

Characterization and tissue-regulated expression of genes involved in pineapple (*Ananas comosus* L.) root development

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Abstract

This study was initiated to increase the molecular genetic resources available for pineapple (*Ananas comosus* L.) breeding and biotechnology. We differentially screened a root cDNA library for mRNAs that are preferentially present in the roots. Over 300 randomly selected cDNAs from the root library were categorized into 14 classes based on their expression characteristics. About 41% of the clones were classified as constitutive, being expressed about equally in roots, fruit and aerial tissues, while 23% of the clones were expressed higher in roots than other tissues. Twenty-four unique clones were sequenced and preliminarily identified based on sequence homology. Genes involved in lignin biosynthesis, cell wall biogenesis, and responses to stress or pathogens were highly represented. Six mRNAs (encoding a germin-like protein, mannose-binding lectin, two lipoxygenases, xyloglucan endotransglycosylase and peroxidase) were verified to be preferentially present in roots by analyzing in detail their abundance during field-grown plant and fruit development. Analysis of mannose-binding lectin gene expression via tissue-prints and micro-dissection of roots showed that its transcript was predominantly present in the protoderm. The occurrence of stress/pathogen-related mRNAs in healthy tissues is discussed in terms of processes that allow CAM plants to adapt to extreme environments. The molecular methods and genetic resources developed in this work will facilitate isolation of root-specific promoters, physical mapping, marker-assisted breeding, genomic studies and bioengineering for pineapple improvement.

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1. Introduction

Cultivated pineapple (*Ananas comosus* L.) is a xerophytic monocot and a member of the Bromeliaceae [1]. It utilizes crassulacean acid metabolism (CAM), a key adaptation of photosynthetic carbon fixation that allows it to be highly productive in limited water availability. However, production is confined by problems such as precocious flowering [2] and susceptibility to numerous diseases and pests [3]. Root-knot (*Meloidogyne* spp.) and reniform nematodes (*Rotylenchulus reniformis*) are the most costly problems in terms of control and lowered fruit production [4]. The chemical

fumigants used to control nematodes are expensive and pose environmental hazards causing governments to ban them.

Genetic variation among pineapple varieties and wild relatives has potential for use in the genetic improvement of pest/pathogen resistance, fruit weight, sugar content, ripening, nutritional value, and post-harvest storage life. Improving pineapple via breeding and genetic transformation can be greatly facilitated by knowledge of its genome sequence, codon usage, tissue-specific promoters, molecular markers, and mechanisms regulating gene expression. However, the only pineapple genes described in literature are for rRNAs [5], ACC synthase [2], polyphenol oxidases [6], ACC oxidase [7], Cu/Zn-superoxide dismutase [8], and ananain [9]. Transposable elements have recently been reported [10] and may explain some of the observed somatic variation [11]. The lack of availability of

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molecular markers that could be used to establish linkage relationships with desirable traits and screen young plants is a major limitation in the breeding programs.

For genetic engineering, tissue-specific gene expression is often a desirable property, because constitutive expression of transgene products may not be acceptable to consumers, can interfere with metabolic or developmental processes and may not provide adequate levels of expression. The promoter is a crucial control element determining the degree and tissue-specificity of transgene expression, but tissue-regulated promoters and genes have not been described for pineapple.

We seek promoters preferentially expressed in roots to drive the expression of nematicidal proteins to control nematodes. Recently, cDNAs encoding proteins with nematode-inhibiting abilities have been cloned from several plant species [12–14]. In addition, previous studies have identified a large number of root-specific genes (e.g. [15–17]). However, most studies were performed on dicots or monocots, such as maize and rice. The dicot, *Mesembryanthemum crystallinum*, is the only CAM plant for which root-enhanced genes have been isolated [18].

As a first step to obtain suitable promoters, we have isolated and characterized cDNAs corresponding to mRNAs that are specifically present in roots during the entire crop cycle. Furthermore, we have identified a large number of cDNAs for mRNAs that have alternative distribution patterns in roots, fruits and aerial tissues. The expression of the corresponding genes was determined in a variety of tissues. A high number of the isolated cDNAs encode proteins that not only function in root development, but may function in stress and/or pathogen responses as well.

2. Materials and methods

2.1. Plant growth conditions

A. comosus L. (cv Smooth Cayenne) field plants were grown at Del Monte (Del Monte Produce Hawaii Inc., Kunia, HI) in central Oahu with a temperature range of 18–32 °C. The same variety was also grown in sand in the greenhouse at 23–30 °C. Water-grown plants were derived from crowns removed from mature fruit. The crowns were placed in water in 1 l beakers at 25 °C and grown for 25 days. Water was renewed every 2 days to avoid rot.

2.2. RNA isolation

Plants were collected, briefly washed, dry-blotted, cut and immediately frozen in the field with liquid N₂ to prevent induction of stress-related genes in the tissues.

Root RNA from field- and sand-grown plants was isolated from fresh tissue at the root apices only (distal 4–5 cm). The woody basal ends gave RNA of poor quality and low yields. Aerial tissues were used either entirely (3.5 months old plants) or partly (older plants) after cutting into equal halves. Fruits and other plant organs were also cut into equal halves to obtain RNA that represented the whole organ equitably. Water-grown roots were dry-blotted before freezing. All RNA was isolated as described [19]. Thorough grinding of fruit samples (20 min) was a prerequisite to obtain acceptable yields of RNA.

2.3. cDNA library construction and reverse northern screening

Total cell RNA from the root apices of 15 month-old sand-grown pineapple plants was enriched for poly(A)⁺ RNA using the PolyATtract mRNA Isolation System III (Promega, Inc., Madison, WI, USA). A cDNA library was constructed using the ZAP-cDNA synthesis kit (Stratagene, Inc., La Jolla, CA). Random plasmids were generated by mass excision according to the manufacturer's instructions. Three hundred and seven random plasmids were digested with *Eco*RI and *Xho*I and equal amounts (~1 µg) of each plasmid were loaded on three gels to make three identical blots. After electrophoresis, the gels were soaked in 0.4 N NaOH for 2 × 15 min and blotted onto GeneScreen Plus (NEN Life Science Products, Boston, MA) [20] using 0.4 N NaOH as transfer buffer.

Single-stranded cDNA probes were synthesized from the root apices of 15 month-old sand-grown plants, the aerial tissue (all above ground tissue excluding the fruit) of 13.5 months old field-grown plants and from shell color 4 fruits (excluding the crown). After poly(A)⁺ RNA isolation, both quantity and quality were checked on a 1.5% 1 × TBE agarose gel [20]. An oligo(dT)₁₅ primer (2 µg) was annealed to approximately 1 µg poly(A)⁺ RNA. Reverse transcription was performed using 100 U M-MLV Reverse Transcriptase (Promega) in 50 mM Tris pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.1 mg/ml BSA, 1.4 U/µl RNasin (Promega), 1 mM dGTP, dATP, and dTTP, 0.01 mM dCTP and 100 µCi [α -³²P]dCTP (ICN, Costa Mesa, CA) (total volume 50 µl). The reaction proceeded for 1 h at 37 °C and another 30 min after the addition of 1 µl 10 mM dCTP. Free label was removed by ProbeQuant G-50 MicroColumns (Amersham Pharmacia, Little Chalfont, UK) and the incorporation of ³²P was determined by spotting on Whatman DE-81 paper [20]. The remaining RNA was degraded by boiling in 0.15 N NaOH. Pre-hybridization and hybridization took place in 5 × SSPE, 5 × Denhardt's, 0.1 mg/ml herring sperm DNA (Promega) and 0.5% SDS at 65 °C for 24 h. The blots were briefly washed twice in 0.5 × SSPE/0.1% SDS at

RT and for 10 min at 65 °C in the same buffer and then exposed to X-ray film (Molecular Technologies, St Louis, MO).

2.4. Northern blot analysis

RNA concentrations were determined spectrophotometrically. 10 µg of total cellular RNA was denatured with glyoxal and loaded on a 1.5% agarose gel [20]. After electrophoresis and transfer to Genescreen Plus, the blots were hybridized to [α^{32} P]dCTP-labeled cDNA inserts. Labeling was performed with Amersham Pharmacia's Oligolabeling Kit and free label was removed subsequently with ProbeQuant G-50 MicroColumns (Amersham Pharmacia). Hybridization and prehybridization were done in 50% deionized formamide, 5 × SSPE and 2.5% SDS at 42 °C for 24 h. After hybridization, blots were briefly washed in 0.5 × SSPE at RT and 3 × 20 min in 0.5 × SSPE/0.1% SDS at 70 °C before exposure to X-ray film or a Cyclone Storage Phosphor Screen (Packard Instrument Company, Inc., Meriden, CT).

2.5. Tissue print hybridization

Tissue-printing was carried out essentially according to [21]. Roots from water-grown plants were dry-blotted before sections were made with a razor blade. The exposed planes were blotted onto GeneScreen Plus (NEN Life Science Products) and hybridized with [α^{35} S]UTP-labeled antisense or sense RNA probes synthesized with Promega's RNA Transcription Kit. After hybridization, blots were exposed to a super resolution Cyclone Storage Phosphor Screen (Packard Instrument Company, Inc.) for 3 weeks before scanning. Ink-stainings (India ink) were performed after scanning for accurate localization of hybridization.

2.6. Micro-dissection of water-grown pineapple roots

Excised whole roots were separated using tweezers into inner and outer explants (Fig. 6C and D). The inner explants were a small cell mass containing the central ground tissue and the metaxylem (Fig. 6C and D, right panels). The outer explants contained the epidermis, phloem, endodermis, cortex and protoxylem (Fig. 6C and D, left panels). The explants were sectioned at 60 µm with a cryotome (Cryocut 1800, Reichert-Jung) at –20 °C and stained with 0.05% Toluidine Blue. Due to the thickness of the outer explants it was necessary to use polarizing optics to depict the tissues photographically.

2.7. Sequence analysis

DNA was isolated using the Qiagen mini kit (Qiagen, Hilden, Germany). Primers were obtained from IDT (Coralville, IA) and sequencing reactions were performed by the Biotechnology/Molecular Biology Instrumentation and Training Facility at the University of Hawaii at Manoa. Sequences were analyzed using the GCG package (Madison, WI), BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>), PROSITE (<http://www.expasy.ch/tools/scnpsit1.html>), SIGNALP (<http://www.cbs.dtu.dk/services/SignalP/>), CLUSTALW (<http://dot.imgen.bcm.tmc.edu:9331/multi-align/Options/clustalw.html>) and links on the CMS homepage (<http://www.unl.edu/stc-95/ResTools/cmsbp.html>).

3. Results

3.1. Isolation of 'root-enhanced' and other cDNAs via reverse northern hybridization

A total of 307 randomly selected recombinants from the root-specific cDNA library were screened with first-strand cDNA probes (normalized for specific activity) that were derived from mRNA of the following three tissues: (i) root apices of 15 month-old plants; (ii) fruits with shell color 4; and (iii) aerial parts of 13.5 month-old plants. Hybridization of each cDNA insert with the three tissue-derived probes was scored (Table 1). The majority of the mRNAs (41.0%) were approximately present at equal levels in all three different tissues. A relatively high number of mRNAs (22.9%) were preferentially present in roots, which is consistent with the

Table 1
Occurrence of randomly picked root mRNAs in roots, fruits and aerial parts

Relative mRNA abundance in the different tissues	Percentage of random cDNAs (number of cDNAs)
roots > fruits > aerial	41.0% (126)
*roots > fruits > aerial	18.6% (57)
roots > aerial > fruits	9.8% (30)
roots > fruits > aerial	6.2% (19)
low-expressed	4.9% (15)
fruits > roots > aerial	4.2% (13)
*roots > aerial > fruits	3.6% (11)
fruits > aerial > roots	3.3% (10)
aerial > roots > fruits	2.6% (8)
fruits > aerial > roots	2.0% (6)
aerial > roots > fruits	1.3% (4)
fruits > roots > aerial	1.0% (3)
aerial > fruits > roots	1.0% (3)
*roots > fruits > aerial	0.7% (2)

The asterisk indicates cDNA clones for mRNAs preferentially expressed in roots.

fact that the library was established from roots, and 15.4% were more abundant in fruits and/or aerial parts.

Eleven cDNAs for mRNAs that were preferentially present in roots and 13 cDNAs representing mRNAs with alternative distribution patterns were selected for further analysis. The differential abundance of the corresponding mRNAs was confirmed using Northern blots containing total cellular RNA from root, fruit and aerial tissues (Fig. 1). The cDNA probes of *PHR30*, *PHR62*, *PHR147*, *PHR181*, *PHR235* and *PHR284* hybridized almost exclusively with root RNA. Two cDNAs, *PCE84* and *PCE245*, had relatively constitutive expression patterns. Transcripts corresponding to clone *PRA116* and *PRA173* were present in both roots and aerial tissues, while mRNAs for *PFE18*, *PFE198* and *PFE258* were more abundant in fruit than either roots or aerial tissues. Other cDNAs represent mRNAs that are also highly abundant in roots, but have low levels in either aerial tissues (*PMR91*, *PMR107* and *PMR117*) or fruits (*PMR272* and *PMR281*). Most notable of the remaining cDNAs are *PMP38*,

PAE122, and *PAE266*. *PMP38* hybridizes to a longer mRNA in roots and fruits than in aerial tissue. The smaller mRNA in aerial tissue either is a closely related, shorter member of the *PMP38* gene family or represents an alternative, tissue-specific processing of the same mRNA. The *PAE122* and *PAE266* mRNAs were rare in roots, but were very abundant in aerial tissues.

3.2. Sequence analysis

All cDNAs (Fig. 1) were sequenced and compared with sequences in the databases. Their predicted protein products are shown in Table 2. Four additional cDNA clones that were initially selected and subjected to northern analysis (data not shown) were found to represent shorter versions of cDNAs already present in Table 2. The isolation of multiple independent cDNAs that represent the same mRNA suggests that the corresponding mRNA is highly abundant. Although the average length of the cDNAs was 814 bp, half of them were presumably full length, indicating that pine-

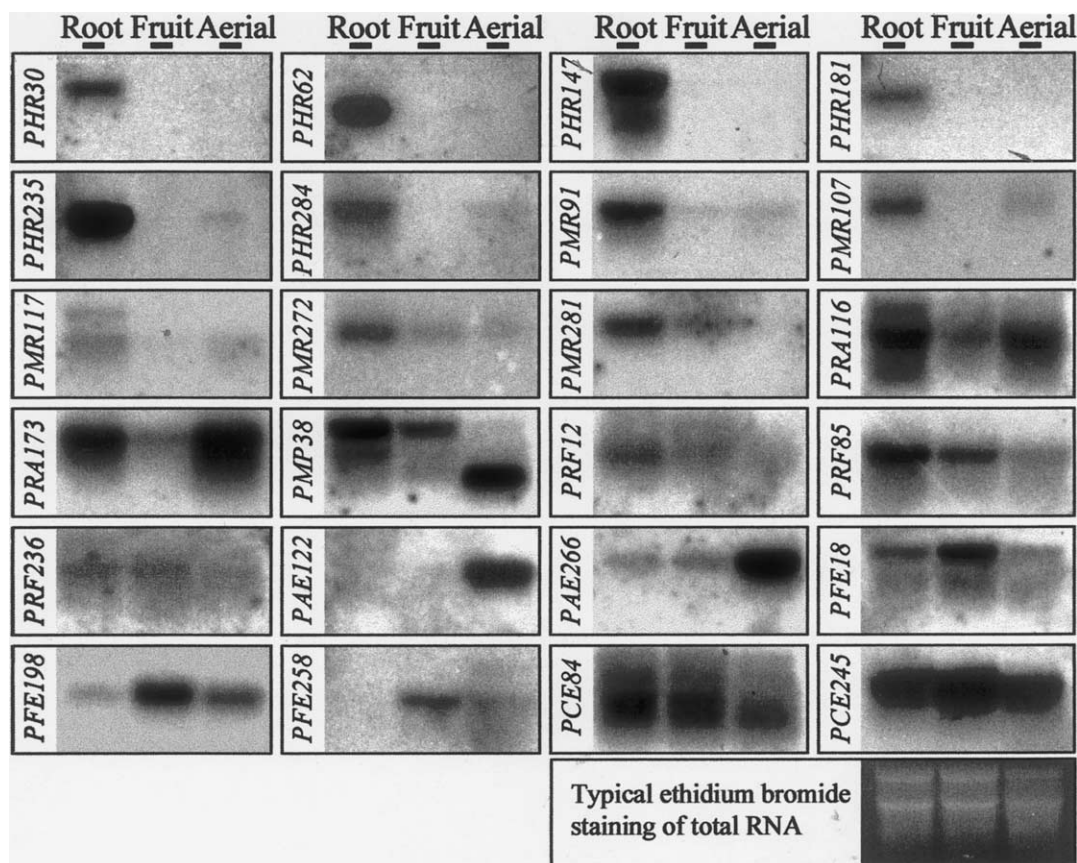


Fig. 1. Detection of mRNAs hybridizing to 24 cDNA probes in roots (apices of 15 month-old sand-grown plants), fruits (shell color 4) and aerial parts (13 month-old field-grown plants), respectively. The cDNA probes used are shown on the left-hand side of each autoradiogram. Exposure times of each individual blot to film were adapted to ensure good comparison of the relative levels in the three different tissues. cDNA inserts were named according to their hybridization patterns and isolation number. Abbreviations: PHR, pineapple highly root-enhanced; PMR, pineapple moderately root-enhanced; PRA, pineapple roots and aerial parts-enhanced; PMP, pineapple mixed pattern; PRF, pineapple root and fruit-enhanced; PAE, pineapple aerial parts-enhanced; PFE, pineapple fruit-enhanced; PCE, pineapple constitutively-expressed. Predicted functions of the deduced amino acid sequences can be found in Table 2.

Table 2
Results of sequence analysis of 24 different pineapple cDNAs categorized according to expression profile

cDNA name	Size*	Similarity/presumed function	Accession number	Reference
<i>cDNAs corresponding to highly root-enhanced mRNAs</i>				
<i>PHR30</i>	783 bp ^c	Germin/germin-like protein	AY098510	[22]
<i>PHR62</i>	686 bp ^c	Mannose-binding lectin	AY098512	[23]
<i>PHR147</i>	1143 bp	Lipoxygenase I	AY098520	[24]
<i>PHR181</i>	742 bp	Xyloglucan endotransglycosylase	AY098522	[25]
<i>PHR235</i>	± 830 bp ^p	Lipoxygenase II	AY098524	[24]
<i>PHR284</i>	1237 bp ^c	Peroxidase	AY098531	[26]
<i>cDNAs corresponding to moderately root-enhanced mRNAs</i>				
<i>PMR91</i>	607 bp	Caffeoyl CoA <i>O</i> -methyltransferase	AY098515	[27]
<i>PMR107</i>	447 bp	Nonspecific lipid-transfer protein	AY098516	[28]
<i>PMR117</i>	± 650 bp ^p	Lipoxygenase III	AY098518	[24]
<i>PMR272</i>	516 bp ^c	Unknown protein with Q-rich domain, similar to BAB19385	AY098529	
<i>PMR281</i>	569 bp	Unknown protein, moderate similarity to non-specific lipid-transfer protein-like protein PvLTP (PVR3)	AY098530	[29]
<i>cDNAs corresponding to root and aerial parts-enhanced mRNAs</i>				
<i>PRA116</i>	324 bp	Novel, E-rich (29%) protein	AY098517	
<i>PRA173</i>	860 bp	RING zinc finger protein, similar to NP_180450, AAK68782 (arabidopsis) and BAB63825 (rice)	AY098521	[30]
<i>cDNA corresponding to mRNAs with a mixed pattern</i>				
<i>PMP38</i>	1110 bp	Phenylalanine ammonia-lyase	AY098511	[31]
<i>cDNAs corresponding to root and fruit-enhanced mRNAs</i>				
<i>PRF12</i>	858 bp ^c	Novel, P-, R-, A-, S-rich (58%) protein	AY098508	
<i>PRF85</i>	460 bp	Caffeic acid <i>O</i> -methyltransferase	AY098514	[27]
<i>PRF236</i>	1606 bp ^c	Novel protein, similar to NP_196563 (arabidopsis), possibly ATP/GTP-binding	AY098525	[32]
<i>cDNAs corresponding to aerial parts-enhanced mRNAs</i>				
<i>PAE122</i>	465 bp	Similar to drought-inducible, late embryogenesis-abundant and senescence proteins	AY098519	[33]
<i>PAE266</i>	911 bp ^c	Class-1 LMW heat shock protein	AY098528	[34]
<i>cDNAs corresponding to fruit-enhanced mRNAs</i>				
<i>PFE18</i>	1174 bp	Unknown protein, similar to NP_194200 (arabidopsis)	AY098509	
<i>PFE198</i>	651 bp ^c	Cytochrome <i>b₅</i>	AY098523	[35]
<i>PFE258</i>	1199 bp ^c	Epoxide hydrolase	AY098527	[36]
<i>cDNAs corresponding to constitutive mRNAs</i>				
<i>PCE84</i>	711 bp ^c	ATP synthase <i>e</i> chain	AY098513	[37]
<i>PCE245</i>	1199 bp ^c	Tetrameric ubiquitin	AY098526	[38]

References to review papers or papers describing the most similar sequence are shown. *PolyA tail excluded. ^cCoding region is presumably complete. ^pAll cDNAs were completely sequenced except those marked.

apple roots produce a high number of relatively short mRNAs.

Our screening revealed six novel cDNAs (*PMR272*, *PMR281*, *PRA116*, *PRF12*, *PRF236* and *PFE18*) with no clearly known function. Two of these, *PRA116* (29% glutamate) and *PRF12* (58% proline, arginine, alanine and serine), have no similar counterparts in any other plant, microbe or animal species and are hitherto unique for pineapple. *PRE272* matched only a glutamine-rich protein derived from a combination of two ESTs of rice. The absence of similarity with any *Arabidopsis thaliana* sequence makes the corresponding *PRE272* gene possibly specific to the monocot lineage. Two deduced protein sequences, *PFE18* and *PRF236*, only show similarity with hypothetical proteins predicted from

Arabidopsis genomic sequences, thus supporting possible function of the *Arabidopsis* genes. A Prosite scan identified a putative bipartite nuclear targeting sequence and ATP/GTP binding site in *PRF236* (data not shown). *PRE281* shows only moderate similarity (Blast E value: 4e-09) to the nonspecific lipid-transfer-like protein, PvLTP, the expression of which is also enhanced in roots [15,29].

The lipoxygenase-like proteins encoded by *PHR147*, *PHR235* and *PMR117* represent three distinct genes with different mRNA levels in the three tissues. In contrast with *PHR235* and *PMR117*, the *PHR147* cDNA did not hybridize to total RNA from aerial tissue (Fig. 1). We used the full-length cDNAs to update the pineapple codon usage table initially created by

Nakamura et al. [39] and now available on <http://agrss.sherman.hawaii.edu/pineapple/pineappl.htm>.

Based on their amino acid similarities, the ‘highly root-enhanced’ cDNAs are predicted to encode a germin-like protein (*PHR30-glp*), mannose-binding lectin (*PHR62-lec*), lipoxygenase (*PHR147-lox*), xyloglucan endotransglycosylase (*PHR181-xet*) and peroxidase (*PHR284-prx*). The alignments of the predicted amino acid sequences of *PHR30-glp*, *PHR62-lec* and *PHR284-prx* with the most similar and relevant sequences in the databases are shown in Fig. 2. *PHR30-glp* possesses all the sequence characteristics of germins and germin-like proteins, including the cupin superfamily signature [40] and a signal peptide sequence [41] (Fig. 2A). *PHR30-glp* shares the highest similarity with GER7 from rice [42] (62% similarity, 57% identity), oxoO1 from *Lolium perenne* [43] (61% similarity, 54% identity) and ‘Germin-like protein subfamily T member 1’ (GLP1At) from *A. thaliana* [44] (64% similarity, 56% identity). The lack of extremely high identity with ‘true germins’ classifies *PHR30-glp* as a germin-like protein that probably lacks oxalate oxidase activity [44,45]. One member of this family, McGER, has been isolated from *M. crystallinum* [18], a facultative CAM plant. Interestingly, both *PHR30-glp* and McGER are root-specific, but share 47% sequence similarity and 40% sequence identity.

PHR62-lec encodes a putative mannose-binding lectin [23]. The deduced amino acid sequence is predicted to have a signal peptide sequence [41], which is cleaved between the amino acids CSA and EN in the N-terminus (Fig. 2B). It was found to be most similar (57% similarity, 50% identity) to a mannose-binding lectin from tulips [46], and other mannose-binding lectins from various bulbs and orchids such as *Galanthus nivalis* agglutinin (GNA) [47] and *Gastrodianin*-MNF, from the orchid *Gastrodia elata* (GMNF) [48]. *Gastrodianin*-MNF has been shown to possess antifungal activity [48] and the three-dimensional structure of GNA has been resolved by X-ray crystallography [49]. The mannose-binding sites of GNA are conserved in all proteins shown in Fig. 2B. The HCA plot of *PHR62-lec* is very similar to that of GNA (data not shown) suggesting a similar tertiary peptide structure.

The deduced amino acid sequence of *PHR284-prx* is highly homologous to class-III cationic peroxidases (Fig. 2C). *PHR284-prx* possesses a putative signal sequence of 23 amino acids in length [41] and two peroxidase signatures [50] consisting of the peroxidase active site and the proximal heme-ligand (Fig. 2C). The eight conserved cysteine residues of *PHR284-prx* are believed to form four disulfide bridges [51]. The protein does not possess the C-terminal pro-sequence, which may function as a vacuolar targeting signal in other peroxidases [52]. *PHR284-prx* is most similar to a rice peroxidase (80% similarity, 73% identity) and is also

homologous to horseradish peroxidase C [53] and a peroxidase from *Arabidopsis* [54] (Fig. 2C).

The cDNAs encoding highly root-enhanced mRNAs, *PHR147-lox* and *PHR181-xet* (Table 1), were also analyzed further, but alignments are not shown because the cDNAs are incomplete. A Prosite scan with the deduced amino acid sequence of *PHR147-lox* revealed the presence of one (the most C-terminal) of the two iron-binding regions that are signatures for lipoxygenases [55]. The deduced amino acid sequence of *PHR181-xet* reveals several features indicative of xyloglucan endotransglycosylases. These include the presence of four cysteine residues, believed to form two disulfide bridges, one putative *N*-glycosylation site and a motif, DEIDFEFLG, which is predicted to form the active center of the enzyme [56].

3.3. Comprehensive analysis of mRNA abundance of root-enhanced genes during development of field-grown plants

The characterization of cDNA sequences and preliminary expression analysis revealed distinct classes of genes with diverse expression profiles. We seek a gene that is not expressed in fruit, but is highly expressed in roots during 6–15 months after transplanting to the field, which is the critical stage at which the root apices are most susceptible to nematode infection [57]. To accurately screen the cDNAs as potential candidates for these features of gene expression, we focused our analysis for practical purposes on field-grown plants during their development. RNA was isolated from root apices (the first 4–5 cm) and aerial tissues of 3.5, 6.5, 13 and 20.5 month-old plants. The latter time point coincides with first fruit harvesting. Fruits of all shell colors (0–5) were examined to confirm the absence of mRNA during all stages of fruit ripening. The results of northern hybridization experiments with the ‘highly root-enhanced’ cDNAs *PHR30-glp*, *PHR62-lec*, *PHR147-lox*, *PHR181-xet*, *PHR284-prx*, the ‘aerial tissue-enhanced’ cDNA *PAE122*, and the ‘constitutively expressed’ cDNA *PCE84*, are shown in Fig. 3. The expression of *PAE122*, which is similar to certain late embryogenesis-abundant protein genes (renamed *PAE122-lea*), was examined in parallel to illustrate a gene that is expressed higher in aerial than root tissues. *PCE84* has high similarity with genes for the e subunit of ATP synthase (renamed *PCE84-ase*). The level of mRNA detected with the *PCE84-ase* probe corresponded with the ethidium bromide-stained rRNA bands on the gel (data not shown). Therefore, we chose *PCE84-ase* as a control for the total amount of RNA loaded in each lane, which was relatively low for the 3.5 and 6.5 month-old root samples (Fig. 3).

The levels of *PHR62-lec* and *PHR284-prx* RNAs remain constant relative to the control, *PCE84-ase*,

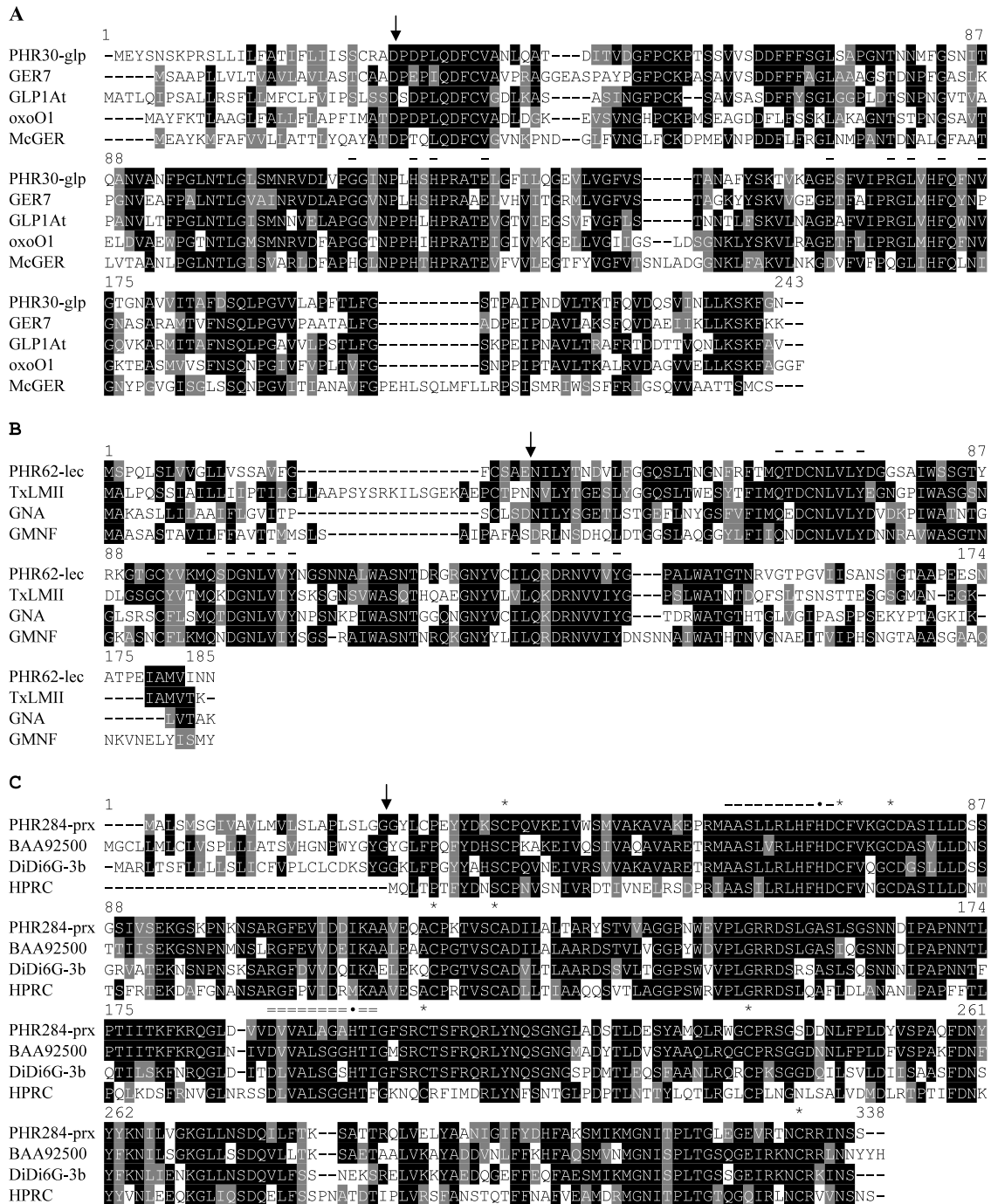


Fig. 2. Multiple sequence alignment of the deduced amino acid sequences of 'highly root-enhanced' cDNAs and related sequences from other plants. (A) GER7 [42], GLP1At [44], oxoO1 [43] and McGER [18] aligned with PHR30-glp. (—) Above the sequence indicates consensus cupin signature residues. (B) TxLMII [46], GNA [47] and GMNF [48] aligned with PHR62-lec. (—) Indicates the mannose-binding sites of GNA, which are conserved in all sequences. (C) BAA92500, DiDi 6G-3b [54] and HPRC [53] aligned with PHR284-prx. The peroxidase active site and proximal heme-ligand signature are indicated by single and double dashed lines above the sequences, respectively. (●) Indicates histidines with a central role within these regions. (*) Indicates conserved cysteines. The signal peptide splicing site for all pineapple proteins are indicated by arrows. All other proteins are also predicted to possess a signal sequence, but only for the germins/germin-like proteins did the predicted splicing sites align with the corresponding pineapple sequence using CLUSTALW.

during root development from 3.5 to 13 months (Fig. 3). At 20.5 months, the mRNA levels of both genes have declined significantly. In aerial tissues, the low levels of these mRNAs gradually decrease during plant develop-

ment. We could not detect any significant expression of *PHR284-prx* and *PHR62-lec* in aerial parts of 20.5 month-old plants and fruits of all shell colors during ripening.

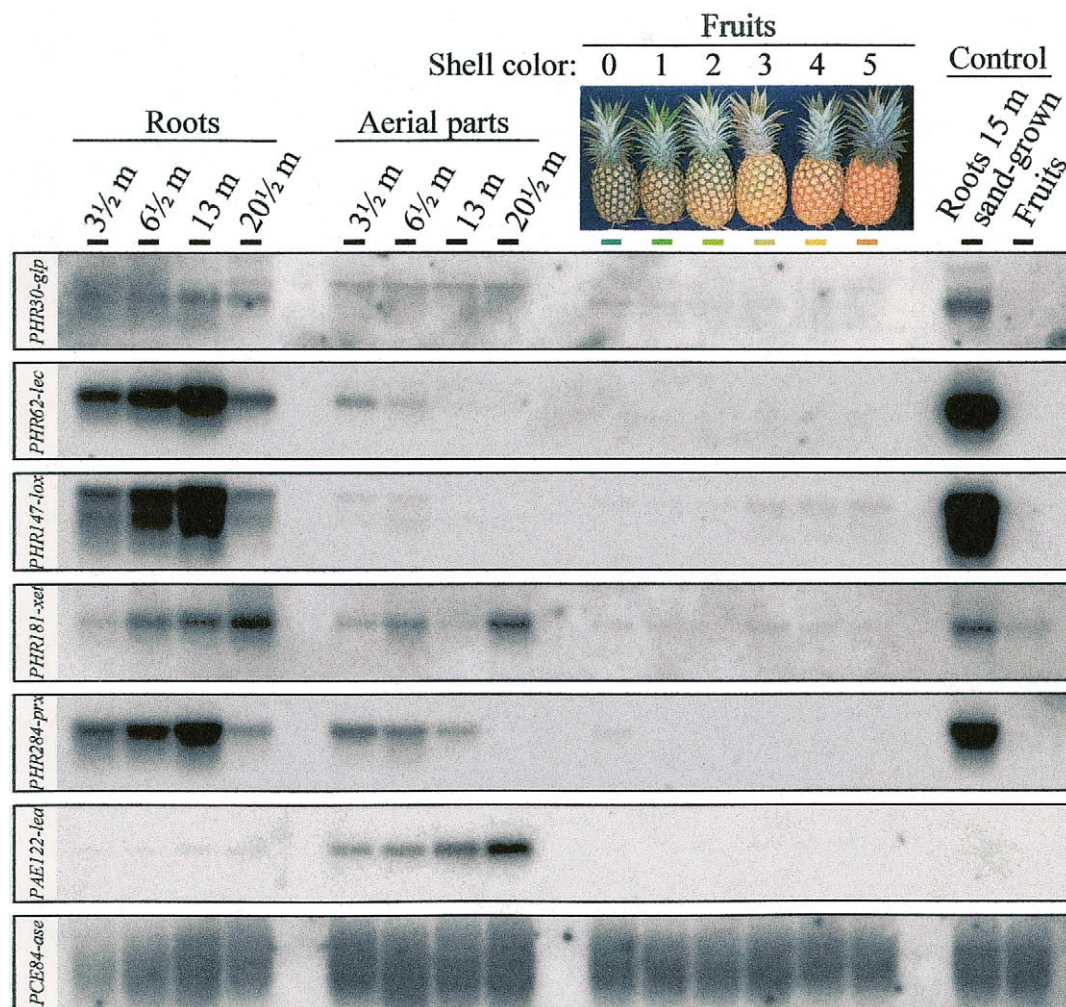


Fig. 3. Northern blot analysis of total RNA from roots and aerial parts of field-grown pineapple plants of different ages (m = months after planting) and fruits at different ripening stages. Probes shown from top to bottom were derived from *PHR30-glp*, *PHR62-lec*, *PHR147-lox*, *PHR181-xet*, *PHR284-prx*, *PAE122-lea* and the control *PCE84-ase*. Controls on the right-hand side contain RNA from roots of 15 month-old sand-grown plants and shell color 4 fruits. The exposure time of each individual blot to film was adapted to ensure good comparison of the relative levels in tissues of different age/shell color.

PHR30-glp hybridizes to a lower abundant mRNA relative to the other mRNAs in roots (Fig. 3). Its mRNA levels in roots decrease slightly with age. In aerial tissues, cross-hybridization takes place with a larger transcript. This larger mRNA could represent transcripts from the same gene, which are spliced/processed differently, or from a different germin-like protein gene. The *phr30-glp* probe hybridizes to a rare mRNA in fruits of shell color 0.

PHR147-lox hybridizes to two mRNAs (Fig. 3). Shorter exposures, repeated experiments (data not shown) and the data in Fig. 1 indicate the second RNA species is reproducible. The ratio of hybridization of the two mRNAs is equal for all tissues and all time points. The mRNA levels of *PHR147-lox* in roots are slightly higher, but follow a similar trend in abundance during development as the mRNA levels of *PHR62-lec*

and *PHR284-prx*. *PHR147-lox* mRNA levels are very low or absent in aerial tissues and fruits.

A different hybridization pattern was observed with *PHR181-xet* (Fig. 3). Expression of this gene in roots increased during plant development with the highest mRNA abundance in 20.5 month-old plants. In aerial tissues, mRNA levels also increased at 20.5 months, after being relatively low in 3.5–13 month-old plants. No significant *PHR181-xet* expression was observed during fruit ripening.

The aerial and fruit tissues consisted of mixtures of specific sub-tissues that differ developmentally and physiologically. The aerial tissue contained leaves, stems, and side shoots, while the fruit consisted of the shell, core, and edible portions. Potential differences in gene expression within distinct sub-tissues could be obscured in the analysis in Fig. 3. Therefore, in Fig. 4,

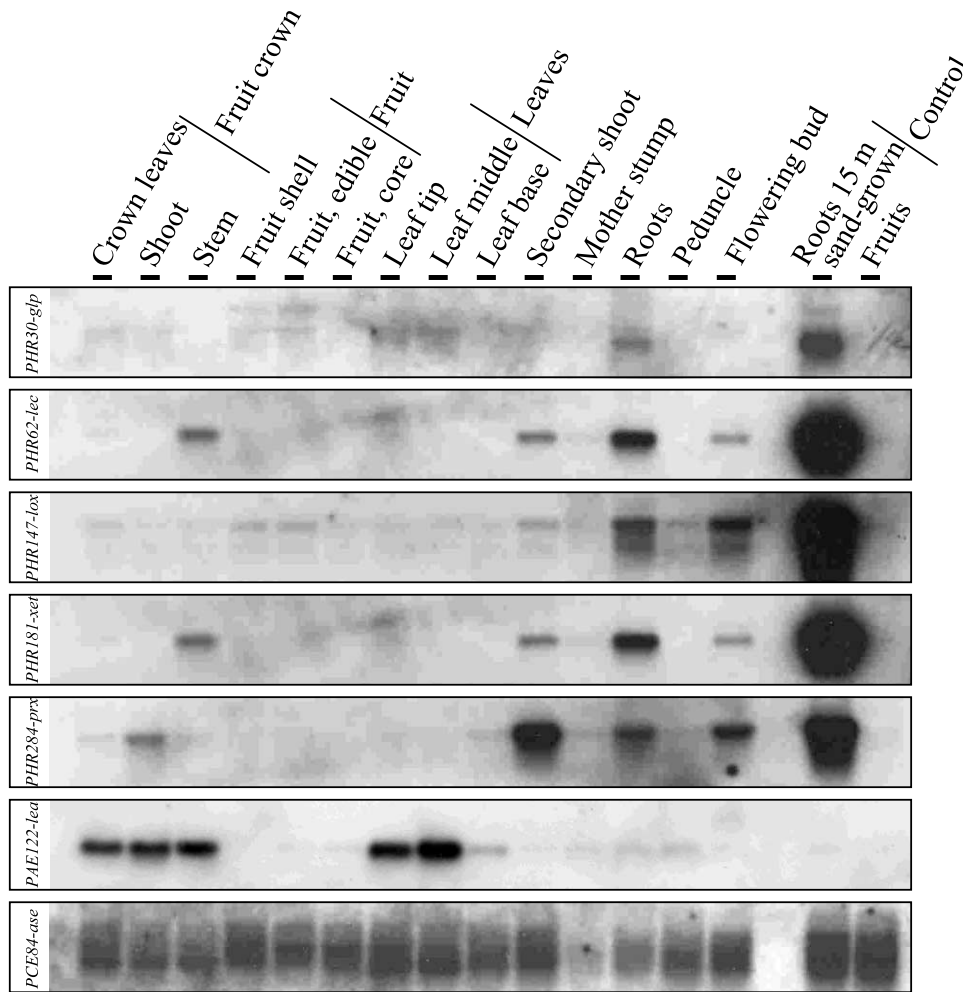


Fig. 4. Northern blot analysis of total RNA from the major organs of field-grown pineapple plants at the time of fruit harvesting (20.5 months after planting) and from a flowering bud (14 months after planting). Leaf tips comprise the distal 12 cm, leaf bases refer to the white basal parts, leaf middle represents the rest of the leaves. Probes shown from top to bottom were derived from *PHR30-glp*, *PHR62-lec*, *PHR147-lox*, *PHR181-xet*, *PHR284-prx*, *PAE122-lea* and the control *PCE84-ase*. Controls on the right-hand side were the same as in Figs. 1 and 3. The exposure time of each individual blot to film was adapted to ensure good comparison of the relative levels in the tissues.

we used northern hybridizations to analyze in detail the expression of *PHR30-glp*, *PHR62-lec*, *PHR147-lox*, *PHR181-xet*, *PHR284-prx*, *PAE122-lea* and *PCE84-ase* in a field-grown plant at the time of fruit harvesting. The 20.5 month-old plant was dissected into 13 different tissues. Gene expression was also examined in a flowering bud from a 14 month-old plant. The smaller transcript hybridizing to *PHR30-glp* (in Fig. 3) was not detected in any other tissue except roots (Fig. 4). The larger and lower abundance mRNA present in aerial tissue (Fig. 3) was also detected in green leaves (leaf tip and middle, Fig. 4). Transcripts for *PHR62-lec* and *PHR181-xet* were detected in the stems of fruit crowns, side shoots, roots and flowering buds. *PHR284-prx* shares this expression pattern, but is also expressed in crown shoots, and not in stems. *PHR147-lox* mRNAs were most abundant in roots and flowering buds. *PAE122-lea* expression is restricted to green leaves and the shoot, stem and leaves of the crown.

3.4. Analysis of gene expression within the root

In the next set of experiments, adventitious roots propagated from a crown were used as a model system to analyze the expression of these genes along the length of the actively growing root. In Fig. 5, the mRNA levels for *PHR147-lox*, *PHR284-prx* and *PCE84-ase* did not change markedly along the length of the root. In contrast, the *PHR30-glp* mRNAs were higher in the base than the middle and tip. Interestingly, *PHR62-lec* mRNA levels were highly abundant in the most apical 7.5 mm of the root, while *PHR181-xet* was more abundant in the more mature tissues of the middle and base of the root.

Lectins can play a role in the interaction of roots and organisms in the rhizosphere. The high level of expression of *PHR62-lec* within the tip of the root prompted us to localize *PHR62-lec* expression in greater detail within the root tip. Tissue-prints of longitudinal sections

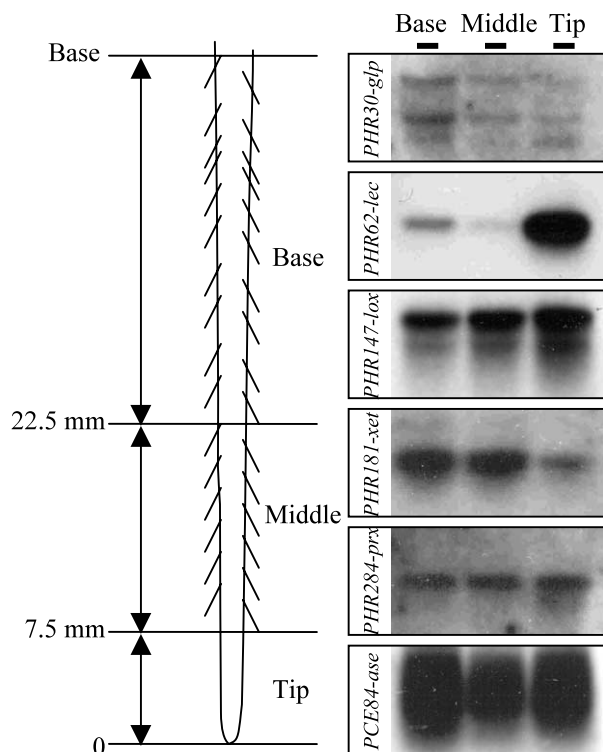


Fig. 5. Northern blot analysis of total RNA from the base, middles and tip of 25 day-old water-grown roots. A diagram of the root (left side) illustrates the relative position of these tissues. Probes shown from top to bottom were derived from *PHR30-glp*, *PHR62-lec*, *PHR147-lox*, *PHR181-xet*, *PHR284-prx*, *PAE122-lea* and the control *PCE84-ase*.

of 25 day-old water-grown plants were hybridized to ^{35}S -labeled antisense *PHR62-lec* RNA (Fig. 6A). The tissue-prints were repeated on over 30 individual roots and reproducibly showed that hybridization was primarily located in protoderm and also in the root apical meristem (Fig. 6A and B). This result was confirmed by tissue-printing experiments on cross-sections of the tip (Fig. 6A). Sense probes of *PHR62-lec*, as negative controls, did not hybridize to the tissue-print (data not shown).

The sensitivity of tissue-printing is limited by the ability of the cells to break and transfer RNA to the membrane. We used *PCE84-ase* as a control for tissue-printing and found that the corresponding mRNA signal was not visible along the entire radius of the root (Fig. 6A). To access and analyze expression of *PCE84-ase* and *PHR62-lec* specifically in the inner tissues of pineapple roots, we micro-dissected the cell layers. Separation of inner and outer tissues of the root was possible from approximately 1.2 cm from the root tip up to the base of the root. Careful examination showed that the inner explant contained most, but not all of the stele, as rupture occurred at the protoxylem. The inner explant contained metaxylem and central ground tissues (Fig. 6C and D). The outer explant

contained the protoxylem, phloem, endodermis, cortex and dermis (Fig. 6C and D). The RNA isolated from the micro-dissected tissues was subjected to northern analysis, which confirmed the presence of *PCE84-ase* mRNA in the inner tissues (Fig. 6C). In contrast, *PHR62-lec* mRNA was truly absent in the inner tissues (Fig. 6C), indicating that *PHR62-lec* expression is indeed highly specific for the regions of the protoderm.

4. Discussion

To expand the molecular genetic tools available for gene mapping, tissue-regulated promoter isolation and genetic engineering of pineapple, we initiated a study to isolate and characterize tissue-regulated cDNAs from this plant. To our knowledge, we present the first characterization of a large number of tissue-regulated cDNAs from a bromeliad. Our analysis of 307 cDNAs resulted in rapid identification of 11 cDNAs preferentially expressed in roots, as well as a high number of other cDNAs with an interesting distribution pattern within root, aerial and fruit tissues (Table 1). We focused on roots because of the pressing need for promoters that are preferentially and highly active in roots, but not in the edible fruits. We have shown that four of the cDNAs isolated in this study (*PHR62-lec*, *PHR147-lox*, *PHR181-xet* and *PHR284-prx*) satisfy these expression criteria. In particular, they are highly expressed during the entire crop cycle (Fig. 3) and especially when nematodes feed [4]. We are currently screening a genomic library to isolate the corresponding promoters. The root-enhanced promoters will enable expression of transgenes to control nematodes, which are the most economically damaging pest of pineapple in Hawaii [58]. Resistance genes encoding proteins that are specifically active against nematodes are available [12–14]. These include the gene encoding cystatin from rice seeds that, when expressed in tomato and *Arabidopsis* roots, provides effective nematode control [59,60].

An obvious problem with using endogenous promoters to drive expression of a nematicidal protein gene is the risk of down-regulation upon infection with nematodes [61]. However, the peroxidase, DiDi 6G-3b, which is very similar to pineapple *PHR284-prx* (Fig. 2), was recently shown to be induced upon infection with root-knot nematodes in *Arabidopsis* [54]. Lipoygenase activity also increases upon infection of oat roots by cyst nematodes [62]. We are currently investigating possible modulation of pineapple *PHR* gene expression by nematodes.

We showed that the methods used for RNA isolation, cDNA synthesis for probe and library construction, and reverse northern screening worked effectively and reproducibly on a variety of diverse tissues, such as flowers, stems, leaves, roots and fruits. This study,

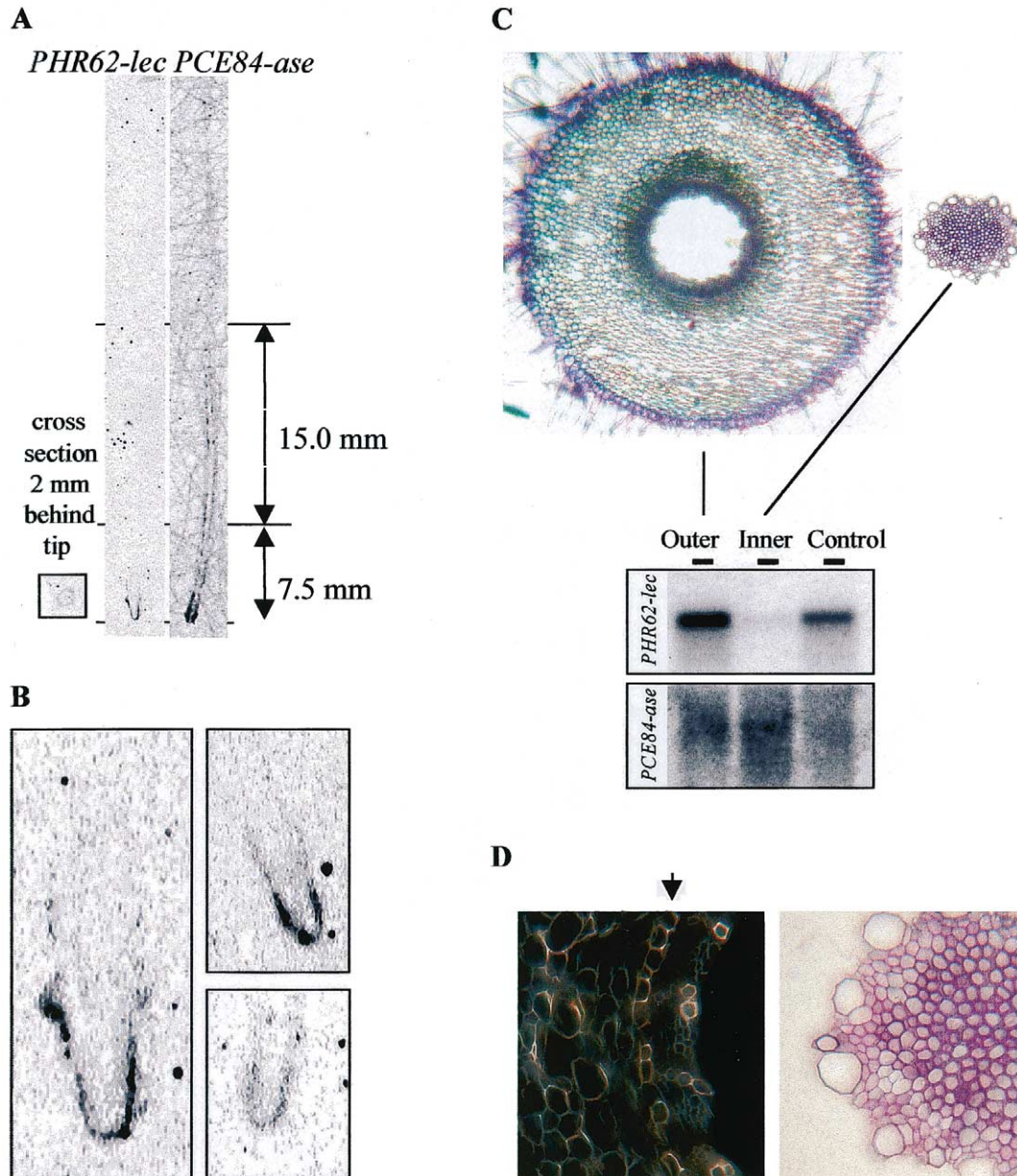


Fig. 6. Distribution of *PHR62-lec* and *PCE84-ase* mRNA in water-grown pineapple roots. (A) Tissue-prints of longitudinal sections with radiolabeled antisense RNA probes of *PHR62-lec* and *PCE84-ase*. Hybridization of a cross section of the root at 2 mm distance from the tip with *PHR62-lec* antisense probe is shown in the left lower corner of panel A. (B) Close-up of three different tissue-printed root apices hybridized to *PHR62-lec* antisense probe. (C) Northern blot analysis of total RNA from the outer cell layers, inner cell layers and complete water-grown root (control). The upper portion illustrates a cross-section of outer and inner root tissues after micro-dissection (D) Detailed pictures of the point of rupture in phloem and protoxylem. Left: the outer tissue; right: the inner tissue. The endodermis is indicated by an arrow.

therefore, provides the opportunity to expand further the range of tissues for which cDNA libraries can be established and characterized on a genomic scale. Microarrays and large-scale characterization of genes involved in CAM and root, flower and fruit development are now feasible. This would allow pineapple to benefit from the genome projects conducted on other plants.

Prior to this work, only ten pineapple genes had been used to establish a codon usage table [39]. The cDNAs

sequenced here greatly improve the codon usage information (available on: <http://agrss.sherman.hawaii.edu/pineapple/pineappl.htm>), which is crucial for engineering of foreign genes to be optimally expressed in pineapple. In addition, the cDNAs and sequence data are also useful for making probes and primers for physical mapping of the pineapple genome, particularly genomic regions maintained as bacterial artificial chromosomes, and to establish linkage relationships with desirable genes. The tools combined with known phe-

notypic markers will enhance RFLP and RAPD studies done in conjunction with cross hybridization to screen segregating progeny in breeding programs for disease resistance, root growth, flower timing, fruit color and sweetness.

Two fruit-enhanced mRNAs, *PFE198* and *PFE258*, encode polypeptides homologous to the membrane-bound electron carrier, cytochrome *b₅*, and a soluble epoxide hydrolase, respectively. Epoxide hydrolase plays an important role in oleic acid metabolism [24]. Oleic acids are the major constituents of cutins, which comprise the cuticle that protects plants against dehydration. In addition, they also serve as signal molecules upon pathogen attack [63]. Both drought [64] and virus-infection [65] can induce epoxide hydrolases expression. *PFE198* is most similar to an olive cytochrome *b₅*, which is also abundant in fruits [35]. Cytochrome *b₅* can stimulate cytochrome P450-dependent reactions in several plant, yeast and animal systems [66,67]. Cytochrome P450 is involved in cutin monomer biosynthesis [24,68], thus leaving a possible role for cytochrome *b₅* together with the co-induced epoxide hydrolase in the oleic acid pathway in pineapple fruit worthy of further investigation.

Several of the cDNAs are predicted to encode enzymes involved in root cell wall biogenesis. Xyloglucan endotransglycosylase (*PHR181-xet*) is a cell wall-modifying enzyme that is thought to be important for cell loosening and wall assembly during cell expansion [25]. Likewise, the enzymes predicted from the cDNAs *PMP38* (phenylalanine ammonia-lyase, PAL), *PRF85* (caffeic acid *O*-methyltransferase, COMT) and *PRE91* (caffeoyl-coenzyme A *O*-methyltransferase, CCoAMT) operate in the phenylpropanoid pathway leading to the biosynthesis of lignin. Mature pineapple roots are extensively lignified. PAL is encoded by multigene families in many plant species. Based on the multiple bands on the northern blots (Fig. 1), we speculate that pineapple contains at least one different isoform of PAL. Differential expression of COMT (PRF85) and CCoAMT (PRE91) is considered to be part of a mechanism that controls specific lignin composition [27,69]. In addition, germin-like proteins (*PHR30-glp*) and peroxidases (*PHR284-prx*) are involved in cell wall reinforcement [22,26,45,70]. Peroxidases can increase cell wall strength by crosslinking phenolic groups in lignin and structural proteins.

Many of the cDNAs isolated here encode enzymes associated with defense responses to abiotic stress and/or pathogens. For example, lignification is a common defense response and PAL, COMT and CCoAMT mRNA levels and/or activity have been shown to be correlated with pathogen and pest resistance [61,71–73], tolerance to heat stress [31], drought [74], ozone [75], and wounding [76,77]. Similarly, lipoxygenases, which catalyze the hydroperoxidation of fatty acids to hydro-

peroxy derivatives [24], are involved in wounding, senescence, and abiotic stress through their role in the biosynthesis of the plant hormone, jasmonate [24,78]. The nonspecific lipid-transfer proteins, which bind and catalyze the transfer of lipids in vitro [28], have antibiotic activity against bacteria and fungi [79,80] and are possibly involved in cutin biosynthesis [81], suggesting multiple roles in plant defense. Peroxidases, which catalyze the oxidation of various organic substrates with H₂O₂ as the final electron acceptor, have been shown to function in wound healing and defense against pathogen infection [26,52,54,82,83]. Lectins are nonenzymatic, highly specific, carbohydrate-binding proteins that play a role in storage, cell wall assembly [23], symbiosis with microorganisms [84] and defense against pathogens [85]. We found that *PHR62-lec* is highly similar to the antifungal lectin, Gastrodinin-MNF (Fig. 2) [48]. We have shown that the pineapple lectin, *PHR62-lec*, is expressed predominantly in the protoderm (Fig. 6). This expression pattern suggests a role for *PHR62-lec* in the rhizosphere in which it possibly interacts with nematodes. Such a possible interaction could inhibit nematode infection, through the antipathogenic activity of lectin [85]. Alternatively, nematodes might be able to recognize their hosts through specific binding of lectins [86].

The expression of *PAE122-lea* and *PAE266-hsp* were high in aerial tissue, but low in roots. *PAE122-lea* is most similar to citrus [33] and cotton *Lea5* [87], which are two late embryogenesis-abundant proteins that are inducible by either drought or heat. *PAE266-hsp* belongs to the family of class-1 low molecular weight heat shock proteins [34]. Both heat shock and *Lea5*-like proteins are believed to be important for thermoprotection [33,34]. Drought-inducible members of non-specific lipid-transfer proteins (*PRE107*) have also been reported (e.g. [88]).

The non-specific lipid transfer proteins [79,80], germin-like proteins [70], lectins [48,85] PAL [89] and peroxidase [90] have successfully conferred resistance against a range of pathogens by transgenic expression in various plant species. Although these results suggest promising applications of the pineapple cDNAs isolated here, it will take a great deal of further research to find out if modifying the expression of these genes in pineapple will ultimately lead to an improved crop.

The similarity of the cDNAs isolated here to proteins involved in stress and pathogen responses in other plants is not sufficient proof of their function in pineapple. However, their proportion within the randomly selected cDNAs from healthy roots is unusually high. The pineapple plants in these experiments were not exposed to temperatures > 32 °C. We suggest that the expression of these genes is part of a general adaptation process that is associated with the high stress tolerance of pineapple and, thus, indirectly with CAM. For

example, high expression of the *PAE122-lea* and *PAE266-hsp* proteins in healthy aerial tissue could be partly responsible for the drought and high temperature tolerance of pineapple. Other highly expressed genes, such as those encoding non-specific lipid transfer proteins and germin-like proteins (Table 2Fig. 1), might enhance stress resistance because they are highly heat stable proteins that have been proposed to play protective roles during drought/dessication in other plants [18,88,91,92].

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