Transcriptional Response of Troyer Citrange, Sour Orange and Alemow Rootstocks to *Citrus viroid IIIb* (CVd-IIIb) Infection

S. Rizza[1,2], C. Capasso[3], G. Catara[3], A. Capasso[3], E. Conte[2], and A. Catara[1,2]

[1]*Department of Phytosanitary Sciences and Technologies, University of Catania, Italy*
[2]*Laboratory of Phytosanitary Diagnosis and Biotechnologies, Science and Technology Park of Sicily, Catania, Italy*
[3]*Institute of Protein Biochemistry, CNR, Naples, Italy.*
srizza@unict.it

ABSTRACT. To study the host plant-viroid interaction, green bark samples of Troyer citrange, sour orange and Alemow seedlings inoculated with a *Citrus viroid-IIIb* (CVd-IIIb) source were processed by a rapid and sensitive RT-SYBR Green I-based real time assay to assess molecular events taking place at an early stage of infection. The messenger RNA (mRNA) differential display technique was applied for the identification and isolation of genes whose transcription was altered significantly in Etrog citron leaves infected by CVd-IIIb. Seven of them, known to be related to plant stress defense, were selected to evaluate the variation of the expression level in Troyer citrange, sour orange, and alemow seedlings by Northern Blot analysis and reverse-transcriptase quantitative real time PCR (RT-qPCR). Both methods were valuable, but the latter was more sensitive. The seven genes were detected, in the three rootststocks, as up- or down-regulated. The inoculation of CVd-IIIb in Troyer citrange seedlings modified the expression of metallothionein (MT), alcohol dehydrogenase (ADH), ethylene-responsive binding protein (EREBP), regulator of gene silencing (RGS), peroxidase (PRX) and CONSTANS-like at a lower level than in Etrog citron but with a similar pattern. Alemow seedlings showed a slight over-expression of MT and ADH; in contrast, PRX was severely down regulated. Sour orange seedlings did not reveal any signal with the exception of PRX and CONSTANS, which were up regulated as in Etrog citron. RT-qPCR allowed the detection of the genes, which escaped the Northern Blot analysis, showing in sour orange the same pattern (over-expression) as Etrog citron for ADH, EREBP and AP; whereas MT and RGS were down regulated. In alemow seedlings, EREBP, RGS, AP and CLP showed a reduction of the expression level as well as AP in Troyer citrange.

Viroids infect economically important herbaceous and ligneous plants, including some ornamentals. Morphological and cytological changes associated with their infection have been well documented (6). Typical symptoms are intensified by high temperatures and include leaf epinasty, chlorosis and stunting, the latter often accompanied by a reduction of the root mass. At the cellular level, the most visible symptom is distortion of cell walls and the plasma membrane, chloroplasts and mitochondria (10).

Citrus are the hosts in which the highest number of viroids has been recovered. Two species are considered disease agents, i.e. *Citrus exocortis viroid* (CEVd) responsible for the citrus exocortis, and *Hop stunt viroid* (HSVd) (variants CVd-IIb and CVd-IIc), the causal agent of cachexia (24). *Citrus viroid III* (CVd-III), causing specific symptoms on the Etrog citron indicator, has the capability of reducing the size of citrus plants grafted on some rootstocks without any other detrimental effect, apart from some reports of growth abnormalities observed under some conditions (17, 23). The CVd-IIIb variant is distributed in all the citrus areas of the world and has been long investigated as a graft-transmissible dwarfing agent of citrus grown on trifoliate orange and its hybrids in order to obtain high density plantings (19); for such an effect, the name *Citrus dwarfing viroid* (CDVd) has been proposed to replace CVd-III because it is more descriptive (22).

The molecular interaction between viroids and their host plant species is largely unknown apart from some accumulation of

pathogenesis-related (PR) proteins (7). Therefore, we previously studied a model system represented by a CVd-IIIb variant isolated in our laboratory (1, 20), and the indicator plant Etrog citron, using the Differential Display technique (DDRT-PCR), a sensitive tool applied with success for identifying genes whose expression level has been altered under different conditions also in citrus (12, 11, 9). Differential Display (DDRT-PCR), is a sensitive tool that has been applied with success for the identification genes of plants including citrus whose expression level have been altered under different conditions (9, 11, 12). In previous work, we used this technique in Etrog citron infected with a CVd-III variant isolated in our laboratory as a model system (1, 20).

Thirteen of the 23 identified genes, activated in response to the CVd-IIIb infection, were up-regulated while five genes were down-regulated. The identified genes were mainly involved in plant defence/stress response, signal transduction, amino acid transport, cell wall structure, and other functions (21).

In order to investigate the response of other citrus species, we tested the expression of some of the previously identified genes on three different rootstocks, namely Troyer citrange, sour orange and alemow, also inoculated with the same CVd-IIIb isolate.

This paper describes the results of the Northern Blot analysis and the quantitative real time PCR (RT-qPCR) assay used to screen the expression level of the selected genes on the three different rootstocks under examination.

MATERIALS AND METHODS

Plant material and viroid isolate. Ten-month-old seedlings of Troyer citrange, sour orange and alemow maintained in a growth chamber were used in this experiment. The seeds were obtained from trees free of virus and virus-like pathogens.

The 294-nt isolate of CVd-IIIb, used in the previous study (20) which was initially found in a mildly stunted Clementine tree grafted on alemow showing no detrimental effects, was used.

Inoculation. The inoculum source plant was a *Poncirus trifoliata* seedling inoculated 20 years ago and showing a 35% reduction of canopy volume without other symptoms. The viroid was graft transmitted to the seedlings by T-cut using bark tissue from the source plant. Ten plants per rootstock species were inoculated using three bark chips each. The same number of plants was left as noninoculated controls. All inoculated and noninoculated seedlings were grown and maintained in a growth chamber with 16 h light (28°C) and 8 h dark (24°C) cycle.

Monitoring of viroid infection. Viroid infection was assessed using a rapid and sensitive RT-SYBR Green I-based real time assay for the quantitative detection of CVd-III in citrus samples (15). CVd-III titer was determined, ten weeks after inoculation, in green bark of sour orange, Troyer citrange, and alemow seedlings inoculated with the CVd-IIIb source.

Isolation of total RNA from plants. Fresh bark samples were collected and immediately dipped in RNAlater (Ambion) to prevent RNA degradation. To normalize variations among individual plants, total RNA was prepared from leaves of a pool of four plants. Pooled leaves from control and viroid-infected samples, taken from four plants were frozen in liquid nitrogen and grinded to a fine powder in a prechilled mortar. The resulting powder was then suspended in 5 mL of Concert Plant RNA Reagent (Invitrogen) and total RNA was isolated according to the manufacturer's instructions. After removal of contaminating genomic DNA using the DNA-free kit

(Ambion), the concentration and purity of RNA samples were determined by UV absorbance spectrophotometry. RNA integrity was checked using formaldehyde-agarose gel electro-phoresis.

Northern blot analysis. According to previous results (21), six of the up regulated genes [metallothionein (MT), alcohol dehydrogenase (ADH), ethylene-responsive binding protein (EREBP), regulator of gene silencing (RGS), peroxidase (PRX) and CONSTANS-like protein (CLP)] and one down regulated gene [aminoacid permease (AP)] were selected among those modulated in Etrog citron after CVd-IIIb infection and used as probes to screen the expression level on sour orange, alemow and Troyer citrange also inoculated with CVd-IIIb. The accession numbers of the selected genes are reported in Table 1.

TABLE 1
NUMBER OF NUCLEOTIDES AND THE ACCESSION NUMBERS OF THE SELECTED GENES

Nucleotide	Identified gene	Accession number
277	Metallothionein	AM906072
296	Alcohol-dehydrogenase	AM906073
242	Ethylene-responsive binding protein	AM906074
219	Regulator of gene silencing	AM906075
364	Amino acid permease	AM906076
350	Peroxidase	AM906077
356	CONSTANS-like protein	AM906053

The screen was performed by Northern Blot analysis and the variation of expression level was calculated by dividing the number of infected samples by the number of healthy controls.

Total RNA (20 µg) was size-fractionated by electrophoresis in denaturing 2.2 M formaldehyde/1.5% (w/v) agarose gel (18), transferred by capillary blotting (16 h) to nylon membrane (Immobilon Ny+, Millipore) and fixed by UV cross-linking. The cDNA fragments of the selected genes were labelled with [α^{-32}P]dCTP (Amersham Pharmacia Biotech) using the random-priming labelling kit (Amersham Pharmacia Biotech) and used as a probe for Northern hybridization. The membrane was prehybridized for 30 min at 42 °C in UltraHyb buffer (Ambion). Hybridization with the labelled probe was performed at 42 °C for 16 h in the same buffer and washed twice with 2× SSC, 0.1% (w/v) SDS at 42 °C for 5 min, followed by two additional washes with 0.1× SSC, 0.1% SDS at 42 °C for 15 min. For detection and quantification of the radioactive signals, the filter was exposed to a PhosphorImager apparatus (Storm Imaging System, Amersham Pharmacia Biotech). After the images were obtained, the membrane was boiled twice in 0.1% SDS for 15 min to remove the bounded probe. Equal loading of RNA was verified by staining the membrane, after UV crosslinking, with 0.02% (w/v) methylene blue in 0.3% sodium acetate (pH 5.5) for 3 min. After washing in 0.2× SSC in 0.1% SDS for 15 min at room temperature, the membrane was ready for hybridization (18).

Reverse-transcriptase quantitative real-time PCR assay (RT-qPCR). On the basis of the nucleotide sequences previously obtained from Etrog citron we designed primer pairs yielding products in the range of 80 to 120 base pairs for each gene to be analyzed.

DNA-free total RNA (1 µg), denaturated in a volume of 12 µl for 5 min at 65 °C in the presence of 0.5 µg of one base anchored oligo(dT) primers (HT_{11}-G) and 10 nmol of each dNTPs, was reverse-transcribed in a 20-µl reaction mixture at 42 °C for 50 min with SuperScript II reverse-transcriptase (Invitrogen).

Real time PCR reactions were performed in a final volume of 20 µl using the Brilliant Sybr Green Master Mix (Stratagene) and 0.4 µM of each primer. Thermocycling conditions were: 95.0ºC for 10 min followed by 40 cycles of 95.0ºC for 30 sec, 60.0ºC for 60 sec and 72.0ºC for 30 sec.

RESULTS

Monitoring of viroid infection. Ten weeks after inoculation, the inoculated viroid was successfully detected in the three species, without significant differences in viroid titer among them. The number of CVd-III copies in the rootstocks ranged from 2.01×10^1 to 1.56×10^2.

Northern Blot analysis. Northern blot analysis was performed to test if the selected genes whose steady-state level of expression changed in Etrog citron following CVd-IIIb infection, had the same response in sour orange, alemow and Troyer citrange inoculated with the same viroid.

Using total RNA from control and viroid-infected samples and radioactively labelled cDNA fragments as probes for hybridization, it was shown that CVd-IIIb infection modified the expression of metallothionein (MT), alcoholdehydrogenase (ADH), ethylene-responsive binding protein (EREBP), regulator of gene silencing (RGS), peroxidase (PRX) and CONSTANS-like (CLP) of Troyer citrange seedlings, but at a lower level than in Etrog citron. Seedlings of alemow showed a slight over-expression of MT and ADH; whereas PRX was strongly down regulated. No signal was obtained for the other genes. Similarly, sour orange seedlings did not reveal any signal for all the genes with the exception of PRX and CONSTANS, which were up regulated as in Etrog citron. (Table 2; Fig. 1).

Reverse-transcriptase quantitative real-time PCR assay (RT-qPCR). In order to confirm the results obtained by Northern blot analysis, we developed a quantitative real-time PCR assay which is more sensitive. The previous results were confirmed but we were able to detect the expression of the genes, which escaped detection with the northern blot analysis. This supports the powerful sensitivity of the real-time PCR.

In alemow seedlings, MT, ADH, and PRX showed the same response observed in the northern blot analysis; EREBP, CLP, AP and, in great measure, RGS, which did not reveal any signal previously, appeared to be down regulated. Sour orange seedlings showed the same response as Etrog citron for all the genes with the exception of MT and RGS, which appeared to be down regulated, and AP, which resulted to be up regulated. For Troyer citrange seedlings, we observed a slight down regulation of AP, whereas the expression pattern of the other genes was confirmed (Table 2; Fig. 1).

TABLE 2
EXPRESSION LEVEL OF SEVEN SELECTED GENES IN FOUR CITRUS SPECIES AFTER INOCULATION WITH CVd-IIIb

Gene	Etrog citron		Troyer citrange		Alemow		Sour orange	
	Northern Blot	Real time PCR	Northern Blot	Real time PCR	Northern Blot	Real time PCR	Northern Blot	Real time PCR
MT	5.31	10.34	3.52	3.46	1.05	1.66	-	- 0.51
ADH	4.63	6.57	1.09	2.09	1.77	6.14	-	1.68
EREBP	2.42	3.45	1.13	1.79	-	- 0.34	-	1.48
RGS	2.72	3.22	1.79	2.1	-	- 2.5	-	- 2.02
AP	- 1.35	- 1.71	-	- 0.78	-	- 0.69	-	- 1.09
PRX	2.9	3.73	1.13	1.28	- 7.9	- 8.54	3.18	2.20
CLP	2.92	2.55	2.41	2.05	-	- 0.60	2.44	3.28

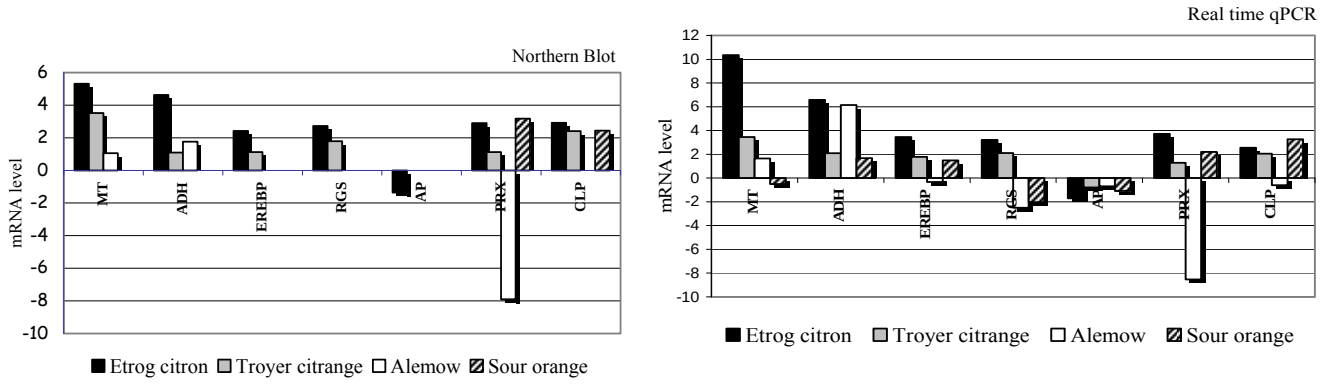

Fig. 1. Graphic representation of the different expression patterns of the seven selected genes in Etrog citron, Troyer citrange, Alemow and sour orange, after the inoculation of CVd-IIIb, detected by Northern Blot (left) and real time qPCR (right).

DISCUSSION

In the recent years, the availability of quite simple and more sensitive techniques has increased the interest for the analysis of the molecular response of citrus to virus, viroid, and cold stress (9, 11, 21).

The molecular mechanisms involved in the interaction between viroids and their host plants are largely unknown, but it appears conceivable that more than one mechanism is responsible for gene activation/inhibition. Using the DDRT-PCR technique, we have identified a number of

plant genes whose expression is altered following CVd-IIIb infection. We isolated cDNAs obtained from mRNAs of viroid-infected Etrog citron plants showing different intensity with respect to cDNA bands of control plants. Only 23 of the differentially expressed bands were validated successively by reverse northern dot-blot analysis and identified by comparison with sequences available in gene bank (21).

We have selected six among the up regulated and one down regulated gene because they were considered the most interesting in their physiological effects.

Metallothioneins (MTs) are low-molecular weight, cysteine-rich heavy metal-binding proteins, which participate in an array of protective stress responses (3) and that may prevent an increase in reactive oxygen species produced.

Alcohol dehydrogenase (ADH) is a ubiquitous zinc-binding enzyme that carries out inter-conversion of short-chain alcohols and their corresponding aldehydes (8). Its conversion to ethanol provides an alternative pathway for energy production during stress conditions.

Ethylene-responsive element binding protein (EREBP) has a crucial role in plant response to pathogen stress by regulating the expression of pathogenesis related genes (5). Plant peroxidases enzymes catalyze the reduction of H_2O_2 by taking electrons to various donor molecules (14). CONSTANS-like proteins (CLP) are plant-specific nuclear regulators of gene expression (16). Amino acid permease 6 (AAP6) mediates transport of a wide spectrum of amino acids (13).

Among them, the regulator of gene silencing (RGS) known as a suppressor of Post Transcriptional Gene Silencing (PTGS), which was upregulated in Etrog citron and Troyer citrange and down regulated in alemow and sour orange was particularly intriguing. The regulator of gene silencing (RGS) is a calmodulin-related protein isolated from tobacco that it is involved in RNA silencing mechanisms. It plays a role in host defense against pathogen infection, as well as in inactivating expression of undesired host genes (2). Viroids can also induce and be the target of host RNA silencing (4). Previous and present data demonstrate that RGS is induced during CVd-III infection not only in Etrog citron but also in commercial rootstocks.

The results further strengthen the hypothesis that viroids could evade host gene silencing by activating an endogenous mechanism that negatively regulate RNA silencing activity.

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