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Putative protease inhibitor gene discovery and transcript profiling during fruit development and leaf damage in grapefruit (*Citrus paradisi* Macf.)

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Abstract

Seven putative protease inhibitor (PPI) cDNAs, representing four protein families, were isolated from a grapefruit (*Citrus paradisi* Macf. Cv. Marsh) immature fruit flavedo cDNA library. Cloned open reading frames encoded proteins with similarity to, and protein signatures for: legume Kuntiz inhibitors (*lkiL-1*, *lkiL-2*, *lkiL-3*), potato trypsin inhibitor I (*ptiIL-1*), serpins (*serpL-1*), cystatins (*cystL-1*), and gamma thionins (*gthL-1*). Response of transcript abundance to fruit development and leaf wounding was determined for all but *lkiL-1* using real-time RT-PCR. Immature leaves had the highest transcript levels for all PPIs. The *gthL-1* transcript in immature leaves was the most abundant transcript but was absent from healthy mature leaves. In fruit flavedo, transcripts for all PPIs were most abundant in youngest fruit (< 15 mm dia. fruit), and declined during development, but displayed different patterns of developmental change. Mechanical or Diaprepes root weevil (DRW) feeding damage to leaves caused a < 10-fold reduction or had no effect on transcript level with the exception of *gthL-1* which, as a result of damage, increased >50-fold in mature leaves and decreased >1400-fold in immature leaves. This developmental control of transcript response to wounding in a woody perennial is opposite of what has been observed for defensive proteinase inhibitors (PIs) in other plants (typically herbaceous and/or annual plants), where younger leaves typically invoke a higher defensive proteinase inhibitor transcript accumulation than older tissues. Except for *gthL-1*, the PPI transcripts were minimally responsive or unresponsive to wounding. Changes in PPI transcript levels suggest diverse roles for the products of these genes in citrus, with only *gthL-1* responding in a defense-like manner. Published by Elsevier B.V.

Keywords: Serine proteinase inhibitor; Cysteine proteinase inhibitor; Wound response

1. Introduction

Plants produce a plethora of proteinases and proteinaceous inhibitors of proteinases (PIs). Members of all four main proteinase classes (serine, cysteine, aspartic and metallo-proteinases) have been described in plants (Estelle, 2001; Savelkoul et al., 1992; Kinoshita et al., 1999; Delorme et al., 2000; Muntz et al., 2001; Wagstaff et al., 2002; Jia et al., 2000; Lam et al., 1999). The majority of the studies on these enzymes in plants have focused on their role in seed storage protein mobilization associated with germination and tissue senescence (Savelkoul et al., 1992; Kinoshita et al., 1999; Delorme et al., 2000; Muntz et al., 2001; Wagstaff et al., 2002) as well as pathogen defense (Jia et al., 2000) and programmed cell death (Lam et al., 1999).

Production of PIs in plants is thought to occur to either modulate endogenous proteinase activity or to interact with foreign proteinases of pathogen origin or digestive enzymes of herbivorous insects (Bode and Huber, 1992). Serine proteinase inhibitors are by far the most commonly described to date; however, inhibitors for cysteine, aspartic and metallo-proteinases are also known (Lawrence and Koundal, 2002). Subfamilies of the serine PIs found in

Abbreviations: *cystL-1*, grapefruit cystatin-like cDNA clone; DRW, diaprepes root weevil (*Diaprepes abbreviatus*); *gthL-1*, grapefruit gamma thionin-like cDNA clone; *lkiL-1*, *-2*, *-3*, grapefruit legume Kunitz inhibitor-like cDNA clones; PI, proteinase inhibitor; PPI, putative proteinase inhibitor; *ptiIL-1*, potato type I proteinase inhibitor-like cDNA clone from grapefruit; *serpL-1*, grapefruit serpin-like cDNA clone.

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plants as identified by conserved protein subdomains include: legume Kunitz; Bowman-Birk; cereal trypsin/alpha amylase inhibitors; potato type I and II; squash-types; serpins; and thionin-type inhibitors as determined using the European Bioinformatics Institute Interpro database (http://www.ebi.ac.uk/interpro/). Because of this great diversity of PIs and their link to plant defense, determination of their function has been a major area of study. A caveate about assigning genes and transcripts to specific functional groups based on protein sequence signatures is that this may not represent their true function in the plant. As an example, we include gamma thionins as proteinase inhibitors even thought there is controversy over their biological function, despite proof of proteinase inhibitor activity (Melo et al., 2002).

Regulation of PI transcript abundance has been a classic measure of induction of the octadeconoid defense pathway in plants (Bowles, 1998; Ryan, 2000; Li et al., 2002; Moura and Ryan, 2001). This pathway is controlled by the action of systemin, an 18 amino acid peptide hormone and leads to the production of jasmonates that are key regulators of stress induced genes (Ryan, 2000; Reymond et al., 2000; Schaller and Frasson, 2001). This work implicates PIs as defense molecules defending against pathogens and herbivorous insects. Thus, wound-stimulation of plant PI transcript accumulation is considered a defensive mechanism. Further proof of this PI function has come from the development of transgenic plants that accumulate PIs, and as a result, are more insect/nematode resistant (Johnson et al., 1989; Urwin et al., 1997; Duan et al., 1996; Urwin et al., 1998).

The basic theme arising from regulation studies on PI transcript and protein abundance indicates that tissue specific, developmentally controlled, and biotic and abiotic stress signals regulate the level of these PIs in plants (Koiwa et al., 1997). The predominant mode of PI regulation is transcriptional control of mRNA abundance. Aside from wounding response, reproductive and storage organs of a number of important crops produce proteinase inhibitors in response to developmental signals, presumably to protect themselves from pathogens and herbivores (Richardson, 1977). These PIs can accumulate to levels reaching 20–80% of the total soluble protein (Atkinson et al., 1990; Hattori et al., 1990).

Proteinase inhibitors have not previously been reported for citrus fruit or other tissues. As part of a large-scale citrus expressed sequence tag (EST) research project, six putative protease inhibitors (PPI) were found initially in a young fruit flavedo library, and subsequently identified in a pool of 34,273 clones isolated from five different citrus species and six different library types. Transcript profiling was performed as a first step in identifying genes that may function to produce defensive proteins in citrus tissues. Results are discussed in relation to the regulation of transcript abundance during fruit development and leaf damage and with respect to differences from those previously described for related PIs.

2. Materials and methods

2.1. Plant treatments and plant tissue harvesting

The fruit development study was performed on grapefruit (*Citrus paradisi* Macf. Cv Marsh) grafted to sour orange (*Citrus auratium* L.) rootstock using healthy mature trees that were planted in a production grove in 1965 located in St. Lucie county, FL. Grapefruits of the appropriate size (indicated in Fig. 2) were harvested at the indicated times throughout the growing season from a group of 24 marked trees, where six groups of trees within four separate rows were groups as four separate replicates. Flavedo was removed in the field from the picked fruit, by combining the flavedo from fruit taken from the six trees within a single row, using a potato peeler and directly stored in liquid nitrogen. Flavedo samples from each of the four groups (rows) were stored at − 80 °C until used for RNA isolation.

For leaf damage studies, 5-year-old *C. paradisi* Macf. Cv Flame grapefruit scions (averaging 1.5 m in height) grafted to US-852 rootstock were provided by Dr. Kim Bowman (USDA, ARS, USHRL, Fort Pierce, FL) and maintained in 36 × 30 cm (w × h) pots containing ProMix BX soil (Premier Horticulture; Quebec, Canada). Flame grapefruit is closely related to Marsh (used in fruit development study), arising from a seed collected from Henderson grapefruit; whereas, Henderson was selected out of three separate branch sports sequentially chosen for fruit flesh color variations initially from Marsh. The plants used for leaf damage studies were maintained in a growth room supplemented with mixed metal halide lighting at an average light intensity at mid canopy height of 800 μE PAR with a 16-h photoperiod and a 27/20 °C day/night temperature regime. Plants were watered every other day, and weekly fertilized by drenching the soil with Peters 20−10−20 Florida Special (The Scotts, Marysville, OH) prepared at 400 ppm N. Prophylactic weekly treatment with Safer™ Insecticidal Soap was performed to prevent insect infestation.

Insect feeding studies were performed using *Diaprepes abbreviatus* (Diaprepes root weevil: DRW) adults, taken from a laboratory maintained colony fed on citrus flush, but starved for three days immediately prior to the experiment. All experiments were performed in triplicate on separate plants (three plants for each group: control undamaged, DRW damaged immature leaves, mechanical damaged immature leaves, and mechanical damaged mature leaves) and each of these experiments was replicated in time three times. Six DRWs were caged on branches containing young expanding leaves (average leaf length of 2 to 3 cm and less than 1 month old). Using this protocol, the DRW would consume approximately 20% of the leaf mass within a 24-h period. After the 24-h period, the DRW were removed. Leaves were left on the tree an additional 8 h and then removed along with caged control leaves on similar plants and immediately placed in liquid nitrogen and stored at − 80 °C until used for RNA isolation. All leaves removed from a

single plant were pooled and represented one sample. Mechanical damage was performed on same stage leaves and greater than 6-month-old mature leaves using a paper hole punch. Single punches were repetitively applied to remove approximately 20% of the leaf area. This was performed 2 h into the photoperiod and the damaged leaves were allowed to remain on the plants for eight more hours, at which time the damaged leaves were harvested as described for the DRW feeding damaged leaves. All control and experimental leaves were harvested 10 h into the photoperiod.

2.2. cDNA libraries used in this study

Sequenced clones were obtained from a cDNA library constructed using mRNA purified from flavedo removed from immature grapefruit less than 3 cm in diameter. The library was constructed in pBluescript II SK(+) using the Uni-ZAP® XR library construction kit from Stratagene (La Jolla, CA) and the provided protocols.

2.3. EST and whole clone sequencing

Expressed sequence tag (EST) sequence information was obtained for 1000 randomly selected clones chosen from the mass-excised unamplified flavedo cDNA library. Individual colonies were picked from the plates to start 1.7 ml overnight LB cultures for automated plasmid isolation. Plasmids were prepared using the Qiagen 9600 liquid handling robot and the QIAprep 96 Turbo mini prep kit following the recommended protocol (Qiagen, Valencia, CA). Five-prime single pass sequencing reactions were performed using the ABI PRISM® BigDye™ Primer Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) at 1/16th the standard total volume reaction and the T7 primer. Reactions were prepared in 96-well format using the Biomek 2000 liquid handling robot (Beckman Coulter). Sequencing reactions were precipitated with 70% isopropanol, resuspended in 15 μl sterile water and loaded onto a 3700 DNA Analyzer (Applied Biosystems). Base calling was performed by TraceTuner™ (Paracel®, Pasadena, CA) and quality trimming, vector trimming and sequence fragment alignments were performed using Sequencher™ Software (Gene Codes, Ann Arbor, MI). Sequence identity was determined based on BLAST® searches using the National Center for Biotechnology Information (NCBI) BLAST® server (http://www.ncbi.nlm.nih.gov/) and comparison to the nt nucleic acid and nr protein databases.

Complete repetitive sequencing of individual PPI clones was performed by initially using both the T7, T3 primers and followed by primer-walking until completely overlapping sequence data from both strands were obtained. Clones were aligned and edited using the Sequencher™ program. Coding region analysis and protein domain analyses were performed using the web based Biology Workbench provided by the San Diego Supercomputer Center, at the University of California, San Diego (http://workbench.sdsc.edu) and the European Bioinformatics Institute Interpro database (http://www.ebi.ac.uk/interpro/).

2.4. RNA detection and quantification

Real time RT-PCR was performed using total RNA as template. Total RNA was isolated using the Rneasy plant RNA isolation kits provided by Qiagen. Specific RNA species were detected and quantified from total RNA samples using the iCycler iQ system (BioRad, Hercules, CA) and the QuantiTect SYBR Green real time RT-PCR kit (Qiagen). Real time RT-PCR reactions were performed in 25-μl volumes following the manufacturer's recommended protocol using 200 ng of total RNA. The one-step reverse transcriptase and PCR cycling conditions were as follows: 50 °C for 30 min, 95 °C for 15 min, and 40 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 40 s. Subsequent to the cycling reactions, fluorescence was monitored during a melting curve analysis. All real-time RT-PCR reaction products were visualized after agarose gel electrophoresis to assure single product amplification and minimal primer-dimmer formation. Primers made for real-time RT-PCR detection of specific RNA species are listed in Table 1. These primers were designed using the web-based Primer3 software provided by the Whitehead Institute at Massachusetts Institute of Technology (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Relative quantification was based on normalization to detection values for citrus 18S rRNA species, and comparing this normalized value among RNA samples. Quantification of transcript amounts relative to total RNA used in an amplification reaction was performed by developing a standard curve of real time RT-PCR threshold cycles (C_t) for serially diluted linearized plasmids containing each PPI clone. The C_t values deter-

Table 1
Real-time RT-PCR primers used for each cDNA clone

Clone name (size of PCR product)	Synthetic nucleotide 5′ primers	Synthetic nucleotide 3′ primers
lkiL-1 (126 bp)	5′-CTATGGCAACCAGGTGGACT-3′	5′-TGGATAACATCCGTTGGACA-3′
lkiL-2 (150 bp)	5′-GGCGTTTCAACTAAGGATGG-3′	5′-TGCTTCAGCAGCTTCAAAAA-3′
ptiIL-1 (151 bp)	5′-GCCAGAGCTGGTTGGTAAAA-3′	5′-CCCTTTCTTGTTGACCCAAA-3′
serpL-1 (158 bp)	5′-TAGACCACCACCTTCCATCC-3′	5′-ATCCACCATCTCTGCCAAAC-3′
gthL-1 (146 bp)	5′-TGCTTTAGCCACCACAACTG-3′	5′-GTGGCAAAAGAGAGCACACA-3′
cystL-1 (150 bp)	5′-AGCCAACAATCAGGAGATCG-3′	5′-ACAGCCTCCAAGGTGATGTC-3′

Table 2
BLAST homologies of cloned proteinase inhibitor-like sequences from citrus EST sequencing of multiple libraries

Clone name (acc. #)	# of clones/ seq.[a]	Seedling[b]	Fruit[b]	Flower[b]	Leaf[b]	Root[b]	Phloem[b]	Size of clone/ contig (bp)	Best match	Bit/E score
lkiL-1 (AF283532)	20	18 (0.13)	1 (0.01)	1 (0.05)	0	0	0	886	gi|7438249| Tumor-related protein, clone NF34-common tobacco	104 (259)/ 9e – 22
lkiL-2 (AF283533)	93	3 (0.02)	84 (0.70)	5 (0.30)	0	0	1 (0.05)	942	gi|7438249| Tumor-related protein, clone NF34-common tobacco	116 (291)/ 2e – 25
lkiL-3 (AF283534)	27	1 (0.01)	23 (0.19)	0	0	0	3 (0.14)	1004	gi|7438249| Tumor-related protein, clone NF34-common tobacco	124 (310)/ 8e – 28
gthL-1 (AF283535)	8	4 (0.03)	4 (0.03)	0	0	0	0	688	gi|2129842| Probable proteinase inhibitor SE60 precursor-soybean	107 (268)/ 2e – 23
serpL-1 (AY158152)	2	1 (0.01)	1 (0.01)	0	0	0	0	1571	gi|15220298| Serpin, putative [*Arab. thaliana*]	496 (1277)/ e – 139
ptiIL-1 (AY158153)	11	5 (0.04)	5 (0.04)	1 (0.05)	0	0	0	590	gi|124984|Inhibitor of trypsin and hagemena factor (CTMI-V)	97.4 (241)/ 2e – 20
cystL-1 (AF283536)	15	9 (0.07)	5 (0.04)	0	0	1 (0.16)	0	1027	gi|8099682|Cysteine protease inhibitor [*Manihot esculenta*]	134 (337)/ 2e – 31

[a] The number of clones representing the indicated sequences (since sequences were from different citrus varieties and species, they were categorized as representing the same related clone if the sequences shared greater than 95% similarity among the different citrus types.
[b] Number of individual clones sequenced. The number in parenthesis is the percent of total clones represented as the specific gene. Total sequenced clones or each library: seedling, 13,654; fruit (whole fruit and flavedo libraries), 12,135; flower, 1937; leaf, 3815; root, 620; phloem, 2112.

mined experimentally for the different total RNA samples (normalized to 18S rRNA C_t values) were fit to the plasmid standard curve to determine specific transcript concentration.

3. Results

3.1. Identification of proteinase inhibitor cDNA clones

Nucleotide sequences from single-reaction 5′-terminus sequencing of cDNA clones randomly selected from a grapefruit (*C. paradisi* Macf. Cv Marsh) immature fruit flavedo cDNA library were compared to the NCBI nr database. Using BLASTX comparisons, clones putatively encoding proteins with significant similarity (Bit scores greater than 100 and E-scores less than 1×10^{-10}) to characterized or putative serine and cysteine proteinase inhibitors were found. The clone containing the longest sequence for each was used for redundant bi-directional sequencing to obtain full-length or near full-length cDNA sequence. Putative proteins encoded by these sequences were compared to NCBI protein database and the best matches are shown in Table 2. Predicted translation products of three of the clones (designated *lkiL-1*, *-2*, and *-3*) were closely related to a tobacco tumor related protein and miraculin-like legume Kunitz inhibitor proteins. The other clones encoded: a cystatin-like PPI (*cystL-1*), a SE60-gamma thionin-like PPI (*gthL-1*), a serpin-like PPI family (*serpL-1*) and one closely related to a cucurbit PI belonging to the Potato Inhibitor I family (*ptiIL-1*). These sequences were deposited in GenBank and used to determine characteristics of the protein putatively encoded by each (Table 3). The *lkiL*

Table 3
Characteristics for proteins encoded by the cloned grapefruit cDNAs

CDNA clone	MW[a] (Da)	Signal peptide[b]	Cell targeting[b]	*N*-glycosyl[c]	cAmp phos.[c]	PKC phos.[c]	CK2 phos.[c]	Protein family signature[d]
lkiL-1	25,253	Yes	Secretory (Vac/extracell.)	90, 128, 180	–	41, 77, 223	25, 77, 137, 156, 193	Legume Kunitz Inhibitor (29)
lkiL-2	25,652	Yes	Secretory (Vac/Extracell.)	155	–	78, 110, 184, 212	26, 41, 76, 124, 184	Legume Kunitz Inhibitor (30)
lkiL-3	22,481	Yes	Secretory (Vac/Extracell.)	51, 91, 114	143	78	25, 40, 63, 76, 78, 157	Legume Kunitz Inhibitor (30)
cystL-1	11,177	No	Cytoplasmic	–	–	45	54	Cystatin Domain (48)
gthL-1	8243	Yes	Secretory (Vac/Extracell.)	–	–	–	–	Gamma Thionin family (27)
serpL-1	~ 43,000	Yes	Secretory (Vac/Extracell.)	159, 179	230	103, 107, 160, 181, 204, 265	61, 211, 247, 327 (TK-phos at 109)	Serpin (359)
ptiIL-1	13,931	Yes	Secretory (Vac/Extracell.)	–	65	–	59, 68	Potato Inhibitor I (69)

[a] Molecular weight was determined using the web based ProtParam tool (http://ca.expasy.org/cgi-bin/protparam).
[b] Signal peptide prediction and cell targeting analysis were performed using the web-based PSORT program (http://psort.nibb.ac.jp).
[c] Amino acid residue number for predicted protein modification signals (*N*-glycosyl, Asparagine glycosylation; c Amp phos., cAMP/cGMP-dependent phosphorylation; PKC phos., protein kinase C phosphorylation; CK2, casein kinase II phosphorylation; TK phos., tyrosine kinase phosphorylation) determined using the web based PPSEARCH program provided by the EMBL European Bioinformatics Institute (http://www2.ebi.ac.uk/ppsearch).
[d] Protein Family Signatures were identified using the PPSEARCH program described in [3] and verified using the HMMPFAM program provided by the San Diego Supercomputer center Biology Workbench (http://workbench.sdsc.edu). Number in parenthesis is the amino acid residue number where the signature site begins.

clones encode a protein with a MW between 22 and 26 kDa containing a predicted N-terminal signal sequence for targeting to the secretory pathway. The predicted MW and secretory targeting are consistent with other plant miraculin-type *lkiL*-like proteins (Masuda et al., 1995; Brenner et al., 1998). The *gthL-1*, *serpL-1*, and *ptiIL-1* clones were also similar in size to their homologs from other organisms, and predicted to enter the secretory pathway. Only the cDNA clone of *serpL-1* appeared to contain an incomplete coding region: by comparison to full-length homologs (GenBank accessions: gi:15220298 and gi:9937311), it is missing at least two N-terminal amino acids including the start methionine. The *cystL-1* sequence putatively encodes a protein similar to other plant cystatins in that it was of the expected size and does not contain an N-terminal signal sequence, and thus is predicted to be a cytoplasmic protein (Table 3).

Of the proteins entering the secretory pathway, the *lkiL-1*, *-2*, *-3* and *serpL-1* have potential *N*-glycosylation sites (Table 3). Miraculin is a glycosylated protein (Takahashi et al., 1990); however, the only well-characterized plant serpins are apparently not glycosylated (Ostergaard et al., 2000). All groups of proteins contained potential phosphorylation sites;

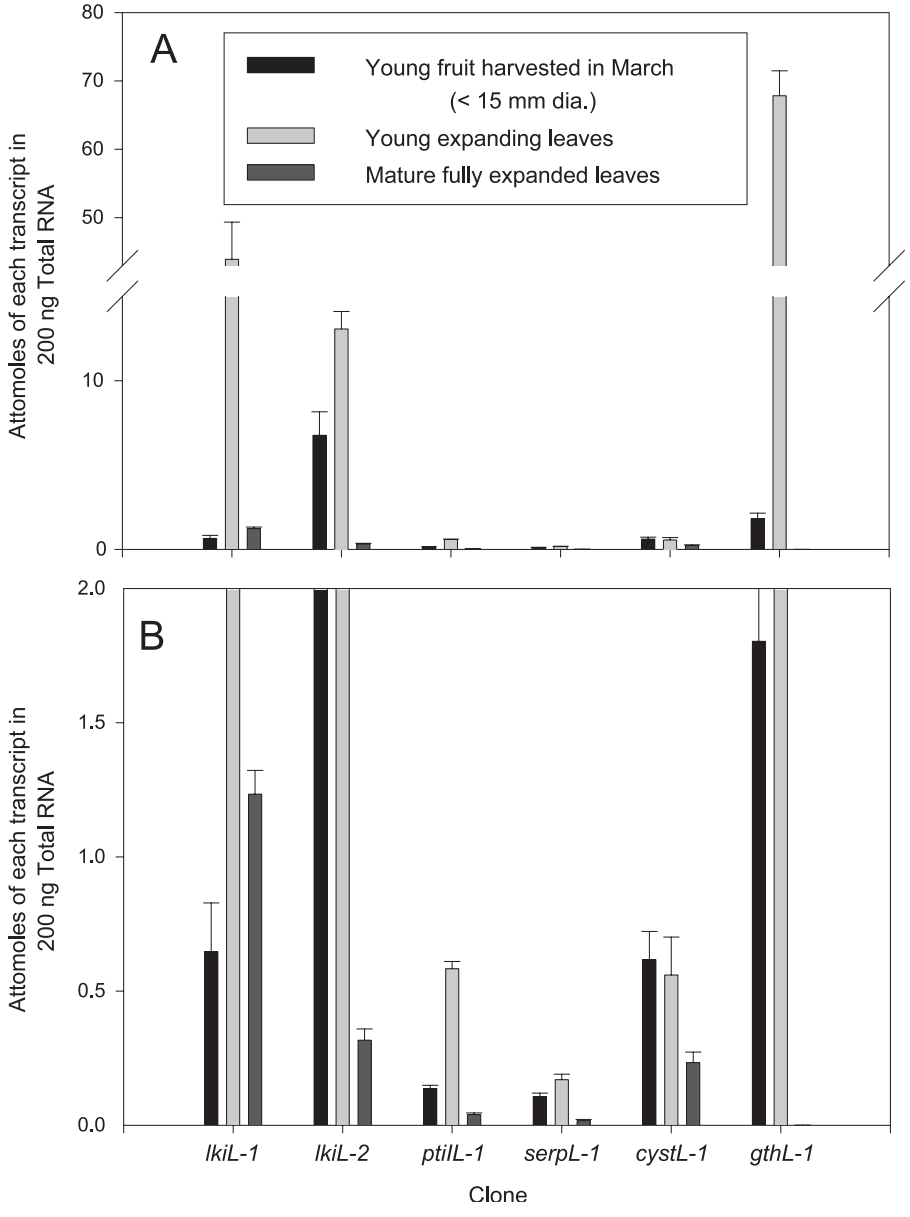

Fig. 1. Real time RT-PCR quantification of mRNA for each PPI clone in grapefruit (*C. paradisi* Macf.) tissues. Comparison is among flavedo from young fruit (< 15 mm diameter). Young immature leaves; and mature leaves (>6 months old). Transcripts were quantified by comparison to standard curves using linearized plasmids containing the indicated clone and the same primers used in the RT reaction. All values are normalized to 18S rRNA content as described in Materials and methods. (A and B) Represent the same data, however the *y*-axis scale is displayed on two different scales to allow visual observation of relative changes in transcript abundance. Error bars represent standard error of the average from triplicate real time RT-PCR analyses of each RNA sample and three replicate samples of plant tissue taken as described in Materials and methods.

however, none of their homologs were reported to be phosphorylated with the exception of mammalian (Isemura et al., 1991) and chicken cystatins (Engh et al., 1993).

These sequences were used to search a citrus EST database developed from numerous citrus species and tissues (unpublished data) to determine if the corresponding genes were expressed in other tissues besides the flavedo, Table 2. The *lkiL*-like clones were by far the most frequently sequenced of the randomly selected clones with a total of 140 separate clones representing three different *lkiL* species (20 from *lkiL-1*, 93 from *lkiL-2*, and 27 from *lkiL-3*) out of a total of 34,273 cDNA clones. The *lkiL-1* transcript was most abundant in the whole seedling library while the *lkiL-2* and *-3* were most abundant in libraries from the whole fruit. The Cystatin-like clone (*cystL-1*) was the next most frequently sequenced PPI clone with 15 independent clones, 9 of which came from whole seedlings and 5 from whole fruit. The *serpL-1* and *ptiIL-1* clones all came from either seedling or whole fruit libraries, with one *ptiIL-1* clone sequenced from a whole flower library. None of the PPIs were present in the 3815 leaf cDNA sequences and only the *ptiIL-1* sequence was observed in the 620 root clones. Direct comparisons of frequency of occurrence in the different EST libraries should be made cautiously since there was a large difference in the total number of clones sequenced among them; however, the most abundant *lkiL*'s should have been observed in the leaf and root libraries if they were as equally abundant between these and the seedling and reproductive tissues.

3.2. Developmental regulation of PPIs in fruit

Real-time RT-PCR was performed to quantify PPI transcript abundance in flavedo during fruit development. We were unsuccessful at finding a primer pair that would amplify the *lkiL-3* transcript specifically so this clone was not studied further. Initial quantification of transcripts in fruit developmental stages indicated that the youngest fruit harvested in March had the highest transcript level for all PPIs analyzed. Therefore, the actual amount of transcript is shown for the youngest fruit (Fig. 1) and then transcript levels in subsequent developmental stages are shown as the log of the fold change compared to the youngest (and smallest < 15 mm dia.) fruit (Fig. 2). The *lkiL-2* transcript was the most abundant in the < 15 mm dia. fruit harvested in March followed by *gthL-1*, *lkiL-1*, *cystL-1*, *ptiIL-1*, and *serpL-1*, respectively (Fig. 1B).

The abundance of *lkiL-1*, *-2* and *ptiIL-1* transcripts continued to decline as the fruit matured, albeit with dynamics unique to the transcript type, while the *cystL-1* transcript level remained constant from June to December fruit. The *serpL-1* transcript level declined after the first (March) stage through to the August samples; but, after that date, transcript levels rose again through the December sampling. Although the *gthL-1* transcript was among the most abundant in the youngest fruit, it was not detected in any flavedo samples taken from fruit harvested after the < 15-mm March harvest.

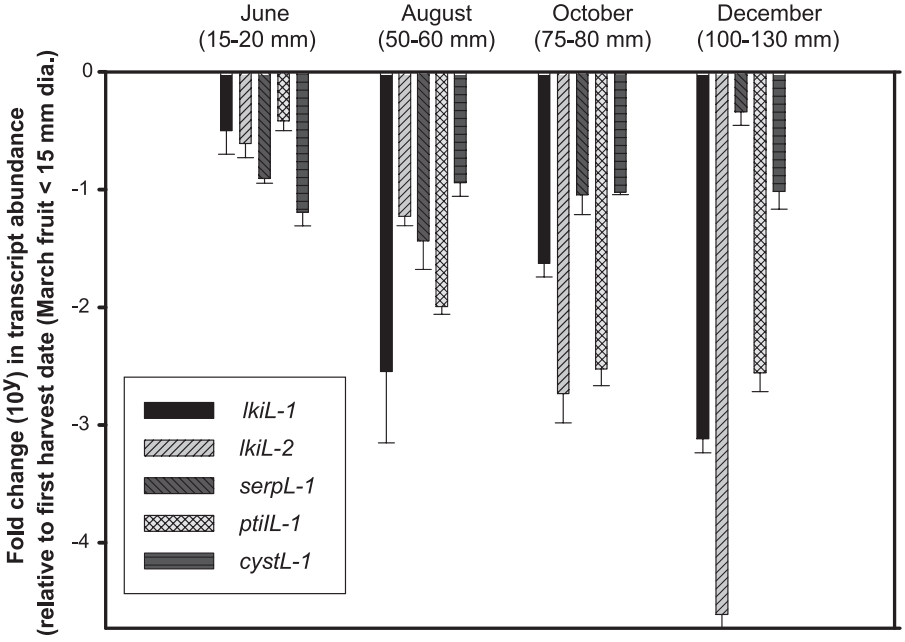

Fig. 2. Changes in grapefruit flavedo PPI mRNA abundance during fruit development. Total RNA isolated from flavedo was used in real-time RT-PCR to quantify PPI transcript levels. Since transcript level for all PPIs was highest in the smallest fruit measured (March harvested fruit smaller than 15 mm diameter, see Fig. 1), results are only shown for subsequent sampling times and presented as fold change in comparison to the smallest fruit. Negative values indicates that the specific RNA population declined relative to the smallest fruit and the fold change is plotted as the log of the actual fold change (i.e. 3 represents a 10^3 fold change in transcript abundance). Error bars represent standard error of the average from triplicate real time RT-PCR analyses of each RNA sample and three separate RNA samples prepared from replicates as described in Materials and methods.

3.3. Comparative regulation of PPIs in citrus leaf tissues

Expression of the PPIs in undamaged vegetative tissue was also examined by comparing Flame grapefruit immature leaves that were not yet fully expanded (sink leaves) to fully expanded mature leaves (source leaves). Transcripts for the seven PPIs were present in both leaf types with the exception of *gthL-1*, which was not detected in mature leaves. All PPI mRNAs were more abundant in the immature leaves (Fig. 1). The most abundant was the *gthL-1* transcript followed by *lkiL-1*, *lkiL-2*, *ptiIL-1*, *cystL-1* and *serpL-1*, respectively. Immature leaf PPI transcript levels were higher than those in the youngest fruit with the exception of the *cystL-1* clone, which was equally abundant in both. In comparisons of PPI transcripts across all fruit and leaf tissues, the *gthL-1* transcript in the immature leaf was the most abundant of all PPIs. Mature leaves had the lowest level of PPI transcripts with the exception of *lkiL-1*, which was more abundant in the mature leaves than in the youngest fruit.

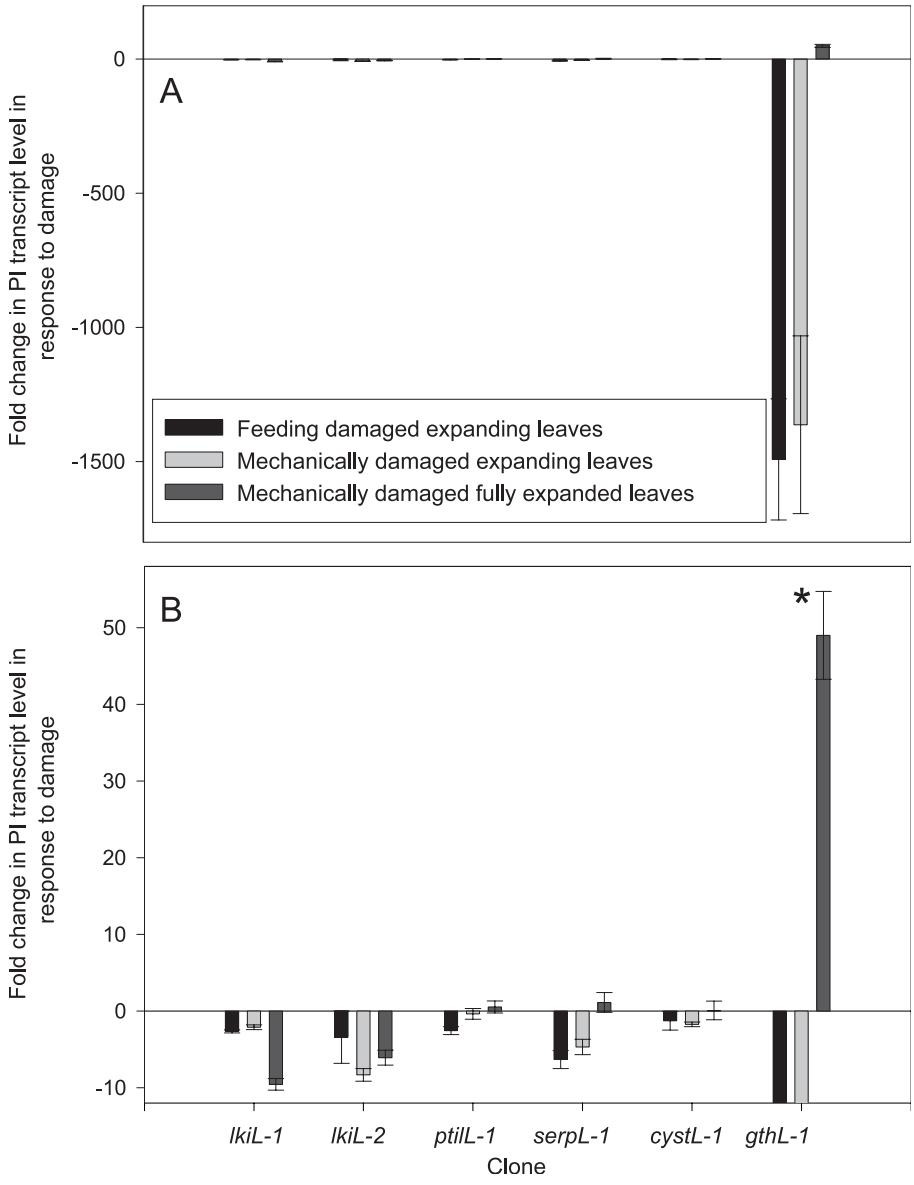

Fig. 3. Damage-induced changes in PPI mRNA abundance in mature and immature leaf tissues of grapefruit. Transcript abundance in total RNA isolated from damaged and undamaged leaves was measured using real-time RT-PCR and is reported relative to the level of each PPI in the undamaged tissue of the same type. Values are reported as a negative value indicates that the specific RNA population declined relative to the undamaged control leaves, and the fold change is plotted on two scales (A and B) to allow visualization of both the large changes associated with the *gthL-1* transcript and the smaller fold changes observed with the other transcripts. The * indicates that *gthL-1* transcript was undetectable in undamaged mature leaves. Error bars represent standard error of the average from triplicate real time RT-PCR analyses of each RNA sample and three separate RNA samples prepared from replicates as described in Materials and methods.

3.4. Wounding response of PPI transcripts

Wounding of immature leaves was performed either mechanically or by insect feeding using DRW adults. Many wounding responsive genes fall roughly into two categories (Ryan, 2000). First are those that accumulate rapidly beginning at 30 min and maximizing at 2–3 h but elevated expression still detectable at 10 h. These are signal pathway genes (i.e. prosystemin, lipoxygenase, allene oxide synthase, etc.). The second category includes the defense genes, the class in which the PIs reside, and are induced beginning at 2 h and maximizing at 10 h. We chose to look at 8 h after the end of the damage period since this time would allow identification of wound responsive genes in both of these classes. Wounding of mature leaves was performed only by mechanical damage, since the DRW would not feed on them. Surprisingly, in immature leaves, none of the PPI transcripts were wounding induced 8 h after either mechanical damage or insect feeding (Fig. 3). However, in mature leaves, mechanical damage stimulated at least a 50-fold increase in *gthL-1* PPI transcript abundance and a small (less than 2-fold) increase in *serpL-1*. The *gthL-1* increase was "at least" 50-fold because undamaged mature leaves did not have a detectable level of *gthL-1* transcript. If mature leaves contained *gthL-1* transcripts at a level slightly below the detection limit of our real-time RT-PCR method, the damage response would be a 50-fold increase. This result was especially interesting since this transcript showed the greatest reduction in abundance (>1400-fold) when immature leaves were damaged, either mechanically or by insect feeding (Fig. 3). Actually, the transcript was undetectable in damaged immature leaves, and the >1400-fold value represent the minimum detectable reduction based on assay detection limits. Other PPI transcript reductions were limited to less than 5-fold.

4. Discussion

The purpose of this work was to use sequence similarity screening and transcript profiling to identify cDNA clones encoding putative proteinase inhibitors that may play a role in plant defense. In other plant systems, transcripts for defensive PIs can respond to either fruit/storage organ development or feeding damage. We presented the cloning and regulation of transcript abundance for seven cDNA clones representing putative grapefruit protease inhibitors belonging to four major protease inhibitor classes within the serine (Legume Kunitz inhibitors, *lkiL-1*, *-2*, *-3*; potato inhibitor I family, *ptiIL-1*; Serpins, *serpL-1*; and gamma thionins, *gthL-1*) and cysteine (cystatins, *cystL-1*) protease inhibitor groups. Since classification of the cDNA clones is based solely on amino acid sequence similarity and functional domain identification, they remain as putative assignments.

Relative transcript abundance (relative to 18S rRNA) analysis using real-time RT-PCR indicated that as a group, the transcript level of the citrus PPIs in leaves was more influenced by developmental stage of the tissue than by environmental stimuli with the exception of the *gthL-1* (encoding a putative gamma thionin with strongest similarity (E score of $2e-23$) to a soybean putative proteinase inhibitor). Interestingly, the steady state level of this transcript in leaves was greatly influenced by wounding, and the response was modulated by leaf developmental stage. Wounding of young leaves caused a drastic reduction in *gthL-1* mRNA, while the same wounding of mature source leaves caused an increase. Previous reports of the influence of leaf age on PI transcripts showed that younger sink leaves induced higher PI transcript levels than older source leaves in response to wounding (Wolfson and Murdock, 1990; Van Dam et al., 2001). Our results with *gthL-1* show an opposite age related response that to our knowledge has not previously been observed in plants.

Although the significance of plant gamma thionin's proteinase inhibitor activity and their biological function is not yet clear, they do appear to play a defensive role against bacterial (Zhang and Lewis, 1997) and fungal pathogens (Bohlmann et al., 1998), and are regulated transcriptionally by the action of the jasmonate (JA) defense response pathway. If the citrus *gthL-1* protein product functions in defense, the wounding induced decline in immature leaf transcript level is not easily explained, unless as a perennial tree, citrus has different strategies for dealing with damage to mature versus immature leaves than classically observed in model plants (potato, tomato, tobacco, and *Arabidopsis*). It is not difficult to speculate that a woody perennial could have different defense strategies than a herbaceous plant that can function as an annual.

There were similarities to the regulation of the citrus PPI mRNAs observed in the developing grapefruit and previously published PI transcript regulation studies. First, a number of plant defensive genes are expressed at high levels in the flower pistil and decline as the flower ages (Van Dam et al., 2001; Gu et al., 1992; Pearce et al., 1993; Karunanandaa et al., 1994; Ausloos et al., 1995; Van Eldik et al., 1996). This is presumed to be a defensive mechanism designed to protect the young reproductive tissues. In developing citrus fruit, the flavedo is derived from the pistil epidermal tissue and all the PPI mRNAs are most abundant in the earliest flavedo tissue taken. Second, the EST sequencing results from different libraries (Table 2) suggested that the PPIs were less abundant in roots, mature source leaves, and phloem than they were in the seedlings, very small immature fruit, and flowers. These results indicate that the highest transcript accumulation occurred in rapidly dividing tissues: a common observation with plant PIs (Van Dam et al., 2001). However, in non-citrus herbaceous plants, specific PI transcript levels that were typically more abundant in the young tissues, also increased in response to damage; whereas, the citrus PPIs abundant in young leaves were not damage-regulated in this fashion.

In light of the large volume of literature presenting evidence for wound-induced elevation of PI mRNA levels in plant tissues, and that many PIs present in young fruit were also wound induced (Van Dam et al., 2001), it was surprising to find that only one out of the six measured PPI clones showed a wound-stimulated increase in mRNA abundance. Similar EST projects using damaged tissues should allow the identification of wound inducible PIs. The unusual regulation of these citrus PPI transcript levels indicates that they responded either to different signals than those reported in other plants or that the same signals (for example, jasmonate level) stimulated different responses in citrus.

This work was initiated to identify PIs that function in defense against insect feeding by comparing regulation of transcript levels to those of well-characterized plant PIs. Only one transcript, *gthL-1*, was wound responsive; however, wound induction was modulated by leaf developmental stage in a manner directly opposite that observed in other plants. Perhaps this difference represents different survival strategies for long-lived woody perennial versus short-lived plants (i.e. *Arabidopsis*, tomato and potato). Because of this unusual regulation pattern, further study of the function of the citrus PPI protein products is warranted and should provide insight into new strategies plants use to support plant growth, development and defense.

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