Sensitive CTV diagnosis using immunocapture, reverse transcriptional polymerase chain reaction and an exonuclease fluorescent probe assay

G Nolasco
Z Sequeira
B Bonacalza
Ucta
Universidade do Algarve
8000 Faro
Portugal

C Mendes
Centro de Citricultura
8000 Faro
Portugal

V Torres
F Sanchez
B Urgoiti
F Ponz
Biol Mol & Plant Virol. Area
Cit Inia
Madrid
Spain

VJ Febres
B Cevik
RF Lee
CL Niblett
Univ Florida
Gainesville
FL 32611
USA

Received 27 April 1997
Accepted 8 October 1997

Fruits, 1997, vol 52, p 391-396
® Elsevier, Paris

***Resumen Español**, p 396*

Sensitive CTV diagnosis using immunocapture (IC), reverse transcriptional polymerase chain reaction (RT-PCR) and an exonuclease fluorescent probe assay.

ABSTRACT

INTRODUCTION. The Elisa technique has been the method of choice for diagnosis of citrus tristeza virus (CTV) during recent years. However, it was sometimes reported to be not sensitive enough to detect the virus before the onset of decline symptoms. The development of diagnostic methods based on genome amplification appears very attractive: recent methods of DNA amplification in vitro, such as the polymerase chain reaction (PCR) have proven to be of a much higher sensitivity than Elisa with various viruses, including CTV. In this work, a diagnostic assay for CTV based on IC/RT-PCR that combines the simplicity of Elisa with the advantages of a DNA-based method is presented. **MATERIALS AND METHODS**. Infected plant material used included selected isolates of CTV from various origins. The primers and fluorescent probes were designed, taking the sequence of the coat protein gene of the well characterised T36 quick decline strain of CTV from Florida. A simplified IC/RT-PCR assay was designed. After PCR amplification, the electrophoretic analysis of the results was substituted for the measurement of the fluorescence. The PCR results were compared with those of the Elisa method used, in parallel, with the same samples. **RESULTS AND DISCUSSION.** The PCR assay enabled the detection of the virus in all the Elisa positive samples, as well as in an additional 20% of the samples that were Elisa negative. There-fore, the new method tested has a greater sensitivity than the Elisa assay and is a better tool for disease diagnosis.

KEYWORDS
Citrus, viroses, diagnosis, immunocapture techniques, Elisa, molecular biology, PCR, tristeza.

Diagnostic sensible de la tristeza des agrumes par immunocapture (IC) suivie de transcriptase inverse et amplification en chaîne de la polymérase (RT- PCR) et hydrolyse enzymatique de sondes fluorescentes.

RÉSUMÉ

INTRODUCTION. Le test Elisa a été la méthode préférentiellement utilisée, ces dernières années, pour diagnostiquer le virus de la tristeza des agrumes (CTV). Cependant, cette technique s'est parfois révélée trop peu sensible pour détecter le virus avant l'observation des symptômes. Les méthodes de diagnostic basées sur l'amplification du génome sont attractives : de récentes méthodes d'amplification du DNA in vitro, comme la technique PCR, se sont révélées beaucoup plus sensibles chez de nombreux virus, dont celui du CTV, que le test Elisa. Dans ces travaux, une technique de diagnostic du CTV, basée sur la méthode IC/RT-PCR, est présentée. Elle combine la simplicité du test Elisa avec les avantages de méthodes utilisant le DNA. **MATÉRIEL ET MÉTHODES**. Des échantillons infectés par des isolats de CTV de diverses origines ont été collectés. Les amorces et les sondes fluorescentes ont été choisies en se basant sur la séquence du gène codant pour la protéine de l'enveloppe du CTV de race T36, de Floride. Une méthode IC/RT-PCR simplifiée a été élaborée. Après amplification par PCR, l'analyse électrophorétique des résultats a été substituée par la mesure de la fluorescence. Les résultats obtenus avec cette technique ont été comparés avec ceux du test Elisa utilisé en parallèle sur les mêmes échantillons. **RÉSULTATS ET DISCUSSION.** La technique PCR a permis de détecter des virus non seulement dans tous les échantillons Elisa-positifs, mais encore dans 20 % de ceux trouvés Elisa-négatifs. La méthode testée, plus sensible que le test Elisa, s'avère un meilleur outil de détection de la maladie.

MOTS CLÉS
Citrus, viroses, diagnostic, technique immunocapture, Elisa, biologie moléculaire, PCR, tristeza.

introduction

Protection against citrus tristeza in low incidence areas depends of early diagnosis and the use of certified virus-free budwood obtained through strictly regulated certification schemes. In both cases, the ELISA technique has been the method of choice for diagnosis of citrus triteza virus (CTV) during recent years. However, it is expensive, due to the high volume of samples that must be analysed, and there are reports in which it was not enough sensitive to detect the virus before the onset of decline symptoms (BEN-ZE'EV et al, 1989).

On the other hand, in areas like Florida and parts of the Mediterranean Basin, the disease is already widely distributed but the prevalence of stem-pitting strains has been low, allowing for a protection strategy based on the use of tolerant rootstocks. In these areas, virus detection and the additional need to be able to quickly differentiate the CTV strains are required. The available ELISA assays are based on coat protein recognition by antibodies and cannot assess genetic variability. Ability of ELISA to distinguish strains has been limited to the discrimination between mild and severe isolates from Florida and the Caribean Basin based on the use of the MCA13 monoclonal antibody (PERMAR et al, 1990).

Within this framework, the development of diagnostic methods based on genomic amplification appears very attractive: recent methods of DNA amplification in vitro, such as the Polymerase Chain Reaction (PCR) have proven to be of a much higher sensitivity than ELISA with diverse viruses, including CTV (NOLASCO et al, 1992, 1993); the PCR product, which is itself a copy of part of the genome, can be used to type the strains present (GILLING et al, 1993). Despite these advantages, this method has not been used in routine diagnosis. It is generally admitted that PCR-based methods require a greater technical skill and are much more time-consuming and laborious than the widely used ELISA assay. There are two main reasons: laborious nucleic acid extractions, and the need to analyse the PCR products by electrophoresis; both are difficult to automate for a high volume of samples.

In this work, we present a diagnostic assay for CTV based on PCR that combines the simplicity of ELISA with the advantages of DNA-based methods (figure 1). Viral particles are captured by solid-phase adsorbed antibodies, and the coat protein gene is amplified by reverse transcriptional PCR. In the reagent mixture is included an additional DNA probe, double labeled with a fluorescent dye and a quencher. In each cycle of the polymerisation reaction, the probe anneals to its target strand and is cleaved by the 5´-exonuclease activity associated with the Taq Polymerase enzyme (HOLLAND et al, 1991). At the end of the reaction, the fluorescence is measured. The hydrolysis of the probe and the resulting physical separation of the fluorescent group from the quencher results in a net increase of the fluorescence of the sample; in healthy samples, the probe is not cleaved and fluorescence remains unchanged at a low level.

materials and methods

virus isolates

Infected plant material used in these studies included selected isolates of CTV from various origins kept under greenhouse conditions, frozen material, and field collected samples which were in most cases of Spanish origin, introduced illegally in Portugal for topworking purposes.

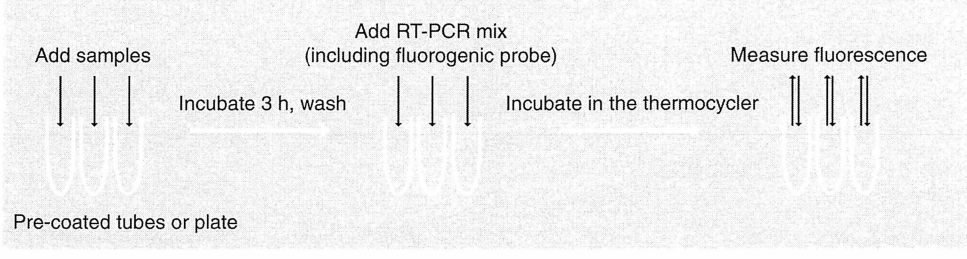

Figure 1
Schematic representation of the IC/RT-PCR-exonulease fluorescent assay.

primers and probes

The primers and fluorescent probes were designed from the sequence (PAPPU et al, 1993) of the coat protein gene of the well characterised T36 quick decline strain of CTV from Florida, as represented in figure 2. In previous work, it was concluded that the primer set used enables the detection of a wide spectrum of strains. Two fluorescent probes were tested. The probes, obtained from Perkin Elmer, were labelled at the 5´ end with 6-carboxyfluorescein (FAM) as a reporter dye and at its 3´ end with 6-carboxytetramethylrhodamine (TAMRA) as a quencher.

one tube - single step IC/RT-PCR[1] assay

Thermoresistant polypropylene strips of PCR tubes or plates (QSP, USA Scientific or Perkin Elmer) were coated with 50 µl IgG (Bioreba, Catalog no 151515). Citrus tissue grinding and extraction was carried out as for ELISA. Antigen trapping on the inner surface of the PCR tubes occurred for 3–4 h at room temperature. At the end of this period, the tubes were washed twice with PBS-T, and once with MiliQ quality water. Fifty microliters of the RT-PCR mix (10 mM Tris HCl pH 9.0 at room temperature, 50 mM KCl, 4 mM $MgCl^2$, 200 mM each dNTP, 200 nM each primer, 40 nM of fluorescent probe containing 7.5 units MMLV RTase, 3.5 units ribonuclease inhibitor and 1.25 units of Taq DNA Polymerase) were added and the tubes incubated in the thermocycler at: 38 °C for 45 min, 94 °C for 2 min, 40 cycles of 92 °C for 30 s and 58 °C for 1 min, with a final step of 58 °C for 10 min.

PCR assays from cDNA clones

Some experiments used cDNA (DNA obtained by reverse transcription on an RNA template) clones. In these cases, the immunocapture and reverse transcription steps and related reagents were omitted. For each reaction, 5 pg were used of the respective template. A non-template reaction was used as negative control.

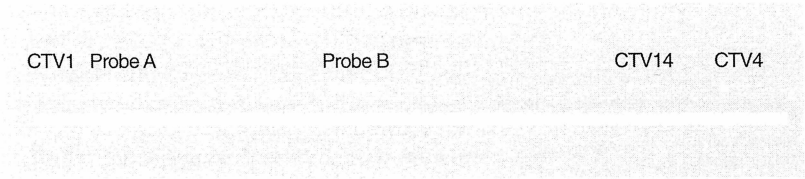

Figure 2
Scheme of the relative position of the primers and fluorescent probes based on the coat protein gene of the T36 strain. CTV 1 is a 20 nucleotide long (mer) forward primer, and CTV 14 and CTV 4 are both reverse primers, 20 mer and 19 mer, respectively. Fluorescent probes A and B are 24 mer and 20 mer, respectively, and are labeled at the 5´ end with FAM (reporter dye) and at the 3´ end with TAMRA (quencher).

fluorescence measurement

After PCR amplification, the samples were transferred to a microtiter plate (96 well format) and the fluorescence measured in a LS50B luminescence spectrometer equipped with a microwell plate reader. The excitation was performed at 488 nm, and the sample emission was recorded at 518 nm (reporter dye) and 583 nm (quencher). After deduction of the plate and buffer background fluorescence, a normalized value for each sample was determined by the ratio (R/Q) between the measurements at 518 nm and 583 nm. Three negative controls (healthy tissue) were included in each experiment. Their mean R/Q was subtracted from each sample to calculate a Delta R/Q value for that sample. A Delta R/Q value was considered to be positive when it was greater than 6.965 times the standard deviation of the negative controls (corresponding to a 99% confidence level assuming a t-student distribution of the R/Q negative values).

ELISA assays

The double antibody sandwich format with commercial antibodies from Bioreba and indirect double antibody sandwich (coating: Ingenasa Mab 3DF1 + 3CA5; detection: antibodies against bacterial expressed coat protein gene) assays were used to compare the performance of IC/RT-PCR and ELISA.

results and discussion

After initial experiments to optimise reaction conditions, the above described protocol was carried out with the primer set 1–14 and fluorescent probe B, which consistently yielded better results. The reasons for that were not fully investigated, but it seems that the length of the amplified product and the

[1] *IC/RT-PCR: immunocapture, reverse transcriptional polymerase chain reaction.*

distance from probe to the forward primer have a marked influence on the ability of the Taq polymerase to cleave the probe.

A preliminary assessment of assay performance was studied through serial dilutions of infected plant extract in buffer or in healthy extracts (figure 3). The results obtained predict that it may be possible to assay with confidence composite samples of extracts of 50–100 trees, significantly lowering the costs of screening.

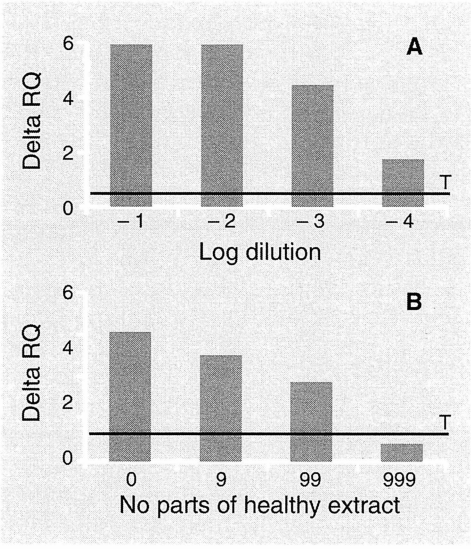

Figure 3
Serial dilutions of an isolate of Spanish origin in extraction buffer (A) or in healthy plant extract obtained 1/10 (B). The bold line, marked with a T, stands for the positive / negative threshold in each experiment.

To assess the capability of the IC/RT-PCR assay under more realistic conditions, two groves where CTV infection had already been detected by ELISA and a nearby grove with manifest decline symptoms, but diagnosed as negative by ELISA, were chosen. Eighty samples were collected from these orchards in January and assayed in parallel by both methods. The results are summarised in table I and figure 4. IC/RT-PCR enabled the detection of virus in all the ELISA positive samples, as well as in an additional 20% of the 50 samples that were ELISA negative. These ELISA negative plants were distributed throughout the three groves tested. Some of the trees were re-tested with the same results.

Sorting of these samples into classes of absorbency or plotting their RQ values against the A_{405} values (figure 4) shows that the correlation of the values of IC/RT-PCR and ELISA is not linear. At very low ELISA values, IC/RT-PCR shows a more intense response with some of the samples. The ELISA response starts only where the IC/RT-PCR values are already well defined as positive values (Delta RQ ~ 2–3). At higher values, there appears to be a more direct relationship between levels detected by the two techniques.

Table I
Comparative assay of field samples by IC/RT-PCR and Elisa. The samples (spl) are distributed into different classes: ELISA negative / PCR negative, ELISA negative / PCR positive, ELISA positive / PCR positive. For each class, the mean value of absorbency at 405 nm (A_{405}) and RQ is presented, with the corresponding value of healthy controls already subtracted. The positive / negative threshold determined as 6.965 times the standard deviation of healthy samples was 1.32 for PCR and 0.1 for Elisa (both protocols).

Mean value	*ELISA –, PCR – (39 spl)*	*ELISA –, PCR + (11 spl)*	*ELISA + $A_{405} < 0.5$ (5 spl)*	*ELISA + $0.5 < A_{405} < 1.0$ (16 spl)*	*ELISA + $1 < A_{405} < 1.5$ (4 spl)*	*ELISA + $1.5 < A_{405}$ (4 spl)*
Double antibody sandwich Elisa technique versus IC/RT-PCR (79 samples)						
A_{405}	– 0.07	– 0.03	0.38	0.72	1.24	1.62
RQ	0.06	2.32	4.04	4.40	5.08	5.17
Indirect Elisa versus IC/RT-PCR (79 samples)						
A_{405}	– 0.03	– 0.01	0.38	0.78	1.29	1.80
RQ	0.04	2.32	3.03	4.36	4.89	5.18

RQ = R/Q; it is the ratio between the measurements of the sample emission at 518 nm (reporter dye) and 583 nm (quencher), deducted from the values of healthy controls.

From the above, it is clear that the IC/RT-PCR assay has a greater sensitivity that enables the detection of infected but ELISA-negative samples. What are the implications for screening programme? Large composite samples would reduce the costs, but it would also reduce the sensitivity to the actual ELISA, risking non-detection of some infected plants. On the other hand, testing each plant by itself would be a waste of sensitivity if there are not plants that have such low virus concentration. Additional work is necessary to define the appropriate relationship between acceptable cost and sensitivity.

To assess the detection spectrum of the system of primers 1–14 and fluorogenic probe B, isolates from various origins and with known biological properties were assayed. It was possible to detect all the isolates whose origin could be traced back to the Mediterranean Basin. However, isolates from regions where *Toxoptera citricida* is present (Madeira Island, Reunion Island) often could not be detected. It was observed that, in these cases, the specific product of PCR amplification was produced, but the Delta RQ values remained low. When correlated to biological data, it was seen that the low Delta RQ values corresponded to isolates causing stem pitting in Madam Vinous sweet orange.

To further elucidate this aspect, several cDNA clones of the coat protein gene of these isolates were sequenced. These isolates had at least one mismatch in the zone of the fluorogenic probe. This was expected as it has been reported that mismatches in the segment between the reporter and the quencher drastically reduce the cleavage of the probe (LEE et al, 1993).

We are now seeking to design a probe specific to stem pitting strains, not present until now at the Mediterranean basin. The capability of detection of these strains is extremely important due to the threat of invasion of citrus in the Mediterranean Basin by *T citricida* and associated stem pitting strains from Madeira Island.

aknowledgements

Portions of this work were supported by the project "New Approach for Diagnosis and Prevention of Tristeza Outbreaks" supported by a grant from the Common Fund for Commodities.

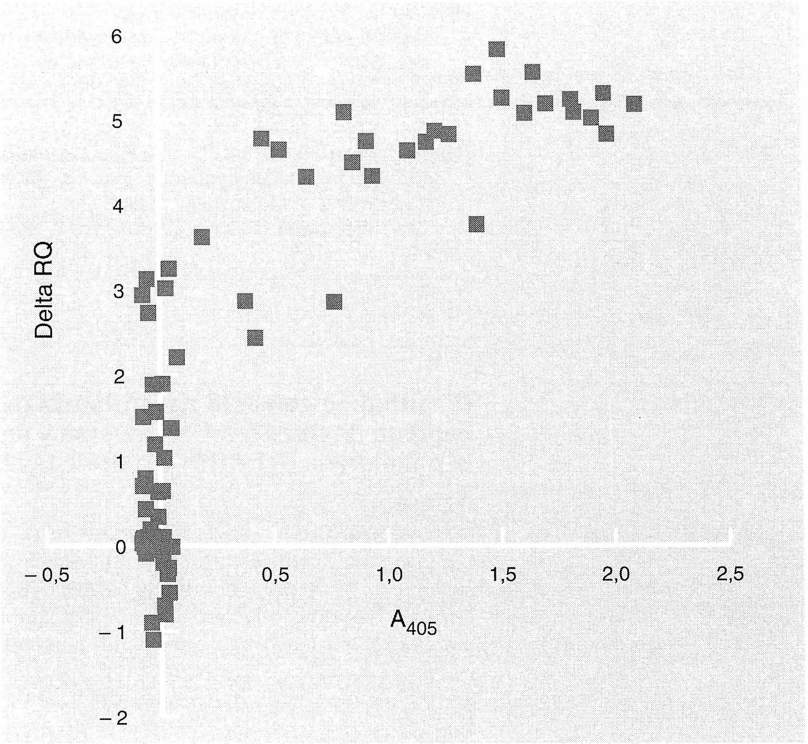

Figure 4
IC/RT-PCR values obtained with field samples plotted against Elisa values. Negative control values have been extracted.

references

Ben-Ze'Ev IS, Bar-Joseph M, Nitzan Y, Marcus R (1989) A severe citrus tristeza virus isolate causing the collapse of trees of sour orange before virus is detectable throughout the canopy. *Ann appl Biol* 114, 293-300

Gillings M, Broadbent P, Insto J, Lee R (1993) Characterization of isolates and strains of citrus tristeza closterovirus using restriction analysis of the coat protein gene amplified by the polymerase chain reaction. *J Virol Methods* 44, 305-317

Holland P, Abramson RD, Watson R, Gelfand DH (1991) Detection of specific polymerase chain reaction product by utilizing the 3'-5' exonuclease activity of Taq DNA polymerase. *Proc Natl Acad Sci USA* 88, 7276-7280

Lee LG, Connell CR, Bloch W (1993) Allelic discrimination by nick translation PCR with fluorogenic probes. *Nucleic acids Res* 21, 3761-3766

Nolasco G, de Blas C, Borja MJ, Torres V, Ponz F (1992) *Procedimiento para la deteccion e identification de patogeneos virales y sub-virales.* Registro de la Propriedad Industrial de España. Patente de Invencion PE2044784.

Nolasco G, de Blas C, Torres V, Ponz F (1993) A method combining immunocapture and PCR amplification in a microtiter plate for the detection of plant viruses and subviral pathogens. *J Virol Methods* 45, 201-218

Pappu H, Pappu S, Niblett CL, Lee RF, Civerolo E (1993) Comparative sequence analysis of the coat protein of biologically distinct citrus tristeza closterovirus isolates. *Virus Genes* 7, 255-264

Permar TA, Garnsey SM, Gumpf DJ, Lee RF (1990) A monoclonal antibody that discriminates strains of citrus tristeza virus. *Phytopathology* 80, 224-228

Diagnóstico sensible de la tristeza de los agrios por inmunocaptación (IC) seguida de transcriptasa inversa y amplificación en cadena de la polimerasa (RT-PRC) e hidrólisis enzimática de sondas fluorescentes.

RESUMEN

INTRODUCCIÓN. Se ha utilizado, durante estos últimos años, preferentemente el test ELISA como método para diagnosticar el CTV. No obstante, esta técnica se ha mostrado a veces muy poco sensible para detectar el virus antes de la observación de los síntomas. Los métodos de diagnóstico basados en la amplificación del genoma son bastante atractivos : recientes métodos de amplificación del DNA in vitro, como la técnica PCR, se han mostrado mucho más sensibles en numerosos virus, y entre ellos el CTV, que el método ELISA. Lo que se presenta en estos trabajos, es una técnica de diagnóstico del CTV basada en el método IC/RT-PCR. Se combina así la simplicidad del test ELISA con las ventajas de métodos que utilizan el DNA. **MATERIAL Y MÉTODOS.** Se tomaron muestras infectadas por aislados de CTV de diferentes orígenes. Los cebos y las sondas fluorescentes se escogieron basándose en la secuencia del gen que codifica la proteína de la envoltura del CTV de raza T36 de Florida. Se elaboró un método IC/RT-PCR simplificado. Tras amplificación por PCR, el análisis electroforético de los resultados se sustituyó por la medida de la fluorescencia. Se compararon los resultados obtenidos mediante esta técnica con los del test ELISA que se utilizó paralelamente en las mismas muestras. **RESULTADOS Y DISCUSIÓN.** La técnica PCR ha permitido detectar virus no sólo en todas las muestras ELISA-positivas, sino también en el 20% de ELISA-negativas. El método probado, más sensible que el test ELISA, se revela como un mejor instrumento para detectar la enfermedad.

PALABRAS CLAVES

Citrus, virosis, diagnóstico, técnicas immunocaptura, ELISA, biología molecular, PCR, métodos, tristeza.