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Molecular cloning of complementary DNAs encoding two cationic peroxidases from cultivated peanut cells

(gene expression/λgt10/λgt11/secondary structure/*Arachis hypogaea*)

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ABSTRACT **We have isolated, cloned, and characterized two cDNAs corresponding to the mRNAs for cationic peroxidases synthesized by cultured peanut cells. The first clone was obtained from a phage λgt11 library screened with antibodies directed against the major secreted isozyme. Its predicted amino acid sequence, deduced from the 1228-base-pair (bp) cDNA, revealed a 22-amino acid signal peptide and a 294-amino acid mature protein (M_r, 31,228). The second clone was isolated from a λgt10 library screened with oligonucleotides corresponding to the regions for acid/base catalysis and the fifth ligand of heme. This cDNA (1344 bp) encodes a protein (330 amino acids) with a mature peptide of 307 residues (M_r, 32,954). The two peanut peroxidases are 46% homologous. The estimated gene copy numbers of these peroxidases might be close to 1 or 2 per haploid genome. A comparison of the amino acid sequence of these peanut peroxidases with other known isozymes shows two already known regions of homology (the region for acid/base catalysis and the fifth ligand of heme). Moreover, some new characteristics appeared such as a glycosylation site identical in five of the seven isozymes, a putative antigenic determinant common to all the isozymes, and a region of the highest homology. A secondary structure prediction showed that it corresponds to a 16-amino acid helix linked to the next one by a long stretch of β strands and coils and might represent a critical structural element.**

Most higher plants possess a large number of peroxidase isozymes, which have been used as convenient enzyme markers in genetic, physiological, and pathological studies (1, 2). Their pattern of expression is influenced by environmental stimuli, is developmentally regulated, and is tissue specific (3), as exemplified in tomato, where anionic isozymes account for nearly 90% of the peroxidase activity in all tissues except for the root tissue (4).

The large number (up to 30) of isozymes could represent gene copy numbers through polymorphism and posttranslational modifications or artifacts of preparation such as cross-linking with phenols (2). These many possibilities of so-called isozyme expression make it difficult to study the actual function of peroxidase. Peroxidase has been implicated in polysaccharide cross-linkages with extensin monomers (3), indole–acetic acid oxidation, lignification, wound healing, phenol oxidation, defense against pathogens, and regulation of cell elongation (1).

Cloning of plant peroxidase could help in elucidating the physiological role that each of the isozymes plays in plant development, and the first report appeared on anionic peroxidase in tobacco (6). Insertion of this cDNA under control of cauliflower mosaic virus 35S promoter into the tobacco genome led to the expected overproduction of the isozyme but also to wilting at the time of flower bud initiation (7). These remarkable results illustrate how complex the analysis of plant peroxidase function is. Under normal conditions the anionic isozyme is associated with cell wall formation, particularly lignin synthesis (4), and is preferentially expressed in immature xylem and epidermis (7).

Cultured cells are able to respond rapidly to many environmental stimuli or to biological compounds such as elicitors (8). Therefore, they may serve as a simpler experimental model to analyze (*i*) the mechanisms by which plant cells perceive these signals and (*ii*) the series of cellular events that lead to modulation of transcription of specific genes. Peroxidases are not only synthesized by cultured cells but also are secreted into the medium (2, 9) from which they may be purified easily. Extracellular peroxidase activity has a strong correlation to growth kinetics and cell dry weight (10).

Peanut cell peroxidases have been studied in detail (2), and it was shown that the released isozymes consisted of two cationic and one anionic species. The major cationic isozyme represents 75% of the medium peroxidase activity. Up to 2% of the total poly(A)$^+$ mRNAs isolated from the cultured cells are engaged in its synthesis (11). These results raised some intriguing questions. Is the extracellular location of the cationic isozyme related to a specific function for the cultured cells? Are its high rate of synthesis and secretion directly related to the mode of growth of these cells? Is there only one major cationic peroxidase synthesized by these cells and, if so, what are the mechanisms of activation and repression of the various peroxidase genes? Answering these questions would broaden our knowledge of the peroxidase function. To this end we undertook the cloning of the major secreted cationic isozyme from peanut cells but also extended our search to other genes to analyze their differential expression. In this communication we present our first set of results devoted to two different cDNAs encoding cationic peroxidases, one of them being the major secreted isozyme.§

MATERIALS AND METHODS

Plant Cells. Culture of peanut cells, *Arachis hypogaea*, was as described (12). Cells were collected (after 6 or 7 days of subculture) by filtration through a sterile 3MM Whatman disk and frozen at −180°C.

Preparation of RNA. Frozen cells (10 g fresh weight) were grown with a Polytron in 25 ml of 6 M guanidinium chloride/0.1 M potassium acetate. Total RNA was extracted by the

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§The sequences reported in this paper have been deposited in the EMBL/GenBank data base (accession nos. M37636 for PNC1 and M37637 for PNC2).

guanidinium salt procedure (13). The poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography.

Construction of the cDNA Libraries. Double-stranded cDNA of poly(A)$^+$ RNA was synthesized by using the Amersham cDNA synthesis system and was used to prepare phage λgt10 and λgt11 libraries (Amersham systems).

Antibody Selection of λgt11 Recombinants. The λgt11 clones were plated on *Escherichia coli* strain 1090 to yield 3 × 10^4 plaque-forming units per 100-mm plate and were screened as described by Huynh *et al.* (14). Immunodetection was carried out as described (15).

Oligonucleotide Screening. Mixed oligonucleotides, synthesized by Appligène (Strasbourg, France) were end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP at 3000 Ci/mmol (1 Ci = 37 GBq) from NEN. The ampicillin-resistant transformants were screened by plaque hybridization with the oligonucleotides mixtures as a probe by the method of Woods *et al.* (16) with hybridization temperatures of 37°C and 42°C for the 17- and 23-mers, respectively. Final washings were done at 47°C and 52°C, respectively.

Subcloning and DNA Sequencing. After the subcloning into M13mp19, deletions of the inserts were prepared using the Cyclone System from IBI. Nucleotide sequences were determined by the dideoxynucleotide chain-termination method {T7 sequencing kit from Pharmacia and deoxy-adenosine 5′-[α-(^{35}S)thio]triphosphate at 1500 Ci/mmol from NEN}.

DNA Filter Hybridization. Peanut cell high molecular weight DNA was isolated as described by Richards (17). DNA (10 μg) was cleaved with various restriction enzymes and subjected to 0.6% agarose gel electrophoresis. The DNA was then transferred to Hybond N (Amersham) filters and hybridized with cDNA inserts (labeled with the Amersham multiprime DNA labeling system and [α-^{32}P]dCTP at 800 Ci/mmol from NEN) at a specific activity of 1.8 × 10^8 cpm/μg (prxPNC1 clone) or 1.8 × 10^9 cpm/μg (prxPNC2) at a temperature of 42°C in 50% formamide according to Amersham's instructions. For washing, high-stringency conditions were used—i.e., the final wash was at 62°C in 0.018 M NaCl/1 mM phosphate, pH 7.4/0.1 mM EDTA/0.1% sodium dodecyl sulfate (SDS). A quantitative analysis was done on the genomic blots with a BIOCOM 200 apparatus.

Computer Analysis. Analyses were made by using the PCGENE software (Genofit) or the methods developed by Biou *et al.* (18) for secondary structure prediction.

RESULTS

Cloning Strategy. At the outset of this work all of the available molecular data consisted of the amino acid sequences from the horseradish (19) and turnip (20) peroxidases. Thus, our strategy was based on the preparation of a λgt11 library, the screening of which was to be conducted with polyclonal antibodies directed against the glycosylated or chemically deglycosylated (21) form of the major cationic peroxidase secreted by the cultured peanut cells.

A significant comparison of some regions that might have been conserved (i.e., the active site) has been made possible through the publication of the cDNA sequences encoding two anionic peroxidases—one from tobacco (6) and the other from potato (22). The deduced oligonucleotide probes corresponding to the regions for acid/base catalysis and the fifth ligand of heme and designated as "acid/base probe" and "heme fixation probe" (Fig. 1) were used to check, first of all, the tentatively positive inserts detected from the λgt11 bank. Moreover, they made possible the use of a λgt10 library to search for related core sequences encoding polypeptides that might not respond to the available antibodies.

Screening of the λgt11 Library. A cDNA library consisting of 5 × 10^6 plaque-forming units/μg of λ DNA was constructed in λgt11. One-fifth of the library was plated; after two rounds of screening with antibodies directed against the native peroxidase, 20 positive clones were identified with inserts ranging from 0.7 to 2 kilobases (kb). As the deglycosylated protein has a M_r of 31,500 (21), nine phages bearing an insert from 0.9 to 1.2 kb were submitted to a new round of immunodetection, this time using the antibodies directed against the deglycosylated peroxidase. Seven of them gave a positive response, and the phage DNAs from four plaque-purified individual clones were isolated for subcloning and DNA sequencing.

A	Horseradish	PHE-HIS-ASP-CYS-PHE-VAL	VAL-ALA-LEU-SER-GLY-GLY-HIS-THR
	Turnip	- - - - - -	- - - - - - ALA - -
	Tobacco	- - - - - -	- - - - - - ALA - -
	Potato	- - - - - -	- - - ALA - ALA - -
B	Tobacco	TTC CAT GAT TGT TTT GTT	GTT GCT CTA TCA GGT GCA CAC ACA
	Potato	--- --- --C --C --C ---	--- --G --- GCT --- --G --- --G
C	Synthesized Oligonucleotides	TTC CAC GAC TGC TTC GT --T --T --T --T --T -- 17 MERS PROBE ("Acid/base Probe")	(a) { GTT GCA CTA TCA GGA GCA CAC AC { --- --T --T --T --T --T --T -- (b) { GTT GCG CTA GCA GGA GCG CAC AC { --- --T --T --T --T --T --T -- 23 MERS PROBES ("Heme fixation Probe")

FIG. 1. DNA sequence of the oligonucleotide probes for screening peanut cDNA clones. (*A*) Direct (horseradish and turnip) or deduced (tobacco and potato) amino acid sequences of the regions corresponding to acid/base catalysis and the fifth ligand of heme. (*B*) The nucleotide sequence of the corresponding tobacco and potato cDNAs. (*C*) The oligonucleotide probes: the acid/base probe is a mixture of 32 combinations of 17-mers; the heme fixation probe consists of two sets (a and b) of 64 combinations of 23-mers synthesized independently but mixed together for the screening.

Nucleotide Sequence Analysis of the prxPNC1 Clone. A strong cross-hybridization was observed between the four selected inserts, and partial sequencing of these four clones indicated that they were derived from the same RNA. The complete nucleotide sequence of the longest cDNA insert [1128 base pairs (bp)], noted as prxPNC1, and the deduced amino acid sequence of this clone are shown in Fig. 2. Hybridization with the oligonucleotide probes (not shown) and comparison with the previously published sequences (6, 19, 22, 23) indicated that this is indeed a peroxidase cDNA.

The predicted size of the mature peroxidase is 294 amino acids with a M_r of 31,228. It is predicted from the cDNA sequence that the protein is synthesized as a preprotein of M_r 33,549 with a predominantly hydrophobic 22 amino acid signal sequence (Fig. 2). The mature protein, of cationic nature (theoritical pI, 7.8), is secreted as a glycoprotein with four N-glycosylation sites (at residues 60, 142, 185, and 275), as also found by protein hydrolysis (21).

Screening of the λgt10 Library and Nucleotide Sequence Analysis of the prxPNC2 Clone. The λgt10 library consists of 4.5 × 10^5 plaque-forming units/μg of λ DNA, and probing was done on half of the library with the "acid/base probe." Seven positive plaques remained after a second screening, corresponding to phages bearing an insert from 0.8 to 2 kb. We selected one plaque-purified phage bearing an insert of around 1.3 kb, designated prxPNC2, that does not cross-hybridize with prxPNC1 in stringent conditions (not shown). The nucleotide and deduced amino acid sequences of the prxPNC2 clone are presented in Fig. 3. The cDNA encodes a preprotein of M_r 35,556 (330 amino acids) with a putative signal sequence of 23 residues (Fig. 3). The mature polypeptide (307 amino acids, M_r 32,954) is also of cationic nature (theoretical pI, 8.5), and it bears only one glycosylation site (at position 189).

The cDNA clone is 1344 bp long and possesses a 199-bp sequence upstream of the starting ATG, which is characterized by a long repeated sequence of 69 nucleotides (boxed in Fig. 3) rich in A+T (73.9%). The 155-bp 3′ untranslated

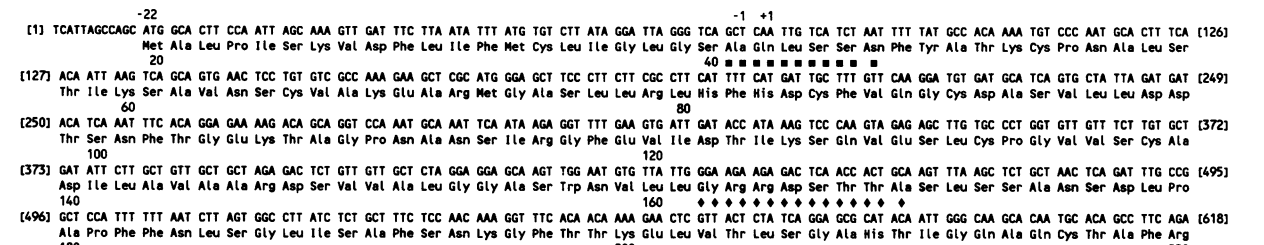

FIG. 2. Nucleotide and deduced amino acid sequence of the prxPNC1 cDNA clone. The nucleotide sequence is presented over the amino acid sequence for the precursor protein, the start of the mature protein being at position +1. The localization of the oligonucleotide probes (see Fig. 1) is indicated by ■ for the acid/base probe and ◆ for the heme fixation probe. A putative polyadenylylation signal is underlined.

region contains a consensus polyadenylylation signal (AATAAA) 27 nucleotides before the addition of poly(A).

Comparison of the Peroxidases. A comparison, based on the matrix used by Risler *et al.* (24), of the mature peanut cationic peroxidases, designated PNC1 and PNC2, respectively, with already published complete sequences (6, 19, 20, 22, 23, 25) is shown in Fig. 4, the partial sequence of wheat peroxidase (26) also being included. The PNC1 was found to be 46% homologous to PNC2, 61% to turnip TP7, and ≈50% to the others. The PNC2 is significantly less homologous to other peroxidases, being from 33% (tomato or potato isozymes) to 47% (turnip TP7) homologous.

All of the peroxidases have eight cysteine residues, which are located in similar positions in the primary sequences and two invariable histidine residues (at positions 42 and 169 in PCN1, Fig. 4) that have been inferred in the active-site structure.

Three rather large domains of homology can be noticed. In the first one (residues 33–56), the peroxidases are 62–83% homologous; the histidine involved in the acid/base catalysis belongs to a subdomain of 100% homology: Phe-His-Asp-Cys-Phe-Val. In the second region (residues 91–110), the homology ranges from 61% to 95% with a sequence of seven residues (Val-Ser-Cys-Ala-Asp-Ile-Leu) present in six of the seven peroxidases and in the wheat sequence; the only discrepancy is that of horseradish peroxidase, in which there is a replacement of the isoleucine by a leucine. The third domain (12 residues from 161 to 172) includes the histidine residue involved in the fifth ligand of heme and shows more variability, with homology ranging from 58% to 75%.

Among the shortest regions (121–127 and 138–142), the first one (Leu-Gly-Arg-Arg-Asp-Ser in PNC1) might be an important structural element, as it corresponds to a tentative antigenic determinant, the location of which is common to all of the analyzed peroxidases.

The number of glycosylation sites varies greatly according to the isozyme (from one in PNC2 and turnip to eight in horseradish). However, a glycosylation site (Asp-185) is common to five of the seven peroxidases.

A separate secondary structure prediction on PNC1 and PNC2, based on the methods of Biou *et al.* (18), is shown in Fig. 5. A striking similarity appears in the relative distribution of predicted helices in both sequences, despite the uncertainties linked to the secondary structure predictions. These predicted secondary structures are in good agreement with the tertiary structure model of Welinder (27). For example, the region of strongest homology (91 to 106 in Fig. 4) corresponds to an helix of 16 residues (helix D) linked to the next one (helix E) by a stretch of around 30 residues consisting of β-strands and coils. This might represent a critical structural element.

Estimation of Gene Copy Numbers. Restriction digests of peanut DNA and reconstruction standards were hybridized with the two peroxidase cDNAs prxPNC1 and prxPNC2. As shown in Fig. 6, a different set of fragments is hybridizable to each of the two probes, confirming the lack of cross-hybridization between the two clones in our conditions. A densitometric comparison of the hybridization signals suggests that the copy numbers per hyploid genome is around 1 for prxPNC2 and 2–3 for prxPNC1.

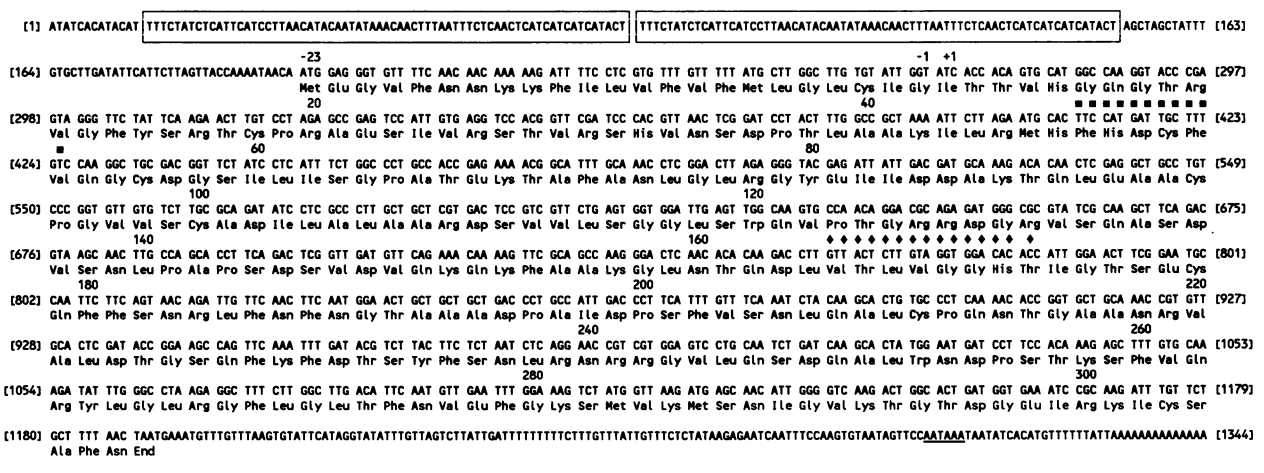

FIG. 3. Nucleotide and deduced amino acid sequence of the cDNA clone prxPNC2. Symbols are as in Fig. 2; the polyadenylylation signal is underlined, and the direct repeat sequence is boxed.

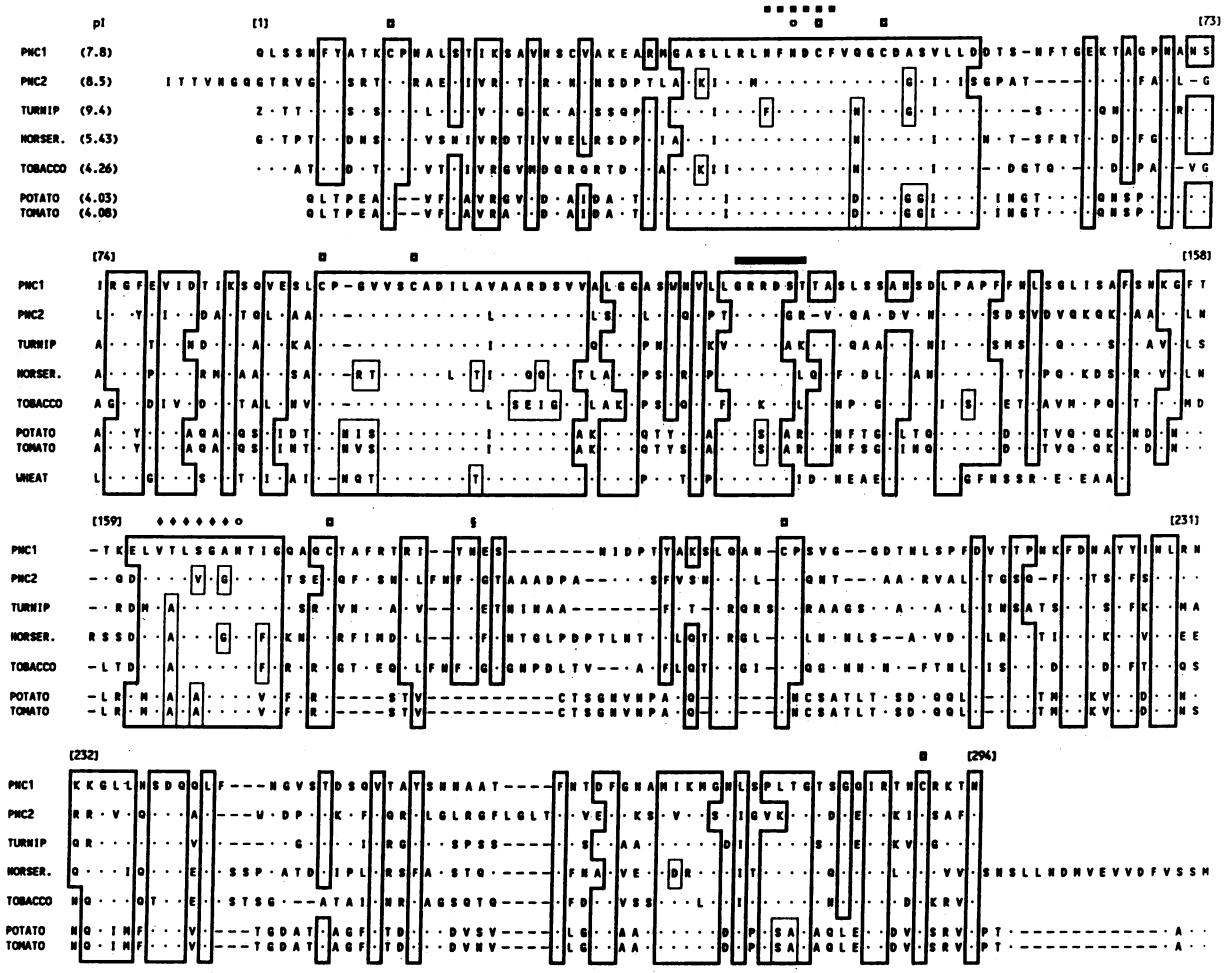

FIG. 4. Amino acid homology between seven mature and complete plant peroxidases. Alignment of the two peanut peroxidases (PNC1 and PNC2) is with the turnip [TP7] (20), tobacco (6), horseradish [C1] (23), potato [AP] (22), and tomato [Tap1] (25) isozymes; the partial wheat sequence (26) is also included for comparison. The calculated pI is indicated for each isozyme. The identical amino acids are designated by a dot, and gaps (introduced to produce the best alignment) are indicated by dashes. The position of the two used oligonucleotide probes is shown by ■ and ◆ (see Figs. 2 and 3). Regions boxed (heavy lines) indicate homology based on equivalent amino acids [F/Y, K/Q/R, S/T, V/I/L/(M)] as described by Risler *et al.* (24); nonequivalent amino acids in these regions are boxed by lighter lines. Conserved cysteines and histidines are noted by ■ and ○, respectively. A putative antigenic determinant present in all sequences is noted by a heavy bar (▬) and a common glycosylation site by §.

DISCUSSION

By using antibodies directed against the major isozyme secreted by peanut cells and oligonucleotide probes (constructed from the comparison with available peroxidase sequences) for the screening of λgt11 and λgt10 libraries, respectively, we have isolated several clones, two of which were thoroughly analyzed. The first clone (prxPNC1) was

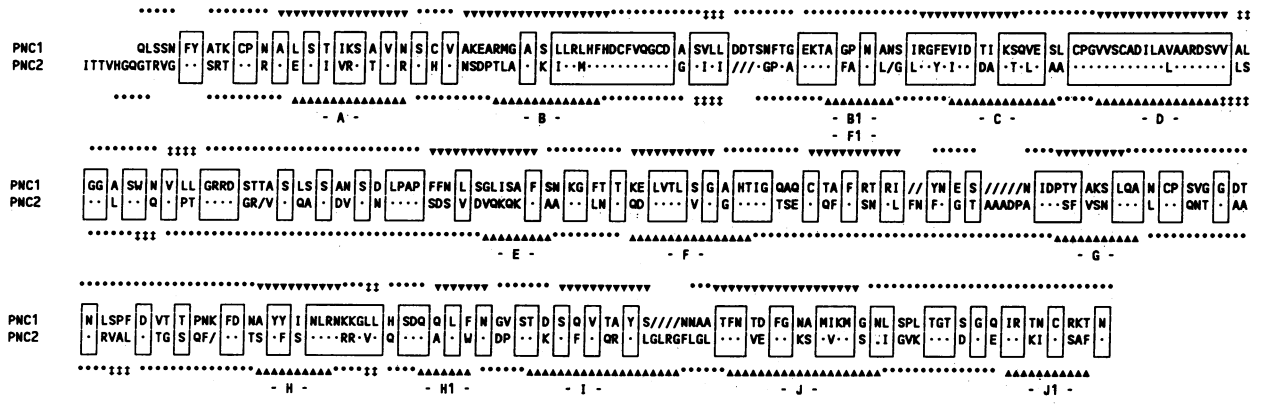

FIG. 5. Alignment of the two peanut peroxidases and predicted secondary structure. Identical amino acids are indicated by a dot, and a gap is indicated by a slash. The homologous regions (defined as in Fig. 4) are boxed. The first and fourth lines show the predicted secondary structure of the PNC1 and PNC2 peroxidases, respectively. Helices are represented by ▼ for PNC1 and ▲ for PNC2, coils by ○, and beta strands by ↕. The numbering of the helices is according to Welinder (27).

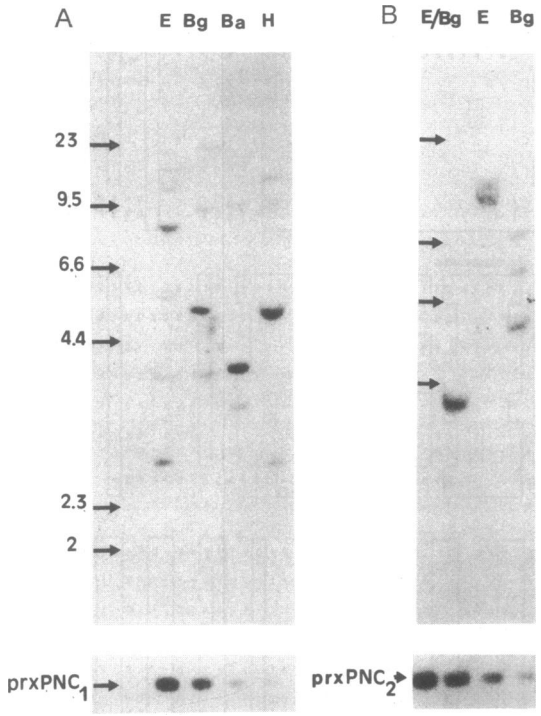

FIG. 6. Southern blot analysis of peanut DNA with peroxidase cDNA clones. Samples (10 μg) of DNA from peanut cells were digested with restriction endonuclease enzymes singly or in combination prior to agarose electrophoresis, blotting, and hybridization with labeled cDNA probes prxPNC1 (*A*) and prxPNC2 (*B*). For copy number standards, purified *Eco*RI inserts [equivalent to 0.5, 1, 2, and 4 (prxPNC1) or 0.175, 0.35, 0.7, and 1.4 (prxPNC2) copies per haploid genome] were loaded onto the gel and were hybridized to the corresponding cDNA. Sizes are shown in kbp. Ba, *Bam*HI; Bg, *Bgl* II; E, *Eco*RI; H, *Hin*dIII.

detected in both libraries and the second (prxPNC2) was isolated from the λgt10 library. They were sequenced in their entirety.

Homology of the deduced amino acid sequences between the two peanut peroxidases, which are synthesized as preproteins with a signal peptide of around 22 residues, is about 44%. The overall homology among the peanut isozymes and the other five known sequences (Fig. 4) ranges from 33% (PNC2 vs. highly anionic peroxidases from potato or tomato) to 61% (PNC1 vs. turnip TP7). However, amino acid alignment around the functional histidine residues (19)—i.e., His-42 and His-168, was conserved in all of the sequences and shows three rather large regions of homology.

The fifth ligand of heme region (residues 161–172) is much more variable than the others, as shown in Fig. 4, and that is confirmed by the published partial sequence of cucumber (5).

Two sets of six or seven amino acids are very highly conserved. The first one (Phe-His-Asp-Cys-Phe-Val) belongs to the acid/base catalysis region (residues 33–56) and includes the functional histidine residue. The second (Val-Ser-Cys-Ala-Asp-Ile-Leu), belonging to the third region of homology (residues 91–110), exists in seven of the eight isozymes, the horseradish C1 having a leucine (Fig. 4) and the C2 and C3 isozymes (not shown) having a valine instead of the isoleucine (23). According to secondary structure analysis (Fig. 5), this region consists of a helix (D) followed by a long stretch of β-strands and coils linking it to the next helix (E). This in excellent agreement with the peroxidase tertiary structure model built by Welinder (27) from a comparison of the horseradish and turnip proteins with the yeast cytochrome *c* peroxidase, which suggests that helix D is deeply buried and that its conserved sequence might indicate essential peptide–peptide contacts. The other two common characteristics noted in Fig. 4 (putative antigenic determinant and glycosylation site) are easily located on the surface of the structural model and correspond to exposed regions. This glycosylation site starting with Asn-185 [Asn-Xaa-(Ser or Thr)] is very close to Arg-182 and Tyr-184 that are putative binding sites of the substrate (5) and are conserved in all the sequences except the highly anionic isozymes from potato and tomato (Fig. 4).

Genomic southern mapping experiments showed that the gene copy numbers might correspond to 1 or 2–3. These values are in reasonable agreement with those available for tobacco [two copies of two parental peroxidase genes (6)] or tomato [two copies each of highly homologous genes (23)].

Unlike the two anionic isozymes from tomato (25) and the three moderately anionic ones from horseradish (23), which correspond in each case to very highly homologous polypeptides, the two peanut cDNA clones are encoding distinct molecular species of cationic nature, one of them being probably the major secreted peroxidase.

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