

A novel protein associated with citrus blight has sequence similarities to expansin

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Abstract

A protein associated with citrus blight (CB), a disease of unknown cause, was partially characterized. The 12 kDa protein, designated p12, is diagnostic of CB and is present in leaves and xylem fluid from roots and stems of CB-affected trees. The protein, and up to six other CB-specific proteins, are readily detected by SDS-PAGE of xylem fluid from CB-affected trees. The partial N-terminal amino acid sequence of p12 was found to be unique based on database searches. A cDNA library from CB-affected root cambium was screened with a 60 bp fragment, obtained by PCR amplification of cDNA with degenerate primers designed using the amino acid sequence of p12, and two clones were selected. These clones were sequenced revealing a 674 nucleotide cDNA with a 393 nt ORF which included sequence predicted by the N-terminal amino acid sequence of p12. The amino acid sequence based on the p12 ORF was found to be up to 49% similar and 31% identical to expansins. Bacterial expression of the cloned ORF, which encodes an 11.8 kDa protein plus an N-terminal hydrophobic signal peptide, produced an immunoreactive protein of the expected size. By northern blot analysis, it was determined that p12 transcripts are present in root and stem cambium, but not in leaves of CB-affected trees, suggesting transport of the protein to leaves. Southern hybridization analysis of citrus genomic DNA indicated that p12 is a citrus encoded protein.

Abbreviations: BAP, blight-associated protein; CB, citrus blight; ICBR, Interdisciplinary Center for Biotechnology Research; p12, 12 kDa BAP; p35, 35 kDa BAP; PR, pathogenesis-related

Introduction

Citrus blight (CB) is a disease of unknown etiology that affects fruit-bearing trees wherever citrus is grown in hot, humid areas worldwide. An estimated 1 million trees per year are lost to CB in Florida, from an industry of roughly 100 million trees. The disease was first described over one hundred years ago [36]. Repeated efforts to transmit CB by grafting were not successful [12, 34, 43] until recently when it was shown to be graft-transmitted through roots [39, 38]. CB causes

zinc deficiency in leaves and accumulation of zinc in bark and wood up to four times the levels found in healthy trees [1]. Xylem vessels become plugged, reducing water flow throughout the tree, resulting in wilting, reduction in leaf and fruit size and eventual limb dieback [13]. Affected trees show symptoms of water stress due to the xylem dysfunction, and not due to changes in stomatal function or gas exchange characteristics [37]. Diagnostic tests for CB include zinc analysis of bark [42], measurement of water uptake [24], analysis of amorphous xylem plugs [7] and, more recently, immunological tests for CB-associated proteins [5, 18]. CB-affected trees can survive for many years and are thus good models for studying prolonged stress.

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CB-affected trees express several proteins that are not observed in either healthy trees or trees with other declines [19]. These blight-associated proteins (BAPs) are found throughout the plant, but most abundantly in the xylem and vascular cambial zone of roots and stems. When crude vacuum extracts from CB-affected root xylem are separated by SDS-PAGE and silver-stained, at least seven proteins are easily distinguished that are not present in identical extracts from healthy roots [19]. The observation that BAPs accumulate in xylem fluid and can be isolated in relatively pure form by SDS-PAGE has led to the production of CB-specific antibodies [18]. These antibodies have become the basis of serological diagnostic tests for CB, and are now being widely used ([17]; Figure 1). One objective of the current work is to discover the functions of the BAPs, which could lead to a better understanding of the disease. One of the diagnostic proteins has been further characterized and described in this report, it is a unique 12 kDa protein (p12) with sequence similarities to expansins.

Materials and methods

Plant material

Field-grown Valencia sweet orange trees (*Citrus sinensis* (L.) Osb.) on rough lemon rootstock (*C. jambhiri* Lush) with CB, as identified by p12 immunoassay [17], were used for protein and nucleic acid extractions. Healthy controls were either field-grown Valencia sweet orange trees on rough lemon for protein extractions, or greenhouse-grown sweet orange or rough lemon trees for nucleic acids.

p12 extraction and N-terminal sequencing

The identification of BAPs in xylem extracts from roots of CB-affected trees was described by Derrick *et al.* [19]. Production of anti-p12 antibodies used in the following section to identify fractions containing the targeted protein was described by Derrick *et al.* [18]. It was later determined that p12 could be partially purified from CB-affected leaf extracts by boiling crude extracts for 10 min, followed by centrifugation for several minutes at 10 000 rpm to remove insoluble proteins; p12 remains soluble after this treatment (unpublished). Therefore crude leaf extracts were used as the starting material in the following protocol for p12 isolation by immuno-precipitation. Figure 1 shows immunospot analyses of CB-affected xylem fluid from

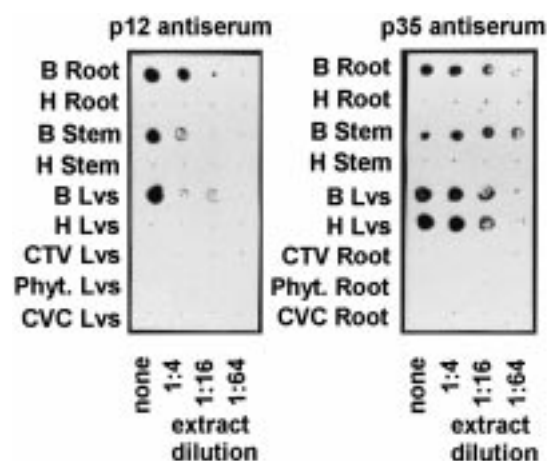


Figure 1. Dot blot immunoassays of citrus tissues. Extracts from leaves, and xylem sap of roots and stems of healthy (H) and blighted (B) trees, or trees affected with citrus tristeza virus (CTV), *Phytophthora* (Phyt.) or citrus variegated chlorosis (CVC, caused by xylem-limited bacteria *Xylella fastidiosa*) spotted and probed with antibodies to p12 protein and p35, a CB-associated β -1,3-glucanase. A widely used diagnostic test for blight involves an immunoassay for p12 in leaves. (Figure modified and reprinted from Derrick *et al.* [17].)

roots and stems and expressed sap from leaves, compared to healthy controls and citrus affected with other diseases. Note the CB-specific nature of p12 and another representative BAP, p35. Anti-p35 antibodies were produced as described by Derrick *et al.* [18]. p12 does not occur in any tissue other than that affected by CB, whereas the CB-associated PR protein, a β -1,3-glucanase (p35) as identified by N-terminal amino acid sequencing and western blot analysis using anti-serum to PR-2 (kindly provided by Dr L.C. van Loon), is constitutive in leaves but specifically expressed in CB-affected root and stem xylem fluid.

CB-affected leaves (5 g) were extracted using a leaf roller and the extract was clarified by centrifugation. To the supernatant, 0.1 mM EDTA and 1% (v/v) 2-mercaptoethanol were added. The sample was boiled 15 min, chilled on ice, centrifuged for 2 min at 10 000 rpm and the supernatant adjusted to $1\times$ PBS. For immunocapture [20], 1 mL of the supernatant was mixed with 10 μ l anti-p12 ascites antiserum [17] and incubated 1 h at room temperature. A 10 μ l portion of protein G sepharose (Pharmacia) was added and incubated 1 h at room temperature. The mixture was centrifuged, the pellet washed twice with $1\times$ PBS, and an equal volume $2\times$ Laemmli [23] treatment buffer added. After boiling, the immunocaptured protein was subjected to 12% SDS-PAGE and blotted onto PVDF

nitrocellulose (Millipore). The N-terminal of the protein was sequenced by the Interdisciplinary Center for Biotechnology Research (ICBR) facility (University of Florida, Gainesville, FL). Homology searches were performed using BLASTP [3].

Tissue prints

Roots of up to 2 cm in diameter, from healthy and CB-affected trees were cross-sectioned and the cut surface immediately pressed onto nitrocellulose membrane (Nitro ME, MSI) for several seconds. The membranes were probed with either p12 (p12 MAB), p35 or dehydrin antisera using the protocol of Derrick *et al.* [17, 18] or Close *et al.* [9] (dehydrin antiserum kindly provided by Dr T.J. Close).

PCR and cloning of probe DNA

Based on the partial amino acid sequence of p12, degenerate primers were designed for PCR amplification of a 60 bp product (p12dF: 5'-TT-(C/T)TA(C/T)ACNCCNCCNTA(C/T)GT-3'; p12dR: 5'-CATNACNCC(G/A)TC(G/A)TT(T/C)TT-3'). A 3 g portion of the root cambial zone, prepared by shaving off the layers between the bark (phloem) and wood (xylem), and 3 g leaves were collected from a CB-affected tree. RNA was extracted using the method of Podivinsky *et al.* [28], modified by adding a second CsCl precipitation and one LiCl precipitation [29]. The resulting RNA pellet was dissolved in DEPC-treated water and ethanol-precipitated. Reverse transcriptase (SuperScript; Gibco-BRL) was used to produce cDNA using the manufacturer's instructions. The cDNA was PCR amplified using primers p12dF and p12dR (0.2 μ M each) and a cocktail consisting of 2 mM Mg PCR buffer (Idaho Technology, Idaho Falls, ID), dNTPs (40 μ M each; Idaho Technology), and *Taq* polymerase (0.05 U/ μ l). The amplification program had three initial cycles of low stringency: 94 °C, 1 min; 37 °C, 1 min; 72 °C, 1 min, followed by 30 cycles of 94 °C, 30 s; 56 °C, 45 s; 72 °C, 1 min, and one final 72 °C elongation period of 12 min. No product was observed when analyzed on a 10% polyacrylamide gel in buffer 'E' (36 mM Tris, 10 mM sodium acetate, 1 mM EDTA pH 7.2) and one final 72 °C stained with ethidium bromide. Therefore, 1 μ l of the reaction mixes were subjected to a second, identical, PCR amplification and the products analyzed as before. A band of the predicted size (60 bp) was seen in the lane corresponding to root cambial cDNA. This band was excised, placed in a microcentrifuge

tube, crushed, diluted in 50 μ l water, and the gel-water mix heated in boiling water then quenched on ice. One μ l of the gel-water mix was used as template in another PCR reaction identical to the previous reactions. A distinct 60 bp band from this reaction mix was observed and subsequently cloned into pGEM-T (Promega) according to manufacturer's directions. Colonies were screened by PCR and selected colonies were sequenced by the ICBR facility. Positive clones were identified by comparison of their translated sequences with the p12 amino acid sequence using SeqAid II software (ver. 3.81, Rhoads and Roufa, 1991; Kansas State University, Manhattan, KS). From this sequence, non-degenerate PCR primers were designed and synthesized for amplification of the 60 bp gene fragment.

cDNA library construction and screening

RNA from CB-affected root cambium was used to produce a cDNA library constructed in λ Zap Express (Stratagene). From the root cambial zone 3 g tissue was collected from the same tree used for PCR analysis, and RNA extractions were performed with the method of Podivinsky *et al.* [28] except that the CsCl precipitation was replaced by LiCl precipitation according to Prescott and Martin [29]. The RNA was ethanol precipitated and dissolved in 0.5 ml DEPC-treated water. The poly(A)⁺ fraction was isolated with streptavidin-paramagnetic particles (Promega) using the manufacturer's instructions. After ethanol precipitation, the pellet was dissolved in 36.5 μ l nuclease-free water and cloned into ZAP Express vector (Stratagene) according to the manufacturer's instructions.

A single-stranded digoxigenin-labeled DNA probe to p12 cDNA was prepared using a method modified from Konat *et al.* [22]. The 60 bp cDNA clone was PCR-amplified as described above from a plasmid containing the gene fragment using non-degenerate primers in limiting quantities (0.02 μ M). The product was quantitated against DNA standards on a 10% polyacrylamide gel. A PCR cocktail was prepared with 2 mM Mg PCR buffer (Idaho Technology), a dNTP/DIGdUTP mix (final concentration 100 μ M of each dNTP; dTTP:DIGdUTP ratio ca. 2:1; Boehringer Mannheim), 4 ng/ μ l template DNA, 3 μ M final concentration p12 DNA reverse primer, and 75 U/ml *Taq* polymerase. The amplification program was taken from Konat *et al.* [22].

The primary library was plated and lifted using the ZAP Express protocol (Stratagene) and probed with

the digoxigenin-labeled single-stranded DNA probe in standard buffer (5× SSC, 1% (w/v) blocking reagent for nucleic acids, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS; Boeringer Mannheim) at 68 °C according to the manufacturer's instructions. After secondary screening, two plaques were isolated and excised into phagemids (ExAssist; Stratagene) according to the manufacturer's instructions. Plasmid DNA minipreps were prepared using the method of Nicoletti and Condorelli [27]. The clones were sequenced at the ICBR sequencing core. The sequences of the cloned cDNA were compared to the available sequence of p12 (SeqAid II, ver. 3.81). Nucleic acid sequences were analyzed using the BLAST [3] program of GCG (Genetics Computer Group, Madison, WI) and PC/Gene (Release 6.8, IntelliGenetics, Mountain View, CA). PCR primers to the full-length open reading frame (ORF) were designed and synthesized (p12F, 5'-ATGGGTGTAGGCACAAAAGTTCTGG-3'; p12R, 5'-TCAAGCCTGATTGAACTCAATC-3').

Bacterial expression

The ORF of p12 was amplified by PCR from a plasmid containing the clone using gene specific primers p12F and p12R with incorporated *Nhe*I and *Eco*RI sites. The product was ligated into the *Nhe*I and *Eco*RI sites of the vector pETH3a (modified from pET [35] by McCarty *et al.* [25]), and the resulting plasmid used to transform *Escherichia coli* BL21 (DE3) pLysS (Novagen) cells. Cells were grown in 3 ml cultures with appropriate antibiotics (Novagen instructions), and induced with 0.4 mM isopropylthio- β -galactoside (IPTG). Whole-cell and periplasmic protein preparations, prepared by the manufacturer's instructions (Novagen) at time 0 and 2 h after induction, were analyzed by 12% SDS-PAGE and a western blot probed with p12 MAB [18].

Southern analysis

DNA from ca. 1 g young leaf tissue of 5 healthy citrus trees was extracted with the method of Dellaporta *et al.* [16]. The DNA was digested with *Eco*RI and *Bgl*II and subjected to electrophoresis on 1% agarose in 1× TBE [31]. The gel was stained with ethidium bromide, photographed, and blotted to nylon (MagnaGraph, MSI) according to standard procedures [31]. After UV-crosslinking the DNA (Stratagene Stratalinker), the membrane was pre-hybridized and probed with the digoxigenin-labeled single-stranded DNA probe in standard buffer (5× SSC, 1% (w/v) blocking reagent

for nucleic acids, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS; Boeringer Mannheim) at 68 °C overnight. The membrane was washed and developed according to the manufacturer's instructions (Boeringer, Mannheim) and exposed to film for analysis.

Northern and western blot analysis

Total RNA was extracted from root and stem cambial zone and leaves of CB-affected trees (as verified by p12 immunoassay) and healthy rough lemon trees as described above. RNA fractions were dissolved in 500 μ l nuclease-free water and mRNA was selected by streptavidin-paramagnetic particles (Promega). The resulting mRNA was electrophoresed on 1% agarose formaldehyde denaturing gels as described in Sambrook *et al.* [31], modified by Chomczynski [8] and transferred onto nylon membrane (MagnaGraph; MSI) in a downward fashion (TurboBlotter; Schleicher and Schuell) for 2 h at room temperature with 3 M NaCl, 8 mM NaOH, 2 mM sarcosyl [8]. The RNA was UV-crosslinked (Stratalinker, Stratagene) and the blots were prehybridized for 1 h in 50% (v/v) formamide, 5× SSC, 2× Denhardt's solution, 1% (w/v) SDS, 100 μ g/ml salmon sperm DNA at 59 °C.

A RNA probe was prepared by restriction digesting plasmid DNA containing the full-length p12 clone 400 bp downstream of a T7 transcription site. Transcription (Ambion, Austin, TX) was performed *in vitro*, labeling p12 RNA with [³²P]CTP. A [³²P]CTP-labeled β -actin transcription product (Ambion) was used as a control probe for mRNA.

The probe was added to the prehybridization buffer and hybridized with the membranes overnight at 59 °C. The membranes were washed using standard procedures [31], with the stringent wash (0.1× SSC, 0.1% SDS) at 59 °C, and exposed to film.

Western blot analyses of proteins extracted from healthy and CB-affected tissues were performed according to Derrick *et al.* [18] using p12 MAB.

Results

Protein sequence and serology

The 34 N-terminal amino acids of p12 had no significant homology to any protein in the GenBank databases.

Tissue print analyses were used to compare the patterns of expression of two BAPs and dehydrin in roots

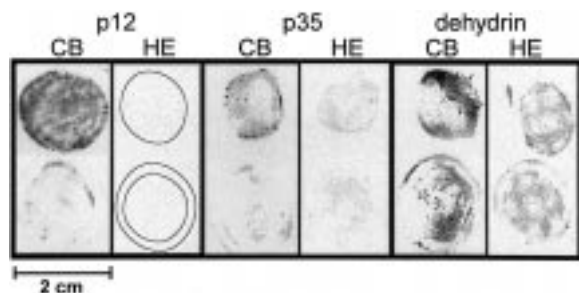


Figure 2. Tissue print analyses. The expression of the BAPs p12 and p35 (a CB-associated β -1,3-glucanase), and dehydrin are shown in CB-affected (CB) and healthy (HE) root tissue. Two cross-sectioned roots for each treatment were pressed onto nitrocellulose, and the membranes probed with the corresponding antiserum. The HE lane of p12 includes a drawing of the tissue layers that can be identified on each blot. The upper panels represent roots which have had the outer layer (phloem) peeled off. The single circle represents xylem and part of the cambial zone. The lower panels represent unpeeled roots; the outer circle is the phloem layer, the inner circle xylem and cambium is at the interface. Photographs are from digitized images.

(Figure 2). An early observation of p12 was that it remained soluble after heating, which is a characteristic of dehydrin [11]. We examined the expression of dehydrin in blighted trees because dehydrin is reported to be produced in response to any stress associated with dehydration [10]. Tissue prints indicated that p12 was generally localized to the outer layers of xylem and the vascular cambial zone of roots, although it occasionally may be found in high concentrations throughout the xylem (Figure 2). The higher levels of p12 in the xylem, (upper panel, p12-CB column, Figure 2) could be related to the degree of infection, or the plant water status. The CB-associated β -1,3-glucanase, p35, is found within the xylem and cambial zone of CB-affected roots (Figure 2). Neither of these BAP's were found in healthy roots. Dehydrin was detectable at low levels in healthy roots, but was present at higher levels in blighted tissues (Figure 2).

cDNA cloning and bacterial expression of p12

Two clones of 674 bp were obtained from the CB-affected root cDNA library, sequenced and found to contain identical ORFs that were identified as encoding p12 by comparison with the amino acid sequence (accession number AF015782). The 393 bp ORF encodes a 14.4 kDa protein, the first 24 amino acids of which compose a hydrophobic signal peptide (PC/Gene, IntelliGenetics). The size of p12 estimated by SDS-PAGE agrees well with 11.8 kDa predicted

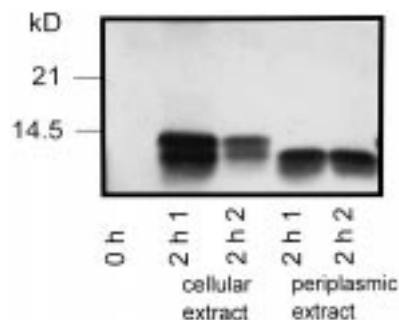


Figure 3. Expression of p12 in *E. coli*. Proteins expressed by two different *E. coli* BL21 (DE3) colonies (labeled 1 and 2) were analyzed by western blotting to verify the immunoreactivity and size of the encoded protein. Samples from left to right are: time 0 (unexpressed); 2 h IPTG-induced whole cell extracts, two colonies; 2 h IPTG-induced periplasmic extracts, two colonies. Molecular weight markers in kDa are to the left of the blot. Photograph is from a digitized image.

from the deduced amino acid sequence of the protein without the signal peptide.

The transformation of bacteria and analysis of expressed proteins verified that the cloned p12 ORF encodes the expected 12 kDa immunoreactive protein (Figure 3). In addition, this protein is transported across the periplasmic membrane, as expected for a protein with a transmembrane signal peptide. Two proteins were seen in whole cell extracts, while only the smaller of the two proteins was present in periplasmic extracts (Figure 3).

A comparison of the p12 cDNA sequence with the GCG (Genetics Computer Group, Madison, WI) database using BLAST [3] revealed a significant similarity of the p12 ORF with expansins from various species. The best match was to an *Arabidopsis thaliana* expansin cDNA (accession number U30476), which was 49% similar and 31% identical (Gap, GCG) at the amino acid level (Figure 4). Moreover, when p12 secondary structure was predicted using PHDsec [30], the structure was found to have homologies to the pollen allergen pI protein of *Lolium* (30% pairwise sequence identity, MaxHom alignment), a protein recently shown to have expansin biological activity in 'creep' assays using grass cell walls as substrate [15]. The areas of greatest homology to both expansin and allergen proteins are within the areas known as the cysteine-rich region (spanning bases ca. 41–102, Figure 4), and the basic region (bases 103–131, Figure 4; [15]).

p12 mRNA is in roots but not in leaves

A stem root
h b h b

B leaf
h b

northern

i

p12

ii

B-actin

western

iii

p12

One of our objectives is to determine the function of p12 [18]. The serological assay for p12 has been widely used and is quite accurate for differentiating producing trees that are either presymptomatic or are declining from CB, from other declines of citrus [17]. Producing grove trees that are declining due to citrus tristeza virus, *Phytophthora*, citrus variegated chlorosis, salinity, nematodes, root weevils, or water stress do not contain the protein (Figure 1; data not shown). Although the p12 assay is the best way to determine if

grove trees have blight, nursery trees will sometimes test positive for p12. We do not yet know if this is due to some type of 'pot stress' or if it is related to a pathogen that may cause the trees to develop blight after they are moved into a grove and brought into production. As part of the p12 immunoassay protocol, crude leaf extracts are incubated at 37 °C for 48 h before spotting on nitrocellulose membranes [17]. A 60 kDa protein which cross-reacts with the p12 monoclonal antiserum is thereby denatured and removed by centrifugation, while p12 remains soluble. p12 also remains soluble when crude extracts are boiled for 10 min and clarified by centrifugation (unpublished results). In all serological tests for p12 the protein must be denatured by heat to expose the epitope to the monoclonal antibody. This is usually done by autoclaving the nitrocellulose membranes in water after samples have been spotted, a treatment which also increases the sensitivity of western blot analysis for p12 [17].

A class of stress-proteins which, like p12, has the characteristic of heat solubility is dehydrin [11], and since CB-affected trees are under considerable water stress [37] we considered that p12 could be related to dehydrins. Dehydrins are known to accumulate in response to any stress that has a dehydration component [10]. Amino acid sequencing and western blot analysis with dehydrin consensus sequence antiserum established that p12 and dehydrin are not related. But dehydrins do accumulate in CB-affected tissues (Figure 2), which is not surprising considering the drought-stress aspect of CB [2, 37].

GCG database comparisons of the p12 cDNA revealed a similarity between p12 and expansin. Expansins are a highly conserved multi-gene family of proteins which have been attributed with loosening cell walls during longitudinal growth [26]. They may also have some connection to growth at low turgor potentials. Wu *et al.* [40] observed that extractable expansin protein as well as wall extensibility increased in the root tips of plants grown under low water potential compared to those under higher water potential. The xylem plugging related to CB infection [6] could trigger a response to extend roots, similar to a plant responding to drought stress. The relationship between p12 and an *A. thaliana* expansin, 49% similar and 31% identical at the amino acid level, is considerably lower than between other known α -expansins, which have 60–87% amino acid identity [33]. Moreover, p12 is significantly smaller than the known expansins which are ca. 30 kDa. p12 and expansin do have similar signal peptides targeting cell walls and levels of

some amino acids (Trp, Cys) which are conserved between expansins [33]. Citrus does have the 30 kDa expansin protein, which can be extracted from walls in a high-salt buffer, as revealed by western analysis using expansin S1 antiserum (kindly provided by Dr D.J. Cosgrove). p12, which is easily extracted by sap expression in a dilute buffer, did not react to the expansin antibody, nor did expansin protein react with p12 antiserum.

Recent work with group I allergens of grass pollen, with distant sequence similarity to α -expansins (20–25% amino acid identity) as well as to p12, has revealed that these proteins induce wall extension and form a new class of expansins, β -expansins [15]. These grass pollen proteins had their strongest activity on cell walls from grasses. The activity of undiluted extract on typically assayed cucumber walls was less than one quarter of that seen on grass walls with a 20% extract. Presumably other expansins could exist that have activity on specific substrates. It has yet to be determined if p12 has expansin activity, since 'creep' assays using cucumber hypocotyl walls as substrate [14] have proved inconclusive (D.J. Cosgrove, personal communication). Similarly to the pollen group I allergens, p12 may only be biologically active on a specific substrate and, if so, may belong to a new class of expansin.

Considering initial difficulties with PCR of leaf DNA, the negative results from northern analysis of leaf mRNA, and the association of p12 with CB, two important questions have been raised. First: is p12 a plant protein? Citrus is host to a variety of plant pathogens and symbiotic organisms, many of them found in roots. Therefore the mRNA cloned from infected roots could have come from the blight pathogen or other microorganism. The results of Southern analyses of the p12 gene from citrus genomic DNA (Figure 5) indicates that it is a citrus gene. Additionally, we have successfully PCR amplified the p12 gene from leaf genomic DNA with p12 primers. Therefore, p12 appears to be a citrus-encoded protein.

The second question is: where is the protein synthesized? The protein is regularly extracted from root xylem and is also present in the vascular cambial zone where the p12 mRNA is found. p12 is also found in high quantities in leaves although its mRNA is not present. When p12-positive branches are removed from a tree and rooted, p12 is no longer detected in the cuttings, although the parent tree in the field remains p12 positive [41]. The absence of p12 mRNA in leaves suggests that the protein is not made in leaves

but transported from roots and stems, possibly via the apoplast, considering its presence in xylem. Sharon *et al.* [32] found that a fungal xylanase protein was transported from cut petiole surfaces both up and down the entire plant. Translocation was via xylem vessels. The authors concluded that some protein elicitors of resistance, ethylene biosynthesis and hypersensitive response in their case, can induce responses without the involvement of intermediary compounds [32]. Since there is no known function for p12 at this time, a plant response cannot be measured, but p12 may be a case of a plant-derived PR or stress protein translocated to perform or induce a resistance response.

We are still trying to answer the question 'what is the function of p12?'. We do not know if it is a component of the plant response to resist CB or a possible cause of symptoms. Another intriguing question comes from considering the role of an expansin in a stress response. Bearing in mind that few things in nature are ever entirely unique, there may be answers in looking for triggers of p12 expression in other species. Current work includes the production of transgenic plants with the gene for p12.

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