Identification of Differentially Expressed Genes from *Poncirus trifoliata* Triggered by *Citrus tristeza virus* Inoculation

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ABSTRACT

***Citrus tristeza virus* (CTV) causes great economic losses to the citrus industries worldwide. Over 100 million citrus trees grafted on sour orange rootstock have been lost or became unproductive worldwide because of CTV. Many citrus trees grafted onto sour orange rootstock in Texas, Florida, California, Italy, and México are highly susceptible to CTV. The presence of the aphid *Toxoptera citricida*, an efficient vector of CTV, in south Mexico and Florida is an imminent threat to the citrus industries of Texas and the rest of México. Studies are needed for better understand on the molecular response of resistant citrus species to CTV. By using Differential Display- RT-PCR, the 3'-ends of 11 cDNA clones were obtained from *Poncirus trifoliata* grafted onto Pineapple sweet orange (*Citrus sinensis*) infected with CTV. Full-length sequences of six of the clones were obtained and compared to sequences deposited at the GenBank database. None of the sequences showed similarity to disease resistance genes, however, four of the full length clones showed similarity with genes related to wounding, stress, attack by insects and pathogens.**

RESUMEN

El virus de la tristeza de los cítricos (VTC) ocasiona grandes pérdidas económicas a la industria citrícola en muchas partes del mundo. Mas de 100 millones de cítricos injertados sobre portainjerto de naranjo agrio se han perdido o se han vuelto improductivos debido a este virus. Muchos de los cítricos injertados sobre portainjerto de naranjo agrio en Texas, Florida, California, Italia y México son bastante susceptibles al VTC. La presencia del áfido *Toxoptera citricida*, un vector eficiente del VTC, en el sur de México y Florida es una amenaza inminente a las industrias citrícolas de Texas y del resto de México. Se necesitan estudios para entender mejor la respuesta a nivel molecular de las especies de cítricos resistentes al VTC. Mediante el uso de la técnica de PCR-Transcripcion Inversa-Expresion Diferencial, los extremos 3' de 11 clones de cDNA fueron obtenidos de plantas de *Poncirus trifoliata* injertadas sobre naranjo dulce cultivar Pineapple (*Citrus sinensis*). Se obtuvieron las secuencias completas de seis de los clones y se compararon a las secuencias depositadas en la base de datos del GenBank. Ninguna de las secuencias fue similar a genes de resistencia a enfermedades, sin embargo, cuatro de los clones secuenciados completamente mostraron similaridad con genes relacionados a heridas, estrés, y ataque por insectos y patógenos.

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Citrus tristeza virus (CTV) has been the cause of important economic losses in the citrus industry worldwide (Mestre et al., 1997a; Bar-Joseph et al., 1981). This disease was first reported in South Africa around 1910 where decline of citrus scions grafted onto sour orange (*Citrus aurantium* L. Osbeck) rootstock was noticed (Marloth, 1938; Webber, 1943).

CTV is an aphid-borne, phloem-limited closterovirus and the incidence of the virus is directly related to the presence of the vector and the use of infected budwood for propagation. There are several aphid vectors for this virus; however, the brown citrus aphid, *Toxoptera citricida* Kirk., is the most efficient one. This aphid has been found in most of the citrus producing areas of the world except in Texas, California, certain Mediterranean areas, and some islands (Lee and Rocha-Peña, 1992). It presents a serious threat to the U.S. citrus industry since its arrival in Florida in 1995 (Hardy, 1995).

Sour orange has been used in favor over other rootstocks due to its tolerance to low temperatures and citrus blight (Brlansky et al., 1986), as well as the adaptability to high pH soils, the acceptable product quality (Lee et al., 1987), and its tolerance to *Phytophthora*-induced diseases (Bar-Joseph et al., 1983). Decline-inducing isolates of CTV have killed over 100 million citrus trees grafted on sour orange worldwide (Roistacher, 1993). For this reason, the use of this rootstock

has been decreasing lately, but nearly all citrus in Texas, 15 to 20% of the trees in Florida and California, and almost 100% in Italy and Mexico are still grafted on sour orange. In Florida, the losses in fruit production and trees due to CTV have been estimated in the range of $500 million over the next 20 years (Gottwald et al., 1999).

Resistance to CTV has been found in the citrus relative, trifoliate orange (*Poncirus trifoliata* (L.) Raf.) in a gene contained at the *Ctv* locus, and probably in one other gene (Mestre et al., 1997b). Trifoliate orange has been important in citrus rootstock breeding due to the tolerance to *Phytophthora* and resistance to nematodes and CTV (Bar-Joseph et al., 1989). Furthermore, hybrids between *Citrus* and *Poncirus* are readily made, and several are used as rootstocks due to its resistance to CTV (Garnsey et al., 1987).

To date, no study has been performed on the molecular response of trifoliate orange to a constant challenge from CTV. This research project comprised the comparison of gene expression in trifoliate orange under constant challenge from CTV with unchallenged control plants.

MATERIALS AND METHODS

Plant material. Trifoliate orange buds were grafted onto two similar Pineapple sweet orange (*C. sinensis* L. Osbeck) and maintained in a greenhouse at approximately 25ºC. One of the Pineapple rootstocks was grafted with a Pineapple sweet orange budwood infected with CTV (labeled as 'infected sample'), the other plant was grafted with a virus-free Pineapple sweet orange budwood (labeled as 'non-infected sample'). New growth flushes were harvested to perform enzyme linked immunosorbent assay (ELISA) to test for the virus presence in the rootstock and scion tissues. Branches from the trifoliate orange scion were cut and cleaned with DEPC (diethyl pyrocarbonate) treated water and the epidermal tissue removed using RNase free forceps and scissors and stored at -80ºC for later use.

RNA isolation. Total RNA from infected and non-infected phloem tissue was isolated using the Totally RNA Isolation kit (Ambion, Austin, TX) according to manufacturer's instructions.

Differential display. Differential display RT-PCR was performed according to Liang and Pardee (1992) as described. Two hundred nanograms of total RNA from CTV infected and non-infected trifoliate orange were reverse transcribed using Omniscript reverse transcriptase (Qiagen, Valencia, CA) and 1 µM of either one base anchor primers series S (5'-AAGCTTTTTTTTTTTM-3' where M represents A, C, or G) or series L (5'-TGCCGAAGCTTTTTTTTTTTM-3' where M represents A, C, or G). Aliquots of 2 µL of the obtained cDNA for each anchor primer series were amplified using 1µM of the correspondent random primer series S or L (Table 1) and HotStartTaq DNA polymerase (Qiagen, Valencia, CA). PCR conditions were according to Rojas et al. (2002). The amplified products were labeled by α-[^{33}P] dATP (3000Ci/mmol; NEN, MA) and separated in 6% denaturing polyacrylamide sequence gels, Sequagel-6, (National Diagnostic, Atlanta, GA). After separation, the gels were dried without fixing and exposed to Kodak Biomax MR (Eastman Kodak Company, Rochester, NY) film at -80ºC.

Re-amplification of differential products. The differential cDNAs were reamplified using the same primer set that produced them, but the total concentration of dNTP was increased to 20 µM in a 40 µL reaction and no isotope was added. The PCR was run at the following conditions: 95ºC for 15 min, followed by 30 cycles of 94ºC for 30 s, 47ºC (53ºC for L series) for 1 min, 72ºC for 1½ min, and a final extension step at 72ºC for 10 min. The PCR products were resolved in 1.5% agarose gel, and the ones showing single transcripts were cleaned using QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and cloned into a pCR®4-TOPO® vector using the TOPO® TA Cloning Kit for sequencing (Invitrogen, Carlsbad, CA). *E. coli* colonies containing the putative differentially expressed fragments were picked and transferred to tubes containing 50 µL of lyses buffer. After boiling for 10 min and

Table 1. Primers used for differential display. All primers were synthesized by Qiagen (Germantown, MD). Variant sequence in italic bold, extra bases underlined.

Primer	Name	Sequence
Series S-random primer	HPC-1	5'-TGCCGAAGCTT***GACT***-3'
	HPC-3	5'-TGCCGAAGCTT***ACAG***-3'
	HPC-4	5'-TGCCGAAGCTT***TCGC***-3'
	HPC-6	5'-TGCCGAAGCTT***TCTG***-3'
	HPC-7	5'-TGCCGAAGCTT***AGAC***-3'
	HPC-8	5'-TGCCGAAGCTT***CGAT***-3'
	HPC-9	5'-TGCCGAAGCTT***AGCT***-3'
	HPC-10	5'-TGCCGAAGCTT***CTAG***-3'
Series L-random primer	DD-11	5'-TGCCGAAGCTTCGAC***ACG***-3'
	DD-12	5'-TGCCGAAGCTTCGAC***AGT***-3'
	DD-14	5'-TGCCGAAGCTTCGAC***CAT***-3'
	DD-16	5'-TGCCGAAGCTTCGAC***CGT***-3'
	DD-17	5'-TGCCGAAGCTTCGAC***GTA***-3'
	DD-18	5'-TGCCGAAGCTTCGAC***TAC***-3'
	DD-19	5'-TGCCGAAGCTTCGAC***TCG***-3'
	DD-20	5'-TGCCGAAGCTTCGAC***TGC***-3'

spun for 2 min, the lysetes were collected to be used for colony-PCR.

Colony-PCR. Colony-PCR was performed using 300 nM of M13 forward (5'-GTAAAACGACGGCCAG-3') and reverse (5'-CAGGAAACAGCTATGAC-3') primers, 200 µM of each dNTP, 1 U of HotStarTaq DNA polymerase (Qiagen, Valencia CA), 10X PCR Buffer, and 4 µL colony lysate in a final volume of 50 µL. The PCR conditions were the same as those used for reamplification but using 5 min less for final extension. The size of the PCR products was determined by comparing the sample with DNA molecular weight marker of 1Kb (Promega, Madison, WI) on a 1.5% agarose gel in 1X TAE buffer.

Reverse northern dot blot. Blotting was performed by mixing 30 µl of each colony-PCR product with sodium hydroxide and EDTA to a final concentration of 0.4 M sodium hydroxide and 10 mM EDTA. The mixture was boiled for 10 min to completely denature the cDNA and an equal volume of cold 2 M ammonium acetate was added to the target solution. Each sample was blotted onto duplicate Zeta-Probe membranes using the Bio-Dot microfiltration system (Bio-Rad, Hercules, CA). After the samples were filtered, the wells were washed with 500 µl of 0.4 M NaOH. The blotted membranes were rinsed in 2 x SSC, air-dried, and crosslinked. Prehybridization and hybridization were carried out in a solution of 1 mM EDTA; 7% SDS; and 0.5 M Na_2HPO_4 at 65ºC. Radioactive-labeled cDNA probe was generated by reverse transcription of total RNA using an oligo dT20 primer and [α-^{32}P]dCTP. After overnight hybridization, the membranes were washed twice for 30 min with wash solution I (1 mM EDTA; 40 mM $NaHPO_4$, and 5% SDS), and twice with wash solution II (1 mM EDTA: 40 mM $NaHPO_4$, and 1% SDS) at 65ºC with gentle agitation. The positive clones were sequenced using an ABI Prism Automatic Sequencing System Model 377 (Applied Biosystems, Foster City, CA). Sequences were compared to the non-redundant database of the National Center for Biotechnology Information (NCBI) using blastn and blastx.

5' RACE (Rapid Amplification of cDNA Ends). Full-length cDNA clones were obtained by 5' RACE using the

Table 2. Nucleotide sequence homology of the full length cDNA clones with sequences deposited at the GenBank database (blastn).

Clone	Similar Genes or Sequences	Organism	E Value[1]	Access
10s-1	Chromosome 3 CHR3v07142002 genomic sequence	*Arabidopsis thaliana*	1E-12	NM112627
	Genomic DNA, chromosome 3, P1 clone: MKP6	*Arabidopsis thaliana*	1E-12	AB022219 BA000014
24s-5	SOS2-like protein kinase mRNA, complete cds	*Glycine max*	4E-12	AF525402
	Cytosolic aldehyde dehydrogenase RF2D (rf2d) mRNA, complete cds	*Zea mays*	4E-12	AF348415
16-1	Saranac nodule Fe-superoxide dismutase precursor sodB) mRNA, complete cds	*Medicago sativa*	1E-18	AF377344
	Iron-superoxide dismutase precursor, mRNA, complete cds	*Vigna unguiculata*	1E-15	AF077224
	Iron superoxide dismutase (FeSOD) mRNA, complete cds	*Glycine max*	1E-15	M64267
18-2	Glutaredoxin (Cl31) mRNA, complete cds	*Tilia platyphyllos*	1E-45	AF406809
	mRNA for glutaredoxin	*Lycopersicon esculentum*	2E-14	Y18346
	Clone 96557 mRNA, complete sequence	*Arabidopsis thaliana*	2E-11	AY088825
	Glutaredoxin mRNA, complete cds	*Vernicia fordii*	2E-11	AF047694
21-2	No similarity to known plant genes			
34-4b	mRNA for amino acid transporter (aap1 gene)	*Solanum tuberosum*	2E-13	Y09825
	SOS2-like protein kinase mRNA, complete cds	*Glycine max*	8E-13	AF525402
	Cytosolic aldehyde dehydrogenase RF2D (rf2d) mRNA, complete cds	*Zea mays*	8E-13	AF348415

[1]E value of 1 assigned to a hit can be interpreted as meaning that in a database one might expect to see 1 match with a similar score simply by chance.

Table 3. Homology of the deduced amino acid sequence of the full length cDNA clones with known sequences deposited at the GenBank database (blastx).

Clone	Protein	Organism	E Value	Access #
10s-1	ppGpp synthase (GTP pyrophosphokinase)	*Arabidopsis thaliana*	7E-82	BAB02036
	Chloroplast RelA homologue 2	*Oryza sativa*	4E-71	BAB21485
	Guanosine-3',5'-bis(diphosphate) 3'-pyrophosphohydrolase	*Borrelia burgdorferi*	2E-13	NP_212332
	RelA/SpoT homolog	*Bradyrhizobium japonicum*	5E-13	AAF04327
24s-5	Inhibitor of trypsin and hageman factor (CMTI-V)	*Cucurbita maxima*	9E-08	P19873
	Chain A, Hydrolyzed Trypsin Inhibitor	*Cucurbita maxima*	9E-08	1HYM_A
	Trypsin Inhibitor V	*Cucurbita maxima*	9E-08	1TIN
	Protease inhibitor 2	*Zinnia elegans*	3E-07	BAC10910
16-1	Superoxide dismutase [Fe], chloroplast precursor	*Glycine max*	6E-36	P28759
	Superoxide dismutase	*Arabidopsis thaliana*	6E-36	CAA73188
	Iron superoxide dismutase	*Zantedeschia aethiopica*	7E-36	AAC63378
	Iron superoxide dismutase precursor	*Vigna unguiculata*	7E-36	AAF28773
18-2	Glutaredoxin	*Tilia platyphyllos*	2E-44	AAL04507
	Glutaredoxin	*Lycopersicon esculentum*	4E-43	Q9ZR41
	Glutaredoxin	*Vernicia fordii*	7E-43	O81187
	Glutaredoxin	*Oryza sativa*	7E-42	JC5445
	Glutaredoxin	*Oryza sativa*	2E-41	P55142

GeneRacer Kit (Invitrogen), following the manufacturer's instructions as briefly described. Five microgram of total RNA from infected trifoliate orange was reverse transcribed using the GeneRacer oligo dT primer, and SuperScript II reverse transcriptase (Invitrogen). In subsequent PCRs, a gene-specific reverse primer based on the sequences of the partial clones was synthesized and used in combination with the GeneRacer 5' primer (5'-CGACTGGAGGCACGAGGACACT GA-3'). The RACE-PCR products were separated on 1.2% agarose gel and cloned into TOPO TA cloning system (Invitrogen).

RESULTS AND DISCUSSION

Before sample collection all plants were assayed by ELISA for the presence of CTV. Virus was detected in all challenged Pineapple rootstocks plants while in the control plants no virus was detected (data not shown). As expected, no virus was detected in the challenged trifoliate orange scions. After differential display, 56 transcripts were found to be differentially amplified. The large amount of differentials was probably due to the fact that transcripts that resulted in weak signals in the control plants and strong signals in the infected (in the differential display polyacrylamide gels), were also considered as differentials. After reverse northern blot, which is a more sensitive technique, 14 transcripts were confirmed as truly differentials. After sequencing, three showed similarity with other transcripts of the group and, therefore, only 11 were considered as different transcripts. No similarity at the GenBank database was found either, at the nucleotide level or at the deduced amino acid for the 11 differentially expressed partial transcripts, but since they had a poly-A tail, they were considered as part of transcribed genes. From the 11 partial transcripts, full-length cDNAs were obtained for six of them, which after sequencing were compared, by BLASTN and BLASTX, to known gene and protein sequences deposited at the GenBank database. Tables 2 and 3 present the results of the similarity search at the nucleotide and deduced amino acid levels, respectively, for the six full length transcripts.

Plants under virus challenge are subjected to stress and may respond at the cellular level in different ways, in addition to the expression of specific genes involved in resistance. The cellular response of citrus plants to a continual challenge by CTV, as in

this study, has never been reported and, therefore, this discussion will be based on the putative function of the full length clones obtained by similarity searches at the GenBank database.

Clone 10s-1 had a reasonable similarity (1E -12) at the nucleotide level to a genomic DNA sequence of *Arabidopsis thaliana* (accession number NM112627), and very high similarity at the deduced amino acid sequence to a ppGpp synthase (GTP pyrophosphokinase- 7E-82, accession number BAB02036), a hypothetical protein (7E-82, accession number NP_188374), both from *A. thaliana*; a chloroplast ReIA homologue 2 from *Oryza sativa* (4E-71, accession number BAB21485), a pyrophosphohydrolase from *Borrelia burgdorferi* (2E-13, accession number NP_212332) and to a RelA/SpoT homolog from *Bradyrhizobium japonicum* (5E-13, accession number AAF04327). Cells respond to nutrient exhaustion in a manner that enables them to survive or adapt to the stress (Mechold et al., 2002). Adjustments in gene expression as well as the complex metabolic and physiological changes are in part mediated by the guanosine di-phosphate (GDP) and guanosine tri-phosphate (GTP) derivatives with a phosphate group on the 3'-hydroxyl position of ribose (i.e., GDP 3'-diphosphate (ppGpp) and GTP 3'-triphosphate (pppGpp), collectively abbreviated as (p)ppGpp) (Chatterji and Ojha, 2001; Cashel et al., 1996).

The involvement of (p)ppGpp in the regulation of antibiotic production in *Streptomyces coelicolor* (Sun et al., 2001) is thought to occur, along with the regulation of colicin synthesis in *E. coli* (Kuhar et al., 2001). These signal nucleotides might contribute to virulence in *Legionella pneumophila* (Hammer and Swanson, 1999) and *Mycobacterium tuberculosis* (Primm et al., 2000). Genes encoding proteins for (p)ppGpp synthesis are common in eubacteria and have been reported in plants as well (Van der Biezen et al., 2000).

The (p)ppGpp synthases (GTP pyrophosphokinases) are governed by two homologous proteins, RelA and SpoT. The first one catalyzes its synthesis while the second one its breakdown by a manganese dependent (p)ppGpp pyrophosphohydrolase activity (An et al., 1979; Heinemeyer and Richter, 1977; Sy, 1977).

Clone 24s-5 showed similarity (4E-12) at the nucleotide level to a SOS2-like protein kinase from *Glycine max*, and to a cytosolic aldehyde dehydrogenase from *Zea mays*. At the deduced amino acid level the clone showed similarity to an inhibitor of tripsin from *Cucurbita maxima* (9E-8, accession numbers P19873), and *Zinnia elegans* (3E-7, accession number BAC10910).

High levels of enzyme inhibitors have been found in the seeds of many plant species and serve as storage or reserve proteins, regulators of endogenous enzymes, and defensive agents against the attack of animal predators, insects and microbial pests (Richardson, 1991). The synthesis of the inhibitor proteins and mRNAs in response to wounding is also regulated at the level of transcription (Palm et al., 1990). Trypsin inhibitor is the most extensively studied enzyme inhibitor. More important is that salicylic acid (SA) and related hydrobenzoic acids (acetylsalicylic acid), that are signals for systemic acquired resistance (SAR) to pathogens, have been shown to inhibit the accumulation of proteinase inhibitors in tomato (*Lycopersicon esculentum*) leaves induced by wounding (Doherty et al., 1988).

Clone 16-1 revealed good similarity at the nucleotide and deduced amino acid levels to iron superoxide dismutases (FeSODs) from *Glycine max* (6E-36, accession number P28759), *Arabidopsis thaliana* (6E-36, accession numbers CAA73188 and NP_199923), *Zantedeschia aethiopica* (7E-36, accession number AAC63378), and *Vigna unguiculata* (7E-36, accession number AAF28773). All the E values are for similarity at the deduced amino acid levels.

Superoxide dismutases destroy free radicals which are normally produced in the cells and are toxic for biological systems by catalyzing the conversion of O_2^- to H_2O_2. FeSODs are found in the cytosol of eubacteria and cyanobacteria and in the chloroplast stroma of photosynthetic plant cells. They are not found in eukaryotes other than plants. In cyanobacteria, FeSOD has been suggested to play a specific role in protecting cellular components from O_2^- formed in the cytosol (Thomas et al., 1998).

Interestingly, the infection process in plants, mostly by necrotizing pathogens, enhances resistance to subsequent attacks by the same, similar or even unrelated pathogens, an event known as SAR (Ryals et al., 1996; Delaney, 1997; Sticher et al., 1997).

Because SAR provides long-term protection in the whole organism against a broad range of unrelated and related pathogens, this led us to believe that in the particular case of *P. trifoliata*, this mechanism might be part of the defense mechanism against CTV.

The development of SAR takes place by the activation of a variable number of plant defense-related genes including five or more families of pathogenesis-related (PR) genes (Ward et al., 1991). It is known that significant levels of exogenous SA induces PR gene expression and enhances resistance to plant diseases (White, 1979). It is also known that SA is a necessary and sufficient signal for SAR because removing it through the expression of salicylate hydroxylase blocks the onset of SAR (Gaffney et al., 1993). Chen et al. (1993) isolated and characterized a SA-binding protein cDNA from tobacco leaves which has a very high similarity at the deduced-amino acid sequence level to plant catalases. Catalases are present in all aerobic organisms and convert H_2O_2 to water and oxygen, opposite to that of superoxide dismutases.

The elevated levels of H_2O_2 may be responsible for the activation of several other biochemical pathways associated with plant disease resistance. Hydrogen peroxide is needed for the increase in the lignification of the plant cell walls at sites of infection and in the oxidative crosslinking of the proteins of the cell wall (Bradley et al., 1992). The ability of superoxide dismutases to induce this crosslinking can be explained by the constant production of H_2O_2, this can also be produced in other plant species by SA by the inhibition of catalase H_2O_2-scavenging activity. Therefore, H_2O_2 could have two functions, an initiator of a physical protection in the cell walls against pathogens and acting as a secondary messenger to activate defense-related PR genes.

During the rapid and intense oxidative burst that occurs in

response to pathogen infection, plants seem to accumulate H_2O_2 rather than producing it. Since the development of SAR requires several days and usually occurs separate from the site of infection, high concentrations of H_2O_2 are not required to kill the pathogens directly, but rather, small amounts are sufficient for activating plant defense genes (Chen et al., 1993).

The full length cDNA 18-2, shows a considerable similarity at the nucleotide and at the deduced amino acid levels to Glutaredoxins from *Tilia platyphyllos* (2E-44, accession number AAL04507), *Lycopersicon esculentum* (4E-43, accession number Q9ZR41), *Vernicia fordii* (7E-43, accession number O81187), and *Oryza sativa* (7E-42, accession numbers JC5445). All E values are for similarity at the amino acid levels.

Glutaredoxins are small heat-stable glutathione-dependent oxidoreductases highly conserved in their reactive site which maintain the redox potential of cells (Holmgren, 1989; Holmgren and Aslund, 1995). They form part of a complex glutaredoxin system consisting of NADHP, glutathione (GSH), and glutathione reductase which is involved in the movement of electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to glutaredoxin through GSH (Holmgren, 1990).

Glutaredoxins can catalyze the cleavage of mixed disulphides which may be formed as a result of oxidative stress (Chrestensen et al., 1995; Raghavachari and Lou, 1996), they also can activate a number of oxidized proteins by reducing the mixed disulphides formed as a result of thiol oxidation (Terada et al., 1992; Terada, 1994; Yoshitake et al., 1994). Glutaredoxins are encoded by two genes, GRX1 and GRX2, in the yeast *Saccharomyces cerevisae*. Grant et al. (2000) reported that the expression of these genes is induced in response to various stress conditions including oxidative, osmotic, and heat stress. Glutaredoxins function as antioxidants in the prevention of damage by active oxygen species (AOS) generated in all aerobic organisms (Halliwell and Gutteridge, 1990).

The initial aim of this research was to isolate genes differentially expressed as a result of the continue challenging of the CTV resistant *P. trifoliata* with the virus. It was expected that if the CTV resistance gene from trifoliate is inducible by the virus, by comparing a CTV challenged plant with a non-challenged one we would be able to detect it by differential expression analysis. However, no gene directly responsible for the resistance was differentially expressed, but as discussed, several genes that could be indirectly involved in the global response of the plant to the virus infection were isolated. Since the putative function of the full length cDNAs was inferred from the comparison with homologous genes from the GenBank database, further studies will be necessary for a better understanding of the role of these genes in the response of trifoliate orange to CTV.

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