotiana sylvestris*, and *Nicotiana tomentosiformis* plants were grown from seed in the greenhouse with 14-hr daily light periods.

Anionic Peroxidase Purification and Protein Sequencing. The anionic peroxidase isozymes were purified from tobacco leaves as described by Mäder (14). Trypsin or cyanogen bromide cleavage was carried out using standard procedures (15). Peptides were isolated by reverse-phase HPLC, and the isolated peptides were sequenced on an Applied Biosystems (Foster City, CA) model 470A gas-phase protein sequencer. NH_2-terminal sequence analysis was obtained by first deblocking with pyroglutamate amino peptidase (15).

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‡The sequence reported in this paper is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J02979).

RNA Isolation and cDNA Library Construction. Poly(A)$^+$ RNA was isolated from either fresh leaf, root, or stem tissue (16). Leaf tissue (10 g) or pith tissue (50 g) was homogenized with a Brinkmann Polytron in equal volumes of grinding buffer (50 mM Tris·HCl, pH 8.0/4% sodium *p*-aminosalicylic acid/1% sodium naphthalene-1,5-disulfonic acid) and water-saturated phenol. After separating the phases by centrifugation, the aqueous phase was extracted again with phenol/$CHCl_3$, and then with $CHCl_3$ alone. The aqueous phase was then made 0.5 M LiCl/1 mM EDTA/0.1% Na_2DodSO_4. Up to 0.5 g of oligo(dT) cellulose was added and shaken for 10 min. The slurry was poured into a small glass column and poly(A)$^+$ RNA was collected (17). Double-stranded cDNA copies were made from leaf poly(A)$^+$ RNA as described by Lapeyre and Amalric (18), ligated into λgt11 arms, and packaged *in vitro* into phage particles (Vector, Burlingame, CA).

Oligonucleotide Screening. Mixed oligonucleotide probes incorporating deoxyinosines were 5′ ^{32}P-end-labeled to a specific activity of 1×10^6 cpm/pmol with [γ-^{32}P]ATP (>5000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) and T4 polynucleotide kinase (17). This was used to probe nitrocellulose filter replicas of the tobacco leaf cDNA library (19, 20).

Subcloning and DNA Sequencing. Restriction fragments were isolated from the cDNA insert and were also subcloned into the appropriately digested vector for DNA sequencing. DNA sequencing was carried out by the dideoxy-chain termination method on alkali-denatured plasmid templates (21, 22).

DNA and RNA Filter Hybridizations. High molecular weight DNA was isolated from tobacco leaves as described by Bendich *et al.* (23). DNA (5 μg) was cleaved with various restriction enzymes and subjected to agarose gel electrophoresis. The DNA was then transferred to nitrocellulose (24) and hybridized with nick-translated cDNA clones (25). Poly(A)$^+$ RNA (1 μg) from leaf, root, and stem tissue was subjected to formaldehyde-agarose gel electrophoresis (26), and transferred to nitrocellulose filters (27). RNA blots were also hybridized with nick-translated cDNA clones.

RESULTS

Peroxidase Purification and Protein Sequencing. The two anionic peroxidase isozymes from tobacco designated $POD_{3.5}$ and $POD_{3.75}$ according to their pI values (6) were purified as a mixture of equal parts from frozen leaf tissue by a modification of the procedure of Mäder (14). The original procedure involved chromatography steps on DEAE-cellulose followed by gel filtration on Sephadex G-75. These purification steps were replaced by single chromatography steps on DEAE-cellulose, Sephacryl S-200, and concanavalin A-Sepharose. Typically, 3 mg of the two anionic isozymes could be obtained from 2 kg of leaves by this method with a recovery of 50% of the initial activity. These isozymes represent ≈0.01% of the total soluble protein in the tobacco leaf. The purity of the isozymes was determined by an *RZ* value of 3.0 (A_{402}/A_{260} ratio) for the preparation, and the presence of only two silver-stained bands on both Na_2DodSO_4/PAGE and isoelectric focusing gels. It was observed that $POD_{3.5}$ had a M_r of 36,000 and $POD_{3.75}$ had a M_r of 37,000 as determined by Na_2DodSO_4/PAGE. It was not possible to separate the two isozymes in the amounts necessary for protein sequence analysis; therefore, peptides were obtained from the mixture for sequencing.

Protein sequence data were obtained from the NH_2 terminus of three tryptic peptides and two CNBr peptides as shown in Fig. 1. No heterogeneous peptides were detected by sequencing as a result of the two isozymes in the mixture. The NH_2-terminal sequencing of the anionic peroxidases failed initially due to a blocked terminus. Following treatment of the proteins with pyroglutamate amino peptidase (15), which removes pyrrolidone carboxylic acid, the NH_2-terminal sequence was determined (Fig. 1). It should be noted that even though NH_2-terminal sequencing was carried out on an equivalent mixture of two anionic peroxidases, they revealed a unique sequence. This is most likely due to the similarity of the two isozymes, although one cannot eliminate the possibility that only one polypeptide was deblocked.

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A.   NH2-terminus   QLSATFYDTTCPNVTSIVRGVMD

B.   Tryptic        1) GVMDQR
     Peptides       2) GMDLTDVALSGAHTFG
                    3) LGNISPLTGTNGQ

     CNBr           1) MIKLGNISPLTG
     Peptides       2) MIPQFTNKG

C.        Met Ile Pro Gln Phe Thr Asn Lys Gly

   mRNA   AUG AUA CCA CAA UUU ACA AAC AAA GGA-3′
                U   G   G   C   G   U   G   G
                C   C           C           C
                    U           U           U

 26 mer   TAC TAT GGI GTT AAA TGI TTG TTT CC -5′
 probe        A       C   G       A   C
              G
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FIG. 1. Protein sequence data and the synthetic oligonucleotide probe used in the cDNA cloning. (*A*) The NH_2-terminal sequence of the anionic tobacco peroxidase after deblocking with pyroglutamate amino peptidase. (*B*) Tryptic and CNBr peptide sequences. (*C*) Partial amino acid sequence of CNBr peptide fragment 2, followed by the deduced mRNA sequence, and the synthetic oligonucleotide sequence from the noncoding strand used as the probe. Amino acids are identified by the single-letter code.

The amino acid sequence of CNBr peptide 2 was used to deduce the corresponding nucleotide sequence. A DNA probe consisting of a mixture of 48 oligonucleotides each 26 bases long was synthesized (Fig. 1). Since inosine can form weak base pairs with all four nucleotides, two inosine residues were inserted at highly redundant positions, lowering the overall redundancy of the oligonucleotide mixture (28).

Construction and Screening of a Leaf cDNA Library. Poly(A)$^+$ RNA was isolated from young tobacco leaves for the synthesis of complementary DNA. A leaf cDNA library consisting of 200,000 recombinant phage was then constructed in λgt11 (18). The entire library was plated onto a single 22 × 22 cm Petri dish from which duplicate nitrocellulose filter lifts were made. Both filters were hybridized with the 5′ ^{32}P-end-labeled 26-mer mixed oligonucleotide probe. By this method, 18 positive clones were identified, which represent ≈0.01% of the cDNA library. Four individual clones were plaque purified, and phage DNA was isolated for subcloning and DNA sequencing (17).

Nucleotide Sequence Analysis of the Peroxidase cDNA. The four cDNA clones chosen for DNA sequence analysis were subcloned into plasmid vectors. The cDNA inserts ranged in size from 1100 to 1254 base pairs (bp), and all had similar restriction maps. Partial sequencing of each clone indicated that they were derived from the same mRNA. The largest cDNA insert was then sequenced in its entirety. DNA sequence was determined from both strands to ensure its accuracy. The complete nucleotide and the deduced amino acid sequence of this clone are shown in Fig. 2.

All of the sequenced peptides can be found in the deduced amino acid sequence shown in Fig. 2, confirming that this is indeed a peroxidase cDNA clone. Further confirmation comes from a comparison of this anionic tobacco peroxidase amino acid sequence with the previously published sequences of horseradish (pI 9.0) (29) and turnip (pI 11.0)

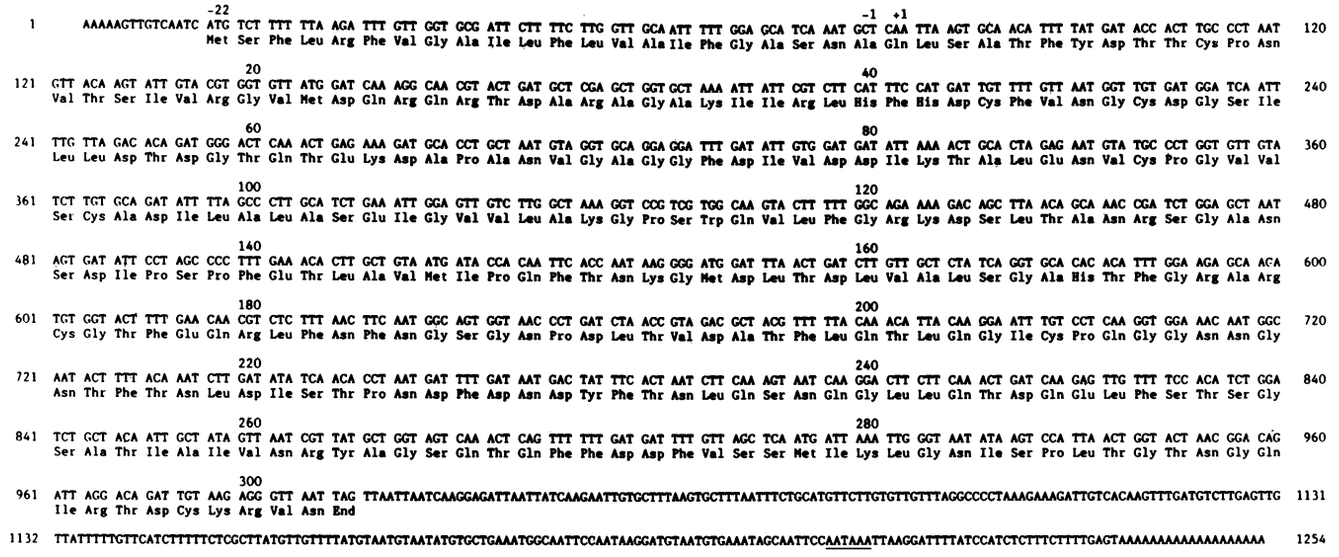

FIG. 2. Nucleotide and deduced amino acid sequence of a cDNA clone for the tobacco anionic peroxidase. The nucleotide sequence of the cDNA is presented over the amino acid sequence of the precursor protein. The start of the mature protein begins at position +1. The predicted polyadenylylation signal is underlined.

cationic peroxidases (30). The comparison of the three sequences is shown in Fig. 3. Tobacco and horseradish peroxidases were found to be 52% homologous, and the tobacco and turnip proteins were shown to be 46% homologous. This is similar to the 50% homology between the turnip and horseradish peroxidases. All three of the peroxidases have eight cysteines, which are located in very similar positions in the primary sequence. These cysteines in the horseradish and turnip enzymes have been shown to be involved in intramolecular disulfide linkages.

The predicted size of the mature tobacco peroxidase is 302 amino acids with a M_r of 32,311. It is predicted from the cDNA sequence that the protein is initially synthesized as a preprotein of M_r 34,635 with a predominantly hydrophobic 22-amino acid signal sequence (see Fig. 2). This was an expected result because the localization of the anionic isozymes to the extracellular space would require secretion. The mature protein is secreted as a glycoprotein, with four potential N-glycosylation sites (Asn-Xaa-Thr/Ser). These sites are at residues 13, 128, 185, and 282. The NH_2 terminus of the mature protein is a pyrrolidone carboxylic acid, as determined by the ability of pyroglutamate amino peptidase to deblock the mature NH_2 terminus. The predicted amino acid sequence contains 32 negative and 23 positive charged side chains at pH 6.0, leaving a net negative charge, which contributes to the acidic pI of the mature protein.

The longest cDNA clone is 1254 bp long, and it possesses 14 bp of the 5'-nontranslated leader and a 245-bp 3'-untranslated sequence before the start of polyadenylylation. It also contains the animal consensus polyadenylylation signal sequence AAUAAA at 38 nucleotides before the addition of poly(A) (Fig. 2) (31). A true consensus polyadenylylation sequence has not yet been determined for plant genes.

Tissue-Specific Expression of Peroxidase mRNA. Poly(A)$^+$ RNA was purified from healthy adult tobacco leaf, stem, and root tissue. These RNAs were fractionated on formaldehyde-agarose gels, transferred to nitrocellulose, and hybridized to the anionic peroxidase cDNA clone. The filters were washed under stringent conditions, which allow only the highly homologous mRNAs to be detected. The results shown in Fig. 4 reveal an abundant transcript of approximately 1300 nucleotides in stem tissue, and virtually no detectable transcript in either leaf or root tissue. The low abundance of the anionic mRNA in leaves is as expected, given the low frequency at which cDNA clones were found (0.01%). It is reasonable to assume that this gene would be highly expressed in stem tissue since the vascular tissue is so highly lignified (2). When mRNA was isolated from wounded stem tissue and probed, there was no appreciable increase in anionic peroxidase mRNA (data not shown).

Genomic Filter Hybridization. The tobacco species used in this work (*N. tabacum*) is allotetraploid and is thought to

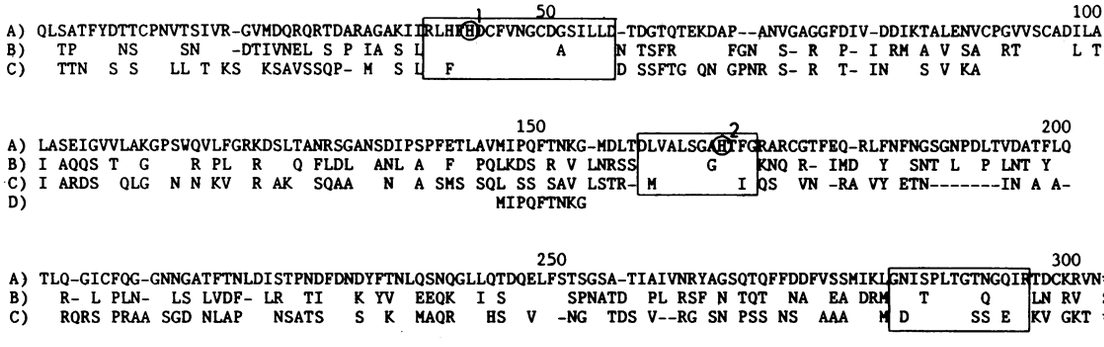

FIG. 3. Amino acid homology between three plant peroxidases. The best alignment of tobacco (*A*), horseradish (*B*), and turnip (*C*) peroxidases is shown. The peptide sequence from which an oligonucleotide probe was made is shown in *D*. Only those amino acids that are not identical to the tobacco sequence are indicated in the lower lines. Gaps in the sequence, which are included to better the alignment, are indicated by dashes. Asterisk marks the end of the protein. Regions boxed-in indicate >85% homology. [1]His residue 42 predicted to be involved in acid/base catalysis. [2]His residue 167 predicted to be the 5th ligand of heme (28). Amino acids are identified by the single-letter code.

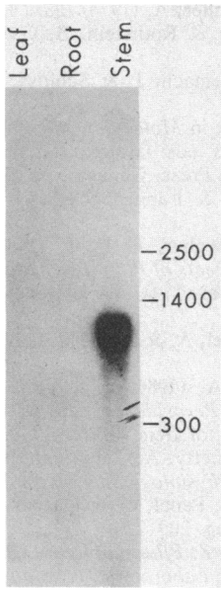

FIG. 4. RNA blot of tobacco leaf, root, and stem poly(A)$^+$ RNA probed with a cDNA clone to the anionic peroxidase. In each lane, 1 μg of poly(A)$^+$ RNA was applied. The blot was hybridized with radiolabeled cDNA (1×10^6 cpm/ml), washed at high stringency, and autoradiographed for 18 hr at −80°C. RNA standards are in nucleotides (Bethesda Research Laboratories).

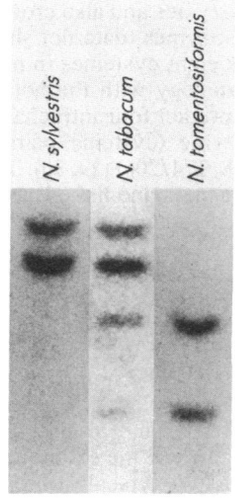

FIG. 5. Genomic DNA blot of three tobacco species using the anionic peroxidase cDNA for a probe. Chromosomal DNA from *N. sylvestris*, *N. tabacum*, and *N. tomentosiformis* (5 μg) was digested with *Eco*RV, subjected to 0.6% agarose gel electrophoresis, and transferred to nitrocellulose. Nick-translated cDNA probe was added at 1×10^6 cpm/ml, and filters were hybridized and washed for 30 min in 0.15 M NaCl/0.0015 M sodium citrate at 55°C (high stringency). Autoradiography was for 2 days at −80°C. From the top of the gel, the four fragments are 11,000, 8000, 5900, and 3700 bp.

have originated as an interspecific hybrid between *N. sylvestris* and *N. tomentosiformis* (32). On isoelectric focusing gels we have found that *N. sylvestris* expresses only $POD_{3.75}$, and *N. tomentosiformis* expresses only $POD_{3.5}$ (data not shown). This result suggests that the two anionic peroxidase isozymes of *N. tabacum* almost certainly serve the same function. To determine whether there is a distinct gene that encodes each isozyme, DNA blots were prepared on genomic DNA from each species.

Nuclear DNA was isolated from young tobacco leaves and digested with a number of different restriction enzymes. The digested DNA was fractionated on agarose gels, transferred to nitrocellulose, and probed with the radiolabeled cDNA clone. Between 2 and 4 bands were seen in restriction digests of *N. tabacum* genomic DNA probed at high stringency (>95% homology) with the anionic cDNA clone. The results of an experiment in which DNA from all three *Nicotiana* species was digested with *Eco*RV (which does not cut within the anionic peroxidase gene) are shown in Fig. 5.

These results indicate that the *N. tabacum* genome consists of four anionic peroxidase genes. It is clear that two copies were contributed from the *N. sylvestris* genome, and two copies were contributed from the *N. tomentosiformis* genome. By comparing peroxidase isozyme patterns with restriction digests, it has been determined that the two larger *Eco*RV fragments (11,000 and 8000 bp) encode $POD_{3.75}$ and originate from *N. sylvestris*. It has also been determined that the smaller fragments (5900 and 3700 bp) encode $POD_{3.5}$ and originate from the *N. tomentosiformis* genome.

When the genomic blots were probed at low stringency, anywhere from 10 to 15 bands could be detected (data not shown). These are likely peroxidase genes from the cationic and moderately anionic isozyme groups.

DISCUSSION

Using a four-step purification scheme, we have purified the tobacco anionic peroxidases to homogeneity. This preparation consists of two anionic isozymes in approximately equal amounts. Our material was similar to that of Mäder (14) with the exception of its migration on Na_2DodSO_4/PAGE. In his preparation, the two isozymes migrated as a single band of M_r 27,000, while we were able to resolve two bands of M_r 36,000 ($POD_{3.5}$) and M_r 37,000 ($POD_{3.75}$) (data not shown). We were not able to purify enough of the individual anionic isozymes for protein sequencing; therefore, presuming that they were similar polypeptides, we sequenced the mixture.

In the present study, we have obtained 20 cDNA clones by hybridization with oligonucleotides based on peptide sequence, and we have characterized four clones in more detail. All of the cDNA clones isolated apparently encode the same isozyme. The absence of clones for the other anionic isozyme may be due to differences in the amino acid sequence from which the oligonucleotide probe used in the screenings was derived. The amino acid sequence used for probe construction did happen to lie in a region of comparatively low homology (33%) when compared to the horseradish and turnip peroxidase sequences (see Fig. 3).

As shown in Fig. 4, the expression of the anionic peroxidase genes shows differential tissue-specific expression. The abundance of this mRNA in stem tissue presumably reflects the degree of lignification. The low abundance of mRNA to the anionic isozymes in leaf tissue was also reflected by the low frequency at which cDNA clones were obtained (0.01%). Furthermore, whereas the activity of the cationic and moderately anionic isozymes increases upon wounding, the activity of the anionic isozymes is unchanged (6), as is the level of mRNA detected with our probe.

Our deduced amino acid sequence for the tobacco anionic peroxidase shows homology with the amino acid sequences of the horseradish cationic peroxidase (29) and the turnip cationic peroxidase (30). The overall homology between tobacco and horseradish is 52%, while tobacco and turnip peroxidases are 46% homologous. As shown in Fig. 3, there are regions where the homology approaches 100%. Two of these conserved regions correspond to domains critical for general peroxidase activity. Homology between the various peroxidase isozymes was also demonstrated with antibodies made to the tobacco anionic peroxidase. By immunoblot analysis these antibodies cross-react strongly with the horse-

radish and turnip isozymes and also cross-react with most of the other tobacco isozymes (data not shown).

Each enzyme has eight cysteines in nearly identical locations (Fig. 3). By analogy with the horseradish and turnip sequences one can predict four intrachain disulfide linkages in the tobacco enzyme (cysteine pairs between residues 11/89, 44/49, 95/298, 174/206) (29, 30). The tobacco enzyme has four potential asparagine-linked glycosylation sites at positions 12, 128, 185, and 283. This compares to eight chemically determined N-linked sugars on the horseradish enzyme and one on the turnip enzyme (29, 30). There are two histidine residues predicted to be involved with catalysis (33). These are His-42, thought to act in acid/base catalysis, and His-167, thought to bind the 5th ligand of heme iron. Both of these are in regions of >90% homology.

Genomic Southern mapping experiments were performed to determine the number and origin of anionic peroxidase genes and also to determine the extent of homology with the other isozyme groups. We typically observed four restriction fragments in the *N. tabacum* genome hybridizing at high stringency to our cDNA clone. Since *N. tabacum* is allotetraploid and is an interspecific hybrid between *N. sylvestris* and *N. tomentosiformis*, we analyzed restriction digests from all three species.

We observed that the fragments present in the *N. tabacum* genome are the sum of the fragments found in the parent genomes. Moreover, by comparison of isozymes, we found that one anionic isozyme originated from each of the parent species. Each of the two isozymes would therefore be encoded by a pair of alleles, giving a total of four anionic peroxidase genes. We presume that each anionic isozyme provides an equivalent function, since it is not necessary to have both isozymes in the parent species. Their similarity is supported by homology at the gene and protein sequence level and their similar physical and biochemical properties (14).

The cloning of this cDNA encoding a plant peroxidase should help in the determination of the physiological role that each of the isozymes plays in the development of the plant. We are currently isolating cDNAs for the cationic and moderately anionic peroxidases and will use these as molecular probes to study the expression of each peroxidase group during developing, wounding, and infection to better understand the functions that peroxidases have in each of these physiological processes.

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