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Research Article

Differential expression of genes identified from *Poncirus trifoliata* tissue inoculated with CTV through EST analysis and *in silico* hybridization

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Abstract

Citrus is the most important fruit crop in Brazil and *Citrus tristeza virus* (CTV) is considered one of the most important pathogens of citrus. Most citrus species and varieties are susceptible to CTV infection. However, *Poncirus trifoliata*, a close relative of citrus, is resistant to the virus. In order to better understand the responses of citrus plants to the infection of CTV, we constructed expressed sequence tag (EST) libraries with tissues collected from *Poncirus trifoliata* plants, inoculated or not with *Citrus tristeza virus* at 90 days after inoculation, grafted on Rangpur lime rootstocks. We generated 17,867 sequence tags from *Poncirus trifoliata* inoculated (8,926 reads) and not (8,941 reads) with a severe CTV isolate. A total of 2,782 TCs (Tentative Consensi sequences) were obtained using both cDNA libraries in a single clusterization procedure. By the *in silico* hybridization approach, 289 TCs were identified as differentially expressed in the two libraries. A total of 121 TCs were found to be overexpressed in plants infected with CTV and were grouped in 12 primary functional categories. The majority of them were associated with metabolism and defense response. Some others were related to lignin, ethylene biosynthesis and PR proteins. In general, the differentially expressed transcripts seem to be somehow involved in secondary plant response to CTV infection.

Key words: citrus, disease resistance, *Citrus tristeza virus*, biotic stress.

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Introduction

Citrus is the most important fruit crop in Brazil. Among all its pathogens, *Citrus tristeza virus* (CTV) is considered one of the most important ones. This virus is an aphid-transmitted, positive sense, single-stranded RNA member of the Closteroviridae. Most citrus species and varieties are susceptible to CTV infection. However, *Poncirus trifoliata*, a close relative of citrus, is resistant to CTV. There are other citrus relatives like *Severinia buxifolia* (Poir) Ten and *Swinglea glutinosa* (Blanco) Merr that are also resistant to infection by most CTV strains (Albiach-Marti *et al.*, 2004).

CTV resistance seems to be a single gene dominant trait (*Ctv*, Gmitter *et al.*, 1996), but according to Albiach-Marti *et al.* (2004) this resistance is modified by a second gene (*Ctm*) (Mestre *et al.*, 1997). Moreover, plants which are heterozygous for *Ctv* are resistant to most CTV isolates, but may allow local movement in the absence of *Ctm* (Mestre *et al.*, 1997). It has been suggested that *Ctv* resistance is constitutive, preventing some early step in the infection process (Albiach-Marti *et al.*, 2004).

To complete its cycle, the virus undergoes a multistep process that includes entry into plant cells, uncoating of nucleic acid, translation of viral proteins, replication of viral nucleic acid, assembly of progeny virion, cell-to-cell movement, systemic movement and plant-to-plant transmission (Kang *et al.*, 2005).

Albiach-Marti *et al.* (2004) showed that a range of biologically and genetically distinct CTV isolates were able to replicate and form infectious viral particles in protoplasts obtained from *P. trifoliata* and *Citrus* x *Poncirus* hybrid plants (which contained the *Ctv* resistance gene) and in protoplasts from *S. buxifolia* and *S. glutinosa* plants. According to the authors, this suggests that the *Poncirus* resistance affects a viral step subsequent to replication and assembly of viral particles. Nevertheless, it should be noted that these data do not eliminate the possibility that resistance is due to a hypersensitive response (HR) since it could

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happen without visible necrosis and also, replication in protoplasts is possible in incompatible interactions (Albiach-Marti *et al.*, 2004).

In order to better understand the responses of *P. trifoliata* plants to the infection of CTV, we constructed expressed sequence tag (EST) libraries from *Poncirus trifoliata* plants inoculated or not with a severe *Citrus tristeza virus* (CTV) grafted on Rangpur lime rootstocks. The reads were analyzed using bioinformatics tools to generate a picture of the defense response of *Poncirus trifoliata* to CTV infection.

Material and Methods

Plant material and construction of libraries

Buds of Pêra sweet orange (*Citrus sinensis* Osbeck) infected with a severe isolate of CTV and free of virus were grafted on *Poncirus trifoliata* cv. Rubidoux previously grafted on Rangpur lime (*Citrus limonia* Osbeck) rootstocks. The infected and non-infected buds of sweet orange were left as a continuous source of inoculum and control, respectively. Approximately 1-3g of the leaf tissue from *Poncirus trifoliata* were collected from each treatment, 90 days after inoculation. Plants were kept under greenhouse conditions.

The libraries were prepared from mRNA isolated from leaves collected from both non-inoculated and CTV inoculated specimens at the same developmental stage. RNA extraction, cDNA and sequencing were done according to Targon *et al.* (in this issue).

EST sequencing and data analysis

Sequencing was carried out using the Big Dye Terminator v.3 Kit as described by the manufacturer (Perkin-Elmer). Products were separated by capillary electrophoresis using an ABI 3700 sequencer (Applied Biosystems).

In silico hibridization and functional annotation

For comparison of the libraries, we performed an *in silico* hybridization analysis. The *in silico* hybridization methodology included a clusterization of all transcripts from both libraries, using the CAP3 tool (Huang and Madan, 1999), with the default parameters. Furthermore, for each tentative consensus (TC) sequence, the relative abundance of transcripts was calculated, using a correction factor of 10,000 for normalization. The differential *in silico* expression was then evaluated using statistic verification (Audic and Claverie, 1997). We considered differential expression as the possibility of a random transcript abundance distribution, for a given TC, to be equal to or lower than 5% (P-value ≤ 0.05). Automatic categorization over the tentative consensi (TC) was performed as well, using the Munich Center for Proteins and Sequences Functional Categories (MIPS) v. 1.3 (http://mips.gsf.de). Comparative genomics was carried out through TC comparison against the GenBank protein database, using the Blastall implementation of the BLAST algorithm (Altschul *et al.*, 1997). More details on the bioinformatics analyses can be found at Reis *et al.* (in this issue).

Results and Discussion

Differential expression and functional annotation

We generated a total of 17,867 ESTs from *Poncirus trifoliata,* with 8,926 reads coming from CTV inoculated and 8,941 reads from the control source. A total of 2,782 TCs (Tentative Consensi sequences) were obtained using both cDNA libraries in a single clusterization procedure. Through the *in silico* hybridization approach, 289 TCs were identified as differentially expressed in the two libraries. A total of 121 TCs out of these were found to be putatively overexpressed in plants infected with CTV (Table 1), while 168 TCs were potentially underexpressed. The 121 overexpressed TCs were grouped in 12 primary functional categories and the 168 underexpressed TCs were grouped in 16 categories (data not shown). An overview of the functional categorization of the putative overexpressed TCs with known or predicted functions is presented in Figure 1.

The largest set of genes (29.75%) was assigned to the metabolism category, while genes involved in transcription constituted the smallest group, comprising less than 2.47% of the genes. Genes involved in signal transduction and protein destination/storage were 4.95%. Genes implicated in stress/defense response constituted 15.70% of the infected cDNA collection. Proteins with unknown functions corresponded to 21.48%. They were similar to already sequenced plant genes of unknown function and might be an additional source of genes participating in the expression of citrus in response to biotic stresses.

General responses to the *Citrus tristeza virus*

Synthesis of phenylpropanoids

We identified several genes involved in the biosynthesis of defense-related secondary metabolites (phenylpropanoids and phytoalexins). Together, these metabolites function in a variety of defense-related processes, including the induction of wound response, antimicrobial and antifungal defense, and antioxidant defense (Verica *et al.*, 2004).

In the present work, we identified six putative proteins encoded by the overexpressed genes related to phenylpropanoids: 4-coumarate-CoA ligase, anthralinate N-benzoyltransferase, flavonol synthase, cinnamoyl CoA-redutase, caffeic acid-O-methyltransferase and anthocyanin 5-aromatic acyltransferase. The 4-coumarate-CoA ligases belong to a group of enzymes necessary for maintaining a continuous metabolic flux for the biosynthesis of plant phenylpropanoids, such as lignin and flavonoids, which are essential for the survival of plants. Thus, hydro-

Table 1 - Functional categories of genes detected as overexpressed in *Poncirus trifoliata* infected with *Citrus tristeza virus* at 90 days after inoculation.

Functional category	Best blast match	Organism	Accession number	% identity	e-value
01. Metabolism					
	Alanine-glyoxylate aminotransferase	*Arabidopsis thaliana*	AT2G13360	86	0.0
	Enolase	*Ricinus communis*	CAA82232.1	92	e-179
	S-adenosylmethionine (adoMetDC2)	*Arabidopsis thaliana*	AT5G15950	67	e-125
	Neutral invertase	*Arabidopsis thaliana*	AT1G56560	74	0.0
	4-Coumarate -CoA ligase	*Arabidopsis thaliana*	AT1G65060	63	3e-78
	Phosphoribulokinase	*Arabidopsis thaliana*	AAN15338	92	e-108
	Proline iminopeptidase	*Arabidopsis thaliana*	AT2G14260	83	1e-71
	Phosphoribosylglycinamide Formyltransferase 2	*Bordetella parapertussis*	NP886172.1	85	3e-13
	Thiazole biosynthetic enzyme precursor	*Citrus sinensis*	cab05370.1	95	e-118
	Thiazole biosynthetic enzyme precursor	*Citrus sinensis*	cab05370.1	84	e-156
	ACC Synthase	*Arabidopsis thaliana*	AT5G51690	67	e-129
	ACC Oxidase	*Arabidopsis thaliana*	AT1G05010	75	e-148
	Ethylene forming enzyme (ACO)	*Arabidopsis thaliana*	AAM613662.1	67	e-85
	Anthranilate N-Benzoyltransferase	*Arabidopsis thaliana*	AT5G01210	65	2e-82
	Flavonol synthase	*Oryza sativa*	XP-482984.1	45	2e-68
	Cinnamoyl CoA reductase	*Arabidopsis thaliana*	AT2G02400	67	e-129
	Caffeic acid O-Methyltransferase	*Arabidopsis thaliana*	AT3G53140	74	e-155
	Xyloglucan endo-transglycosylase	*Arabidopsis thaliana*	AT1G14720	69	e-135
	Fructose-biphosphate aldolase	*Arabidopsis thaliana*	AT4G26530	75	1e-22
	Anthocyanin5-aromatic acyltransferase	*Arabidopsis thaliana*	AT3G29590	39	3e-72
	UMP synthase	*Arabidopsis thaliana*	AT3G54470	85	e-111
	Putative glyoxysomal malate dehydrogenase	*Arabidopsis thaliana*	AT2G36790	50	4e-61
	Cobalamin Synthase	*Arabidopsis thaliana*	NP-173974	77	6e-82
	Methylthioadenosine/S-adenosyl homocysteine nucleosidase	*Oryza sativa*	NP-910292.1	71	4e-84
	Phosphate/phosphoenolpyruvate	*Arabidopsis thaliana*	AT5G33320	47	2e-35
	Phosphoenolpyruvate carboxylase	*Glycine max*	AAS67005.1	86	0.0
	Putative Prolyl endopeptidase	*Arabidopsis thaliana*	AAL86330.1	72	0.0
	Sucrose synthase	*Citrus unshiu*	BAA88904.1	98	e-117
	Sedoheptulose-Biphosphatase	*Arabidopsis thaliana*	AT3G55800	82	0.0
	3-ketoacyl-CoA thiolase	*Arabidopsis thaliana*	AT2G33150	89	e-174
	Inositol 1,3,4-Triphosphate	*Arabidopsis thaliana*	AT4G08170	90	1e-75
	Flavonol synthase	*Oryza sativa*	XP-482984.1	45	2e-68
	Sucrose synthase	*Citrus unshiu*	BAA88904.1	98	e-117
	UDP-Glucoyl transferase	*Arabidopsis thaliana*	AT2G36790	50	4e-61
	UDP-Glucose Glucosyltransferase	*Rhodiola sachalinensis*	AAS55083.1	56	e-110
	Glycosyltransferase NTGT5a	*Nicotiana tabacum*	BAD93689.1	65	1e-87
02. Energy					
	Chlorophyll A/B-binding protein	*Arabidopsis thaliana*	AT4G10340	83	5e-96
	Photosystem II Polypeptide	*Arabidopsis thaliana*	AAM20194.1	82	2e-57
	Ribulose 1,5-Biphosphate Carboxylase	*Manihot esculenta*	AAF06101.1	81	1e-42
	NADP-Dependent Glyceraldehyde-3-phosphate Dehydrogenase	*Arabidopsis thaliana*	AT2G24270	90	0.0
	Protein I Photosystem II oxygen-evolving	*Arabidopsis thaliana*	AT3G50820	79	e-155
04. Transcription					
	Ein3-like	*Cucumis melo*	BAB64345.1	55	5e-78
	RNA polymerase Sigma 70	*Arabidopsis thaliana*	AT2G36990	58	1e-44
	Homeobox-leucine zipper protein HAT5	*Arabidopsis thaliana*	AT3G01470	39	1e-44

Table 1 (cont.)

Functional category	Best blast match	Organism	Accession number	% identity	e-value
05. Protein synthesis					
	Translation initiation factor eIF-2 Beta chain	*Arabidopsis thaliana*	AT5G20920	76	e-113
	30 Ribosomal protein S5	*Arabidopsis thaliana*	AT2G33800	69	e-109
	60S Ribosomal protein L1	*Arabidopsis thaliana*	AT3G09630	87	e-104
	50S Ribosomal protein L13	*Arabidopsis thaliana*	AAD30573.1	66	3e-90
06. Protein fate (folding, modification, destination)					
	Hydroxypyruvate reductase (HPR)	*Arabidopsis thaliana*	AT1G68010	90	0.0
	FtsH chloroplast protease	*Arabidopsis thaliana*	AT2G30950	86	0.0
	Expressed protein	*Arabidopsis thaliana*	NP 178048.1	33	3e-21
	Ketoacyl-CoA	*Arabidopsis thaliana*	AT2G33150	89	e-174
	ATP-dependent Clp protease	*Arabidopsis thaliana*	AT5G50920	92	0.0
	Transformer-SR ribonucleoprotein putative	*Arabidopsis thaliana*	AT1G07350	51	1e-40
08. Cellular transport and transport mechanism					
	ABC transporter	*Arabidopsis thaliana*	NP-188762.2	78	0.0
	ABC Transporter protein 1-Like	*Arabidopsis thaliana*	AT5G64840	86	e-132
	Peroximal membrane related protein	*Arabidopsis thaliana*	NP564615.1	87	7e-84
	Rieske iron-sulfur protein	*Nicotiana tabacum*	AAA20832.1	73	1e-94
	Sulfate transporter 2	*Lycopersicon esculentum*	AAK27688.1	80	0.0
	ADP-rybosylation factor-like protein	*Arabidopsis thaliana*	AT3G62290	97	e-100
10. Cellular communication/Signal transduction mechanism					
	BIS (5-Adenosyl triphosphatase; histidine triad)	*Arabidopsis thaliana*	AT5G58240	67	3e-55
	Rab-type small GTP-binding	*Arabidopsis thaliana*	AT5G45750	100	7e-12
	Ras-related GTP-binding	*Arabidopsis thaliana*	AT5G45130	56	8e-99
	CONSTAINS- like- B Box Zinc Finger	*Arabidopsis thaliana*	AT5G57660	54	e-103
	Zinc Finger	*Arabidopsis thaliana*	NP-197938.2	72	e-133
	14-3-3 Protein GF14	*Arabidopsis thaliana*	AT5G65430	86	e-117
11. Cell rescue, defense and virulence					
	NADPH Oxydase	*Arabidopsis thaliana*	AT5G49730	59	3e-59
	Germin-like protein	*Arabidopsis thaliana*	AT1G72610	68	6e-76
	Papain-like Cysteine proteinase	*Gossypium hirsuntum*	CAE54306.1	75	e-113
	Chitinase	*Citrus sinensis*	CAA938471	89	e-107
	Ankyrin	*Vitis aestivalis*	AAQ96339.1	66	4e-85
	NADPH-Ferrihemoprotein reductase (ATR2)	*Arabidopsis thaliana*	AT4G30210	81	0.0
	N-Rich protein	*Glycine max*	CAI44933.1	60	e-110
	SRG1	*Arabidopsis thaliana*	AT1G17020	56	e-116
	Miraculin-like protein 2	*Citrus paradisi*	AAG38518.1	44	7e-42
	Miraculin-like protein 3	*Citrus paradisi*	AAG38519.1	39	4e-31
	DNAJ	*Arabidopsis thaliana*	AT3G44110	84	0.0
	High molecular weight heat shock protein	*Malus x domestica*	AAF34134	93	e-147
	TCP1-chaperonin cofactor A	*Arabidopsis thaliana*	AAM63030.1	82	6e-46
	Cytochrome P450	*Arabidopsis thaliana*	AT3G52970	40	4e-50
	Peroxidase prxr1	*Arabidopsis thaliana*	AT4G21960	83	e-159
	Type I proteinase inhibitor-like protein	*Citrus paradisi*	AAN76363.1	97	4e-65
	Resistance protein	*Arabidopsis thaliana*	AT5G52780	45	3e-29
	Putative auxin-induced protein	*Arabidopsis thaliana*	AT1G23740	73	e-125
	LLS1-like protein	*Arabidopsis thaliana*	AAR05798.1	61	e-140

Table 1 (cont.)

Functional category	Best blast match	Organism	Accession number	% identity	e-value
40. Subcellular localization					
	CP12 protein	*Arabidopsis thaliana*	AT3G62410	56	3e-31
	Coatomer complex subunit	*Arabidopsis thaliana*	AT1G52360	78	e-156
63. Protein with binding function or cofactor requirement					
	NADP/NADP binding	*Arabidopsis thaliana*	AT1G42970	85	0.0
	DNA binding protein	*Arabidopsis thaliana*	AAN13013.1	60	e-156
	Arabidopsis dynamin like protein ADL2	*Arabidopsis thaliana*	AT4G33650	59	3e-34
	RNA binding protein	*Arabidopsis thaliana*	AT1G09340	78	e-143
67. Transport facilitation					
	Aquaporin	*Arabidopsis thaliana*	AT3G01280	73	e-115
	Aquaporin	*Arabidopsis thaliana*	AT2G45960	89	e-150
	Aquaporin	*Arabidopsis thaliana*	AT2G36830	80	e-115
	Aldo/Keto reductase	*Fragaria x ananassa*	AAV28174.1	61	e-115
99. Unclassified protein					
		Arabidopsis thaliana	AAM63493.1	59	1e-39
		Arabidopsis thaliana	AT3G09050	63	1e-86
		Arabidopsis thaliana	AT1G44920	69	5e-80
		Arabidopsis thaliana	AT2G46820	64	2e-57
		Arabidopsis thaliana	AT2G39570	59	1e-11
		Arabidopsis thaliana	AT3G58900	52	3e-07
		Arabidopsis thaliana	AT2G16350	38	7e-22
		Arabidopsis thaliana	AT4G11570	77	3e-16
		Arabidopsis thaliana	AT3G52740	52	2e-25
		Arabidopsis thaliana	AT1G48090	74	e-119
		Arabidopsis thaliana	AT1G63610	72	e-129
		Arabidopsis thaliana	AT3G57890	71	0.0
		Arabidopsis thaliana	AT2G03440	50	4e-26
		Arabidopsis thaliana	AT1G74640	81	e-130
		Arabidopsis thaliana	AT4G32020	41	7e-25
		Arabidopsis thaliana	AT2G44310	80	5e-61
		Arabidopsis thaliana	AT3G07760	91	6e-64
		Arabidopsis thaliana	AT1G15340	47	9e-54
		Arabidopsis thaliana	AT2G35330	69	1e-64
		Arabidopsis thaliana	AT3G56360	42	1e-40
		Arabidopsis thaliana	AT3G22850	75	e-110
		Arabidopsis thaliana	AT1G09930	58	e-112
		Arabidopsis thaliana	AT5G53450	66	0.0
		Arabidopsis thaliana	AT3G21360	74	e-146
		Arabidopsis thaliana	AT5G23950	43	1e-49
		Arabidopsis thaliana	AT3G06190	74	e-108

xycinnamoyl-CoA:shikimate /quinate hydroxycinnamoyl transferase seems to control the biosynthesis and turnover of major plant phenolic compounds such as lignin and chlorogenic acid. Benzoyl-CoA:anthranilate N-benzoyl-transferase catalyzes the first committed reaction of phyto-alexin biosynthesis in carnation (*Dianthus caryophyllus* L.) (Reinhard and Matern, 1989).

Only a few studies have demonstrated the antiviral activity of phenylpropanoids against plant viruses (Chong *et al.*, 2002). However, a range of flavonoids inhibit the infectivity of *Tobacco mosaic virus* (TMV) (French *et al.*, 1991). Up-regulation of the flavonol synthase encoding gene reported here may be involved in such a resistance mechanism in *P. trifoliata* against CTV.

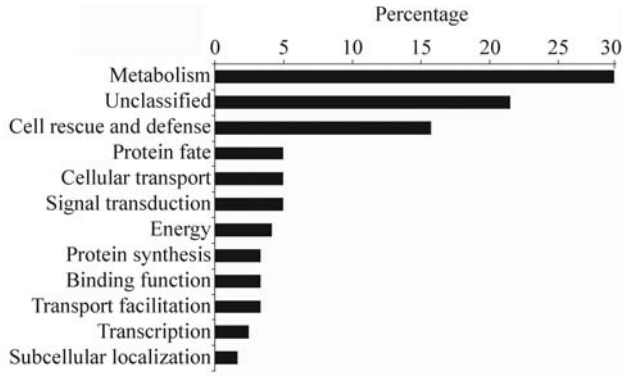

Figure 1 - Functional classification following MIPS categories (Munich Information Center for Protein Sequences) of expressed sequence tags (ESTs), identified as overexpressed in the library of CTV inoculated plants.

Cell wall changes

Cell wall reinforcement and thickening are associated with plant defense during resistance responses. In this study, we found two TCs (cinnamoyl-CoA reductase and caffeic acid-O-methyltransferase) whose expression is associated with lignification in several dicot species (Ye, 1997; Ye *et al.*, 2001). Lignin is a complex phenolic polymer that reinforces the walls of certain cells in higher plants. Such reinforcement is an effective defense response against infection by pathogens (Kawasaki *et al.*, 2006). In addition, many antimicrobial substances such as phytoalexins are known to be produced by the monolignol synthetic pathways. It is therefore likely that lignin and lignin-related compounds with antimicrobial activities cooperatively play important roles in disease resistance of various plant species. Lignin synthesis was induced in soybean leaves inoculated with *Soybean mosaic virus* (SMV) (Hajimorad and Hill, 2001).

According to Jaeck *et al.* (1992) the regulation of an enzyme involved in lignin biosynthesis, an O-methyltransferase occurred during the hypersensitive reaction of tobacco in interactions with TMV.

Phytohormones

In the present work we also identified four TCs (S-adenosylmethionine adoMetDC2) (SAM), ACC synthase, ACC oxidase and ethylene forming enzyme (ACO) whose expression is putatively overexpressed under conditions inducing ethylene biosynthesis (Gomez-Gomez and Carrasco, 1998). SAM serves as a precursor of the plant hormone ethylene, implicated in the control of numerous developmental processes (Kende, 1993). In a cDNA library, prepared from leaves of TMV-infected tobacco after TMV infection and subsequent recognition of the pathogen by the host, ethylene is produced by the conversion of S-adenosyl-L-methionine (SAM) into ACC. ACC is then converted into ethylene, carbon dioxide and cyanide. Ethylene production generates a molecular and genetic cascade of responses that lead to the induction of host defense-related genes (Knoester *et al.*, 1995). In the present work, the up-regulation of ACC enzymes suggests a possible TMV-like interaction and, thus, indicates that ethylene participates in the response to CTV infection.

Additionally, a TC coding for a putative EIN3-like protein (an important component in ethylene signal transduction pathway) was identified, corroborating the possible participation of ethylene in the plant response to CTV infection.

Defense-related genes

The third largest functional category (accounting for 15.70% of the differentially expressed genes) was cell rescue, defense, cell death and ageing. This group includes putative homologs of ankyrin, NADPH oxydase, germin, papain-like cysteine proteinase, chitinase, NADPH-ferrihemoprotein reductase (ATR2), N-rich protein, SRG1, miraculin-like protein 2, miraculin-like protein 3, DNAJ, TCP1-chaperonin cofactor A, cytochrome P450, peroxidase prxr1 PR9, type I proteinase inhibitor-like protein, resistance protein, putative auxin-induced protein and LLS1-like protein.

A N-rich protein was found overexpressed in CTV inoculated plants in the present work. According to Ludwig and Tenhaken (2001), the NRP gene appears to be a new marker in early responses in plant disease resistance. The protein is located in the cell wall, with a very high content of asparagines and was, therefore, termed N-rich protein (NRP). The NRP-gene is not directly induced by salicylic acid or hydrogen peroxide, indicating a distinct and specific signal transduction pathway which is only activated during programmed cell death.

One of the putative TCs related to cell defense showed similarity to LLS1 (Lethal leaf spot-1) that has a role in cell death-suppression. LLS1 may act to prevent reactive oxidative species formation or serve to remove a cell death mediator to maintain chloroplast integrity and cell survival. Yang *et al.* (2004) demonstrated that the LLS1 protein is present constitutively in all photosynthetic plant tissues and that a transient increase in Lls1 gene expression by about 50-fold upon physical wounding of maize leaves indicates that the function of Lls1 is regulated in response to stress.

We also found genes encoding miraculin-like protein 2 and miraculin-like protein 3 of *Citrus paradise* in the CTV infected libraries. In *Citrus jambhiri,* two distinct transcripts of miraculin-like proteins accumulated to higher levels in leaves after wounding, inoculation with conidia of *Alternaria alternata*, or treatment with methyl jasmonate vapors (Tsukuda *et al.*, 2006). Stress-inducible genes such as pathogenesis-related class Chitinase (PR3), PR10 (SGR1), Peroxidase prxpr1 (PR9) and a germin-like protein (PR16) were also observed as potentially overexpressed transcripts in CTV inoculated plants.

An important common feature of most PRs is their antifungal effect. Some PR also exhibit antibacterial, insec-

ticidal, nematicidal and, as recently shown, anti-viral action. PR-10 induced in hot pepper by incompatible interactions with TMV pathotype (TMV-Po) and *Xanthomonas campestris* was shown to function as a ribonuclease. A hot pepper (*Capsicum annuum*) cDNA clone encoding pathogenesis-related protein 10 (CaPR-10) was isolated by differential screening of a cDNA library prepared from pepper leaves inoculated with TMV-Po (Park *et al.*, 2004). The inoculation and subsequent phosphorylation of CaPR-10 increased its ribonucleolytic activity to cleave invading viral RNAs, and this activity should be important to its antiviral pathway during viral attack *in vivo*. In the present work, one TC was *SRG1*, a gene of unknown function that is a member of the PR-10 family (Truesdell and Dickman, 1997) that was also represented in ESTs libraries from cacao leaves treated with inducers of defense response like methyl jasmonate/ethylene (Verica *et al.*, 2004). Xu *et al.* (2003) showed for the first time that multiple defense responses are specifically induced in *Cucumber mosaic virus* (CMV) and D satRNA (CMV/D satRNA)-infected tomato plants, but not in mock-inoculated or CMV-infected plants. These responses include callus deposition and hydrogen peroxide accumulation in infected plants. Furthermore, the transcription of several tomato defense-related genes (*e.g.*, PR-1a1, PR-1b1, PR-2, and PR-10) was activated, and the expression of tomato PR-5 and some abiotic and biotic stress-responsive genes are enhanced.

The germin-like protein (PR16) was also observed as overexpressed in CTV inoculated plants. The multifaceted functionality of PR-15 and PR-16, including a cell wall remodeling ability, can be directed against pathogens and may have protective role (Park *et al.*, 2004). Germins and germin-like proteins (GLPs) have been classified as PR-15 and PR-16. PR-16 has been isolated from hot pepper during the resistance response to bacterial and viral infection (Edreva, 2005). Another overexpressed gene in the presence of the CTV was the peroxidase encoding prxr1. Peroxidase prxr1 is considered a PR9 peroxidase that probably strengthens plant cell walls by catalyzing lignin deposition in reaction to microbial attacks (Scherer *et al.*, 2005).

In *P. trifoliata* plants, *PR-2*, *PR-3*, *PR-15* and *PR-16* gene families were highly expressed within leaves after infection by CTV, whereas no expression was found for other *PR* gene families (Campos *et al.*, in this issue). According to these authors, the differential *PR* gene expression profiles vary between infected and healthy tissues, as well as between different pathogen infections. 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 suppressed in steam bark after *P. parasitica* infection. This indicates that it is also possible that *PR* gene expression profiles may vary among tissues.

BAC clones of Ctv

Citrus tristeza virus (CTV) is an important pathogen of Citrus. A single dominant gene *Ctv*, present in *Poncirus trifoliata,* confers broad spectrum resistance against CTV (Gmitter *et al.*, 1996). BAC clones and their use as anchors localized *Ctv* to a 282,699 bp region, comprising 22 predicted genes (*Ctv*.1 to *Ctv*.22) (Yang *et al.*, 2003). Refinement of genetic maps delimited this gene to a 121 kb region, comprising ten candidate *Ctv* resistance genes. The ten candidate genes were individually cloned in an *Agrobacterium* based binary vector and transformed into three CTV susceptible grapefruit varieties (Rai, 2006). The authors found that two of the candidate R-genes, R-2 and R-3 were exclusively expressed in transgenic plants and in *Poncirus trifoliata*, while five other genes are also expressed in non-transformed Citrus controls.

In the present work, no significant differences could be observed in the expression profiles of the *Ctv* regions (*Ctv.*1 to *Ctv*.22) of *Poncirus trifoliata,* challenged or not with CTV. Homologs of *Ctv.2, Ctv.3, Ctv.10, Ctv.12, Ctv.15, Ctv.20,* and *Ctv.22* were identified in inoculated and non-inoculated *Poncirus* leaf libraries, as well as in other libraries constructed from *Poncirus* bark and seeds (data not shown). Moreover, *Ctv* homologs were also present in libraries constructed from all tissues (leaf, bark, fruit, flower, root, and seed) and all *Citrus* species analyzed in the CitEST database (*C. aurantifolia, C. aurantium, C. latifolia, C. limettioides, C. limonia, C. reticulata,* C. *sinensis,* and *C. sunki*). It is possible that the *Ctv* BAC clone regions may be involved in resistance to CTV in *P. trifoliata*, as suggested by Yang *et al.* (2003) and Rai (2006). Nevertheless, the observation that *Ctv* homologs seem to be expressed in the related genus *Citrus*, including in highly susceptible species such as *C. aurantium*, indicates that they are not a major component in resistance, or that they behave in a very unexpected fashion. The *Ctv* BAC clone regions may not exhibit the characteristics of a typical resistance gene, and it has not been unequivocally shown that they confer resistance to CTV. Hence, further experiments will need to address whether or not the *Ctv* regions play an important role in CTV resistance, and which of them are responsible for the major component of such resistance.

Concluding Remarks

CTV resistance in *P. trifoliata* prevents viral proliferation in plants by an undetermined mechanism, essentially resulting in immunity. Lack of a visual hypersensitive response in inoculated plants or in rootstocks with infected susceptible scions suggests that resistance is associated with the interruption of some step in viral multiplication.

Assuming that CTV resistance is monogenic and dominant (Gmitter *et al.*, 1996), we had expected to find evidence of a differentially expressed resistance gene within the CTV-infected library, yet, we could not identify any typical resistance gene. This may be explained by the fact that the libraries were constructed with tissues collected 90 days after infection. In this case, we probably detected only secondary responses to CTV infection. Alternatively, we

would have to assume an atypical mechanism of resistance that would have to be investigated in further experiments.

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Internet Resources

MIPS Functional Categories (FunCat), http://mips.gsf.de/projects/funcat (August 15, 2006).

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