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Research Article

Identification of citrus expressed sequence tags (ESTs) encoding pleiotropic drug resistance (PDR)-like proteins

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Abstract

Pleiotropic drug resistance (PDR) proteins, a subfamily of the ATP-binding cassette (ABC) transporters, have been recently shown to play a role in plant defense against biotic and abiotic stresses. However, nothing is known about their expression in citrus. To investigate the occurrence of PDR homologues in citrus species, we have surveyed EST sequences from different tissues and conditions of the Citrus Expressed Sequence Tags (CitEST) database, through sequence similarity search analyses and inspections for characteristic PDR domains. Multiple sequence alignments, prediction of transmembrane topology and phylogenetic analysis of PDR-like proteins were additionally performed. This study allowed the identification of nine putative proteins showing characteristic PDR features in citrus species under various conditions, which may indicate a potential correlation between PDRs and stress and metabolism of citrus plants. Moreover, a tissue-specific putative PDR-like protein was found in sweet orange fruits. To our knowledge, this is the first report regarding the identification of citrus ESTs encoding PDR-like proteins as well as the first to identify a putative full ABC transporter with specific expression in fruits.

Key words: ABC transporters, CitEST, expressed sequence tag, transcriptome, stress.

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Introduction

The ABC transporters, found in bacteria, fungi, plants and animals, form one of the largest protein families and are central to the transport of diverse substances in plants, including those related to resistance to biotic and abiotic stress (Martinoia *et al.*, 2002). In *Arabidopsis*, the ABC transporter family is considered to be relatively large and composed of 131 members (Jasinski *et al.*, 2003).

Although the ABC genes have been divided into several subfamilies according to structural features, most of them belong basically to three subfamilies: multidrug resistance (MDR), multidrug resistance-associated protein (MRP), and pleiotropic drug resistance (PDR). Currently, however, very little is known about their roles in plants. These ABC transporters were named according to the initial observation that they confer resistance to various drugs. Such nomenclature can be extremely restrictive, since they may also transport other substrates and be involved in functions other than detoxifying cells. In reality, some members do not even directly participate in drug transport (Jasinski *et al.*, 2003; Crouzet *et al.*, 2006).

All of these ABC transporters subfamilies are highly represented in *Arabidopsis* and rice: 22 and 17 members of MDR, 15 and 12 members of MRP, and 15 and 23 members of PDR, respectively (Sánchez-Fernández *et al.*, 2001; Jasinski *et al.*, 2003; Crouzet *et al.*, 2006).

MDR proteins are widely distributed from prokaryotes to eukaryotes. Although only a few members have been functionally characterized in plants, their function in human and rodent tissues has been well investigated (Schiengold *et al.*, 2006). In plants, these transporters are highly involved in translocation of alkaloids (Otani *et al.*, 2005) as well as auxin and, as a consequence, in lateral root and root hair development in *Arabidopsis* (Santelia *et al.*, 2005; Terasaka *et al.*, 2005) and height reduction of maize plants (compact lower *stalk* internodes) (Multani *et al.*, 2003).

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MRP genes may function in a number of processes, including salt (NaCl) tolerance and detoxification (Lee *et al.*, 2004, Klein *et al.*, 2006), regulation of guard cells aperture and root development (Gaedeke *et al.*, 2001; Klein *et al.*, 2004), and vacuolar flavonoid transport (Kitamura *et al.*, 2004).

Representatives of the PDR subfamily have been found in plants and fungi but not in prokaryote or animal species. Most of the PDR genes from plants so far characterized have been shown to be related to responses to abiotic and biotic stress; in fact, pathogens induce their expression, probably resulting in transport of antimicrobial secondary metabolites to the cell surface (Crouzet *et al.*, 2006). Members of the plant PDR subfamily have been implicated in the transport of various compounds, including antifungal agents (van den Brûle and Smart, 2002).

The first plant PDR cDNA to be cloned, SpTUR2, coding for a PDR5-like ABC transporter from the aquatic plant *Spirodela polyrrhiza* (Smart and Fleming, 1996), was also identified in *Arabidopsis,* as a response to the diterpenoid antifungal agent sclareol (van den Brûle *et al.*, 2002). Most interestingly, in members of this subfamily of PDR transporters, the nucleotide binding domain (NBD) precedes the hydrophobic transmembrane domain (TMD), as opposed to other full ABC transporters (Crouzet *et al.*, 2006).

TMDs are two highly hydrophobic protein domains that form the channel through which the substrate passes during translocation. They are poorly conserved, which reflects the large diversity of substrates transported; on the other hand, the NBDs show high sequence identity, bound to the cytosolic face of the TMDs and act in both substrate translocation by ATP-binding and hydrolysis (Biemans-Oldehinkel *et al.*, 2006).

ABC transporters can be divided into two categories: half-size and full-size transporters. Members of the former share the same structural organization as other ABC transporters, but harbor a single copy of the fusion between NBD and TMD, a category in which the White-Brown Complex subfamily is included (Schmitz *et al.*, 2001; Garcia *et al.*, 2004). On the other hand, full transporters show contiguous core domains on a single multidomain polypeptide (Sánchez-Fernández *et al.*, 2001; Biemans-Oldehinkel *et al.*, 2006).

Although a significant number of PDR genes were found throughout the complete genome of *Arabidopsis* and rice, only a few members of this subfamily have been recently investigated in plants. These include: NtPDR3 (iron-deficiency inducible) and NtPDR1 (microbe-elicited) ABC transporters in *Nicotiana tabacum* (Sasabe *et al.*, 2002; Ducos *et al.*, 2005); NpPDR1, which is involved in both constitutive and jasmonic acid-dependent-induced pathogen defense in *N. plumbaginifolia* (Stukkens *et al.*, 2005); AtPDR8 and AtPDR12, which, respectively, induce or contribute to hypersensitive cell death upon pathogen infection and resistance to lead in *Arabidopsis thaliana* (Lee *et al.*, 2005; Kobae *et al.*, 2006; Stein *et al.*, 2006); AtPDR9, which is involved in detoxification of herbicides (2,4-D) in *A. thaliana* (Ito and Gray, 2006); GmPDR12, which was identified as a salicylic acid-induced gene from soybean cell suspensions (Eichhorn *et al.*, 2006) and Ospdr9, which is induced by the heavy metals cadmium and zinc and hypoxic stress in rice roots (Moons, 2003).

In this study, we identified citrus ESTs encoding PDR-like proteins by search analyses and annotation of the CitEST database.

Materials and Methods

We have surveyed clones from citrus EST libraries from the CitEST database, supported by MCT-CNPq-Millennium. Altogether, 33 cDNA libraries were constructed from nine different citrus species and diverse conditions (including environment- and pathogen-challenged plants), from which 5'-sequenced ESTs from 242,790 valid reads (transcripts) were generated. Because EST databases may contain many overlapping sequences from the same gene, the ESTs were clustered using the CAP3 program with a 40-base pair overlap and 80% identity criterion (Huang and Madan, 1999). Consensus sequences were periodically compared using BLASTX (Altschul *et al.*, 1990) ($E = 10^{-5}$) to the NCBI translated nonredundant (nr) database.

Initially, the CitEST database was searched for PDR homologues, based on sequence similarity to the deduced amino acid sequences from the SpTUR2 protein, the first plant PDR to be cloned (Smart and Fleming, 1996), and all PDR members of *A. thaliana* (AtPDR1 through AtPDR15) through BLAST analyses. As an additional procedure, the database was repeatedly searched during the course of this inventory with the keyword "PDR." The presence of Walker A and B motifs and ABC signature was used as complementary identification parameters: sequences (clusters) that did not show at least a single copy were rejected.

The multiple sequence alignment of the obtained sequences and other plant PDRs was performed by the CLUSTAL W program (Higgins *et al.*, 1996). Final alignment was visually inspected and manually corrected. In addition, the HMMTOP program (Tusnády and Simon, 2001) was used for prediction of transmembrane helices and topology of proteins.

Phylogenetic analysis of the citrus putative PDR-like transporters along with 15 representative sequences from *A. thaliana* was conducted with the Molecular Evolutionary Genetics Analysis (*MEGA*) software version 3.1 (Kumar *et al.*, 2004). The minimum evolution method was used, with a total of 1,000 bootstrap replicate trees.

To calculate a probability value for each gene in each two-library comparison, the AC test (Audic and Claverie, 1997) was used; *i.e.*, a "digital analysis of gene expression" was performed taking into account the total number of transcripts retrieved from each of the libraries and the statistical significance, with a confidence of 95%.

Results and Discussion

Overall, the analysis of the CitEST database revealed 230 PDR-related sequences, which were assembled into 29 contigs and 30 singlets (sequences that did not fit into any contig). The selected transcripts were then grouped on the basis of citrus species. All groupings were performed manually. The species-directed approach rendered nine clusters (sequences in a cluster are assumed to represent the same gene) that showed high BLAST similarity values ($E = 2e^{-98}$) and presence of the characteristic domains. The clusters included four citrus species (*C. sinensis*, *C. reticulata*, *P. trifoliata*, and *C. limonia*) and various EST libraries and showed best matches with PDR homologues from *N. tabacum* [NtPDR1 (Q76CU2), NtPDR2 (BAD07484, AB109389), and NtPDR3 (Q5W274)] and *A. thaliana* [AtPDR4 (DAA00872), AtPDR6 (AAD24623), AtPDR11 (DAA00879), and AtPDR12 (NP 173005)] (Table 1). Indeed, two of the citrus clusters were formed by only one transcript; *i.e.*, they represent singlets. The multiple alignment of all clusters and PDR homologues did not reveal any full-length PDRs (Figure 1). The alignment showed that all citrus PDR-like clusters as well as the NtPDR2 homologue lacked the Walker A and B boxes and the ABC signature in N-terminal region. On the other hand, they contain perfectly conserved Walker A motifs with the Arabidopsis C-terminal consensus of GVSGAGKT. Also, the C-terminal PDR Walker B box [I(ILV)F(ML)D] and ABC signature [L(ST)TEQRLTIA] fit the consensus perfectly, with the exception of CsPDR4, where one residue does not fit the consensus. Obviously, the topology of motifs predicted by the computer program was affected by incomplete sequences (Figure 2).

Since there is no report on PDR-like proteins in citrus to date, and in line with a recently suggested nomenclature system (van den Brûle and Smart, 2002; Eichhorn *et al.*, 2006), we suggested a nomenclature for these homologues which took into consideration the citrus species and the phylogenetic tree (Figure 3), which was generated with predicted PDR proteins from *A. thaliana* and all CitEST PDR-like candidates as follows: CAS-CS-107920, CsPDR12; CAS-CS-111457, CsPDR11; CAS-CS-104504, CsPDR4; CAS-CS-109837, CsPDR13; CAS-CR-202176, CrPDR12; CAS-CR-209514, CrPDR11; CAS-CR-204968, CrPDR13; CAS-PT-307851, PtPDR9; and CAS-CL-701595, ClPDR6.

Only seven of the nine citrus clusters displayed typical TMD of ABC transporters. In fact, unlike other full ABC transporters (MDR and MRP), all citrus clusters with TMD showed the reverse configuration expected from PDR proteins (NBD preceding TMD). Although neither CrPDR11 nor PtPDR9 showed TMDs, we assumed both sequences as representative of PDR-like proteins due to the high homology to the PDR transporters NtPDR2 and NtPDR3, respectively, as well as due to the presence of the complete sequences of Walker A and B motifs and the ABC signature.

Alignment of two sequences using BLAST engine for local alignment (Tatusova and Madden, 1999) between all 29 AtWBC (*A. thaliana* White-Brown Complex) transporters from the GenBank and all citrus PDR candidates found much lower similarities as compared to AtPDR transporters.

A quantitative method for the analysis of transcript frequencies and detection of differences among libraries was used (Audic and Claverie, 1997) during systematic pairwise comparisons conducted between CitEST libraries, which revealed some very interesting characteristics regarding gene expression in sweet orange (Table 2). From this analysis, we identified two genes with tag differences between libraries (Pera sweet orange varieties and developmental stage of fruits) that were significant at $p \leq 0.05$. In fact, three additional comparisons with p values between 0.05 and 0.07 also were considered for further analysis.

Two clusters of the citrus database (CsPDR12 and CsPDR13) were constructed from sweet orange (*C. sinensis*) transcripts that matched the NtPDR1 gene. These clusters comprise transcripts from different conditions (Table 3). Remarkably, the citrus homologue CsPDR13 was constructed exclusively with cDNA from sweet orange fruits. Although NtWBC1, a half transporter of the White-Brown subfamily found in tobacco, was the first ABC

Table 1 - Pleiotropic drug resistance (PDR) transporters found in the CitEST database.

Cluster	Species	Length (aa)	Accession	Gene name and organism	E-value	Identity (%)
CsPDR12	CS	624	Q76CU2	NtPDR1 (*Nicotiana tabacum*)	0.0	81
CsPDR11	CS	522	Q7PC84	AtPDR11 (*Arabidopsis thaliana*)	0.0	68
CsPDR4	CS	468	DAA00872	AtPDR4 (*A. thaliana*)	$8e^{-139}$	89
CsPDR13	CS	552	Q76CU2	NtPDR1 (*N. tabacum*)	$4e^{-127}$	89
CrPDR12	CR	337	NP_173005	AtPDR12 (*A. thaliana*)	0.0	73
CrPDR11	CR	310	AB109389	NtPDR2 (*N. tabacum*)	$2e^{-102}$	72
CrPDR13	CR	687	NP_173005	AtPDR12 (*A. thaliana*)	0.0	73
PtPDR9	PT	304	Q5W274	NtPDR3 (*N. tabacum*)	$4e^{-110}$	79
ClPDR6	CL	486	DAA00874	AtPDR6 (*A. thaliana*)	0.0	73

1- CS - *Citrus sinensis*, CR - *C. reticulata*, PT - *Poncirus trifoliata* and CL - *C. limonia*.

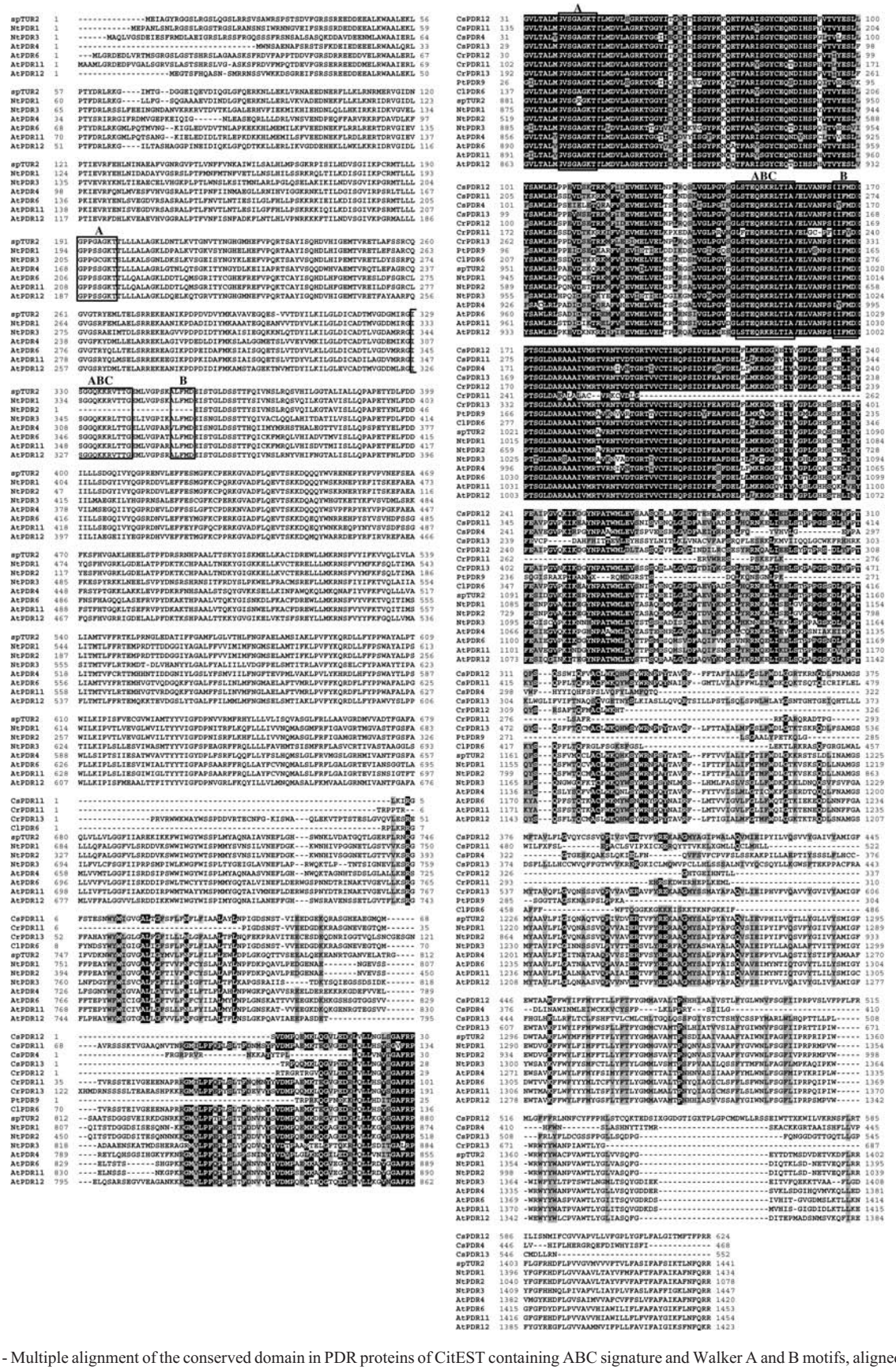

Figure 1 - Multiple alignment of the conserved domain in PDR proteins of CitEST containing ABC signature and Walker A and B motifs, aligned to plant PDRs SpTUR2 (accession no. O24367), NtPDR1 (Q76CU2), NtPDR2 (BAD07484), NtPDR3 (Q5W274), AtPDR4 (DAA00872), AtPDR6 (AAD24623), AtPDR11 (DAA00879), and AtPDR12 (NP_173005), using the CLUSTAL W program (Higgins *et al.*, 1996).

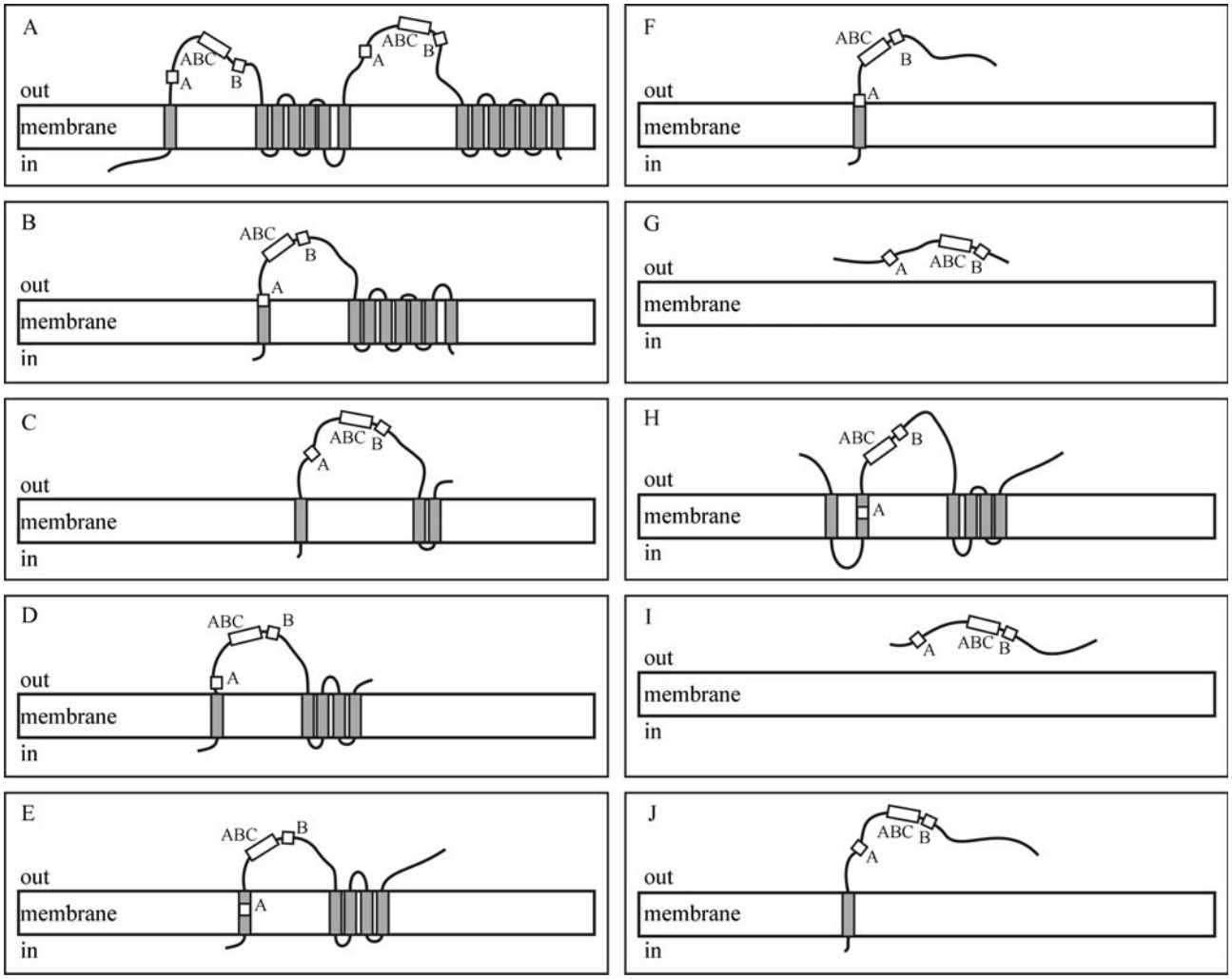

Figure 2 - Organization of typical PDR proteins and their comparison with the CitEST clusters, showing the transmembrane domains (TMD) and the nucleotide binding domain (NBD), which includes the Walker A and B motifs and the ABC signature. [**A**: SpTUR2 (accession no. O24367), **B**: CsPDR12, **C**: CsPDR11, **D**: CsPDR4, **E**: CsPDR13, **F**: CrPDR12, **G**: CrPDR11, **H**: CrPDR13, **I**: PtPDR9, and **J**: ClPDR6].

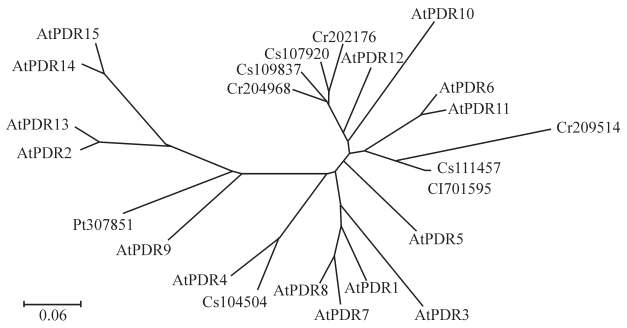

Figure 3 - Phylogenetic interrelationships of nine predicted PDR-like transporters identified in citrus species and 15 PDR-like representatives from *A. thaliana*. The unrooted tree was constructed by the *MEGA* 3.1 software (Kumar *et al.*, 2004), according to the minimum evolution method (Rzhetsky and Nei, 1993) with 1,000 bootstrap replicate trees, based on a preliminary neighbor - joining tree (Saitou and Nei, 1987). The scale bar represents the number of nucleotide substitutions per site.

transporter gene with specific expression in plant reproductive organs to be identified (Otsu *et al.*, 2004), to our knowledge, CsPDR13 is the first full ABC transporter candidate with specific expression in fruit identified to date.

The deduced amino acid sequence of CsPDR13 is homologous to NtPDR1 with 89% identical residues, which rates the highest identity to plant PDR proteins as compared to the other citrus PDR-like proteins. However, this citrus homologue originated from a cDNA library not stress-challenged (*i.e.*, apparently healthy plant) thus suggesting a function of this homologue which might be not related to stress response. Although it is well known that levels of many compounds accumulate during citrus fruit ripening and in different stages of growth (Kato *et al.*, 2004; Cercos *et al.*, 2006; Kato *et al.*, 2006), specific ABC transporters that participate in this process have not previously been identified. Additionally, the analysis of the transcript frequencies suggests that a higher expression of this gene occurs early during fruit growth.

On the other hand, the cluster CsPDR12 was formed with transcripts originating from a number of tissues and

Table 2 - Differentially expressed PDR-like genes in citrus EST libraries detected by electronic subtraction.

Cluster	Libraries		Number of ESTs				p value[1]
			Per cluster		Per library		
	N1	N2	N1	N2	N1	N2	
CsPDR12							
	Leaves from field-grown Pera Olimpia orange trees	Leaves from greenhouse-grown Pera sweet orange trees	3	1	2,603	9,536	0.02434
		Leaves from greenhouse-grown Pera sweet orange trees infected with *Xylella fastidiosa*		1		6,408	0.04876
		Leaves from 8-month old Pera sweet orange trees, 30 days after inoculation (dai) with *X. fastidiosa*		2		9,243	0.05040
		Peel of fruits, 2.5 cm in diameter, from greenhouse-grown Pera sweet orange trees		1		8,760	0.02858
		Peel of fruits, 7 cm in diameter, from greenhouse-grown Pera sweet orange trees		2		8,195	0.06124
		Peel of fruits, 8 cm in diameter, from greenhouse-grown Pera sweet orange trees		1		7,330	0.03920
		Peel of fruits, 9 cm in diameter, from greenhouse-grown Pera sweet orange trees		2		8,653	0.05618
CsPDR13							
	Peel of fruits, 9 cm in diameter, from greenhouse-grown plant	Peel of fruits, 2.5 cm in diameter, from greenhouse-grown plant	1	5	8,653	8,760	0.04833
		Peel of fruits, 7 cm in diameter, from greenhouse-grown plant		5		8,195	0.04081

1 - p value for the Audic and Claverie test.

environmental conditions, including fruits, *Xylella*-infected and healthy leaves, grown either under greenhouse or in the field. Although CsPDR12 shares high similarity of deduced amino acid sequences with NtPDR1, a microbe-elicited ABC transporter in tobacco and the first plant PDR to be induced by a microbial elicitor (Sasabe *et al.*, 2002; Ducos *et al.*, 2005), the role played by this putative protein in citrus is still unclear, especially because CsPDR12 includes transcripts from various conditions. However, remarkably, the highest contribution in number of transcripts for this cluster originated from adult plants of Olimpia, a clonal variation of Pera sweet orange that is regarded as a productive plant in the Northern region (warm climate) of the citrus industry in São Paulo State, the largest area of citrus production in Brazil. In contrast, the clone shows a very low yield in the South (temperate weather) of the same state. The relationship, if any, between CsPDR12, Olimpia sweet orange and environmental conditions is still not clear and merits more studies.

The PDR-candidate CsPDR11 matched the AtPDR11 gene and was constructed with transcripts originating from the following libraries: leaves from 8-month old plants, 30 days after inoculation (dai) with *Xylella fastidiosa*, leaves from greenhouse-grown plants inoculated with CiLV, and peel of fruits 2.5 cm and 8 cm in diameter, both from greenhouse-grown plants. Although there is no experimental information regarding AtPDR11 function, its similarity with another PDR-homologue (OspDR9) found in rice and induced by heavy metals such as zinc (Zn), nickel (Ni) and cobalt (Co) (Moons, 2003) supports a hypothesis that it might be a general defense protein. In fact, the same similarity was found for CsPDR4, a PDR-like sequence found in *C. sinensis* libraries, which showed best match with AtPDR4. The cluster included transcripts from diverse libraries of plants grown under greenhouse conditions: leaves from trees infected with *X. fastidiosa*, the second pair of leaves from seedling and fruits.

The clusters CrPDR11 and CrPDR12, found in Ponkan mandarin trees (*C. reticulata*), matched the NtPDR2 gene from *N. tabacum*. CrPDR12 is a cluster constructed with transcripts from leaves collected from plants 30 dai with *X. fastidiosa*, grown in phytotron and a transcript from peel of fruits 1 cm in diameter, from field-grown trees. On the other hand, the singlet CrPDR11 was formed from leaves of a healthy Ponkan mandarin grown in phytotron. Although there is no function identified for NtPDR2 to date, its high identity with NtPDR1 might indicate a participation in stress response (Sasabe *et al.*, 2002). As a matter of fact, the putative PDR protein NtPDR2 lacks the first conserved domains containing ABC signature and Walker A and B motifs.

Transcripts from three *C. reticulata* libraries (leaves from healthy plants grown in phytotron, leaves collected from plant 60 dai with *X. fastidiosa*, grown in phytotron, and peel of fruits 1 cm in diameter from field-grown trees) rendered the cluster CrPDR13 and matched the AtPDR12 gene, which is phylogenetically close to GmPDR12, a salicylic acid-induced gene from soybean cell suspensions (Eichhorn *et al.*, 2006). However, the transcript expression

Table 3 - List of citrus cDNA libraries and PDR homologues (normalized for 10,000 ESTs per library).

Species[1]	Library	Number of ESTs per PDR homologue								
		CsPDR12	CsPDR11	CsPDR4	CsPDR13	CrPDR12	CrPDR11	CrPDR13	PtPDR9	ClPDR6
CS	Leaves from greenhouse-grown trees	1.05								
CS	Leaves from greenhouse-grown trees infected with *Xylella fastidiosa*	1.56		1.56						
CS	Leaves from 8-month old plants, 30 days after inoculation (dai) with *X. fastidiosa*	2.16	2.16							
CS	Leaves from greenhouse-grown plants inoculated with CiLV		2.06							
CS	Second pair of leaves from greenhouse-grown seedling			2.03						
CS	Bark from greenhouse-grown plants									
CS	Peel of fruits, 1 cm in diameter, from greenhouse-grown plant									
CS	Peel of fruits, 2.5 cm in diameter, from greenhouse-grown plant	1.14	1.14		5.7					
CS	Peel of fruits, 5 cm in diameter, from greenhouse-grown plant									
CS	Peel of fruits, 7 cm in diameter, from greenhouse-grown plant	2.44			6.1					
CS	Peel of fruits, 8 cm in diameter, from greenhouse-grown plant	1.36	1.36							
CS	Peel of fruits, 9 cm in diameter, from greenhouse-grown plant	2.31		1.16	1.16					
CS	Flowers at different developmental stages									
CS	Leaves from field-grown trees									
CS[2]	Leaves from field-grown trees	11.52								
CR	Leaves from healthy plant, grown in phytotron						1.18	2.36		
CR	Leaves from plants 30 dai with *X. fastidiosa*, grown in phytotron					2.37				
CR	Leaves from plants 60 dai with *X. fastidiosa*, grown in phytotron							2.35		
CR	Peel of fruits, 1 cm in diameter, from field-grown trees					1.07		2.14		
CR	Peel of fruits, 2.5 cm in diameter, from field-grown trees									
CR	Peel of fruits, 5 cm in diameter, from field-grown trees									
PT	Leaves from greenhouse-grown plants									
PT	Leaves from plants inoculated with CTV, grown in greenhouse								1.12	
PT	Bark from greenhouse-grown plants									
PT	Bark from plants inoculated with *Phytophthora parasitica*, grown in greenhouse									
PT	Seeds from fruits at different developmental stages									
CA	Leaves from field-grown plants									
CG	Leaves from greenhouse-grown plants									
LT	Leaves from greenhouse-grown plants									
CL	Roots from seedlings grown in nutritive solution									5.52
CL	Roots from seedlings after drought stress									3.57
CM	Leaves collected from greenhouse-grown plants 2 dai with CiLV									
TS	Bark from greenhouse-grown plants									
Total		23.54	6.72	4.75	12.96	3.44	1.18	6.85	1.12	9.09

1- CS - *Citrus sinensis* var. Pera, CR - *C. reticulata* var. Ponkan, PT - *Poncirus trifoliata*, CA - *C. aurantium*, CG - *C. aurantifolia*, LT - *C. latifolia*, CL - *C. limonia*, CM - *C. limettioides*, TS - *C. sunki*. 2- *C. sinensis* var. Olimpia.

analysis indicates no differences between "healthy" and *Xylella*-infected libraries.

The AtPDR12 and its homologues are the experimentally best-studied PDR proteins from plants to date (Campbell *et al.*, 2003; Lee *et al.*, 2005; Crouzet *et al.*, 2006). AtPDR12 expression in *Arabidopsis* is induced by fungal- and bacterial-pathogen inoculation (*Alternaria brassicicola* and *Sclerotinia sclerotium* and the bacterium *Pseudomonas syringae* pv. tomato) or defense-related signal molecules like methyl jasmonate, ethylene, salicylic acid and jasmonic acid (Campbell *et al.*, 2003).

Although little is known about the substrates that are transported by this protein, in ABC transporter AtPDR12-knockout *A. thaliana* plants, a higher (and toxic) content of lead was identified in the roots, while wild plants showed a lead detoxification mechanism (Lee *et al.*, 2005). It is suggested that, in contrast to fungal PDR transporters, which show a large substrate spectrum, plant PDR transporters might have much higher substrate specificity (van den Brûle *et al.*, 2002).

Interestingly, the cluster PtPDR9, which matched the NtPDR3 gene, originated from a single transcript from a li-

brary constructed from leaves of *P. trifoliata* plants inoculated with CTV and grown in greenhouse. As a matter of fact, *P. trifoliata* is a relative of *Citrus* that shows a putative single dominant gene (Ctv) that is highly associated to the resistance against CTV (Bernet *et al.*, 2004; Rai, 2006). In tobacco, NtPDR3 expression was shown to be induced by iron deficiency and plant defense-related compounds, such as salicylic acid (SA) and methyl jasmonate (MJ) (Ducos *et al.*, 2005). Moreover, in other plants, PDR transporters were also found to be responsive to these chemical signals: AtPDR12 (SA), NtPDR12 (MJ), NpABC1 (MJ), and GmPDR12 (SA and MJ) (Sasabe *et al.*, 2002; Campbell *et al.*, 2003; Grec *et al.*, 2003; Eichhorn *et al.*, 2006). Both SA and MJ molecules have been shown to play a role as chemical signal compounds during plant defense against pathogens, by regulating the signaling pathways associated with defense responses (Dong, 1998; Reymond and Farmer, 1998). In fact, these PDR transporters could function by transporting substrates that are excreted onto the plant surface and act as antimicrobial agents (Jasinski *et al.*, 2003; van den Brûle and Smart, 2002).

ClPDR6 matched the AtPDR6 gene from *A. thaliana*, to which no functional role has been assigned so far. The cluster was formed by transcripts originating from roots of Rangpur lime (*C. limonia*) seedlings grown either in nutritive solution or under drought stress. *C. limonia* is the main rootstock used in the Brazilian citrus industry, especially due to its high tolerance to water deficit. However, there is no clear evidence of PDR6 participation during response to stress or differential expression of these libraries.

These analyses provide critical information to help identify new target genes for further investigation and functional analysis, which will lead to a better understanding of the mechanisms that govern a number of cellular processes, including stress tolerance, and may open up new ways to improve the agronomic properties of citrus plants. Therefore, further studies are necessary to gain a better understanding of the substrates of these citrus PDR-like proteins under specific conditions.

Finally, taking these data together we conclude that at least nine putative PDR proteins may be present in citrus tissues. Given the current paucity of knowledge on PDR proteins activity in citrus, additional analyses will be conducted to clarify the roles played by them in plant cell physiology.

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Internet Resources

CitEST database, http://citest.centrodecitricultura.br/.

PDR members of *A. thaliana*, http://www.arabidopsis.org/info/genefamily/pdr.html.

Prediction of transmembrane helices and topology of proteins (HMMTOP), http://www.enzim.hu/hmmtop/.

GenBank BLAST, http://www.ncbi.nlm.nih.gov/blast.

Alignment of two sequences (GenBank), http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi.

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