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Characterization of a defensin in bark and fruit tissues of peach and antimicrobial activity of a recombinant defensin in the yeast, *Pichia pastoris*

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Herein the cloning and characterization of a defensin gene (*PpDfn1*) from a cDNA library made from peach (*Prunus persica* [L.] Batsch) winter bark tissues is described. A partial clone obtained from the library was extended to full length by 5′ Rapid Amplification of cDNA Ends (RACE). The open reading frame of 237 bp codes for a 79 amino acid peptide related to the defensin family of proteins. Sequence comparison of the encoded protein using BLAST analysis revealed significant homology to defensins from other plant species. RNA gel blot analysis indicated that the gene is seasonally expressed in bark tissues of 1-year-old shoots, and is also expressed in early fruit development. Results of quantitative RT-PCR and protein blot analysis were similar to those of RNA gel blot analyses for the bark tissues. A recombinant version, rDFN1 was expressed in the yeast, *Pichia pastoris*. It was found that rDFN1 inhibited germination of the fungal pathogens *Penicillium expansum* and *Botrytis cinerea*, but not the Gram-negative bacterium *Erwinia amylovora*. The potential physiological role of PpDFN1 and its antimicrobial properties are discussed.

Introduction

Considerable research in our laboratory has focused on identifying seasonally regulated proteins and their genes in peach (*Prunus persica* [L.] Batsch) that may be related to cold hardiness. We have previously reported on a 60-kDa dehydrin protein (PCA60), that has both cryoprotective and antifreeze properties (Arora et al. 1992, Arora and Wisniewski 1994, Artlip et al. 1997, Wisniewski et al. 1999), as well as on the accumulation of two putative bark storage proteins (Arora et al. 1992, 1996). In the course of isolating a seasonally regulated dehydrin gene (Artlip et al. 1997), we identified a cDNA fragment with high nucleotide sequence homology to the plant defensin gene family. In the present study, we have characterized both transcript and protein expression.

Defensins are a cysteine-rich family of antimicrobial, low-molecular-weight polypeptides (45–54 amino acids) found in insects, mammals and plants (Thomma et al. 2002). They are part of a larger group of proteins that are Cys-rich, with two to six disulphide bridges that stabilize the structure (Garcia-Olmedo et al. 1999). Plant defensins, however, present a specific Cys arrangement involving a globular structure stabilized by four disulphide bridges (Meyer et al. 1996). In addition to the eight conserved Cys residues, there are two Gly, one Glu, and an aromatic residue that are also conserved (Artlip and Wisniewski 2001). A signal peptide domain which targets the protein for extracellular secretion has been noted or predicted for all plant defensins (Broekaert et al. 1997). Although plant defensins share structural similarities with mammalian and insect defensins, the latter polypeptides tend to be generally much smaller, of the order of 5 kDa (Selitrennikoff 2001).

As reviewed by Thomma et al. (2002), plant defensins were initially identified and studied in seeds. In radish,

Abbreviations – PCR, polymerase chain reaction.

defensins accounted for 30% of the proteins released from radish seed coats by imbibition (Terras et al. 1995). Seeds of numerous other plant species in many taxonomic divisions are also known to contain defensins (e.g. Osborn et al. 1995, Liu et al. 2000). Defensins or the transcripts encoding them have also been found in other tissues including bell pepper fruit (Meyer et al. 1996), radish leaves, pea pods, tobacco leaves, tubers and flowers (as reviewed by Garcia-Olmedo et al. 1999). There have been no published reports of defensin transcript accumulation or expression in woody plants. However, two GenBank accessions (AB052687 and AB052688) indicate that pear (*Pyrus pyrifolia*) pollen contains defensin-like transcripts.

In the present article, we describe the cloning and sequence analysis of a peach defensin. In addition, we examine transcript and protein abundance in bark tissues during a complete annual cycle and determine transcript abundance during peach fruit development and ripening. Finally, we describe the antimicrobial properties of a recombinant version of the protein expressed in the yeast, *Pichia pastoris*.

Materials and methods

Isolation of the defensin cDNA clone

A 500-bp fragment of a defensin cDNA clone was recovered during screening of a Uni-ZAP (Stratagene, La Jolla, CA, USA) peach cDNA library (Artlip et al. 1997). Primers were designed from the sequence, and 5′ RACE (Life Technologies, Inc., Gaithersburg, MD, USA) performed as per the manufacturer's protocol, with final PCR parameters: 1 min at 94°C, 1 min 20 s at 59°C, 2 min at 72°C (30 cycles), followed by 7 min at 72°C. The resulting amplicon was fractionated via agarose gel electrophoresis, the appropriate band removed, and then purified using GlassMax (Invitrogen, Carlsbad, CA, USA). The purified product was cloned into pCR-2.1 using the TA-TOPO kit (Invitrogen) as per the manufacturer's protocol.

Sequencing

Sequencing reactions were conducted with the dRhodamine kit (Applied Biosystems, Foster City, CA, USA) essentially as per the manufacturer's protocol. An ABI 310 (Applied Biosystems, Inc.) was used to analyse the reaction products and compile the sequence data. A BLAST comparison (Altschul et al. 1997) confirmed the sequence as a member of the defensin gene family.

Copy number analysis

Genomic DNA was isolated from 'Loring' leaves according to Kobayashi et al. (1998), and 5 μg aliquots were digested with *Bam*H I, *Eco*R I, *Hin*d III, and *Pvu* II. The restricted DNA was fractionated by agarose-gel electrophoresis (0.8% agarose), and transferred to a Nytran membrane (Schleicher & Schuell, Keene, NH, USA) according to the manufacturer's protocol. The membrane was pre-hybrized (4 h) and hybridized (12 h) in 5× standard saline phosphorus EDTA (SSPE)/5× Denhardt's/50% formamide/0.1% SDS/100 μg ml^{-1} DNA at 42°C as recommended by the manufacturer. The probe (25 ng of the full-length cDNA clone) was random-primer labelled (Life Technologies, Inc.) with [α-^{32}P]-dCTP. The membrane was subsequently washed at increasing stringency, culminating in a 1-h incubation at 65°C in 0.1 SSPE/0.1% SDS; blots were exposed to film for 1 week at −80°C.

Transcript level analyses

Peach bark tissue was collected on a monthly basis from current-year twigs. Peach fruit were collected at various times throughout development. The tissues were immediately plunged into liquid N_2 and stored at −80°C until RNA extraction. Total RNA from bark tissues was extracted using the PureScript kit (Gentra, Gentra Systems, Minneapolis, MN, USA), whereas the method of Callahan et al. (1993) was used for fruit. Ten micrograms of total RNA were fractionated by formaldehyde-gel electrophoresis (Sambrook et al. 1989), followed by transfer to Nytran (Schleicher & Schuell) according to the manufacturer's instructions. The membranes were pre-hybridized and hybridized as per the DNA blot. The membranes were washed as before, culminating in a 30-min incubation at 65°C in 0.1 SSPE/0.1% SDS; blots were exposed to film for 1 week at −80°C. A template consisting of the soybean 18 s rRNA was prepared and used as above to re-probe the current year bark RNA blot to confirm equal loading. Previous hybridizations with the fruit blot indicated that it had equal loading (data not shown).

Quantitative RT-PCR was also performed to confirm trends observed by RNA blot analysis. In brief, 2 μg total RNA was converted to cDNA using Thermoscript Reverse Transcriptase with oligo-dT, according to the manufacturer's protocol (Invitrogen). One hundred nanograms of the cDNA was then subjected to PCR on a ABI Prism 7700 Sequence Detection System (Applied Biosystems) using the Taqman Universal PCR Master Mix according to the manufacturer's specifications. The following primers and probe were used: 3b 211 Forward 5′-CAACTGTGCATCTGTTTGCCAA-3′, 3b 292 Reverse 5′-AATCACGTTTTCGTAGACGCCG-3′, Probe 5′-GGTGGCCATTGTCGTGGTGGCTTT-3′. Comparative data was also obtained for 18S rRNA as an endogenous standard, using the following primers and probe: 18S Forward primer 5′-AGGAATTGACGGAAGGGCA-3′, 18 s Reverse primer 5′-ATTTGACTCAACACGGGGAAA-3′, Probe 5′-CACCAGGAGTGGATCCTGCGGC-3′. Relative transcript abundance of *PpDfn1* was calculated as per User Bulletin no. 2 for the ABI Prism 7700 Sequence Detection System, using the standard curve method (http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf). In this procedure, the calculated amounts of *PpDfn1* were divided by the calculated amounts of 18S rRNA to normalize them. One sample was then

chosen as a calibrator and the remaining samples then expressed relative to that value.

Protein analysis

Bark proteins were extracted in borate buffer (50 m*M* borate, 50 m*M* ascorbic acid, 1 m*M* PMSF, pH 9.0 at 4°C as reported by Arora et al. (1992), with immunoblotting as reported by Artlip et al. (1997). The blotted proteins were probed with a 1 : 300 dilution of a polyclonal antibody from rabbits directed against a synthetic peptide of the first 14 amino acids of the mature defensin peptide (EARTCESQSNRFKG; Alpha Diagnostic Intl., Inc., San Antonio, TX, USA). Immunoreactive bands were visualized by alkaline phosphatase conjugated to a goat-anti-rabbit antibody.

Defensin constructs

The pGAPZα vector series (Invitrogen) was chosen to provide recombinant peach defensin protein for further characterization. This system allows for the in-frame insertion of the gene of interest behind the α signal peptide, as well as optional in-frame addition of c-myc and poly His tags at the carboxy-terminus of the expressed protein. The pGAPZαA vector was chosen for overall sequence compatibility with the defensin gene. A mutagenic PCR primer was designed to introduce an *Eco*R I site at the 5′ end of the defensin coding region (DFNcodEcoF, 5′GTGAGTTCAGAATTCATGGAGCGC3′). Two PCR primers were designed to introduce an *Xba* I site at the 3′ end of the defensin gene, with or without provision for the c-myc/poly His tag coding region in frame (DFNxbaR, 5′ACCACAAGCCTCTAGACAATGTTTAG3′; DFNtermXbaR, 5′ACCACAGCCTCTAGATTAACAATGTTTAG3′, respectively). An additional 5′ primer was designed to incorporate an *Eco*R I site at the start of the mature peptide coding region (DFNmatEcoF, 5′TTGTAGTTGAATTCAGGACCTGTGAG3′), as it was unknown whether the *Pichia* system would recognize the endogenous signal peptide of the defensin peptide. PCR conditions were as follows: 5 min at 94°C, 2 min at 55°C, 3 min at 72°C (1 cycle); 1 min at 94°C, 1 min at 60°C, 1 min at 72°C (30 cycles). Four products were created in this manner, varying as to the presence of the endogenous signal peptide and inclusion of the C-terminal tags. The manufacturer's instructions were followed, and five *Pichia* strains were created, including one with the vector alone (no-insert control). The sequences of the constructs were verified by sequencing as above.

Antimicrobial assays

The efficacy of recombinant defensin (rDFN) against the fungal pathogen *Penicillium expansum* was assayed using the system of Janisiewicz et al. (2000).

In brief, 24-well tissue culture plates were containing 1.0% apple juice were inoculated with 0.6 ml of strains of transformed *Pichia pastoris* with or without a defensin gene construct. Polystyrene cylinders with a 0.45-μm polytetraflouroethylene (PTFE) membrane on one end were placed into the wells of the tissue culture plate. Dilutions of *Penicillium expansum* conidia (0.4 ml) were then placed inside the cylinder. The PTFE membrane allowed free exchange of the apple juice and rDFN, but not the micro-organisms. The plates and cylinders were then incubated at 26°C, and germination of the conidia was then assessed at various times via microscopic observation. The yeast strains were used at two different concentrations, 95% transmittance and 50% transmittance, based on transmission readings at 420 nm of yeast that had been pelleted, washed twice in water, and re-suspended in water.

Purified rDFN1 was also tested against *P. expansum* as well as *Botrytis cinerea*. Briefly, yeast cultures were grown for 5 days in YM broth. Supernatants were then harvested by centrifuging the yeast out of suspension at 3000 *g* for 1 h, and concentrated using a Millipore Minitan tangential flow dialysis system with a 3000 MW cutoff filter unit. Culture medium was exchanged for Tris-buffered saline (TBS; pH 7.0) by replacing the volume three times. The crude protein suspension was filtered through a 0.45 μm sterile filter and stored at −20°C. An aliquot was lyophilized and re-suspended at one-quarter the original volume as a sample of even higher concentration (1 × and 4 ×, respectively). Tests of the effect of the protein suspensions on the germination of *Botrytis cinerea* and *Penicillium expansum* were conducted using 24-well plates. Five hundred microlitres of either control (TBS) or protein suspension were added to the wells. Apple juice of various concentrations was added (as above), and fungal spores suspended in water with a trace of Tween 20 were added as a 10-μl droplet. The plates were stirred on an orbital shaker designed for microtitre plates to prevent the accumulation of spores in the centre of the well and incubated at 25°C overnight. The wells were examined and counts of total and germinated spores were conducted. Results were reported as percentage germination. Assays were replicated in three plates with three microtitre wells per treatment. Spore counts ranged between 100 and 200 spores per well. All spores in each of the three wells were counted as part of the germination assessment. Each plate was treated as a replicate (n = 3).

Antibacterial activity of recombinant proteins was determined by mixing 2×10^6 colony-forming units (CFU) of *E. amylovora* strain Ea273 in 150 μl of test solutions and incubating at room temperature for 1 h. Bacteria were then plated, incubated, counted and the proportion of cells surviving exposure calculated. Each sample was replicated three times and tests were repeated. Partially purified preparations of recombinant protein and culture filtrates of yeast expressing recombinant proteins were evaluated. Positive control was 50 μg streptomycin ml^{-1} and negative controls were TBS (partially purified preparations) or culture medium

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CATCGATATACAACCTAAGCTAGCTTTGCTACCTCATCAGATACATATACATTAGAAAGA   60
TATGGAGCGCTCCATGCGTTTATTTTCAACTGCCTTCGTCTTCTTTCTGCTTCTGGCAGC  120
  M  E  R  S  M  R  L  F  S  T  A  F  V  F  F  L  L  L  A  A
TGCTGGGATGATGATGGGGCCAATGGTTGCTGAGGCTAGGACCTGTGAGTCTCAGAGTAA  180
  A  G  M  M  M  G  P  M  V  A  E  A  R  T  C  E  S  Q  S  N
TCGGTTCAAGGGAACTTGCGTGAGTACAAGCAACTGTGCATCTGTTTGCCAAACTGAGGG  240
  R  F  K  G  T  C  V  S  T  S  N  C  A  S  V  C  Q  T  E  G
CTTCCCTGGTGGCCATTGTCGTGGCTTTCGCCGCAGATGCTTTTGCACTAAACATTGTTA  300
  F  P  G  G  H  C  R  G  F  R  R  R  C  F  C  T  K  H  C
ATTAGCGATGATCGTGATACTCCTGCATGCACATTTCTATGTGGGCCTAGGAGGAGACGA  360
TTCTGAATGATCCCTTATATCAGTGGCCGTACTTGCTACTAAAATAAATTATTATATGAT  420
GAGCCAATTAACAGTTGGGTTATTATTATTATATCCTTGTTATTGTTCTTGTTCTTGTTT  480
GTTAATTAAGTACCACCCTCGTGAGTTGCCTTGCCTTGTTTTGTCAACGATGTCTTTGTA  540
ACACCCCTACGTTTTGTAATAATGCAATATTATTGATTTCCTCAACCATACATGTAGTCA  600
TTTTGCAAAAAAAAAAAAAAAAAA
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Fig. 1. Nucleotide sequence and conceptual translation of *PpDfn1*. The predicted translation is in the one-letter amino acid code. The putative signal peptide is underlined, conserved cys-residues are boxed and other conserved residues are shaded.

(culture filtrates). Differences between treatments were evaluated using Tukey's Studentized Range Test (SAS Institute Inc., Cary, NC, USA).

Results

The defensin 5′ RACE clone is 624 bp in length, with the largest ORF (237 bp) encoding a putative protein of 79 amino acids, with a Mr of 8.7 kDa (Fig. 1). A 30 amino acid signal peptide is predicted, with cleavage between amino acids 30 and 31 (Fig. 1). A BLASTP (Altschul et al. 1997) search of the Swissprot database using the conceptual translation product of the cDNA clone indicated that it was a member of the defensin family of plant peptides. We propose that the gene corresponding to this clone be named *PpDfn1*, which is consistent with homologues in other plant species. The nucleotide sequence has been deposited in GenBank under Accession Number AY078426.

The predicted mature, peach defensin was aligned with several other plant defensins (Fig. 2) using CLUSTAL X (Thompson et al. 1994, 1997). The predicted product of *PpDfn1* showed appreciable homology to members of the proposed B1 subfamily of plant defensins (Harrison et al. 1997), such as bell pepper and petunia which were 83 and 79% identical to peach, respectively. Similar identities were noted between the peach peptide and defensins from soybean (81%) and oil palm (75%). There was considerably less identity shared between two pear defensins and the peach peptide (51–62% identity).

The DNA blot analysis of the peach defensin suggests that at least one copy of the gene exists (Fig. 3). The two *Pvu* II restriction fragments that hybridize with the probe

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Petunia    RT__________CESQSHRFHGTCVRESNCASVCQTEGFIGGNCRAFRRRCFCTRNC
Palm       RT__________CESQSHKFQGTCLRESNCANVCQTEGFQGGVCRGVRRRCFCTRLC
Peach      RT__________CESQSNRFKGTCVSTSNCASVCQTEGFPGGHCRGFRRRCFCTKHC
Pepper     RT__________CESQSHRFKGLCFSKSNCGSVCHTEGFNGGHCRGFRRRCFCTRHC
Soy        RT__________CESQSHRFKGPCLSDTNCGSVCRTERFTGGHCRGFRRRCFCTKHC
Pear3      RT__________CEAASGKFKGMCFSSNNCANTCAREKFDGGKCKGFRRRCMCTKKC
Pear1      RTESSKAVEGKICEVPSTLFKGLCFSSNNCKHTCRKEQFTRGHCSVLTRACVCTKKC
           **          **  *  *:* *.  .**  .*  * *  * *  . * *.**: *
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Fig. 2. Alignment of several plant defensins. Predicted translations of the indicated polypeptides were obtained from GenBank at NCBI. The sequences were aligned with CLUSTALX (Thompson et al. 1997). Petunia: *Petunia inflata* thionin-like peptide (Karunanandaa et al. 1994) [Ac. No. L27173]; Palm: *Elaeis guineensis* defensin (Tregear et al. 2002) [Ac. No. AF322914]; Peach: this paper; Pepper: *Capsicum annuum* defensin, ji (Meyer et al. 1996) [Ac. No. X95730]; Soy: *Glycine max* defensin (Maitra and Cushman 1998) [U12150]; Pear1: *Pyrus pyrifolia* defensin-like protein, pdn-1 [Ac. No. AB052688]; and Pear3: *P. pyrifolia* defensin-like protein, pdn-3 [Ac. No. AB052687]. Asterisks below the one-letter amino acid code indicate conserved residues; colons indicate substituted residues with strong similarity; periods indicate substituted residues with weak similarity. The hallmark conserved cysteines are shaded.

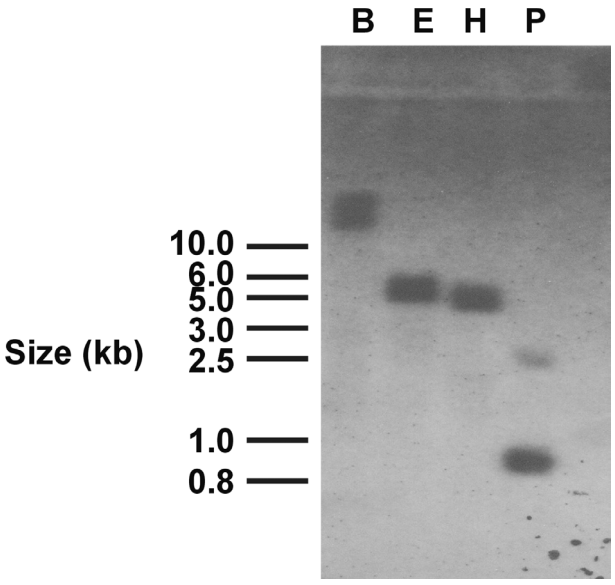

Fig. 3. DNA blot of genomic peach DNA probed with *PpDfn1*. Five micrograms of genomic DNA was isolated from cv. 'Loring' leaves, digested with the indicated restriction endonucleases, and blotted to nylon membrane. The blot was then probed, hybridized with a radioactively labelled *PpDfn1* probe and washed stringently (as described in 'Materials and methods)'. B, *Bam*H I; E, *Eco*R I; H, *Hin*d III; P, *Pvu* II.

are consistent with a predicted restriction site within the coding region of *PpDfn1* cDNA clone. The single *Hin*d III and *Eco*R I fragments are also consistent with the nucleotide sequence; that is, there are no predicted sites for either of these restriction endonucleases within the coding region. It is difficult to discern whether there are two *Bam*H I restriction fragments. Two such fragments is inconsistent with the known nucleotide sequence.

In current-year bark tissue, peach defensin transcript levels vary on a seasonal basis with a peak during the autumn and early winter months (Fig. 4). This trend was also seen in scaffold limb- and trunk-bark tissues (data not shown). Duration of peak dehydrin expression varies between the ages of bark sources, with the defensin transcripts persisting longest in current-year-bark tissues (data not shown).

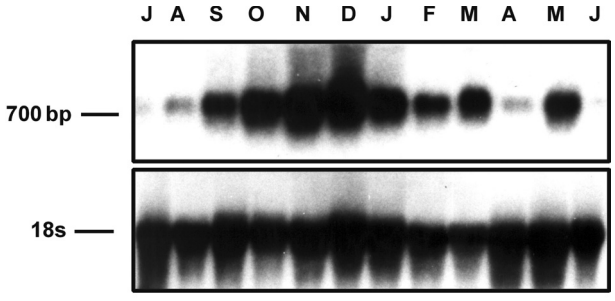

Fig. 4. Seasonal expression of *PpDfn1* as determined by RNA blot analysis. Total RNA was isolated from current-year bark tissue sampled monthly, blotted to nylon membrane and probed as described. Months are abbreviated as the first letter of the month, starting with July (J, July; A, August; S, September; etc.). Top panel: total RNA hybridized with a radioactively labelled *PpDfn1* probe. Bottom panel: same blot, stripped and rehybridized with a radioactively labelled 18 s probe to insure equal loading. Hybridization and washing conditions were the same in each case.

Quantitative RT-PCR was also used to investigate *PpDfn1* transcript accumulation on a seasonal basis in bark tissue (Fig. 5). The general trend was similar to that observed by RNA blot analysis (Fig. 4).

Defensin peptide accumulation appeared to lag behind changes in transcript abundance (Fig. 6). Defensin protein began to accumulate in late summer (August), reached a maximum in early winter (December) and maintained high levels until late spring (May). No protein was observed in samples collected in June or July. It is unclear why little protein was observed in October.

Peach defensin transcript levels also display a distinct pattern during fruit development (Fig. 7). High transcript levels are associated with early development (during roughly 60 days after bloom). Whether or not the mature peptide continues to be present in the fruit tissue after transcription ceases was not determined.

In order to study the antimicrobial activity of the peach defensin, the constitutive pGAPZα vector and *Pichia pastoris* yeast expression system were selected (Cereghino and Cregg 2000). This system should theoretically permit the correct removal of the endogenous PpDFN1 signal peptide. However, if the signal peptide was not cleaved, the subsequent antimicrobial properties might be altered compared to a properly processed, mature peptide. In addition, the vector allows for the addition of c-myc and poly His tags for purification that might also interfere with antimicrobial properties. Therefore, a series of four recombinant peptides (rDFN1s) were created and transformed into *Pichia pastoris* (Fig. 8A). Protein blots of rDFN1s isolated from growth media, and probed with a polyclonal antibody directed against a portion of the mature peptide, indicated that cleavage and processing appeared to occur properly (Fig. 8B).

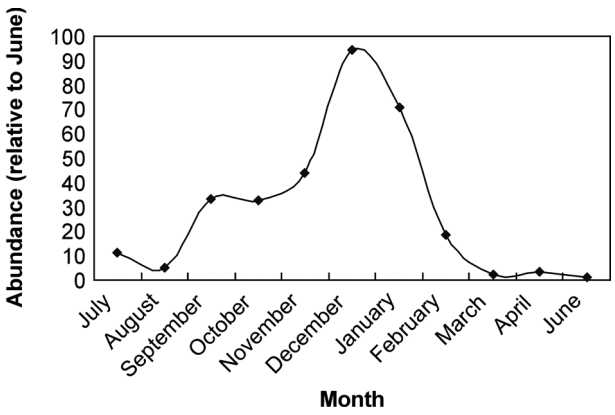

Fig. 5. Seasonal expression of *PpDfn1* transcript in bark tissues as determined by quantitative PCR. The same RNA from the samples used in the RNA blot analysis (Fig. 4) was subjected to quantitative PCR using primers and probes specific to *PpDfn1* and 18S RNA. Transcript levels of *PpDfn1* were normalized against 18S rRNA, and then expressed relative to the June values, which was used as a calibrator due to its low abundance.

Fig. 6. Seasonal expression of *PpDfn1* in bark tissues. Proteins were fractionated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with an antibody directed against a synthetic peptide containing the first 15 residues of the mature *PpDfn1*. Similar blots probed with pre-immune serum had no signal (data not shown).

It should be noted that one yeast strain, 15-0, did not properly express rDFN1. As can be seen in Fig. 8B, strains 26-2 and 41-3 had strong signal intensities compared to 9-3, suggesting that they expressed more of the rDFN1. Strains 26-2, 41-3, and a no insert control were chosen for subsequent antimicrobial experiments.

Several attempts were made to determine whether any of the rDFN1 peptides had antibacterial activity. Purified rDFN1 from 26-2 and 41-3 was tested against *Erwinia amylovora*, the Gram-negative causative agent of fire blight in pome fruits. The effect of rDFN1 preparations against *E. amylovora* were not significantly different than that of water, and were significantly less effective than the antibiotic, streptomycin, in in-vitro experiments (data not shown).

The antimicrobial properties of the recombinant strains of *Pichia pastoris,* and/or partially purified extracts containing the recombinant peach defensin protein were also evaluated against two post-harvest pathogens, *Botrytis cinerea* and *Penicillium expansum*. *Pichia* strains containing the vector alone (no-insert control), the peach defensin gene (26-2), or the peach defensin gene plus a myc epitope (41-3), were coincubated with spores of *P. expansum*, in 1% (v/v) apple juice. The spores and yeast were physically separated by a membrane which allowed free exchange of apple juice and anything it might contain between the two compartments. After 24 h, germination of the spores was recorded. Representative micrographs are presented in Fig. 9. Results presented in Table 1 indicated that germination using the no-insert yeast at 95% transmittance was 7.23 ± 5.4%, yeast strain 41-3 was 6.6 ± 4.7%, and yeast strain 26-2 was 0.7 ± 0.9%. Germination in the absence of any yeast strains was 83.11 ± 4.79%. Similar results were obtained when higher concentrations of yeast (50% transmittance) were used except that a significant difference was observed between the use of the no-insert and the 41-3 yeast strains that was not observed at the lower concentration of yeast. It is important to note that the suppression of spore germination by the non-transformed yeast was due to nutrient competition as evidenced by the lack of an effect of supernatant obtained from non-transformed yeast on spore germination (Fig. 10). Nutrient competition is recognized as the principal mode of action for many yeasts used as biocontrol agents (Wilson and Wisniewski 1994, El Ghaouth et al. 2002). The difference between the efficacy of the 26-2 and 41-3 strains at different yeast concentrations, compared to the no-insert control, may reflect differences in copy number of the defensin gene in each strain resulting in lower production of rDFN in the 41-3 strain as seen in Fig. 8B.

Observations indicate that the effect on spore germination was static in that if the mini-cell containing the spores of *P. expansum* was lifted from the larger cells containing the *Pichia* strains and placed in fresh 1% apple juice, germination would proceed normally (data not shown). Additionally, no morphological changes were observed in the hyphae of *P. expansum* as a result of being exposed to the defensin-producing strain of *P. pastoris* (Fig. 9), as have been reported for other defensins (Broekaert et al. 1997, Garcia-Olmedo et al. 1999).

Using extracts of partially purified, recombinant peach defensin obtained from liquid cultures of 26-2 and 41-3,

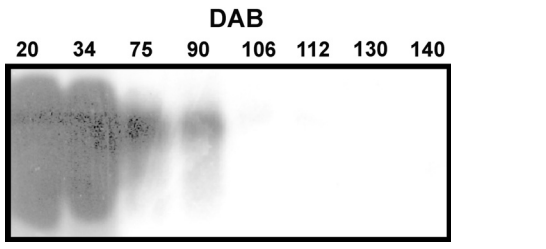

Fig. 7. Developmental expression of *PpDfn1* in peach fruit as determined by RNA blot analysis. Total RNA was isolated from fruit sampled at various days after bloom (DAB), blotted to nylon membrane and hybridized with a radioactively labelled *PpDfn1* probe as described.

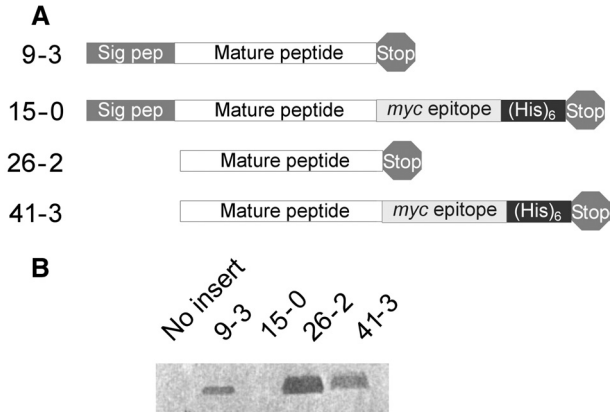

Fig. 8. Recombinant *Pichia pastoris* strains. (A) Schematic depiction of the four rDFN1 constructs. The transformed no-insert control, consisting of the vector alone, is not shown. (B) Expression of rDFN1 constructs in liquid culture. The four rDFN1 *Pichia pastoris* strains and the no-insert strain were grown in liquid culture as described, pelleted, and aliquots of the supernatant taken. The aliquots were subjected to SDS-PAGE, and immunoblotted as described. Equal volume aliquots were used.

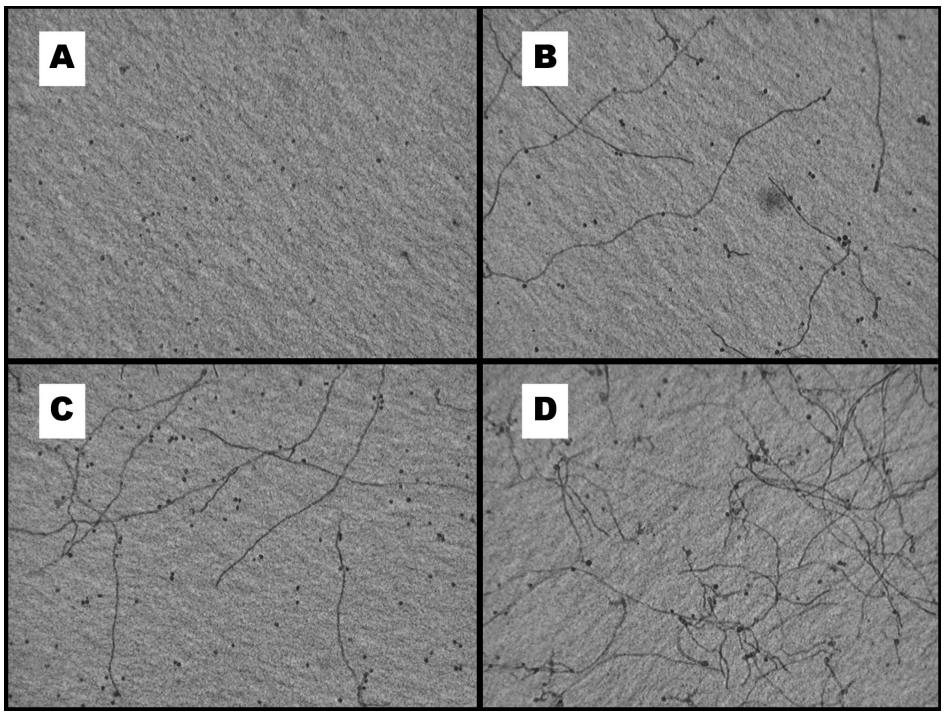

Fig. 9. Inhibition of *Penicillium expansum* conidia germination by rDFN1 *Pichia pastoris* strains. All trials shown were conducted as described by Janisiewicz et al. (2000), and are representative of results obtained using *Pichia pastoris* strains at 95% transmittance in 1% apple juice, 24 h. (A), Strain 26-2; (B), strain 41-3; (C), no-insert control; (D), no yeast control.

significant inhibition of both *Botrytis cinerea* and *P. expansum* spore germination was observed (Fig. 10). Nearly 100% inhibition was observed with both $1\times$ and $4\times$ concentrations of the partially purified preparations of recombinant peach defensin. In this in-vitro assay of antifungal activity, the presence of the *myc* epitope did not seem to affect the activity of the partially purified protein. The actual concentrations used, approximately 20 and 80 $\mu g\,ml^{-1}$, are on par with concentrations cited by other researchers using other systems (e.g. Osborn et al. 1995; Meyer et al. 1996).

Discussion

The cDNA representing *PpDfn1* and its encoded protein are consistent with examples of defensins from other plant species and is, to our knowledge, the first report of a defensin from woody vegetative tissues. The predicted protein would have a Mr of 8.7 kDa, and a 30 amino acid signal peptide, with cleavage between amino acids 30 and 31 (Fig. 1). Proper cleavage of the signal peptide would result in a mature peptide with a Mr of 5.2 kDa. All plant defensins described to date have a signal peptide marking the protein for extracellular secretion. The predicted mature peptide contains all the conserved residues reported for plant defensins (Thomma et al. 2002) thus far, with eight cysteines, two glycines at positions 12 and 33, a glutamate at position 28, and an aromatic residue (phenylalanine) at position 10 (numbering relative to the first residue of the mature peptide).

Comparison of the predicted *PpDfn1* amino acid sequence with other plant defensins shows that the peach peptide is more similar to the petunia, oil palm, bell pepper and soy defensins, than it is to two pear defensins. These defensins appear to be members of the B1 subfamily of plant defensins, as defined by Harrison et al. (1997). Since oil palm is a monocotyledonous plant, it is surprising to see greater similarity between its defensin and the peach peptide, than between peach and the two pear defensins. The pear defensin sequences were from pollen cDNAs, and no detailed information on these genes or peptides is available outside of GenBank accession data (AB052687 and AB052688). Despite a short stretch of additional amino acids in the pear *pdn-1* product, all the defensin peptides compared here appear to be homologous.

It is possible that *PpDfn1* is a member of a small family of defensin genes in peach, given the potential anomaly in the number of *Bam*H I digested fragments hybridizing to the *PpDfn1* probe. In contrast, the fragments resulting from the other restriction endonucleases hybridized to the *PpDfn1*

Table 1. Inhibition of *Penicillium expansum* by recombinant *Pichia pastoris* strains. Incubations were conducted in 1% (v/v) apple juice. 95% T and 50% T are yeast re-suspended in water to 95% and 50% transmittance at 420 nm, respectively. A minimum of 30 conidia were counted per replicate. Results are reported as percent germination (mean ± SE), n = 3.

	Percentage germination ± SE
95% T	
No-insert	7.23 ± 5.40
26-2	0.65 ± 0.92
41-3	6.62 ± 4.70
50% T	
No-insert	6.97 ± 2.24
26-2	0.00 ± 0.00
41-3	3.24 ± 0.77
No yeast control	83.11 ± 4.79

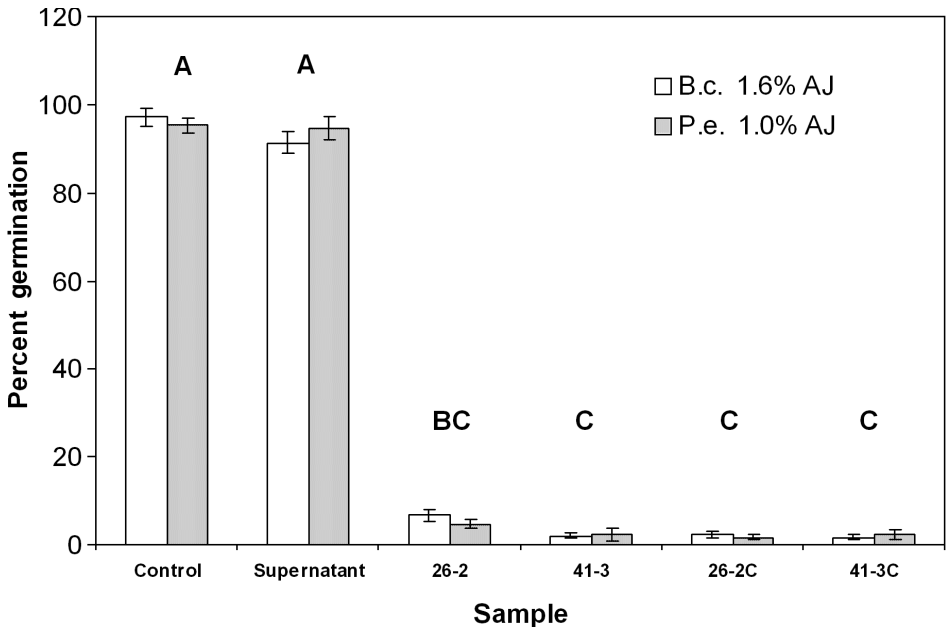

Fig. 10. Effect of semi-purified rDFN1 constructs on germination of *Botrytis cinerea* and *Penicillium expansum*. Apple juice at either 1.6% (v/v) or 1.0% (v/v) was inoculated with spores from *Botrytis cinerea* (Bc) or *Penicillium expansum* (Pe), respectively. Semi-purified 26-2 or 41-3 rDFN1 at 1 × or 4 × (26-2C and 41-3C) concentrations were co-incubated with the fungal spores. Control consisted of TBS, as the no-insert *Pichia pastoris* strain does not produce any rDFN1. Three replicate microtitre plates were used with three microtitre wells per treatment in each plate. Each plate was considered a replicate. Results are reported as percentage germination (mean ± SE). n = 3.

probe in a predictable fashion, based on known sequence. The most logical explanation for this would be that *PpDfn1* is duplicated, and that the *Eco*R I, and *Pvu* II restriction sites have been conserved, whereas the *Bam*H I site has not. Several other plant species are known to harbour two or more defensin genes. For example, radish (*Raphinus sativa*) and bell pepper (*Capsicum annum*) both have two homologues of a particular defensin gene, and *Arabidopsis* apparently contains at least five (Terras et al. 1995, Meyer et al. 1996, Epple et al. 1997).

Defensins have been shown to accumulate in tissues, especially seeds, as part of normal development or maturation, perhaps as a static defence against pathogens (Thomma et al. 2002). Defensins also differentially accumulate in response to challenge to tissues by microbial pathogens, indicating that at least some members have a role in active defence (Meyer et al. 1996, Epple et al. 1997). Overall, differential expression patterns and sequence/biological activity correlations (e.g. Broekaert et al. 1997) suggest that defensins play a role both in the specificity and pathogenicity of fungal pathogens and in the plant's defence response.

In peach, it appears that *PpDfn1* transcript and protein accumulate as part of a developmental programme in bark and fruit, as evidenced in Figs 4–7. In the case of bark tissues, the accumulation of transcript and protein reach a maximum during the winter and early spring. The seasonal pattern of expression of peach defensin is very similar to the pattern of other seasonally regulated genes and peptides previously reported in peach as bark storage proteins (Arora et al. 1996) and a dehydrin (Artlip et al. 1997). In the case of peach dehydrin (*PpDhn1*), maximum transcript accumulation occurred in November and December, as did expression of *PpDfn1*. In contrast, however, maximum dehydrin protein accumulation occurred in the period December to February. In the present study, maximum accumulation of peach defensin transcript also occurs in the period December to May, with some variable expression occurring in late spring. Such fluctuation is not uncommon for seasonally regulated proteins, and is viewed as a dynamic process responding to environmental cues such as rapid changes in temperature (Sauter and van Cleve 1990). Accumulations of the bark storage proteins were also similar to that of the dehydrin. In the case of all the seasonally regulated genes and proteins thus far characterized in peach, there was no evidence of either transcript expression or protein in the summer months of June and July

It has been postulated that dehydrins are cryoprotective and ameliorate freezing injury (Artlip et al. 1997, Artlip and Wisniewski 2001) and purified peach dehydrin (PCA60) has been shown to have both cryoprotective and antifreeze activity in vitro (Wisniewski et al. 1999). Therefore, it appears that dehydrin accumulates as an adjustment to a predictable period of adverse environmental conditions. In the case of the accumulation of the peach defensin, it is possible that a similar evolutionary adaptation has occurred. During the time period of late October to mid-March, peach trees are dormant and tissues in general are not responsive to brief periods of warm temperatures. Accumulation of an extracellular antimicrobial peptide, such as a defensin, may serve as a protection against pathogens at a time when other direct responses are not possible. Pathogen spores may be more responsive to brief episodes of warmer temperatures whereas fruit tree tissues remain dormant. If this is the case, the presence of water-soluble antimicrobial peptide in the extracellular space of bark tissues may play an important role as a defence mechanism, as apparently it does in seeds (Thomma et al. 2002). Once active growth occurs, presumably, other defence mechanisms may become available.

In the case of fruit tissue, the pattern of transcript expression is strongly developmental, occurring during the early to mid-stages of fruit development. Again, the pattern is similar to that observed for dehydrin gene expression (unpublished data). Unfortunately, data is not available on the accumulation of defensin protein so it is not known if the protein accumulates and persists in the tissue after transcript expression has ceased. The early expression of the defensin gene in fruit tissues, however, may relate to the evolutionary role of the fruit, which is to disperse seed. As the fruit tissue needs to break down, either by natural decay or ingestion by an animal, to release the seed, perhaps selection of defence mechanisms in the latter stages of fruit development were not as important. Alternatively, accumulation of large amounts of antimicrobial compounds may not be associated with fruit quality attributes and persistence of these compounds in mature fruit may have been an attribute that was inadvertently selected against during the domestication and breeding of peach. Comparison of defensin expression patterns in fruits of peach (large, fleshy fruit) and the closely related almond (a small, hard fruit) may provide information to evaluate the above-stated hypotheses.

The recombinant yeast strains, as well as the extract containing partially purified peach defensin, exhibited antimicrobial activity against two common post-harvest pathogens of fruit, *Penicillium expansum* and *Botrytis cinerea*. In contrast, no activity against the fire blight bacterium, *Ewinia amylvora,* was observed. This is not unexpected as the defensin family of proteins exhibit a great deal of variability in the organisms they affect (Broekeart et al. 1997, Garcia-Olmedo et al. 1999). In addition, the lack of any observed morphological changes in the *P. expansum* hyphae is consistent with observations of the B1 defensin subfamily (Harrison et al. 1997).

The ability of the yeast strain transformed with the vector alone to also inhibit spore germination is to be expected, as the yeast would compete with the germinating spores for available nutrients. The antimicrobial activity of the recombinant strains is of particular interest in that yeast species have been used as post-harvest biocontrol agents (Wilson and Wisniewski 1994, El Ghaouth et al. 2002, Janisiewicz and Korsten 2002) and our laboratories are developing ways to enhance their efficacy (Janisiewicz et al. 1998, El Ghaouth et al. 2000a, 2000b, 2001, Yehuda et al. 2001, Droby et al. 2003). To our knowledge, this is the first report that recombinant strains of yeast expressing a defensin gene have been shown to have direct antimicrobial activity. Constitutive expression of a defensin in a selected yeast antagonist may be a suitable approach to augment post-harvest biocontrol efficacy. Recently, Jones and Prusky (2002) over-expressed a cecropin antimicrobial peptide in *Saccharomyces cerevisae* and demonstrated that spores incubated with the yeast prior to inoculation of tomato fruit exhibited decreased pathogenicity. Perhaps one drawback with the use of peach defensin in such a system, however, is that it exhibited fungistatic rather than fungicidal activity. This is not uncommon (e.g. Meyer et al. 1996), and differences in the type of antimicrobial activity exhibited by defensins have been previously noted and used as part of several classification schemes (Broekeart et al. 1997, Harrison et al. 1997, Garcia-Olmedo et al. 1999). In addition, this approach would require the development of a transformation system for a yeast already identified as having antagonistic activity as the antagonistic yeast must be extremely well adapted to compete against the pathogen in the ecological niche of fruit wounds. Such a transformation system exists for the biocontrol yeast, *Candida oleophila* (Yehuda et al. 2001), and recombinant studies utilizing the peach defensin gene are in progress.

In summary, we have identified a defensin gene in bark tissues of peach and demonstrated that it exhibits a seasonal pattern of expression. The pattern of expression was similar to other seasonally regulated genes and/or proteins that we have studied. The peach defensin was also expressed developmentally in fruit tissues of peach, although no information was gathered on expression of the protein. It is suggested that the accumulation of an extracellular, water-soluble, antimicrobial peptide in bark tissues may serve as a static, defence mechanism at a time when tissues are dormant and may not be able to respond to pathogen attack by active response mechanisms. Furthermore, given the occasional low levels of specific transcripts found in bark tissues, the quantitative PCR should be a useful tool to augment observations made by the more traditional RNA blot technique. The demonstration of antimicrobial activity directly by the recombinant strains of yeast, rather than with purified protein, may provide an avenue to enhance the efficacy of yeasts used as biocontrol agents against post-harvest diseases of fruit. Transformation of the biocontrol yeast, *C. oleophila,* is being conducted and isolation of defensin genes from other species of fruit trees is also in progress.

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