Genetics and Molecular Biology, 30, 3 (suppl), 917-930 (2007)

www.sbg.org.br

Research Article

PR gene families of citrus: Their organ specific-biotic and abiotic inducible expression profiles based on ESTs approach

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Abstract

In silico expression profiles, of the discovered 3,103 citrus ESTs putatively encoding for PR protein families (PR-1 to PR-17), were evaluated using the Brazil citrus genome EST CitEST/database. Hierarchical clustering was displayed to identify similarities in expression patterns among citrus *PR*-like gene families (*PR*lgf) in 33 selected cDNA libraries. In this way, *PR*lgf preferentially expressed by organ and citrus species, and library conditions were highlighted. Changes in expression profiles of clusters for each of the 17 *PR*lgf expressed in organs infected by pathogens or drought-stressed citrus species were displayed for relative suppression or induction gene expression in relation to the counterpart control. Overall, few *PR*lgf showed expression 2-fold higher in pathogen-infected than in uninfected organs, even though the differential expression profiles displayed have been quite diverse among studied species and organs. Furthermore, an insight into some contigs from four *PR*lgf pointed out putative members of multigene families. They appear to be evolutionarily conserved within citrus species and/or organ- or stress-specifically expressed. Our results represent a starting point regarding the extent of expression pattern differences underlying *PR*lgf expression and reveal genes that may prove to be useful in studies regarding biotechnological approaches or citrus resistance markers.

Key words: citrus, functional genome, ests, gene expression profiles, pathogenesis-related proteins, defense genes.

Received: July 21, 2006; Accepted: June 22, 2007.

Introduction

Plants evolved mechanisms that enable them not only to resist drought and wounding but also to oppose attacks by pathogenic microorganisms. One of the ways that plants respond to biotic and/or abiotic stress factors is in the accumulation of pathogenesis-related proteins (PR proteins) (van Loon and van Strien, 1999). Plant PR proteins are defined as proteins encoded by host plants that are induced in pathological or related situations, and represent major quantitative changes in soluble protein during the defense response (van Loon *et al.*, 1994; Stintzi *et al.*, 1993). Originally described in tobacco leaves upon virus infection, PR proteins were first classified into PR-1 to PR-5 families, based on serological properties and later on sequence data. They generally have two subclasses: an acidic subclass, secreted to cellular space, and a vacuolar basic subclass (van Loon and van Kammen, 1970; Stintzi *et al.*, 1993; Kitajima and Sato, 1999). Subsequently, several other protein groups were recommended for inclusion into this class. Now plant PR proteins comprise a large group of 17 protein families, even though PR-15 to PR-17 families have been recognized only recently (van Loon *et al.*, 1994; van Loon and van Strien, 1999; van Loon *et al.*, 2006).

Direct antimicrobial activities for members of PR protein families have been demonstrated *in vitro* through hydrolytic activities on cell walls and contact toxicity; whereas indirect activities perhaps bypass an involvement in defense signaling (van Loon *et al.*, 2006). There are at least ten PR families whose members have direct activities against fungi pathogens (PR-1, PR-2, PR-3, PR-4, PR-5, PR-8, PR-11, PR-12, PR-13, PR-14 families). However,

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PR-1 and PR-5 proteins also show activity directed specifically against oomycetes (van Loon *et al.*, 2006). While for members of PR-1 family the molecular mechanisms remain unclear, several mechanisms have already been ascribed to members of PR-5 protein family, such as membrane permeabilizers, glucan binding and hydrolysis and apoptosis (Melchers *et al.*, 1994; Abad *et al.*, 1996; Narasimhan *et al.*, 2001; Osmond *et al.*, 2001; van Loon *et al.*, 2006). Hydrolytic activities were demonstrated in members of the PR-2 protein family; β-1,3-glucanases that can hydrolyze glucan present in fungi and oomycetes cell walls. Members of the PR-3, PR-4, PR-8 and PR-11 protein families are also endochitinases which can hydrolyze chitin from fungal cell walls, but members of PR-8 family also exhibit lysozyme activity with antibacterial activity (Métraux *et al.*, 1989; Melchers *et al.*, 1994; Abad *et al.*, 1996; García-Olmedo *et al.*, 1998). PR-12, PR-13 and PR-14 protein families are defensins, thionins and lipid-transfer proteins, respectively, putative membrane permeabilizers with antifungal and antibacterial activities (Terras *et al.*; 1992; Molina *et al.*, 1993; Epple *et al.*, 1995; García-Olmedo *et al.*, 1995).

Plant protection against nematode and herbivorous insect attacks has been associated with members of the PR-6 protein family, which are proteinase inhibitors (Ryan, 1990). The PR-7 proteins are endoproteinases which act as an accessory to antifungal activity in cell wall dissolution (van Loon and van Strien, 1999). A very interesting role has been ascribed to members of the ribonuclease-like PR-10 protein family, apparently the only PR protein family possessing antiviral activity (Somssich *et al.*, 1986; Zhou *et al.*, 2002). The PR-9 proteins are lignin-forming peroxidase with peroxidase activity, implicated in the oxidative cross-linking of plant cell wall components in order to prevent pathogen penetration (Lagrimini *et al.*, 1987, 1997). The remaining PR-like proteins, classified as PR-15 and PR-16 protein families, are pathogen-induced germin-like oxalate oxidases and germin-like oxalate oxidase-like with superoxide dismutase activity. These are thought to be involved in signal transduction pathway for the regulation of HR (Hypersensitive Response) as the members of PR-17 protein family that are possible proteolytic enzymes presenting sequences for a putative active site of zinc-metalloproteinases, pathogen-induced transcript and protein accumulation patterns (Zhang *et al.*, 1995; Wei *et al.*, 1998; Okushima *et al.*, 2000; Zhou *et al.*, 1998; Christensen *et al.*, 2002; Park *et al.*, 2004; van Loon *et al.*, 2006).

High levels of *PR* gene expression during local HR and systemic plant defense (Systemic acquired resistance, SAR) have been suggested as markers for both defense responses (Ahl Goy *et al.*, 1982; Lawrence *et al.*, 1996; Tornero *et al.*, 1997). Signaling molecules mediate induction of PR proteins in plants during pathogen infection including SA (salicylic acid) for acidic *PR* genes as well as ethylene and methyl jasmonate for basic *PR* genes (Kitajima and Sato, 1999). In addition, *PR* genes (basic in general) also are present constitutively in some plant organs or tissues, including roots, leaves and floral tissues. Citrus *PR* gene families have been poorly reported so far. Citrus chitinase and glucanase proteins were associated with fruit development (McCollum *et al.*, 1997) and pathogen response (Porat *et al.*, 2001; Porat *et al.*, 2002; Fanta *et al.*, 2003; Porat *et al.*, 2003) as well as with constitutive expression (Recupero *et al.*, 1997). *PR* gene expression in citrus was shown to be promoted by hot water (Pavoncello *et al.*, 2001), UV irradiation and wounding (Porat *et al.*, 1999) and ß-aminobutyric acid (Porat *et al.*, 2003). Moreover, Fagoaga *et al.* (2001) has reported that a tobacco PR-5 protein, constitutively overexpressed within *Citrus sinensis* transgenic plants, confers enhanced resistance towards the *Phytophthora citrophthora* pathogen. This suggests that PR proteins can also be used successfully in citrus genetic engineering approaches.

Citrus is the main fruit crop in the world and, as such, an important commodity. For this reason, the sequencing in large scale of expressed sequence tags (ESTs) from citrus organs was performed as an approach to fill some of the gaps in knowledge concerning the genetic and molecular factors involved in several citrus diseases and fruit development. cDNA libraries were constructed using tissues from different plant organs, such as leaf, flower, fruit, bark, seed and root, from twelve citrus species (*Citrus sinensis*, *C. limonia*, *C. reticulata*, *C. aurantium, C. limettiodes, C. aurantifolia, C. sinensis x C. reticulata, C. sunki, C. latifolia, C. reshni, Citrumelo swingle, Fortunella margarita, Poncirus trifoliata*). These plants were submitted to diverse situations of biotic stresses caused either by bacteria (*Xylella fastidiosa*), viruses (*Citrus leprosis virus*, *Citrus tristeza virus*) or *Phytophthora parasitica,* or abiotic stress caused by environmental factors, such as drought. Therefore, Brazilian citrus genome EST database (CitEST) covers a wide diversity of gene sequence information for the study of components of citrus defense response pathways to pathogen, wounding and other abiotic stresses, which have often been used as marker resistance onset. In addition to providing an efficient method for gene discovery, the ESTs data set can also provide information about gene expression. However, the challenge is to extract biological knowledge from large amounts of gene expression data deposited in databases.

Tools for *in silico* analysis of the gene expression allow comparison of expression profiles of specific genes in plant tissues, based on the frequency of sequence tags in cDNA libraries. According to Ohlrogge and Benning (2000), gene expression analysis is based on the rationale that an abundance of mRNAs synthesized from a particular gene highly expressed in a given tissue can be estimated by the counting of the number of ESTs corresponding to the cDNA of this gene, which is present in cDNA library constructed from the tissue. A natural basis for organizing gene expression data is to group together genes with similar pat-

terns of expression (Eisen *et al.*, 1998). In plants, hierarchical clustering has been used routinely to identify genes highly expressed from rice (Ewing *et al.*, 1999), sugarcane (Lambais, 2001), soybean (Shoemaker *et al.*, 2002), barley (Zhang *et al.*, 2002), wheat (Ogihara *et al.*, 2003) and eucalypt (Domingues *et al.*, 2005) transcriptome; and it is expected that this number will continue to increase. A combination of clustering methods with a graphical representation of the primary data, by representing each data point with a color, may quantitatively and qualitatively reflect the original experimental observations and allow an understanding of the data in a naturally intuitive manner (Eisen *et al.*, 1998).

By using this strategy, we explored the CitEST database to analyze the expression of the putative *PR* gene families in citrus plants, since the proteins encoded by them have been described as associated with all of the different conditions cited for citrus library construction. Albeit *in silico,* the results here presented may provide insights about the expression differences underlying *PR* gene family expression patterns and reveal genes that may prove to be useful in studies regarding biotechnological approaches or citrus resistance markers. Furthermore, a similarly compiled study concerning expression profiles from all of the recognized 17 *PR*-like gene families has never been reported for citrus before.

Material and Methods

Identification and *in silico* expression analysis of citrus *PR*-like homologous ESTs

Search analyses by comparison were performed within the CitEST database (http://citest.centrodecitricultura.br) against amino acid sequences of members (preferentially '*type member*') of each pathogenesis-related (PR) protein family (http://www.bio.uu.nl/~fytopath/PRfamilies.htm), which were found in public databases, in attempts to identify homologous sequences in citrus. Additionally, searches using keywords and more than one amino acid sequence (from different PR isoforms or classes) in BLAST were also used for each PR family. Afterwards, by using the Basic Local Alignment Tool (blastx) program (Altschul *et al.*, 1997) with the cut-off value of e-10^{-05} and BLOSUM62 matrix criteria, a total of 3,103 ESTs-reads were selected from CitEST in order to analyze the *PR*-like gene expression profiles in citrus cDNA libraries, which cover 173,967 useful reads (Table 1).

As an initial consideration in constructing expression profiles, the frequency of reads in the selected libraries was computed and normalized by the whole number of useful reads from each library, corrected to 1,000 ESTs. Hierarchical clustering was used to group EST-contigs and librar-

Table 1 - *PR* gene sequences used as query for searching the homologous *PR* genes within the citrus genome EST data bank - CitEST and distribution of the number of citrus *PR*-like ESTs within the 17 *PR* gene families.

PR family	Query sequences	Accession number	Organism	Number of ESTs	Clusters		Ref.
					Contigs	Singlets	
PR-1	PR-1a	gi|722274	*Brassica napus*	5	2	1	1
PR-2	B-1,3-glucanase	gi|8980815	*Castanea sativa*	264	33	38	2
PR-3	Chitinase class I, II, IV, V, VI, VII and endochitinase	gi|23496447	*Citrus jambhiri*	444	24	13	3
PR-4	Chitinase Hevein-like	gi|19962	*Nicotiana tabacum*	5	2	1	4
PR-5	Thaumatin-like	gi|4586372	*Nicotiana tabacum*	40	6	6	5
PR-6	Proteinase inhibitor	gi|170484	*Lycopersicon esculentum*	107	18	6	6
PR-7	Aspartic proteinase	emb|CAC86003.1	*Theobroma cacao*	178	15	5	7
PR-8	Chitinase class III	gi|167515	*Cucumis sativus*	12	1	3	8
PR-9	Lignin-forming peroxidase	gi|170316	*Nicotiana tabacum*	562	22	16	9
PR-10	Ribonuclease-like	gi|15811629	*Gossypium arboreum*	63	4	4	10
PR-11	Chitinase, class V	gi|899342	*Nicotiana tabacum*	20	4	7	11
PR-12	Defensin	gi|11386628	*Raphanus sativus*	11	1	2	12
PR-13	Thionin	gi|1181531	*Arabidopsis thaliana*	2	1	0	13
PR-14	Lipid transfer protein	gi|1045201	*Hordeum vulgare*	347	12	5	14
PR-15	Oxalate oxidase	gi|2266668	*Hordeum vulgare*	503	14	5	15
PR-16	Oxalate oxidase-like or germin-like	gi|1070358	*Hordeum vulgare*	539	12	6	16
PR-17	NtPRp27	gi|5360263	*Nicotiana tabacum*	1	-	1	17

[1]Hanfrey *et al.*, 1996, Antoniw *et al.*, 1980; [2]Antoniw *et al.* 1980; [3]Gomi *et al.*, 2002, van Loon, 1982; [4]Linthorst *et al.*, 1991, van Loon, 1982; [5]Kuboyama *et al.*, 1998, van Loon, 1982; [6]Lincoln *et al.*, 1987, Green and Ryan, 1972; [7]Vera and Conejero, 1988; [8]Métraux *et al.*, 1989, Métraux *et al.*, 1988; [9]Lagrimini *et al.*, 1987; [10]Zhou *et al.*, 2002, Somssich *et al.*, 1986; [11]Melchers *et al.*, 1994; [12]Terras *et al.*, 1992; [13]Epple *et al.*, 1995; [14]García-Olmedo *et al.*, 1995; [15]Zhou *et al.*, 1998; [16]Wei *et al.*, 1998; [17]Okushima *et al.*, 2000.

ies by similarities, displayed in a Cluster program and Tree View software (http://rana.lbl.gov/EisenSoftware.htm). To obtain the hierarchical clustering, the calculation of the distance between all pairs of objects was performed using an un-centered correlation matrix and the pairwise average-linkage method (Eisen *et al.*, 1998). The reordering of data matrix was performed according to similarities in the pattern of gene expression and graphically displayed as color arrays of EST-contigs, using a color scale representing the number of reads from a particular library in each EST-contig.

Computer subtraction analyses were also performed by subtracting the stress libraries from non-stress libraries, thus getting the positive, negative and co-regulation of each *PR* gene family in both abiotic and biotic stress situations. EST-contigs, expressed in non-stressed organs compared to the stressed organs, were calculated as described in Lambais (2001). Graphical representation of each data was highlighted with a color, using a color scale representing the range between suppression and induction gene expression, in relation to the counterpart control library.

Phylogenetic analysis

EST clusters were built only for each *PR-2*, *PR-3*, *PR-5* and *PR-7* gene families separately (Table S1), by alignment using the Contig Assembly program (CAP3) (Huang and Madan, 1999). A consensus sequence from each cluster used in phylogenetic analysis was compared with the amino acid sequences from PR-3 protein homologous sequences deposited in the public GenBank database, using the TBlastN and BlastX algorithms (Altschul *et al.*, 1997). Final alignment was obtained with Clustal X 1.83 (Thompson *et al.*, 1997) and afterwards used for phylogenetic analysis. Phylogenetic analysis of amino acid sequences was performed with the Neighbor joining method and Dayhoff Matrix Model with the substitution method, with 1,000 bootstrap replicates, using the MEGA 3 program for construction and visualization of trees (Kumar *et al.*, 2004).

Description and identification of CitEST cDNA libraries

All citrus sequences used in this work correspond to sequenced EST-reads. Cluster consensi were obtained from the Genetic Breeding, Functional Genome and Comparative of Citrus project (http://citest.centrodecitricultura.br) and derived from cDNA libraries specific for different citrus species, organs or growth and stress conditions, as described in Table 2 and by Targon *et al.* (in this issue).

Results and Discussion

Selection of CitEST *PR*-like gene homologous ESTs and expression profiles in citrus

The use of *in silico* methods to search homologous sequences of known genes is an important approach for the discovery of new genes. Comparison searches using the blast program (Altschul *et al.*, 1997) and preferentially '*type member*' amino acid sequences of each PR protein family as query led to identification of a total of 3,538 citrus *PR*-like EST-reads with a cut-off value of e^{-05} in CitEST database (Table 1). This number represents around 2% of the whole CitEST database, which contains about 173,967 ESTs obtained from all citrus plant libraries.

In this study, we reported, for the first time, the presence of homologous ESTs encoding for all of the recognized 17 *PR* gene families in citrus plants (van Loon and van Strien, 1999; van Loon *et al.*, 2006). A graphical representation of expression profiles of the 17 gene families encoding PR proteins was generated by using 3,103 *PR*-like EST-reads (Figure 1). The cDNA library construction conditions in which the *PR*-like EST-reads were isolated are described in Table 2. In a general view, transcripts coding for members of eight PR protein families (PR-2, PR-3, PR-6, PR-7, PR-9, PR-14, PR-15 and PR-16) were found to be highly expressed within 24 to 30 of the 33 citrus libraries studied, whereas members of the PR-13 and PR-17 families were present in only one library each (Figure 1). Even though *PR-15* and *PR-16* gene families display similar nomenclature and activities, the expression profiles were notably distinct. These *in silico* expression profiles that were preferentially induced under the different situations indicate conserved functions in citrus species.

Within the libraries, the majority of putative PR protein families encoded by highly expressed citrus transcripts were found in those constructed from healthy organs during normal plant growth, such as in non-drought-stressed roots and flowers, and fruit peel. Transcripts coding for members of eleven of the 17 PR-like protein families (PR-1, PR-2, PR-3, PR-4, PR-6, PR-7, PR-8, PR-9, PR-10, PR-15, and PR-16) seem to be highly expressed within the CL06-C4-500 library, which was constructed from roots of *Citrus limonia* 'Cravo' non-drought-stressed. Likewise, transcripts coding for members of twelve PR-like protein families (the highly expressed PR-2, PR-3, PR-5, PR-6, PR-7, PR-9, PR-14, PR-15, PR-16 and the moderately expressed PR-1, PR-8, PR-12 families) were found in the healthy flowers of the *Citrus sinensis* 'Pêra IAC' (CS00-C5-003) library. PR proteins present in apparently healthy organs during normal plant growth are thought to play additional unsuspected roles in morphogenesis or in symbiosis (Datta and Muthukrishnan, 1999). The reason why transcripts encoding members of PR-11, PR-13 and PR-17 protein families were not expressed in these libraries needs to be investigated.

Only members for the putative *PR-3*, *PR-10* and *PR-14* gene families were highly expressed in stem bark tissues of *Citrus sunki* BAG cv. 200 RG 23 (TS27-C2-300). Similarly, only *PR-3*, *PR-9* and *PR-14* gene families were strongly expressed in leaves of *Citrus sinensis* cv. Pêra IAC induced by the *Citrus leprosis virus* (CiLV) (CS00-C1-

Table 2 - EST libraries used in this study.

Citrus library	N of ESTs	Plant material	Organ	Condition
LT33-C1-003	5484	*C. latifolia* BAG cv 304 source/779	Leaf	Healthy from greenhouse
CG32-C1-003	6621	*C. aurantifolia* BAG cv 224 RG 23	Leaf	Healthy from greenhouse
CA26-C1-002	5950	*C. aurantium* BAG cv 299 source/2380	Leaf	Healthy from field
CM30-C1-401	1679	*C. limettiodes* BAG cv 300 source/5555	Leaf	Infected by CiLV
CS00-C1-401	945	*C. sinensis* cv 'Pêra IAC'	Leaf	Infected by CiLV
CR05-C1-100	7490	*C. reticulata* cv 'Ponkan'	Leaf	Non-infected *X. fastidiosa* mock
CR05-C1-102	6891	*C. reticulata* cv 'Ponkan'	Leaf	Infected by *Xylella fastidiosa,* 30 d.a.i.
CR05-C1-103	5853	*C. reticulata* cv 'Ponkan'	Leaf	Infected by *X. fastidiosa,* 60 d.a.i.
CS00-C1-100	7185	*C. sinensis* cv 'Pêra IAC'	Leaf	Non-infected *X. fastidiosa* mock
CS00-C1-101	5899	*C. sinensis* cv 'Pêra IAC'	Leaf	Infected by *X. fastidiosa* 270 d.a.i
CS00-C1-102	7231	*C. sinensis* cv 'Pêra IAC'	Leaf	Infected by *X. fastidiosa,* 30 d.a.i
CS00-C1-650	2865	*C. sinensis* cv 'Pêra IAC'	Leaf	Young plant
CS12-C1-001	3465	*C. sinensis* cv 'Pêra Olimpia' STG plants	Leaf	Infected by CTV (from field)
CS13-C1-001	2217	*C. sinensis* cv 'Pêra IAC' STG plants	Leaf	Infected by CTV (from field)
PT11-C1-900	6917	*Poncirus trifoliata* var *Rubidoux* BAG 835 CN RG 035	Leaf	Non-infected CTV mock
PT11-C1-901	6968	*P. trifoliata* var *Rubidoux* BAG 835 CN RG 035	Leaf	Infected by CTV
PT11-C2-300	4204	*P. trifoliata* var *Rubidoux* BAG 835 CN RG 035	Bark	Non-infected *Phytophthora parasitica* mock
PT11-C2-301	2955	*P. trifoliata* var *Rubidoux* BAG 835 CN RG 035	Bark	Infected by *P. parasitica***
TS27-C2-300	1421	*C. sunki* BAG cv 200 RG 23	Bark	Non-infected *P. parasitica* mock
CS00-C2-003	5451	*C. sinensis* cv 'Pêra IAC'	Bark	Healthy from greenhouse
CR05-C3-700	5398	*C. reticulata* cv 'Ponkan'	Fruit*	1 cm in diameter*
CR05-C3-701	6793	*C. reticulata* cv 'Ponkan'	Fruit	2.5 cm in diameter
CR05-C3-702	7056	*C. reticulata* cv 'Ponkan'	Fruit	5 cm in diameter
CS00-C3-700	8454	*C. sinensis* cv 'Pêra IAC'	Fruit	1 cm in diameter
CS00-C3-701	7052	*C. sinensis* cv 'Pêra IAC'	Fruit	2,5 cm in diameter
CS00-C3-702	7909	*C. sinensis* cv 'Pêra IAC'	Fruit	5 cm in diameter
CS00-C3-703	6387	*C. sinensis* cv 'Pêra IAC'	Fruit	7 cm in diameter
CS00-C3-704	6242	*C. sinensis* cv 'Pêra IAC'	Fruit	8 cm in diameter
CS00-C3-705	6712	*C. sinensis* cv 'Pêra IAC'	Fruit	9 cm in diameter
CL06-C4-500	2075	*C. limonia* 'Cravo'	Root	Non-drought stressed mock
CL06-C4-501	2110	*C. limonia* 'Cravo'	Root	Drought stressed by PEG
CS00-C5-003	4330	*C. sinensis* cv 'Pêra IAC'	Flower	Healthy from greenhouse
PT11-C9-005	3368	*P. trifoliata* var *Rubidoux* BAG 835 CN RG 035	Seed	Pool of developmental stages

*Pericarp from fruits with different size diameters (cm). ***Phytophthora parasitica* (= *Phytophthora nicotiana* Breda de Haan var. *parasitica* (Dast.) Waterh.) IAC 0195 isolate. CiLV, *Citrus leprosis virus*; CTV, *Citrus tristeza virus*; D.A.I., days after inoculation; STG plants, Shoot-tip grafted virus free plants.

401). In the case of the former, despite expression of members for only few (*i.e.*, three) *PR* gene families, this expression pattern may be associated with wounding, given that wounding was mimicked here as a mock control library for the *P. parasitica* infection library. Interestingly, a very similar experiment carried out with *Poncirus trifoliata* plants, using the same organs and conditions (PT11-C2-300 library), was able to induce not only transcripts coding for members of these same three PR-families (PR-3, PR-10 and PR-14) but also for members of seven PR protein families extra within *Citrus sunki*. It is noteworthy that in addition to differences of environmental stimuli, differences between *P. trifoliata* and *C. sunki* could also be noted in the constitutive expression pattern of *PR* genes. This is an indication of an effective defense response to stresses, which appears to be more elaborated in *P. trifoliata* than in *C. sunki* organs. Since *P. trifoliata* is a citrus rootstock plant known to be resistant to several citrus diseases, including *P. parasitica* and CTV (*Citrus tristeza virus*) among others (Yang et *al.*, 2003; Siviero et *al.*, 2006), in contrast to *C. sunki* which is known to be susceptible, these results contribute to an understanding of the defense responses to stresses involved in both plant species. Using real time-PCR, it has already been reported by our group that high quantitative levels of constitutive and timing induced expression of *PR* genes in *P. trifoliata* leaves and stem bark tissues, respectively, were associated with resistance to *P. parasitica* (Teixeira *et al.*, 2005).

In the latter case, the number of members of the three *PR* gene families expressed in *C. sinensis* CiLV-induced

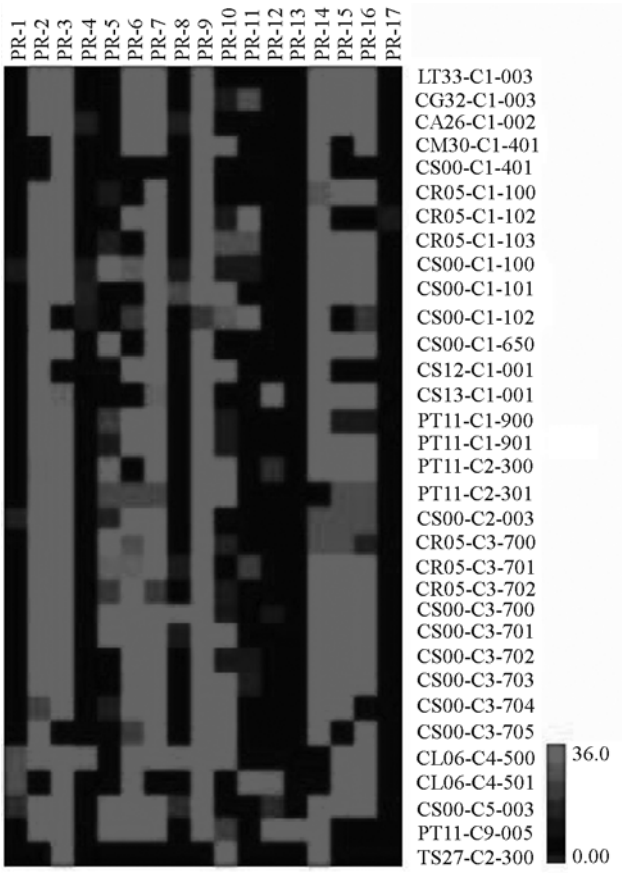

Figure 1 - Expression patterns of 3,103 citrus ESTs encoding 17 putative PR protein families (PR-1 to PR-17) in 33 selected cDNA libraries from the CitEST database. Each library is represented by a single row of colored boxes, whereas each *PR*-like gene family is represented by a single column. Data represent the relative number of *PR* reads from a specific library in each EST-contig per 1,000 reads.

leaves contrasts with the amount of members for unexpected fifteen *PR* gene families found within the counterpart uninfected control library (CS00-C1-100). One explanation for this fact could be that *C. sinensis* plants used to construct these libraries were pre-immunized with a non-virulent CTV strain, about 48 h before CiLV infection, as an attempt to mimic *in vivo* conditions occurring in the field groves, since that is a standard procedure carried out in our nurseries. Because of this, a constitutive expression profile in leaves could not be inferred for this CiLV susceptible citrus species. It was apparent, however, that *C. sinensis* susceptible plants have genetic potential for resistance, but the success of the defense may also depend on the pathogen's ability to overcome it. It is possible that pre-immunized leaves either provided high *PR* gene expression profiles, which were suppressed by presence of CiLV, or that a large number of *PR* gene family transcripts could not be detected within *C. sinensis* leaves at 48 h.a.i. (hours after inoculation) with CiLV. Further analysis could confirm these hypotheses, using time points larger than 48 h.a.i., and this is the subject of our current research projects.

Interestingly, within *P. trifoliata* leaves infected with a virulent CTV strain (PT11-C1-901) or uninfected control leaves (PT11-C1-900), members of the same groups of ten *PR* gene families were apparently expressed in both libraries (Figure 1), even though changes in the relative amount of gene expression can be noted. In this case, a high level of *PR* transcripts constitutively expressed in the uninfected leaves apparently suggests a possible role preformed barrier. High levels of constitutive expression of *PR* transcripts have been associated with high levels of natural non-specific quantitative resistance to pathogens (Ahl Goy *et al.*, 1992; Vleeshouwers *et al.*, 2000). Likewise, within *C. reticulata* uninfected leaves (CR05-C1-100), high levels of gene expression for members of *PR-2*, *PR-3*, *PR-7*, *PR-9* and *PR-14* gene families could also represent a putative constitutive expression pattern; however, members of these same five *PR* gene families were also highly expressed in *C. reticulata* leaves at 30 and 60 days after infection with *X. fastidiosa.* Detailed *in silico* cluster analysis (as discussed from Table S1) accomplished by quantitative experimental methods could provide concrete insights to elucidate if the same or different members, *i.e.*, PR protein isoforms belonging to each of the five *PR* gene families, could be playing a role in both situations. These analyses could contribute to understanding unclear molecular mechanisms of the resistance of *P. trifoliata* and *C. reticulata* to CTV and *X. fastidiosa* pathogens, respectively.

Differential expression profiles of *PR*-like gene families within citrus organs upon pathogen infection and drought stresses

The recruitment of different *PR* genes for conserved functions in response to pathogen and drought stresses in citrus was analyzed based on expression profiles of all of *PR*-like gene families. For all situations studied, citrus plant samples were collected in specific non-stressed and stressed organs as an effort to determine, by using gene expression patterns, if a relevant putative local response is occurring. This is an important approach for determining if a particular response to infection is truly a defense against pathogens, at the time and location of the stress. Thus, in an attempt to gather more evidence for changes of *PR* gene activity upon biotic and abiotic stress conditions, the differential expression profiles of pathogen and drought-induced citrus *PR* ESTs were subtracted from uninfected or non-drought-stressed mock controls, respectively (Figure 2). As a result, unexpectedly, few *PR* gene families showed relative expression 2-fold higher in pathogen-induced than in uninfected organs, even though the differential expression profiles displayed have been quite diverse among studied species and organs.

It appears that transcripts of *PR* gene families found have converged from different defense responses triggered against virus pathogens in leaves of different citrus species. In *C. sinensis* leaves, putative suppression of expression for

most of the *PR* gene families was observed in the presence of the CiLV, when only the *PR-3* gene family showed high expression patterns. Here, the supposed effect of the pre-immunization with the non-virulent CTV strain, previously discussed, is thought to have been eliminated when the subtractive analysis was performed, since both uninfected (mock control) and CiLV-infected *C. sinensis* leaves have been subjected to the same pre-immunization conditions. In *P. trifoliata*, *PR-2*, *PR-3*, *PR-15* and *PR-16* gene families were highly expressed within leaves after infection by a virulent CTV strain, whereas no expression was found for seven *PR* gene families. Although PR-10 protein family has been associated with antiviral activity, no expression of *PR-10* transcripts was found in *P. trifoliata* leaves. On the other hand, the differential expression pattern of *PR* genes, assembled within *C. sinensis* leaves as part of the defense responses against *X. fastidiosa* pathogen, include the high expression of *PR-7, PR-9, PR-10* and *PR-14* gene families

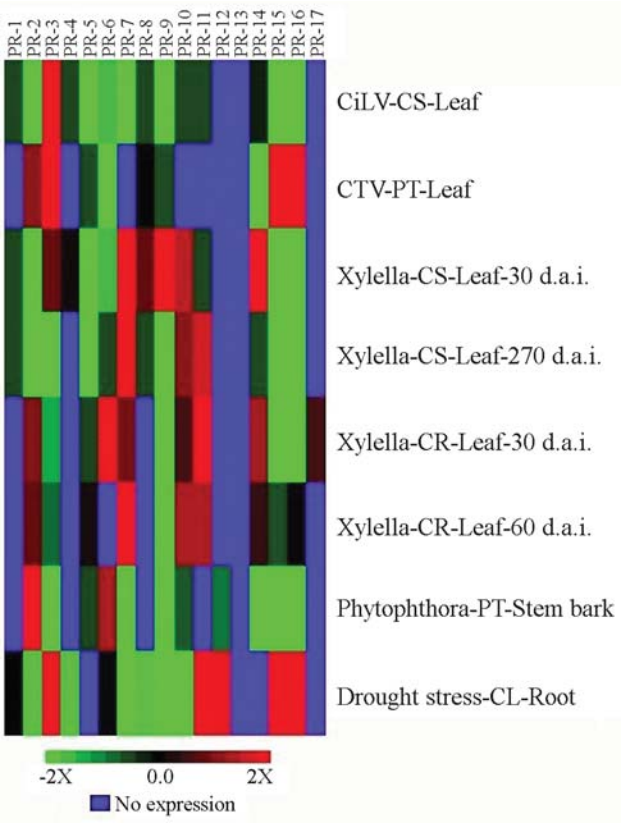

Figure 2 - *In silico* differential expression profiles of putative *PR* gene families (PR-1 to PR-17) in cDNA CitEST libraries from citrus organs induced by pathogen or drought stresses. Each subtracted stress situation (treatment *vs.* control) is represented by a single row; each *PR*-like gene family is represented by a single column. The color bar represents the color scales between relative induction (intense red for larger than 2) or suppression (intense green for lower than -2) ranges in relation to the counterpart control library. Black means no change (ratio 0) and blue no expression. CiLV, citrus leprosis virus; CTV, citrus tristeza virus; Xylella, *X. fastidiosa*; Phytophthora, *P. parasitica*; CS, *C. sinensis*; CR, *C. reticulata*; CL, *C. limonia*; PT, *Poncirus trifoliata*. D.A.I., days after inoculation.

at 30 d.a.i (days after inoculation) and of *PR-7, PR-10* and *PR-11* gene families at 270 d.a.i. These data indicate that the same citrus species appears to trigger different defense responses in leaves against different pathogens. The differential expression pattern of *PR* genes upon *X. fastidiosa* infection of *C. reticulata* leaves was different from these of *C. sinensis*, including the expression of *PR-2, PR-6* and *PR-17* gene families at 30 d.a.i.

Within the same species, the differential *PR* gene expression profiles vary between infected and healthy organs as well as varying between different infections caused by different pathogens. For instance, the high expression of the *PR-3*, *PR-15* and *PR-16* gene families within *P. trifoliata* leaves upon CTV inoculation was found to be putatively suppressed in stem bark after *P. parasitica* infection. It is also possible that *PR* gene expression profiles may vary among organs and/or different pathogens may lead to induction of different PR protein set. Citrus *PR-17* gene family expression was found only in *Citrus reticulata* leaves induced by *X. fastidiosa.* This data suggests that the *PR-17* gene family might be involved in a defense response of *C. reticulata* species to *X. fastidiosa* infection. Molecular mechanisms involved in *C. reticulata* resistance to *X. fastidiosa* were discussed by Souza *et al.* (in this issue). Moreover, high expression of the *PR-7* family was observed only within leaves of both *C. reticulata* and *C. sinensis* upon *X. fastidiosa* infection*,* whereas the *PR-11* gene family was observed within leaves of both species upon *X. fastidiosa* infection and also in *C. limonia* drought-stressed roots. Taken together, members of *PR* gene families identified with altered expression by the presence of a particular pathogen may participate in an effective response against these pathogens, as a component of a highly specialized signaling pathway against pathogen infection. Induction of *PR-1* genes is typical of the onset of SAR. However, *PR-1* gene family expression observed here was not significantly found within any pathogen infection condition. No expression of PR-13 gene family members was observed within any stressed or non-stressed organ.

Members of *PR-3*, *PR-11*, *PR-12*, *PR-15* and *PR-16* gene families showed expression 2-fold higher in drought-induced roots than in non-stressed roots of *C. limonia*. Suppression in expression for members of six *PR* gene families, which were preferentially expressed in non-stressed roots, indicates that more *PR* gene families were repressed than induced in artificial drought-stressed roots. Additional experiments to show the difference in expression between the drought sensitive and the drought resistant cultivars will contribute to elucidate the dynamic response capacity to stress in citrus roots with the participation of *PR* gene families.

Expression pattern of *PR*-like gene families within citrus fruits

The expression patterns of *PR*-like ESTs from CitEST fruit libraries were also analyzed. These fruit librar-

ies were constructed from pericarp, using fruits of different diameters (1, 2.5, 5, 7, 8, 9 cm). Interestingly, at least members of nine *PR* gene families were expressed in fruits of different sizes from two studied citrus species, *C. reticulata* and *C. sinensis,* and also, apparently, the same *PR* gene families were found within both plant species (Figure 3A). With the exception of the *PR-1*, *PR-4*, *PR-13* and *PR-17* gene families, which were not expressed in fruits, members of other *PR* gene families were present throughout all the different fruit size stages in both species. However, it was possible to observe changes in the relative amount of expression patterns among different fruit stages and also between citrus species. An overview of changes in expression profiles of *PR* gene families in fruits of *C. reticulata* and *C. sinensis* is shown in Figure 3B. The greatest number of *PR*-like ESTs was found within *C. sinensis* fruits of 2.5 cm of diameter. Accumulation of defense-related mRNAs in citrus fruits has been studied in the context of fungal and ethylene perception (Marcos *et al.*, 2005). Therefore, the high expression level of different *PR* gene families observed in fruits of citrus might indicate constitutive or developmental defense responses, as a preformed barrier to pathogen infection or hormone-induced putatively playing unsuspected roles in fruit development.

Organ-specific expression profiles of citrus *PR*-like gene families

Genome wide transcriptome analysis with histological information can provide insights into candidate genes that are differentially expressed in certain organs. At the transcriptome level, differential expression of *PR* genes may play key roles in maintaining resistance functions in plants during the development or, similarly, in normal developmental processes. It is worth mentioning that devel-

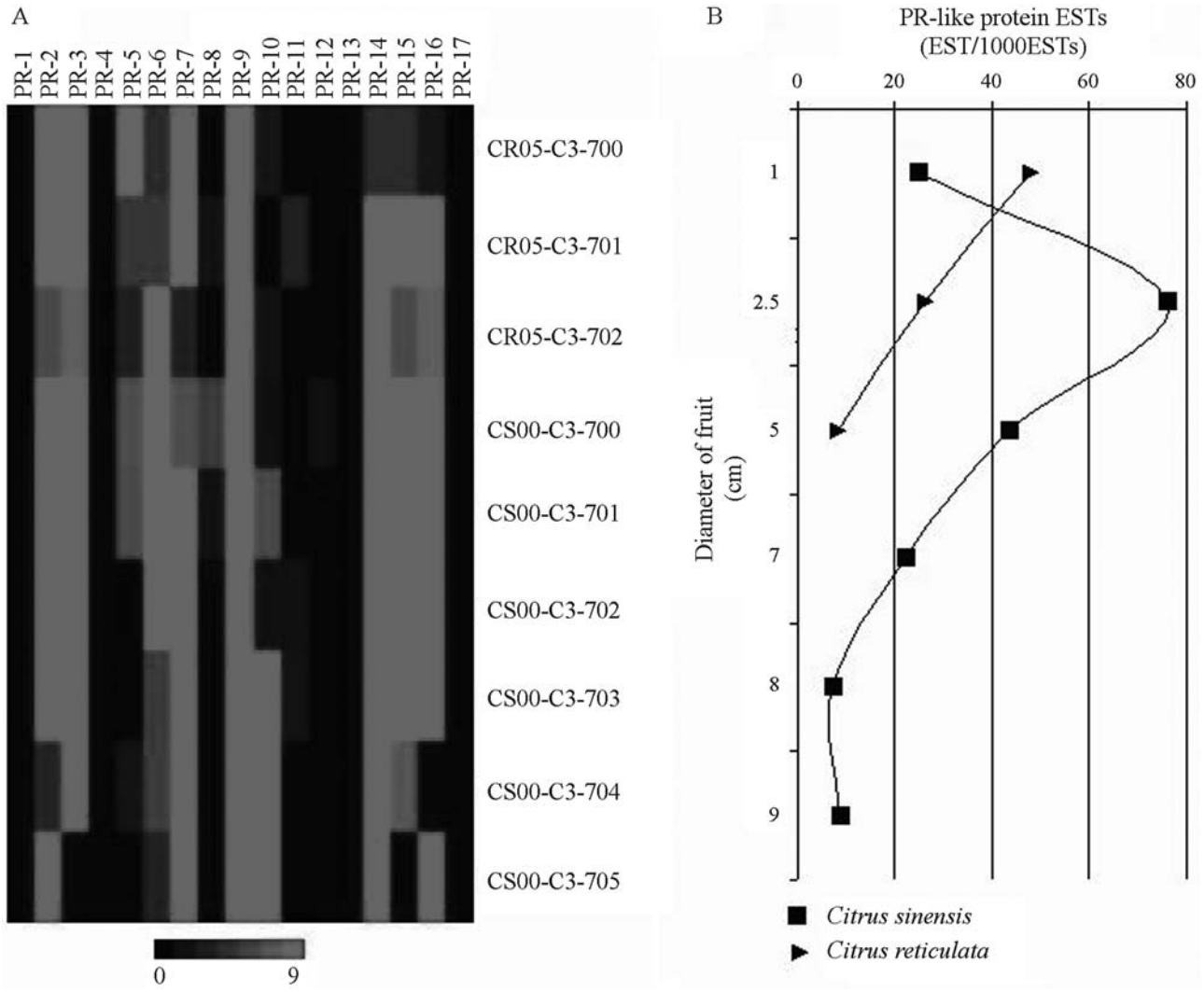

Figure 3 - Expression patterns of putative citrus *PR* gene families (PR-1 to PR-17) in fruit cDNA libraries (A). Data represent the relative number of *PR* reads from a specific library in each EST-contig per 1,000 reads. Graph of the number of *PR*-like ESTs expressed in fruits of *C. reticulata* and *C. sinensis* with different sizes (expressed in diameters: cm) (B). *C. reticulata* libraries: CR05-C3-700, -701 and -702. *C. sinensis* libraries: CS05-C3-700, -701, -702, -703, -704 and -705. Size of fruit per centimeter of diameter is indicated by 700 (1 cm), 701 (2.5 cm), 702 (5 cm), 703 (7 cm), 704 (8 cm) and 705 (9 cm).

opmental-induced PRs are accumulated in an organ and tissue-specific manner (van Loon and van Strien, 1999; Edreva, 2005). In order to speculate whether the preferential expression of some *PR* gene family could be associated with particular organ-specificity within citrus plants, we performed an expression profiles analysis for different organs (Figure 4). The results show that, with the exception of the *PR-13* gene family, which was found only in seeds of *P. trifoliata*, members of the other *PR* gene families seem to be expressed in citrus leaf tissue. Even though present in all plant organs, PR proteins are particularly abundant in leaves, where they can amount to 5%-10% of total leaf proteins (Edreva, 2005). In this organ, PRs were reported present both in epidermal and mesophyll cells, as well as in the vascular bundles (van Loon *et al.*, 2006).

PR-17 gene family expression was observed exclusively within leaves, whereas *PR-13* gene family was presented only within seeds among the studied citrus organs. Further molecular and biochemical characterization of *P. trifoliata PR-13* EST-contigs will provide information about a putative seed-specific expression pattern. In addition, construction of libraries using seeds of other citrus species will provide the answer as to whether this *P. trifoliata PR-13* gene family is present in other citrus seeds. Organ-specific expression of certain *PR* genes suggests that the proteins also play roles in normal developmental process (Edreva, 2005). However, the *PR-17* gene family expressed in *C. reticulata* was induced only upon *X. fastidiosa* infection, as shown in Figure 2. It has already been demonstrated for two barley (*Hordeum vulgare* L.) proteins belonging to the PR-17 family that they accumulated in the mesophyll apoplast following inoculation with *Blumeria graminis* f.sp. *hordei*, as well as in the leaf epidermis, the only tissue to be invaded by the fungus (Christensen *et al.*, 2002). Thus, whether the *PR-17* gene family expression in leaves could to be associated more closely to the pathogen-induced than an organ-specific pattern needs to be tested, focusing on the *C. Reticulata-X. fastidiosa* interaction.

Transcripts of *PR-4* gene family were found only in leaves and roots. Members of *PR-1* gene families were not expressed in citrus fruit peel and seed, whereas *PR-8* transcritpts were not found in stem bark and seed organs. Members of four *PR* gene families were expressed in most of the studied organs, except in root for *PR-5* and *PR-14*, in flower for *PR-10* and in seed for *PR-15* and *PR-16* transcripts. Finally, members of six *PR* gene families (*PR-2*, *PR-3*, *PR-6*, *PR-7*, *PR-9* and *PR-12*) were expressed in all of the studied citrus organs: leaf, stem bark, fruit peel, root, flower and seed.

Occurrence of *PR*-like gene families in citrus species

The occurrence of transcripts putatively encoding PR protein families in all of the studied citrus species was also investigated (Figure 5). Our first question was whether it

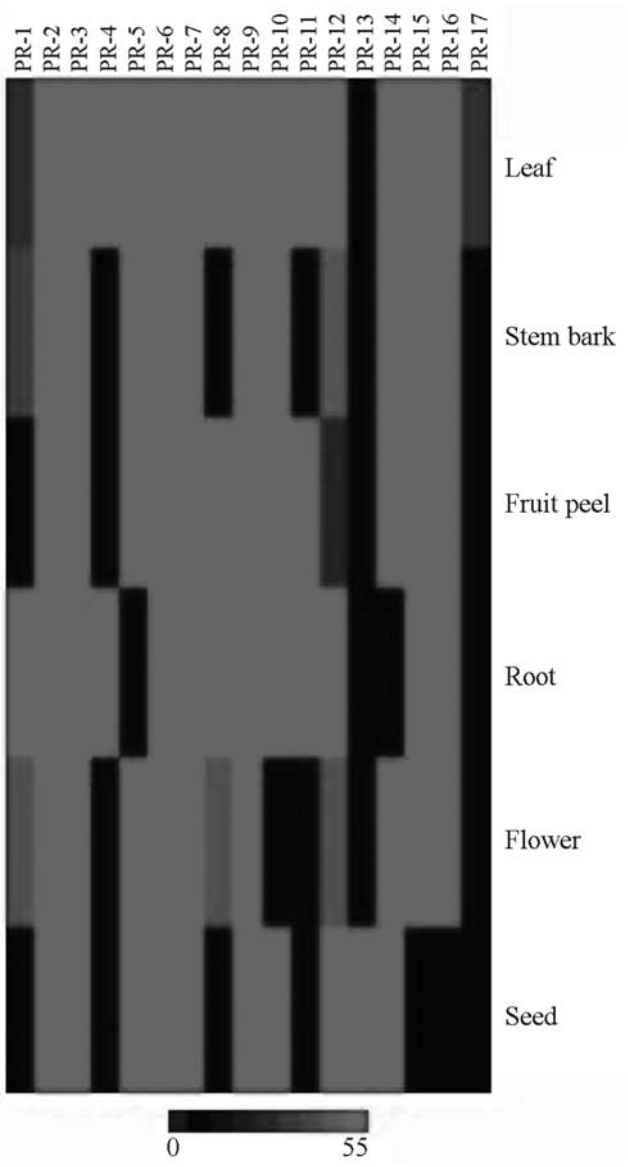

Figure 4 - Graphical representation of the expression profiles of putative *PR* gene families (PR-1 to PR-17) in different citrus organ cDNA libraries. Data represent the relative number of *PR* reads from a specific library in each EST-contig per 1,000 reads. Each organ library is represented by a single row; each *PR* gene family is represented by a single column.

was possible that all of the recognized *PR* gene families could be present in a single plant genome, but here we were not able to show this. Regulation of the different *PR* transcripts requires different stimuli within the same plant; however, the analyzed libraries had not been constructed using several different organs and stimuli for a single citrus species. In addition, the analyzed libraries also had not been constructed using the same organs and conditions varied in a considerable manner for each species, as well as the whole number of ESTs among citrus species libraries (Table 2). Therefore, the data shown in Figure 5 is not supposed to reflect the representative number of *PR* gene family members within the genome of each citrus species.

Furthermore, comparisons among species could not be made. Nevertheless, it can bring to light which types of *PR* gene families were found in the CitEST database expressed within each species under analyzed conditions.

A total of sixteen out of seventeen *PR* gene families were found in the *C. sinensis* species, a plant that had the greatest number of ESTs sequenced from four different organ libraries (leaf, fruit, bark and flower). Hence, it is possible that different EST-contigs from the same *PR* gene family have been expressed in all of the four organs; and also it is expected that some EST-contigs of different *PR* gene families have had co-regulated expression within the same *C. sinensis* organ. The lowest number the *PR* gene families (3) was observed in a *C. sunki* stem bark library, wich also contained the lowest number of sequenced ESTs. In summary, 3 to 15 *PR* gene families/species were found expressed in analyzed citrus species, and their expression profiles were similar to those found for *PR-3*, *PR-6*, *PR-7*, *PR-9* and *PR-16* gene families among all of citrus species.

Members of the *PR-14* gene family were found to be expressed in all of the studied species with exception for *C. limonia*, whereas the PR-*13* gene family was found only in *P. trifoliata* species. Nevertheless, it is noteworthy that the CitEST libraries using root or seed tissues were constructed only for *C. limonia* and *P. trifoliata*, respectively. Members of the *PR-17* gene family were found only in the *C. reticulata* species and it was also observed that the *PR-1* gene family was found only within *C. sinensis* and *C. limonia* species.

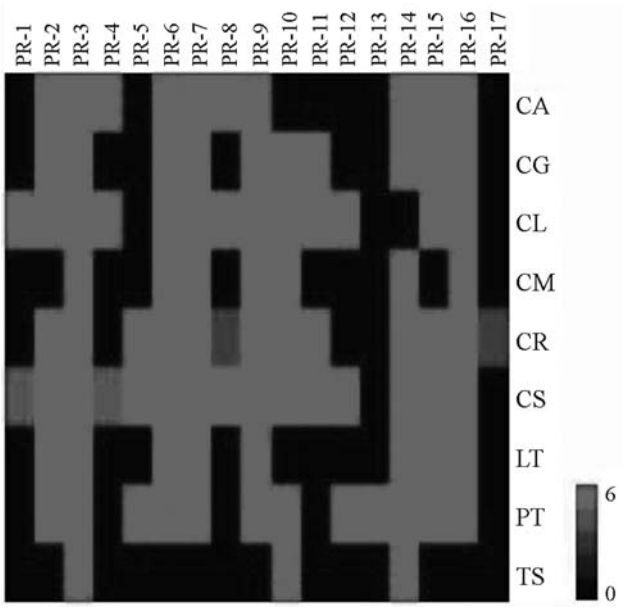

Figure 5 - *PR*-like gene families expression patterns present in each citrus species. Each citrus species is represented by a single row; each *PR* gene family (*PR-1* to *PR-17*) is represented by a single column. Data represent the relative number of *PR* reads from a specific library in each EST-contig per 1,000 reads. CA, *Citrus aurantium*; CG, *C. aurantifolia*; CL, *C. limonia*; CM, *C. limettiodes*; CR, *C. reticulata*; CS, *C. sinensis*; LT, *C. latifolia*; PT, *Poncirus trifoliata*; TS, *C. sunki.*

Citrus *PR* multigene families: an insight into the clusters

In this work, we have studied expression profiles of citrus *PR* gene families. Additionally, in order to speculate on the diversity of *PR* genes in a particular *PR* gene family, we have analyzed the different *PR* ESTs and their grouping into clusters generated for four *PR* gene families separately. Distribution of the total number of citrus *PR*-like ESTs within the 17 *PR* gene families was indicated in Table 1. Here, we have focused only in contigs belonging to the *PR-2*, *PR-3* (largely expressed), *PR-5* (poorly expressed) and *PR-7* (moderately expressed) gene families (Table S1). Two of the best represented contigs in the *PR-2* gene family (contigs 2 and 8) were found to be expressed in leaf, fruit and stem bark tissues from *Citrus aurantium* (CA), *C. aurantifoilia* (CG), *C. latifolia* (LT), *C. reticulata* (CR) and *C. sinensis* (CS) species. Similar patterns of gene expression were found for two contigs (contigs 1 and 2) of the *PR-3* gene family as well as for two contigs (contigs 3 and 6) of the *PR-7* gene family, which comprise ESTs expressed in leaf, fruit, stem bark, flower and seed tissues from CA, CG, LT, CR, CS and *P. trifoliata* (PT) species. In other words, several putative *PR-3* genes were clustered within chimerical contigs comprising EST-reads isolated from various cDNA libraries of several different organs, species and conditions. This may suggest the occurrence of the same *PR* genes with evolutionarily conserved functions among different citrus species genomes. The report that the origin of *Nicotiana tabacum PR* genes was confirmed from their wild progenitors *N. sylvestris* and *N. tomentosiformis*, based on *PR* gene patterns (Ahl Goy *et al.*, 1982), represents strong evidence that *PR* genes can be distinguished by species specificity, thus allowing their application as general markers in taxonomic, phylogenetic and evolutionary studies (Edreva, 2005). Moreover, it has been proposed that genes with similar functions, or cDNA libraries expected to share similar patterns of gene expression, cluster together (Ewing *et al.*, 1999). Hence, a related function could be implicated in each particular group of *PR* genes that was clustered together suggesting a common mechanism controlling their regulation.

Categorizing *PR* genes into clusters or regulons based on the similarity of *PR* gene expression profiles in organs from a particular citrus species under several conditions could also point to *PR* gene members of a multigene family. For instance, clustering of *PR-3* gene family ESTs provided a total of 24 contigs and 13 singlets (Table 1), among them 6 contigs and 2 singlets possess ESTs derived from *Poncirus trifoliata* species. In theory, these cited 6 contigs and 2 singlets may be pointing to the occurrence of at least 8 *PR-3*-like genes within the *P. trifoliata* genome. However, this hypothesis needs to be confirmed by Southern genomic hybridization analysis, for example, using a *PR-3* gene as probe. The occurrence of *PR* genes organized in plant genomes as multigene families has already been demon-

strated for the *PR-5* gene family in *Solanum* species (Zhu *et al.*, 1995; Vleeshouwers *et al.*, 2000; Campos *et al.*, 2002) and oat (Lin *et al.*, 1996), among other *PR* gene families. Therefore, the citrus *PR* gene families putatively comprise several members.

Likewise, *PR* genes belonging either to the same or to different *PR* gene families that share similar pattern of gene expression within the same plant organ library may possibly indicate a coordinated expression control of multiple *PR* genes playing roles together in a given biochemical pathway. In this context, two *PR-3* contigs (contigs 22 and 24) were found to be co-expressed only in drought-stressed *C. limonia* roots as well as the *PR-3* contigs 3 and 11 comprise ESTs isolated only from *C. reticulata* fruits (Table S1). Nevertheless, more detailed analyses regarding the expression pattern of these clusters/genes will be necessary in order to gain evidence for a possible organ and/or species expression specificity. Most of the different *PR* contigs, coordinately expressing all of analyzed *PR-2*, *PR-3*, *PR-5* and *PR-7* gene families, were found in both *C. reticulata* and *C. sinensis* fruit libraries (Table S1). It has been postulated that the co-regulation is associated with the presence of the same promoter *in cis* elements, such as SA-responsive element (SARE), GCC box, G-box, W-box, and MRE-like sequence (Zhou, 1999; Chakravarthy *et al.*, 2003; Edreva, 2005), leading to coordinated expression control of multiple *PR* genes. In this context, coordinated expression for multiple *PR* genes was correlated with the onset of SAR (Ward *et al.*, 1991). Additionally, enhanced defense actions have already been demonstrated by the synergistic effect of the combinatorial expression of PR protein classes in transgenic plants (Zhu *et al.*, 1994, Jach *et al.*, 1995).

A BLAST search was performed for proteins with amino acid sequences similar to the deduced citrus chitinase proteins from selected contigs; the best hits were found to be PR-3 homologues from *Citrus jambhiri*, *Gossypium hirsutum* and *Sambucus nigra* plants, which were used in phylogenetic analysis. The neighbor-joining tree (Figure 6) shows that the ten studied citrus *PR-3* gene sequences were grouped into three major clusters containing, interestingly, different members. Four citrus PR-3 contigs (3, 11, 12 and 16 contigs) comprise the first cluster which covers ESTs that were expressed within several organs (fruit, root, stem bark, leaf and seed) from *C. reticulata, C. limonia* and *P. trifoliata* species. They share 95%, 84%, 88% and 86% of amino acids identity with *Citrus jambhiri* acidic class I chitinase (gi|23496445), respectively. In the second cluster, there are four citrus PR-3 contigs (1, 2, 10 and 21 contigs) that share sequence identity (73%, 71%, 73% and 75%, respectively) with *Gossypium hirsutum* basic class VII chitinase (gi|32401255). All of these contain *P. trifoliata* ESTs, which are 1 and 2 chimeric contigs. The remaining two contigs (contigs 22 and 24) belonged to a third cluster, in

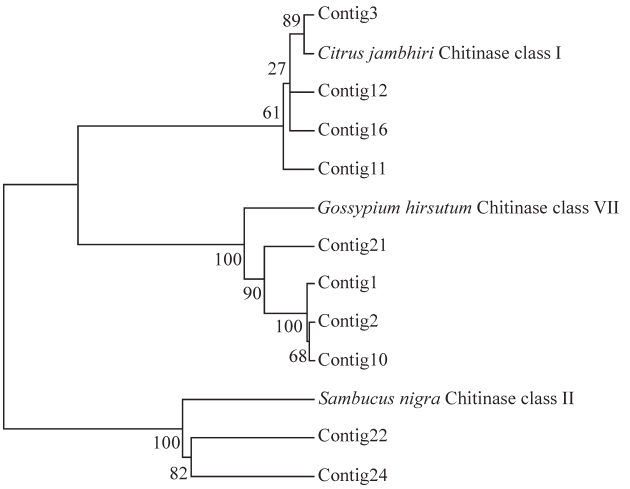

Figure 6 - A neighbor-joining tree of PR-3 homologues from Citrus constructed and visualized in MEGA program from deduced amino acids sequences alignment in Clustalx. The numbers indicate in percentage bootstrap analysis (1,000 replicates) of the data supporting the branches. The following sequences were taken from the EMBL GeneBank: *Citrus jambhiri* acid class I chitinase (gi|23496445); *Sambucus nigra* class II chitinase (gi|603884) and *Gossypium hirsutum* basic class VII chitinase (gi|32401255).

which only ESTs from *C. limonia* drought-stressed roots that share sequence identity (only 62% and 53%, respectively) with *Sambucus nigra* class II chitinase (gi|603884) were placed. These findings correlate with the presence of three different PR-3 chitinase classes with citrus species, two of which (I and VII classes) can be found within the *P. trifoliata* species, while the class II chitinase was found within the *C. limonia* species, based on sequence similarities. Interestingly, PR-3 contigs 16 and 21, which were co-expressed within *P. trifoliata* stem bark tissue, were grouped into two different major clusters. This is an indication of the presence of the two PR-3 chitinase I and VII classes co-expressed within a same organ of a single plant, putatively in response to an identical signal.

Members of the PR-3 family belong to family 19 of glycoside hydrolases (EC 3.2.1.14), which catalyses the hydrolysis of beta-1,4-N-acetyl-D-glucosamine linkages in chitin polymers, a major component of the cell wall of most fungi. It has been demonstrated that the *Citrus jambhiri* acidic *class I chitinase* transcripts were not constitutive but accumulated within leaves after wounding or inoculation with non-pathogenic or pathogenic isolates of the *Alternaria alternata* fungus (Gomi *et al.*, 2002). The putative citrus *class I chitinase* genes studied herein that make up the major first cluster were expressed under fruit development (from *C. reticulata*), CTV-infection (from *P. trifoliata*), stem bark wounding or wounding/*P. parasitica*-infection (from *P. trifoliata*), root drought-stress (from *C. limonia*) and in seed tissue (from *P. trifoliata*) conditions. Likewise, the *Gossypium hirsutum* basic *class VII chitinase* gene was inducible by salicylic acid in seedlings, with transcript accumulation in root and cotton fibers, associated

with the cotton's resistance to diseases (Li and Liu, 2003). Whether the studied putative citrus *class VII chitinase* genes were induced by salicylic acid remains to be investigated. Similarly, the *Sambucus nigra class II chitinase* gene was found to be expressed during ethylene-promoted leaflet abscission (Coupe *et al.*, 1997), but the putative citrus *class II chitinase* genes analyzed herein were associated with root drought-stress. Whether they are involved in ethylene pathways needs to be studied.

Concluding Remarks

In this paper, we present a large-scale analysis of gene expression profiles to identify citrus *PR* candidate genes that may participate in environmental and developmental responses. This can now be examined further in experimental studies regarding biotechnological approaches or citrus resistance markers. Albeit *in silico*, the data presented in this work represent a starting point to elucidate the complex responses of citrus plants to biotic and abiotic stresses. The identified *PR*-gene families may be useful to verify innate immunity mechanisms in citrus species possessing basal or induced/nonhost or host resistances to certain pathogens taking place with a participation of PR proteins, and this is the subject of current research.

Acknowledgments

We are grateful to Dr. Marilia Santos Silva (Embrapa Cerrados) and Maria Fátima Grossi de Sá (Embrapa Genetic Resources and Biotechnology) for valuable suggestions. A research fellowship granted to the first author, M.A.C., by CNPq/Institutos do Milênio/Citrus during two years is gratefully acknowledged, as well as financial support by CAPES/PRODOC/UFLA, Brazil.

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Supplementary Material

The following online material is available for this article:

Table S1

This material is available as part of the online article from http://www.scielo.br/gmb.

Associate Editor: Marco Aurélio Takita

Libraries	PR-2 Protein Family EST-Contigs																			
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
LT33-C1-003	1	3	-	-	-	2	-	3	-	-	-	-	-	-	-	-	-	-	-	1
CG32-C1-003	-	3	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-
CA26-C1-002	4	1	-	-	1	4	-	1	1	-	-	-	-	1	-	-	-	-	-	-
CM30-C1-401	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CR05-C1-100	-	-	2	-	-	-	-	4	-	-	-	-	-	-	-	-	-	-	2	-
CR05-C1-102	-	-	2	-	1	-	-	2	-	-	-	1	-	-	-	-	-	-	-	-
CR05-C1-103	-	-	-	-	3	-	-	1	-	2	-	-	-	1	-	-	1	-	-	-
CS00-C1-100	3	6	2	1	1	-	1	2	-	-	-	-	-	-	1	-	-	1	-	-
CS00-C1-101	-	2	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
CS00-C1-102	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	2	-	-	-	-
CS00-C1-401	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CS00-C1-650	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-
CS12-C1-001	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CS13-C1-001	2	1	-	2	-	-	-	-	1	-	-	-	-	3	-	-	-	-	-	-
PT11-C1-900	-	-	1	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
PT11-C1-901	-	-	3	-	-	-	-	1	-	1	-	-	2	-	-	-	-	-	-	-
PT11-C2-300	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PT11-C2-301	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TS27-C2-300	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CS00-C2-003	-	1	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	2	-	-
CR05-C3-700	3	1	-	1	-	-	-	4	1	-	-	-	-	6	-	-	-	-	-	-
CR05-C3-701	2	1	-	-	-	-	1	3	-	-	-	-	-	8	1	-	1	-	-	-
CR05-C3-702	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CS00-C3-700	2	2	-	1	2	-	-	1	-	-	-	-	-	4	2	1	1	-	1	-
CS00-C3-701	2	4	-	-	2	1	1	-	-	-	2	-	-	2	-	-	-	-	-	-
CS00-C3-702	1	1	1	-	1	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-
CS00-C3-703	-	-	-	-	1	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
CS00-C3-704	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CS00-C3-705	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-
CL06-C4-500	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CL06-C4-501	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CS00-C5-003	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PT11-C9-005	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1
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EST Length (pb)	1795	1782	1736	1645	1546	1358	1358	1350	1312	1306	1193	1078	1068	1033	1032	1020	984	969	966	923

													PR-3 Prot										
21	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
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3	2	3	2	3	8	13	2	2	6	2	2	2	53	31	26	70	20	6	70	2	3	3	21
922	922	918	894	893	892	882	863	833	820	742	716	332	1556	1410	1351	1346	1271	1266	1259	1254	1249	1196	1138

ein Family EST-Contigs														PR-5 Protein Family EST-Contigs										
12	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**
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17	30	6	14	8	2	3	12	4	3	4	3	2	12	6	10	7	2	3	33	26	25	20	17
1118	1101	1075	956	936	922	908	898	862	850	753	696	433	960	954	855	805	756	731	2200	1926	1891	1832	1473

PR-7 Protein Family EST-Contigs									
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1240	1154	1063	1060	903	754	724	708	680	315