Immunocapture RT-PCR detection and partial nucleotide sequence of the coat protein gene of citrus psorosis virus from Egypt

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ABSTRACT

Citrus psorosis virus (CPsV) is a multipartite virus with ssRNA genome present in very low concentration in infected citrus tissues. Diagnosis is made by biological indexing on indicator citrus seedling but it is time consuming and expensive procedure; therefore it is not used generally. Direct tissue blot immunoassay (DTBIA) technique which requires very little sample manipulation, has been used for rapid detection of CPsV proved to be more sensitive and cheaper than ELISA. Attemptes for the detection of CPsV by DTBIA technique using flower explant tissues from CPsV infected isolates with a conjugate CPsV polyclonal antibody showed high signals especially with the ovary referring to the high titre of the virus in the ovary. A high reliable sensitive immunocapture – reverse transcription-polymerase chain reaction (IC- RT-PCR) assay followed by nested-PCR was developed for the detection of CPsV. Two sets of primers were used to amplify the full length coat protein ORF (1365 bp) and a core region of 600 bp of the viral coat protein. The 600 bp core- CP fragment from the isolate, CPsVE1, was cloned and sequenced. The sequence comparative analysis of nucleotides showed 98% similarity with CPV4 and CPV-4e isolates belonging to Argentina and Florida, respectively. Therefore, the CPVE1 isolate is probably considered the type strain of CPV especially citrus ring spot virus.

Key words: Citrus CPsV, IC-RT-PCR, nested-PCR, Cp-gene, sequence comparative analysis.

INTRODUCTION

Citrus psorosis (CPsV) is a widespread and damaging disease of citrus in many parts of the world especially South America and the Mediterranean basin. CPsV is associated with a serious disease causing the death of citrus trees (Roistacher, 1993). In Argentina, the disease seems to be spread by an unknown vector (Beñatena and Portillo, 1984), and continues to cause serious losses (Danos, 1990). However, due to low concentration of the virus and poor stability of the particles, its genome has not been fully characterized. Psorosis was characterized by different forms like psorosis A , psorosis B and ring spot , related by cross protection (Derrick *et al.,* 1991 and Roistacher and Calavan 1965).There are large variations among psorosis isolates with respect to their mode of transmission, apart from grafting , since some are mechanically transmissible whereas others like the severe Argentine and Urug Uayan strains may be possibly vector transmitted (Roistacher, 1991 and 1993) , although the putative vector is unknown.

Partial characterization of several isolates in the USA (Derrick *et al.;* 1988, 1991), Spain (Navas-Castillo and Moreno, 1993; 1995; Navas-Castillo *et al.,* 1993), Israel (da Graça *et al.,* 1993) and Argentina (García *et al.,* 1991) indicated that at least two sediment components (top or T and bottom or B) are required for infectivity. In both components, circular filamentous particles are observed, with similar morphology although the B particles are about five times larger than the T particles (García *et al.,* 1994). Both particles share a single coat protein (CP) (Derrick *et al.,* 1988, García *et al.,* 1991). More recent results have confirmed that one type of RNA (RNA 1) is in the B component, but Northern blot hybridization indicated that in the T component there are two different segments of RNA (RNA2 and RNA 3) of approximately 1650 and 1500 nucleotides respectively and RNA 3 codes for the coat protein (Sanchezdela Torre *et al.,* 1988 and Barthe *et al.,* 1998). CPV is a multicomponent ssRNA virus with a coat protein of approximately 48 KDa (Derrick *et al.,* 1988 ,1991 and Garcia *et al.,* 1991).

For many years, laborious and costly indexing on citrus indicator was only the diagnostic method available (Roistacher, 1993). Sensitive laboratory procedures such as enzyme linked immunosorbent assay (ELISA) (D`Onghia *et al.,* 1998 & 2001a and Alioto *et al.,* 1999 & 2000) and RT-PCR ( Garcia *et al.,* 1997 ; Legarreta *et al.,* 2000; D`Onghia *et al.,*. 2001b and Barthe *et al.,* 1998) and Direct tissue blot immunoassay (DTBIA) was used for the rapid detection of CPsV (D`Onghia *et al.,* 2001b). The biological indexing requires a temperature- controlled greenhouse facility and 2-4 months for the evaluation of the indexed materials. Even under optimum conditions, many isolates of CPsV give faint, transitory local symptoms on indicator plants, making them difficult to detect biologically. There is a need for sensitive non-biological techniques of detecting CPsV, which is one of the few remaining important graft transmissible disease agents of citrus that can only be detected by biological indexing. In order to provide a more rapid and effective detection systems, we have used the DTBIA using polyclonal antibodies and develop detection methods for CPsV using Immunocapture-RT-PCR and nested-PCR.. Furthermore, we report here the partial nucleotide sequence of the coat protein gene of an Egyptian isolate of CPsV deposited at the Genbank (accession No.DQ304108).

MATERIALS AND METHODS

Source of virus isolates

CPsV isolates showing typical Psorosis – A symptom (bark scaling and ring spots leaf pattern) were collected from Horticulture Research Institute Orchard, Agricultural Research Center (ARC), and were serologically and biologically characterized. Budwood from these isolates were used for indexing by the method of Roistacher (1991). A total of four indicator seedlings of sweet orange (*C sinensis*) were graft inoculated from each tree. Plants were grown in a greenhouse at about 24-27 °C and inspected at least once each week for symptoms.

Direct tissue blots immunoassay (DTBIA)

Direct tissue blot immunoassay (DTBIA) was used for detection of CPsV as described by Garnesey *et al.* (1993) and D`Onghia *et al.* (2001b).

Immunocapture nested RT-PCR assay

Two sets of primers (Table 1) were designed to amplify the full coat protein gene (CPV3 &CPV4) and the core region of the coat protein gene (CPV1 & CPV2) according to Barthe *et al.* (1998). The tested samples were prepared in a dilution of 1/10 by grinding

0.3 g of infected and uninfected tissues in 3 ml sample extraction buffer, (4 mM $NaHPO_4$, 1.8 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, PVP, pH 7.4). Eppendorf tubes were pre-coated with 50 µl of 1: 500 dilution of virus IgG in coating buffer (11 mM Na_2CO_3 and $NaHCO_3$). The tubes were incubated for 3 hr at 37°C, then washed 3 times each 5 min with 150 µl of sterile washing buffer (135 mM NaCl; 15 mM KH_2PO_4; 20 mM $Na_2 HPO_4.12 H_2O$; 25 mM KCl and 0.05 % Tween-20). Aliquots of 50 µl of clarified tissue extracts were added to each tube, then Antigen trapping on the inner surface of the PCR tubes occurred through incubating overnight at 4 °C. The tubes were then washed three times with PBS -Tween-20 buffer. The RT-PCR mixture containing 1 X PCR buffer; 4 mM $MgCl_2$; 0.2 mM dNTPs ; 10 pmol of each primers CPV3 and CPV4; 10 U AMV ; 2.5 U of Taq DNA Polymerase were completed to a final volume of 50 µl by demonized water. The RT was performed in a thermocyler (Biometra Co.) with 42°C for 45 min, then the PCR was performed with 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 30 sec ; 50 °C for 30 sec and 72°C for 1 min. For nested - PCR, 0.3 µl from the IC-RT- PCR products was added to nested PCR mixture containing for each sample 1 XPCR buffer; 10 pmol of each complementary and homologous primer (CPV1 and CPV2) , 0.2 mM dNTPs , 2.5 U Taq DNA polymerase were added to a final volume of 50 µl by deionized water. Tubes were placed on the thermocycler and processed as described previously for PCR with final extension at 72°C for 10 min.

Table (1): Oligonucleotide sequences of the used primers.

Primer	Sequence 5`.....3`	Position
CPV1	GCTTCCTGGAAAAGCTGATG	654
CPV2	TCTGTTTTTGTCAACACACTCC	1253
CPV3	CCAACGCTAGCATGTCGATTCCTATTAAAG	1
CPV4	GACGAATTCTTAAAAGCATAACATGCAAGC	1365

Cloning and Nucleotide Sequencing

An amplified DNA fragment of expected size (600 bp) of the core coat protein gene (CP), obtained after nested-PCR amplification of CPVE1 isolate was directly ligated with linearized and thymidylated pGEM-T-easy vector. The ligation was transformed into *Escherichi coli* DH5α competent cells. Recombinant colonies were screened and tested for the presence of the CPV-CP fragment using the PCR technique. Clones from transformed cells were purified using the Wizard minipreps DNA purification system (Promega Corporation MD) and analyzed on 1% agarose gel followed by restriction digestion with *EcoR1* endonuclease enzyme. The core CP region of CPV in the purified recombinant pGEM-T-easy plasmid was sequenced in one direction with the CPV1 specific forward primer (Vaccera Company, Egypt) using ABI PRISM model 310 version 3.4 semi-adaptive version 3.2. Sequence analyses were performed using BioEdit program version 5.0.6 and the homology tree analyses were done using DNAMAN trial version 5.2.10 program.

RESULTS AND DISCUSSION

The present study demonstrates the successful use of DTBIA, IC-PCR and nested–PCR to directly detect CPsV in infected citrus trees. Citrus psorosis Egyptian isolate-1(CPsVE1) induced shock reaction symptoms

in the emerging young shoots (Fig.1-A), young leaf mottle and yellows in sweet orange (*C. sinensis*) under cool condition (Fig. 1-B). Psorosis is partly controllable by bud wood certification and indexing through grafting inoculation to citrus indicator plants and examining the resulting symptoms under controlled conditions. Such indexing requires special skills and facilities, in addition it is slow, costly and not applied in many countries. Thus there is a need to develop more sensitive tools for diagnosis such as molecular and serological methods.

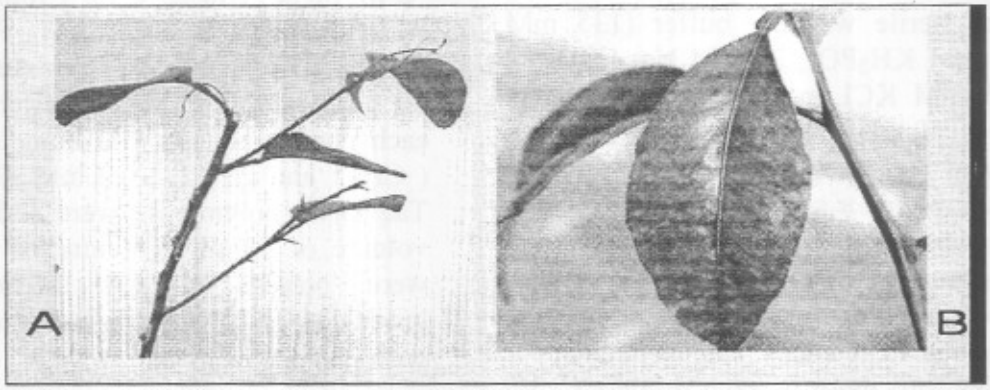

Fig. (1): A. Psorosis –A shock symptom in inoculated seedlings of orange showing the shock remain on the plant and do not drop off.. B: leaf mottle and yellows symptoms in sweet orange under cool condition.

Direct tissue blot immunoassay (DTBIA) was carried out for the detection of CPsV in several types of tissue (young leaves, old leaves, flowers and stems) from different collected isolates (Fig. 2) - E1- 5 & E7 -11). CPsV was clearly detected in tissue blots of ovary and stigma from CPsV infected trees giving strong signals as illustrated by the arrows, whereas blots from old infected leaves and stems gave weak signals (Fig. 2) and detection was less consistent. No reaction was observed in equivalent prints from healthy plants (Fig. 2 grid E6 & E12).

The result implies that CPsV can be readily and specifically detected by DTBIA when flower tissues are available. This would limit the use of DTBIA in early stage of infection when a suitable tissue is not available. However, DTBIA has the advantage of being simple, cheaper and faster than ELISA (Cambra *et al.,* 2000) and can give the same specificity and sensitivity if tissue is selected appropriately (flower tissue). Tissue print can be prepared in the field and stored for long periods without loss of reactivity. This possibility is very convenient for epidemiological studies in which large areas away from the laboratory are to be sampled in a short period. DTBIA is a technique that requires very little sample manipulation, in addition it has been used for rapid detection of several viruses proving to be more sensitive and cheaper than ELISA (Lin *et al.,* 1990; Hsu and Lawson, 1991; Garnsery *et al.,* 1993; Makkouk and Comeau , 1994; Hsu *et al.,*. 1995; D`Onghia *et al.,*. 2001b and Martin *et al.,* 2002).

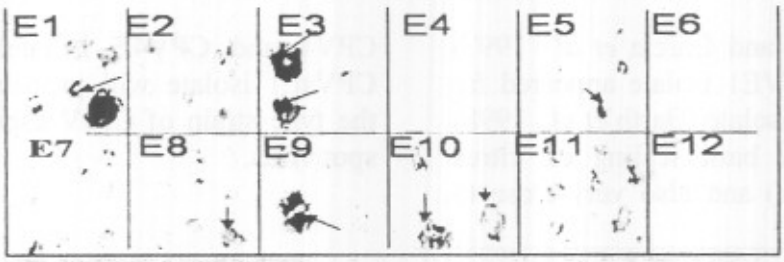

Fig. (2): Immunodetection of field CPsV isolates, collected from Horticulture Research Institute Orchard, with polyclonal antibodies (grids E1-E11). Tissue prints were prepared by gently pressing the cut surface of transversal sections from young leaves, old leaves, flowers and stems of infected and healthy (grid E6 & E12) tissues on nitrocellulose membrane. The arrows show the tissue print from the ovary and stigma.

The CPV3 and CPV4 primers were used to amplify a fragment of 1365 bp representing the complete Open Reading Frame (ORF) of the coat protein (CP) gene using IC/RT-PCR technique, followed by nested PCR using another two set of primers (CPV 1 and CPV2 primers) to amplify a core region of 600 bp of the CPsV CP- ORF according to Barthe *et al.* (1998) and Garcia *et al.* (1997). The amplification of CPsV cDNA from only selected three CPsV infected citrus trees (CPVE1, CPVE3 and CPVE10, previously gave sharp positive signals by the DTBIA) using IC-RT-PCR followed by nested PCR was positive. A major DNA product of approximately 1365 bp and 600 bp were amplified as a product of captured RNA preparations from various isolates of CPsV as shown in Fig (3-A).

The non-radioactive DIG –labelled CPsV cDNA probe, amplified from the full length coat protein gene, was used for detection of the CPsV using Southern blot hybridization technique. The analysis revealed intensive signals as shown in Fig. (3 - B) when hybridized with the RT-PCR products (1365 bp and 600 bp) from different CPsV infected trees. No hybridization was detected in capture RNA from uninfected citrus seedling.

One of our research objectives was to clone and sequence the coat protein gene of CPsV. The 600 bp amplified CP gene product of the isolate CPVE1 was ligated into the pGEM-T-easy vector and cloned. Only one clone of CPVsE1 isolate was sequenced revealing 574 bases as expected. The terminal part (25 bases long) corresponding to the forward primer were absent due to the use of the forward primer in sequencing reaction as shown in Fig. (4). A multiple alignment was done along with previously obtained sequences by Barthe *et al.*, 1998; Garcia *et al.*, 1997 and from the GenBank sequence data. The following sequences were used in the comparisons: Florida isolates CPV-4E & CPV-6 (AFO36338 and AF036926), CPV4-Argentina citrus ringspot viruses isolate (AF060855), NA88-Italy isolates (AY194918) and the CP sequence part from P121- Spanish strain (AY654894) from the GenBank. The partial nucleotide sequence alignment (Fig. 4) showed 98 % similarity with CPV4 and CPV-4e isolates belonging to Argentina and Florida respectively. In the meantime the Egyptian isolate showed 86 % similarity with P121, NA88 and CPV-6 of Spain, Italy and Florida, respectively. However, The phylogenetic homology tree based on multiple sequence alignments (Fig. 5) of the CPVE1- Egyptian isolate, reveled that the (CP) of the CPVE1 was closely related to CPV-4 and CPV-4E of Argentina and Florida citrus ringspot virus

(Barthe *et al.*,, 1998 and Garcia *et al.*, 1997) revealed that the CPVE1 isolate appeared far from CPV-6 Florida isolate (Barth *et al.*, 1998) which causes severe bark scaling on citrus (CPV-A- Scally bark) and also very close to CPV4 and CPV4E isolates. Therefore, the CPVsE1 isolate will probably be considered the type strain of CPsV especially citrus ring spot virus.

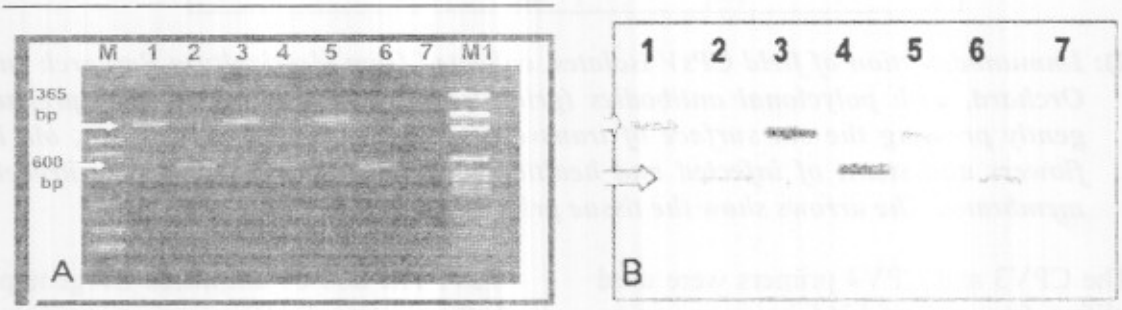

Fig. (3)-A :1 % agarose gel electrophoresis for IC-RT-PCR amplified products (1365 bp fragment) of the complete coat protein gene of three CPsV isolates (CPVE1, CVPE3 and CPVE10) using primers 3 and 4 (lane 1 , 3 and 5 for CPVE1, CPVE3 and CPVE10, respectively) and nested –RT-PCR amplified products of core coat protein gene (600 bp fragment) for the amplified CP-ORF of isolates using primers 1 and 2 (lane 2, 4 and 6).No RT-PCR amplification from uninfected tissues (Lane 7). Lane M: PCR DNA marker (Promega).Lane M1: pGEM DNA marker (Promega).B. Southern hybridization analysis using non-radioactve DIG – labelled cDNA probe, showing corresponding hybridization signals with the nucleic acid amplified products (1365 bp fragment) of the complete coat protein gene and Nested –RT-PCR amplified products (600 bp fragment) of three CPsV isolates (CPVE1, CVPE3 and CPVE10) as illustrated in (Fig A) .

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1   AAAGAAGTTCTGAATTTGTCAAGTCCAACTGTATGATGGTGACGTGAGTTTGTCACAAATCAGTGATGAG 70

71  TTGAGCCATGCTCCAACCAAGAAATTCCCTGCAAGGGTATTTCTCAAGATTGATATTGATAACTTGCCAA 140

141 GTGCTGTTTGCTCCAGATGCAAACTGAATATTGCTGGAAATCGATCTGTGAGGTATGCCAGCTTTGCAGG 210

211 CAGCTTTCAAACAAAGCAAAAGTTGTCCCCCTGCTGTTGGTGCAACTCCTGAGTCCTTGATGCCTTTACT 280

281 AGAAACAAACCAGAAGAT.GAGAAGGCAATTGCAATTAGGGATTTCTTAAAAACGATGGAAGGTCAATGG 350

351 AAAAACCAGAAGCGACTCCACCCCTTATCTGATGAGAAACCGACAATCAAGAACTTCACCTTGAAGCTAA 420

421 CATGCGCTATAATCTACAGCCTCACTCCAGATGGAAGAACTGACATGGCAGAGAGGATAATATCCGACAA 490

491 TAATAAGGGGTTTCAGAATGATAGGAACTTTTTTGGTGATGGGTGAAAGGGCCAATAAGGACTGAATGGT 560

561 GTTGACAAAACAG 573
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Fig. (4): Nucleotide sequence of cloned CPsVE1 core coat protein gene.

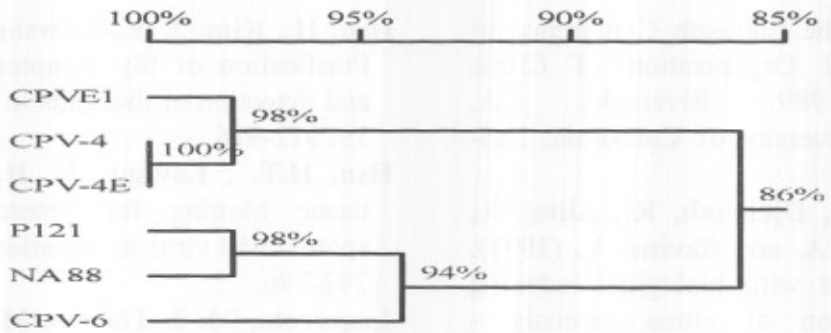

Fig. (5): The phylogenetic homology tree based on multiple sequence alignments of the CPVE1-Egyptian isolate compared to previously sequenced isolates.

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الملخص العربي

الكشف باستخدام تفاعل الأدمصاص المناعىالمتبوع بتفاعل البلمرة المتسلسل للنسخ العكسى و التتابع النيوكليوتيدى الجزئى لجين الغلاف البروتينى لفيروس قوباء الموالح المعزول من مصر

محمود أحمد عامر و هالة عبدالله آمين و عزه جلال فرج
معهد بحوث أمراض النباتات- مركز البحوث الزراعية- الجيزة- مصر

يعتبر مرض قوباء الموالح من أهم الأمراض الفيروسية التى تصيب اشجار الموالح فى مصر والذى يحتل المرتبة الثانية بعد مرض التدهور السريع فى الموالح . هذا الفيروس من الفيروسات متعددة الجينوم ذات الحمض الريبوزى (ر ن أ) والذى يتواجد فى الأنسجة النباتية المصابة بتركيزات منخفضة .و قد استخدمت كثير من الطرق سواء الفحص البيولوجى أو السيرولوجى أو الطرق الحديثة التى تعتمد على استخدام الحمض النووى فى الكشف عن العزلة المصرية تحت الدراسة. ولقد تم الفحص البيولوجى على واحد من أهم العوائل المشخصة لهذا الفيروس ولكن يعاب على هذه الطريقة أنها تستغرق وقتا طويلا. ولذلك تم اللجوء الى استخدام الطرق الأكثر تطورا وهي طريقة البصمة النسيجية كطريقة سيرولوجيية وذلك باستخدام الأجسام المضادة المتعددة الكلون للكشف عن الفيروس فى الأنسجة المختلفة للاشجار المصابة وجد أن انسجة مبايض الزهرة هى أكثر الأنسجة التى تحتوى على أعلى تركيز من جسيمات الفيروس. و بأستخدام الطرق الأكثر تطورا مثل طريقة تفاعل البلمرة التسلسل والتى تعتمد على ادمصاص الأجسام المضادة وذلك باستخدام بادئ متخصص فى منطقة الغلاف البروتينى لهذا الفيروس وبادئ أخر متخصص داخل نفس المنطقة من الغلاف البروتنى فى الكشف عن الفيروس وقد وجد أن هناك حزمتين من الحمض النووى المتخصصة لهذا الفيروس حجمها حوالى و 600 و1365 قاعدة نوكلوتيدية باستخدام البادئ الرئيسى والبادئ الداخلى كامل التحديد على التوالى.تم دراسة التتابع النوكلتيدى لجزء من الغلاف البروتينى (600) نوكلوتيدة وتم مقارنة التاتج بعزلات أخرى تم الحصول عليها من بنك الجينات ووجد أن العزلة المصرية تحت الدراسة أقرب تماثلا مع العزلة لنفس الفيروس والتى تنتمى الى الأرجنتين وفلوريدا والتى تنتمى الى سلالة التبقع الحلقى حيث وصلت نسبة التماثل الى 98 % .