A New Approach for the Rapid Detection and Genetic Discrimination of Mild and Severe Isolates of *Citrus tristeza virus* Based on ELISA/CE-SSCP

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ABSTRACT. A new approach for the rapid discrimination of mild and severe *Citrus tristeza virus* (CTV) isolates was developed by a sequential process made up of the DAS-ELISA immunological test followed by fluorescence-based Capillary Electrophoresis-Single Strand Conformation Polymorphism (CE-SSCP) analysis. The new method helps both in locating CTV infected trees and in preliminarily typing of the virus isolates, thus saving both time and resources. Partial p18 gene amplification products of CTV-RNAs directly extracted from infected leaves or recovered from ELISA plates are presented. Specific profiles of forward and reverse strands were obtained when biologically distinct CTV isolates were analysed both directly and following ELISA. The CE-SSCP method is simple, highly sensitive and highly reproducible, and can process a large number of samples for a variety of needs. Applied in a combined process, ELISA and CE-SSCP helped simultaneously to detect the virus and to collect useful information on the genetic diversity and structure of the CTV population present in a territory.

Index words: CTV, characterization, CE-SSCP analysis, capillary electrophoresis, serological detection

Citrus tristeza virus (CTV), the causal agent of tristeza disease, has been dispersed by propagation of infected buds to most of the world's citrus-growing areas and locally spread by several aphid species (3). Considerable biological and genetic diversity has often been reported for CTV isolates, with some causing severe losses to trees grafted on sour orange while infections of sweet orange trees grafted on sensitive rootstock – including the sour orange – might remain symptomless for rather long periods (2, 3, 9). For effective management of CTV epidemics, especially during disease emergence, it is imperative to eradicate or to confine any trees infected with aggressive isolates after proper characterization of the aggressiveness. Several diagnostic protocols based on serology and molecular hybridization have been proposed over the years (9, 16, 17, 20).

Despite the advances in molecular testing, biological indexing on a battery of CTV-sensitive indicator plants remains the most reliable method for the in-depth characterization of CTV strains (12). However, biological assays are not only costly but also time-consuming and therefore less suitable for situations of an emerging CTV epidemic, such as in the recent case in Sicily (6).

One of the most popular techniques for rapid differentiation of CTV strains is based on conformational variation of single-stranded DNA sequences (SSCP) (21). This method has been applied in many countries, including Italy (1, 7, 11). It can be used to detect mixed infections of virus isolates (21). However, the method is time-consuming and labor-intensive, mostly because of the work involved in preparing, running and recording non-denaturing polyacrylamide gel analyses, and is thus unsuitable as a routine procedure for large-scale analysis.

In the last decade, the use of capillary electrophoresis SSCP (CE-SSCP) has been widely reported in human pathology for mutation detection (14) and

also for the genetic typing of influenza virus isolates (18). The method combines the potential of single strand conformation analysis with automation and standardization of electrophoresis run conditions, providing improved resolving power and sensitivity, as well as simultaneous analysis of multiple samples (up to 96 in a single run). In plant pathology, CE-SSCP has recently been applied for screening genetic diversity in a *Plum pox virus* population (8). In a previous study, we reported the use of CE-SSCP for the characterization of CTV isolates using the Applied Biosystem 3130 capillary electrophoresis apparatus in "non denaturing" conditions (19), modifying the running protocol which had previously only been calibrated for human pathology studies (14).

This paper reports on an attempt to further improve the CE-SSCP technique by applying it as a follow-up for an earlier step of CTV detection using the indirect double-antibody sandwich, Enzyme Linked Immunosorbent Assay (DAS-ELISA). By combining the two technologies in a sequential protocol, we were able to simultaneously monitor CTV infections and also collect useful information on the genetic diversity of the CTV isolates and the genetic structure of the virus populations in a CTV-infected grove or area.

MATERIALS AND METHODS

Source of virus isolates. A total of eight different CTV isolates obtained from field trees in Sicily were used to test the applicability of this new technology. Five of the isolates, classified as biotype 4 (severe), were obtained from local sweet orange trees grafted on sour orange: isolates SG29, S24 and S25 were from a 25-yr-old declining 'Sanguinello' sweet orange (7), while isolate S9 was from a declining "Tarocco comune" sweet orange

and isolate S13 from a "Tarocco Gallo" sweet orange. Three isolates belonged to biotype 2 (mild): isolate TDV was collected from a 5-yr-old symptomless 'Tarocco' sweet orange tree grafted on Troyer citrange (19), M1 from a sour orange seedling naturally infected by aphids and M2 from a "Tarocco Sant'Alfio" grafted on sour orange.

DAS-ELISA and post-ELISA virus release. The plant samples were first tested for the presence of CTV by the indirect double-antibody sandwich, Enzyme Linked Immunosorbent Assay (DAS-ELISA), using the Ingezim CTV 2.0. kit (Ingenasa, Madrid, Spain), while ELISA positives produced by well testing were used for continued analysis by the OEPP/EPPO protocol (16). Plant extracts were prepared by homogenising 200 mg fresh weight of leaves in a small plastic bag with 1.5 ml of extraction buffer (phosphate-buffered saline, pH 7.4, with 2% polyvinylpyrrolidone). Duplicate assays were performed, using both healthy leaves and buffer control as negative samples and infected leaves as positive samples. ELISA results were recorded using the Ultramark Microplate Imaging System, 110/230 V (Bio-Rad Laboratories Inc., Milan, Italy).

Following ELISA, the trapped virus particles were eluted as described by Harju et al. (13). Positive or potentially positive ELISA plate wells were washed with phosphate-buffered saline (PBS), tapped dry and processed soon or stored at -20°C for at least 3-4 weeks. The wells were then filled with 50 μl of virus release buffer (VRB) (10mM Tris–HCl, pH 8.0 with 1.0% (v/v) Triton X-100), covered to prevent evaporation and the plates were shaken for 5 min at 65°C. After the elution process was completed, the extracts were decanted and stored at 5°C prior to testing at the day of elution or at a later stage after storing samples at −80 C. Aliquots of extract (5 μl) were directly used for cDNA synthesis.

One step RT-PCR. RT-PCR amplification was performed in a single closed tube in a final volume of 25 µl. The reaction cocktail contained 1.6 X Nova Hot Start Taq Buffer (Novagen®), 2.5 mM MgCl₂, 0.4 mM dNTPs, 400 nM of primer PM44 (5'-TTCTATCGGGATGGTGGAGT-3') (21) labeled with the fluorescent dye 6-FAM (Applied Biosystems), and 400 nM of primer PM45 (5'- GACGAGATTATTACAACGG-3') (21), labeled with the fluorescent dye NED, 0.4 U RNase inhibitor (Applied Biosystems), 0.4 U Multiscribe reverse transcriptase (Applied Biosystems) and 1.25 U of Nova Hot Start Taq DNA Polymerase (Novagen®) and 5 µl of RNA target from immobilized samples or purified RNA .

PCR conditions were: 50°C for 30 min, 95°C for 7 min, 35 cycles each of 94°C for 20 s, 56°C for 30 s, 72°C for 40 s, and a final extension of 72°C for 4 minutes (Eppendorf, Mastercycler ep gradient Thermocycler, UK). The size of the expected amplicon of p18 was 425 bp.

CE-SSCP analysis. The experiments were performed on an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA) equipped with four 36-cm long capillaries, a window of detection for fluorescent emissions with a spectrum of 480 nanometres and a software to control the temperature, the volume and the injection time of the sample. Non-diluted PCR product (if coming from ELISA released templates) or up to 128 fold dilution (if coming from pure RNA templates) was mixed with 0.25µl of GeneScan-500 ROX Size Standard and 10 µl of formamide Hi-Di (Applied Biosystems). The sample mixture was denatured at 95°C for 5 min and immediately chilled on ice before loading on the instrument. The injection time and voltage were set to 10s and 3.5 kV, the migration time was set at 1600s at 24°C so as to test different mobility patterns. The non-denaturing polymer, consisting of 5%

CAP (POP polymer Conformational Analysis, Applied Biosystems), 10% glycerol and 10X Genetic Analyzer buffer (Applied Biosystems), was used to fill up the capillaries. Sample runs were performed three times under the same conditions. CE-SSCP profiles were analysed with the GeneMapper™. Software version 4 (Applied Biosystems). Variations in run-to-run time were compensated for by normalizing each run with the internal size standard, according to which fragments migrated depending on conformation. For each run, standard fragments surrounding the sample were selected to 'size' for the forward and reverse strands, expressed as migration time data points. Differences in migration times between the peaks of respective samples were expressed as standard deviations. Each sample was tested in three to six replicates and standard deviation calculated so as to evaluate repeatability within or between runs.

RESULTS

Optimization of sequential ELISA/CE-SSCP. During the initial development of the sequential ELISA/CE-SSCP protocol, eight CTV-infected plants were tested by DAS-ELISA for serological detection and in a continuous process genotypically characterized by direct amplification using fluorescent primers and CE-SSCP.

The mobility values and peak profiles of the forward and reverse strands on a partial p18 gene (Fig. 1) were used for typing CTV isolates. Peak conformation was clear and reproducible and the profiles supported by the given information (size, conformation, data point and migration). Migration of sample peaks was calculated and normalized relative to the internal size standard in order to eliminate capillary-to-capillary or run-to-run variability. Peak profiles of forward and reverse strands could be individually

valued by the different colours of the fluorophore. The CE-SSCP profiles showed at least two different patterns of migration, allowing differentiation of mild and severe isolates (Fig. 1). In particular, TDV, M1 and M2 isolates showed a two-peak profile, with mobility values of around 70.5 and 73.8, for forward and reverse primers respectively (Table 1). The migration pattern of SG29, S24, S25, S9 and S13 severe isolates was different as the peak mobility had a shifted profile with values around 72.3 and 75.9 for forward and reverse strands.
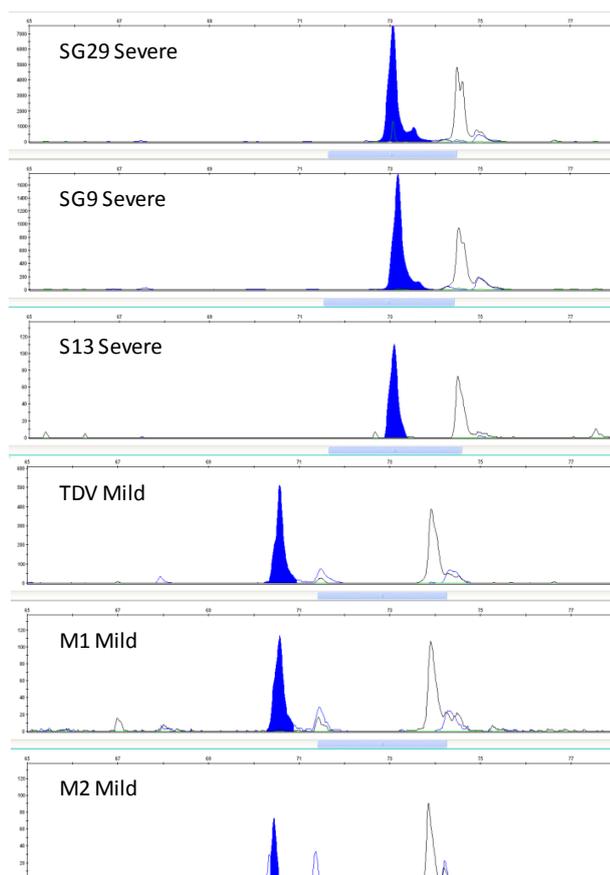
Fig. 1. Chromatograms of CTV isolates obtained by sequential ELISA/CE-SSCP analysis on the p18 gene. Peaks correspond to the fluorescent label of the single-strand DNA fragments detected by the automated sequencer according to the migration from right to left. Filled and empty peaks represent forward and reverse strand positions, respectively.

To validate this new protocol, we compared the migration patterns obtained by sequential ELISA/CE-SSCP protocol with the profiles obtained using Trizol® Reagent (Invitrogen, Milan, Italy) for total RNA extraction. Data points and profiles were in good correlation, clear and reproducible, revealing the robustness of the new methodology (Table 1).

Moreover, when we applied the sequential ELISA/CE-SSCP protocol to samples which after DAS ELISA showed an optical density near or under the cut-off value (OD<0.200), clear migration profiles were obtained after CE-SSCP, suggesting that the ELISA sensitivity was successfully increased thanks to the PCR amplification process applied directly to the microtiter plate (data not shown). Reproducible results were also obtained with samples stored in washed and dried microtiter plates for at least 3-4 weeks at -20°.

TABLE 1
RELATIVE MIGRATION DATA FOR FORWARD AND REVERSE STRANDS IN THE CTV P18 GENE
AND GENETIC PROFILE DETECTED BY SEQUENTIAL ELISA/CE-SSCP OF EIGHT CTV ISOLATES
BELONGING TO BIOTYPE 2 AND 4

CTV Isolates	Biotype*	Relative migration (size)			
		Sequential ELISA/CE-SSCP		CE-SSCP	
		FW	RW	FW	RW
SG29	4	72.52 ± 0.08	75.81 ± 0.17	72.43 ± 0.18	75.78 ± 0.25
S24	4	72.47 ± 0.12	75.69 ± 0.23	72.44 ± 0.32	75.76 ± 0.07
S25	4	72.59 ± 0.38	75.77 ± 0.09	72.39 ± 0.27	75.78 ± 0.22
S13	4	72.31 ± 0.04	75.83 ± 0.30	72.77 ± 0.05	75.39 ± 0.07
S9	4	72.54 ± 0.03	75.74 ± 0.05	72.68 ± 0.12	75.47 ± 0.23
TDV	2	70.35 ± 0.21	73.91 ± 0.18	70.27 ± 0.15	73.85 ± 0.21
M1	2	70.48 ± 0.07	73.32 ± 0.01	70.65 ± 0.12	73.58 ± 0.03
M2	2	70.54 ± 0.12	74.02 ± 0.14	70.41 ± 0.04	73.29 ± 0.01

* According to Garnsey et al., (12), biotype 4 stands for severe isolates and biotype 2 for mild

DISCUSSION

Characterization of CTV isolates is of considerable importance, since it could provide the means to predict the outcome of infections and to establish optimal disease control strategy. This would facilitate proper decision-making in recently-invaded areas often populated by different isolates that vary in their pathogenicity. Furthermore, typing could also help to monitor changes in CTV populations in areas where CTV isolates have been deliberately spread, such as in the case of cross protection (15) and could also be used to follow the emergence of resistance-breaking isolates, such as that reported for the Troyer citrange rootstock in New Zealand (10). Therefore, fast identification of potentially virulent isolates would be helpful for managing CTV.

Although SSCP analysis of different gRNA regions has been widely used to characterize the population structure of CTV isolates (21), further improvements could be obtained by the higher resolving power of CE-SSCP due to the capillary length and the properties of the polymer. The use of 36-cm capillaries with a 50 μM diameter as well as accurate temperature control and an internal standard improves the resolving power of strand separation and provides good reproducibility. Moreover, the fluorescent detection of single strands is useful to clearly localize the position of forward and reverse strands. So it can happen that Fw strands migrate much more rapidly than Rw strands, as usually appeared in the electropherograms revealed in this study, or that peaks can overlap, or even that the

peak corresponding to the Rw strand can be localized before the Fw one. These data can be evaluated only by CE-SSCP and not through conventional SSCP analysis and provide added value which is useful for improving genotypic characterization.

The present paper reports on a new approach to potentiating CE-SSCP analyses based on combining with it the ELISA procedure which is commonly applied for CTV detection (4). This new procedure helps first in locating trees infected by the pathogen and then could potentially type the disease agent and – in cases with a previously defined history of isolate characterization – also discriminate between mild and severe CTV isolates.

Comparison between migration profiles obtained using samples directly from the microtiter plates or after conventional RNA isolation revealed good data correlation and closely similar migration profiles. The new technology is very suitable for preparing and processing large numbers of samples using microtiter plates, increasing the rapidity of sample processing and reducing the manpower input required. It can be routinely used in CTV monitoring programs, while further analysis on other genes could be helpful for CTV genotype analysis.

Moreover, thanks to the flexibility of the ELISA test, through the use of different CTV antisera, this procedure could be differently targeted to help identify specific (i.e. severe) groups of isolates using the MCA13 antibody (17).

Furthermore, the amplification step directly into the microtiter plate and after the serological test increases the sensitivity of the ELISA test.

We expect that this technological development will considerably facilitate epidemiological analyses and improve management of large-scale screening of infected trees, with much lower costs and much greater precision. The method will turn out to be a useful tool for CTV epidemiological studies, quarantine stations and citrus nurseries, which need to certify the virus-free status of plant materials.

ACKNOWLEDGEMENTS

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