

DETECTION OF *Citrus psorosis ophiovirus* IN CITRUS CV. NAVEL ORANGE IN EGYPT

R. E. SALEM¹, FATMA BADAWEY², M. I. SALAMA¹, HANAN NOUR EL-DIN¹, KH. A. EL-DOUGDOUG³ AND EMAN M. FAHMY²

1. Agricultural Genetic Engineering Research Institute (AGERI), ARC, Giza, Egypt

2. Dept. Genetics, Faculty of Agric., Ain Shams University, Shoubra El-Kheima, Egypt

3. Dept. Agric. Microbiol., Faculty of Agric., Ain Shams University, Shoubra El-Kheima, Egypt

Citrus is one of the most important fruit crops worldwide. Most of the citrus cultivars are grown as grafted plants. Virus pathogens transmitted by grafting as well as insect vectors could cause economic problems. Among graft transmissible diseases that have been reported from Egypt as well as in the rest of the Mediterranean countries; citrus psorosis disease that is of the most serious and remain the most spread disease in Egypt (Roistacher, 1991; Sofy, 2008).

In infected trees, scaly bark symptoms appears on the trunk, staining of interior wood of branch and gummy as well as shortened leaf internodes and mottling patterns on leaves (Ghazal *et al.*, 2008; El-DougDoug *et al.*, 2009).

Citrus psorosis virus (CPSV), the type species of the genus *Ophiovirus* (Vaira *et al.*, 2005), is the putative causal agent of psorosis, a major graft-transmissible disease of citrus (Roistacher, 1993).

Virus particles are formed by three single-stranded, negative sense RNAs and a coat protein (CP) of 48 kDa (Derrick *et*

al., 1988; Martín *et al.*, 2005). RNA1 contains 8184 bp and its complementary strand has two ORFs potentially encoding a 24 kDa protein of unknown function and a 280 kDa protein with motifs characteristic of RNA-dependent RNA polymerases (RdRp). RNA2 contains 1644 bp and its complementary strand encodes a 54 kDa protein without similarities to other known proteins and RNA3 contains 1454 bp and its complementary strand encodes the CP (Barthe *et al.*, 1998; Sańchez de la Torre *et al.*, 1998 & 2002; Naum-Onganía *et al.*, 2003; Martín *et al.*, 2005).

The sensitive, reliable and rapid identification of plant viruses is essential for effective disease control. For many years, laborious and costly indexing on citrus indicators was the only diagnostic method available (Roistacher, 1993).

The first breakthrough in the development of a laboratory diagnostic testing technique came in 1977 with the development of the ELISA (Clark and Adams, 1977).

DAS-ELISA (García *et al.*, 1997) and TAS-ELISA (Alioto *et al.*, 1999) are

methods developed and applied for psorosis diagnosis in field trees.

Polymerase chain reaction (PCR) was developed in the early 1990's and has the ability to selectively amplify a part of the target deoxyribonucleic acid (DNA). Reverse transcription-polymerase chain reaction (RT-PCR) (Garcia *et al.*, 1997; Legarreta *et al.*, 2000; D'Onghia *et al.*, 2001), are now being utilized and several primers have been designed for the CPsV detection by the RT-PCR providing an alternative method for diagnosis (Barthe *et al.*, 1998; Legarreta *et al.*, 2000).

This study aimed to detect CPsV in citrus trees cv. Navel orange *via* biological, serological and molecular methods.

MATERIALS AND METHODS

Samples collection

A set of 28 samples showing psorosis virus like symptoms were collected from citrus trees (cv. Navel orange) grown in El-Qanater area-El-Qaluobia Governorate, Egypt. Between 10-40 gm of mature and young leaves from each sample were stored at 4°C.

ELISA detection

Double antibody sandwich (DAS)-ELISA was used as described by Clark and Adams (1977); using monoclonal antibodies (MAbs) specific to CPsV which purchased from Valanzano, Italy.

Biological indexing

One from the +ve-ELISA citrus tree samples were indexed by graft inocu-

lation with two blind buds on Dweet tanger (indicator host). The grafted plants were kept under an insect-proof greenhouse indexing at 24-27°C (max. day)/18-21°C (min. night) and the symptoms development for 1 month was recorded according to Roistacher (1991).

Molecular detection

RNA extraction: According to the procedure described in Tripure Isolation Reagent Manual (Roche Diagnostics Corporation, IN, USA), total RNA was extracted from 50 mg leave tissues of citrus trees reacted with biological indexing and serological test, in addition to leave tissues of virus-free citrus tree as a control.

Primer design: Two sets of primers specific to CPsV were designed according to CPsV sequences which are available in the GenBank database of the national centre of biotechnology information (www.ncbi.nlm.nih.gov), for amplification of full and core sequence of *cp*-gene, CPsVR1 (GGC GGG *ATC* *CTC* *GAT* *TCC* *TAT* *TAA* *AGT* *GT*) and CPsVR2 (CGA *AAG* *CTT* *TTA* *CAT* *AGT* *CGC* *AGC* *CA*) to amplify the full length and CPsVN1 (*GTC* *ATC* *AAA* *CAG* *AAA* *ATG* *G*) and CPsVN2 (*GTT* *GAG* *TGA* *TTT* *AGT* *GTC*) to amplify core sequences of the gene (Fig. 1). The consensus nucleotides were underlined, while the italic ones refer to *Bam* HI and *Hind* III restriction sites in the CPsVR1 and CPsVR2, respectively. These oligonucleotides were synthesized at AGERI, Giza, Egypt. The oligonucleotides were desalted, ethanol pre-

cipitated, dissolved in dH₂O and quantitated by determining the OD at 260 nm.

RT-PCR: In this experiment, the protocol of the QIAGEN one step RT-PCR kit was successfully used for RT-PCR of CPsV-*cp* gene using 2 µg of the extracted total RNA, 10 µl of 5x Qiagen one step RT-PCR buffer, 2 µl of dNTP mix (containing 10 mM of each dNTP), 2 µl of QIAGEN Onestep RT-PCR enzyme mix, and 0.6 µM from each primer, then the total reaction volume was completed to 50 µl by RNase free d.H₂O.

The mix was subjected to one cycle at 50°C for 30 min; one cycle at 94°C for 15 min and 30 cycles, each consists of 94°C, 50°C and 72°C for 45 sec, 45 sec and 1 min, respectively, and the final cycle was extended for 10 min at 72°C. The PCR product was analyzed by agarose gel electrophoresis of 1.4% in TAE buffer stained with ethidium bromide and the product was visualized by transillumination as recommended by Sambrook *et al.* (1989).

RESULTS AND DISCUSSION

The 28 citrus trees exhibited virus-like symptoms of CPsV were tested against MAbs specific for CPsV Italian isolate. Results in Table (2) showed that a number of 3 out of 28 samples represent 10.07% gave positive. The positive ELISA values ranged between 0.621 and 0.740 compared to the negative (Healthy, 0.300) sample.

These citrus trees were visually inspected for leaf viral like symptoms (oak leaf pattern, mottling, ringspot, chlorosis, vein enation, crinkly), bark (bark scaling, gummies, and concavities) and fruit symptoms (acornshaped and ringspot).

CPsV isolate was detected in infected citrus trees which exhibited bark scaling and gum symptoms (Fig. 2) by DAS-ELISA. Also, it was detected by grafting on healthy citrus Dweet tangor seedlings which gave oak leaf pattern (OLP) symptoms on Dweet after 28 day from grafting (Fig. 3). The plants were maintained on the same host for further detection.

The total RNA was extracted from one of the three trees which reacted with biological indexing and gave positive result with DAS-ELISA (0.740). Total RNA was also extracted from virus-free citrus tissues and the quality of the RNA was assessed by ethidium bromide staining agarose gel electrophoresis and (Fig. 4) used as a template for RT-PCR.

Four reactions of RT-PCR were done by using the extracted RNA from each sample (negative and positive). The first reaction of one-step RT-PCR was done by the primers CPsVR1 and CPsVR2. Results in Fig. (5) showed that a fragment of 1320 bp was amplified from the +ve ELISA citrus tree sample, but no DNA band was amplified from the CPsV-free citrus tree sample.

To confirm that the amplified fragment is specific for *cp*-gene, nested RT-

PCR in addition to a combination of the designed primers were used. RT-PCR was conducted to amplify partial sequence of CPsV-*cp* gene using CPsVN1 and CPsVR2 primers producing the expected fragment of 1020 bp (Fig. 6). While DNA band of 540 bp was obtained when using the CPsVR1 and CPsVN2 primers in RT-PCR with +ve-ELISA citrus tree but not with CPsV-free citrus tree (Fig. 7).

For amplifying the core region of the *cp*-gene, the primers CPsVN1 and CPsVN2 were successfully to amplify a fragment of 240 bp from the +ve-ELISA citrus tree (Fig. 8).

For the detection of citrus viruses, ELISA is the most commonly employed procedure used because of its sensitivity, efficacy and economy (Garnsey and Cambra, 1991). However, many citrus viruses may be difficult to detect because of their low titer or uneven distribution. For these reasons, the use of rapid, sensitive and accurate detection methods were recommended.

Polyclonal antisera and monoclonal antibodies are now available for the sensitive detection of CPsV in virus-infected citrus tissues (Garcia *et al.*, 1997; D'Onghia *et al.*, 1998; Alioto *et al.*, 1999; Potere *et al.*, 1999; Djelouah *et al.*, 2000).

MABs specific to the Italian isolate of CPsV were used for the ELISA detection. A positive ELISA reaction was found between our viral isolate and the used MABs.

CPsV was detected in naturally infected citrus exhibited bark scaling and gum symptoms at Qanater, Qaluobia, Egypt. CPsV isolate was detected on the basis of biological assay which gave oak leaf pattern (OLP) on Dweet tangor and serological assay using MABs specific to CPsV by DAS-ELISA. These results agree with that found by Alioto *et al.* (1999) and Ghazal *et al.* (2008).

The CPsV isolate was indexed by graft inoculation on Dweet tangor which gave OLP symptom. The development of OLP was developed as vein clearing (hyperplasia), vein flecking and complete OLP 28 day post grafting. This was in harmony with that of Roistacher (1991) and Ghazal *et al.* (2008).

Molecular techniques have revolutionized the way of plant virus detection and identification. RT-PCR is the 'gold standard' method for virus detection in most laboratories worldwide because of its sensitivity, specificity, simplicity and rapidity. The PCR is a powerful technique, which is sensitive and highly specific and has been used to detect citrus viruses.

In this study, RT-PCR was successfully used for detection of CPsV in citrus tree(s) showed +ve-ELISA and reacted in biological indexing using four primer pairs flanking the full length and partial sequence of *cp* gene. These results are in agreed with that found by Garcia *et al.* (1997).

SUMMARY

Citrus psorosis ophiovirus (CPsV) was detected from naturally citrus cv. Navel orange trees exhibited virus-like symptoms (bark scaling and gum) at Qanater, Qalubia governorate, Egypt. CPsV isolate was detected by serological assay using monoclonal antibodies (Mabs) specific to CPsV by double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA), results showed that three trees out of twenty eight trees gave positive reactions with ELISA, values ranged between 0.621 and 0.740 compared to the negative (Healthy, 0.300) sample. The results were biologically confirmed by showing oak leaf pattern (OLP) on Dweet tangor after 28 days of inoculation. These plants were maintained as a suitable propagative host for the CPsV. Molecular detection was carried out by reverse transcription-polymerase chain reaction (RT-PCR) with the extracted RNA from CPsV infected trees and tissues virus-free as a negative control and two sets of primers. Four reactions of one-step RT-PCR were done using the combination of the four primers, i.e., CPsVR1 & CPsVR2, CPsVN1 & CPsVR2, CPsVR1 & CPsVN2 and CPsVN1 & CPsVN2. Results showed that four DNA fragments of 1320, 1020, 540 and 240 bp were amplified respectively.

ACKNOWLEDGMENT

This project was supported financially by Science and Technology Development Fund (STDF), Egypt, Grant #34.

REFERENCES

- Alioto, D., M. Gangemi, S. Deaglio, P. Sposato, E. Noris, E. Luisoni and R. G. Milne (1999). Improved detection of citrus psorosis virus using polyclonal and monoclonal antibodies. *Plant Pathol.*, 48: 735-741.
- Barthe, G. A., T. L. Ceccardi, K. L. Manjunath and K. S. Derrick (1998). Citrus psorosis virus: nucleotide sequencing of the coat protein gene and detection by hybridization and RT-PCR. *J. Gen. Virol.* 79: 1531-1537.
- Clark, M. F. and A. N. Adams (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology*, 34: 475-483.
- Derrick, K. S., K. S. Bransky, R. F. Lee, L. W. Timmer and T. K. Nguyen (1988). Two components associated with citrus ringspot virus. In *Proc. 10th Conf. International Organization of Citrus Virologists*, p. 340-342. Edited by L. W. Timmer, S. M. Garnsey and L. Navarro. Riverside: Univ. of California IOCV.
- Djelouah, K., O. Potere, D. Boscia, A.M. D'Onghia and V. Savino (2000). Production of monoclonal antibodies to Citrus psorosis associated virus. *Proc. 14th Conf. International*

- Organization of Citrus Virologists, Campinas, 1998: 152-158.
- D'Onghia, A. M., K. Djelouah, D. Alioto, M. A. Castellano and V. Savino (1998). ELISA correlates with biological indexing for the detection of citrus psorosis-associated virus. *J. Plant Pathol.*, 80: 157-163.
- D'Onghia, A. M., K. Djelouah, D. Frasheri, and O. Potere (2001). Citrus psorosis virus detection by direct tissue blot immunoassay. *J. Plant Pathol.*, 83: 139-142.
- El-Dougdoug, Kh. A., S. A. Ghazal, A. A. Mousa, H. Fahmy and A. R. Sofy (2009). Differentiation among three Egyptian isolates of *Citrus psorosis virus*. *International Journal of Virology*, 5: 49-63.
- Garcia, M. L., M. E. De La Torre, E. Dal Bo, K. Djelouah, N. Rouag, E. Luisoni, R. G. Milne and O. Grau (1997). Detection of Citrus psorosis ringspot virus using RT-PCR and DAS ELISA. *Plant Pathology*, 46: 830-836.
- Garnsey, S. M. and M. Cambra (1991). Enzyme-linked immunosorbent assay (ELISA) for citrus pathogens. Pages 193-216 in: *Grafttransmissible diseases of citrus-A handbook for detection and diagnosis*. C.N Roistacher, ed. Food and Agriculture Organization of the United Nations, Rome.
- Ghazal, S. A., Kh. A. El-Dougdoug, A. A. Mousa, H. Fahmy and A. R. Sofy (2008). Isolation and identification of *Citrus psorosis virus* Egyptian isolate (CPsV-EG). *Commun. Agric. Appl. Biol. Sci.*, 73: 285-295.
- Legarreta, G. G., M. L. Garcia, N. Costa and O. Grau (2000). A highly sensitive heminested RT PCR assay for the detection of Citrus psorosis virus targeted to a conserved region of the genome. *Journal of Virological Methods*, 84: 15-22.
- Martín, S., C. Lo'pez, M. L. García, G. Naum-Onganía, O. Grau, R. Flores, P. Moreno and J. Guerri (2005). The complete nucleotide sequence of a Spanish isolate of Citrus psorosis virus: comparative analysis with other ophioviruses. *Arch. Virol.*, 150: 167-176.
- Naum-Onganía, G., S. Gago-Zachert, E. Peña, O. Grau and M. L. García (2003). Citrus psorosis virus RNA 1 is of negative polarity and potentially encodes in its complementary strand a 24 K protein of unknown function and 280 K putative RNA dependent RNA polymerase. *Virus Res.*, 96: 49-61.
- Potere, O., D. Boscia, K. Djelouah, V. Elicio and V. Savino (1999). Use of monoclonal antibodies to citrus psorosis virus for diagnosis. *J. Plant Pathol.*, 81: 209-212.
- Roistacher, C. N. (1991). Graft transmis-

- sible Diseases of Citrus. Handbook for Detection and Diagnosis. FAO, Rome, Italy.
- Roistacher, C. N. (1993). Psorosis: A review. In: Moreno P., da Graca J.V. and Timmer L.W. (eds). Proc. 12th Conf. International Organization of Citrus Virologists IOCV, Riverside 1993: 134-139
- Sambrook, J., E. F. Fritsch and T. Maniatis (1989). Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory, Cold Spring Harbor New York.
- Sánchez, de la Torre, E., O. Riva, R. Zandomeni, O. Grau and M. L. García (1998). The top component of citrus psorosis virus contains two ssRNAs, the smaller encodes the coat protein. Mol Plant Pathol On-Line. <http://www.bspp.org.uk/mppol/1998/1019sanchez/index.htm>
- Sánchez, de la Torre, M. E., C. López, O. Grau and M. L. García (2002). RNA 2 of Citrus psorosis virus is of negative polarity and has a single open reading frame in its complementary strand. J. Gen. Virol., 83: 1777-1781.
- Sofy, A. R. (2008). Biological and molecular studies on *Citrus psorosis virus* in Egypt. M. Sc., Fac. of Science, Al-Azhar Univ., PP. 195, Egypt.
- Vaira, A. M., G. P. Accotto, S. Gago-Zachert, M. L. Garcia, O. Grau, R. G. Milne, T. Morikawa, T. Natsuaki, V. Torok, M. Verbeek and H. J. Vetten (2005). Genus *Ophiovirus*. In: Fauquet C. M., Mayo M. A., Maniloff J., Desselberger U., Ball L. A. (eds.). Virus Taxonomy. Eight Report of the International Committee on Taxonomy of Viruses, p 673-679. Elsevier Academic Press, San Diego, USA.

Table (1): DAS-ELISA detection of CPsV in citrus samples using the MABs specific to the Italian isolate.

Samples #	ELISA detection		Samples #	ELISA detection	
	EV	R		EV	R
1	0.300	-	15	0.387	-
2	0.222	-	16	0.344	-
3	0.254	-	17	0.430	-
4	0.345	-	18	0.367	-
5	0.242	-	19	0.393	-
6	0.298	-	20	0.403	-
7	0.298	-	21	0.305	-
8	0.207	-	22	0.265	-
9	0.621	+	23	0.273	-
10	0.298	-	24	0.740	+
11	0.256	-	25	0.399	-
12	0.223	-	26	0.692	+
13	0.277	-	27	0.391	-
14	0.256	-	28	0.305	-

Positive control = 0.582.

Negative control = 0.280.

EV: ELISA values

R: Result.

+ : Positive

- : Negative

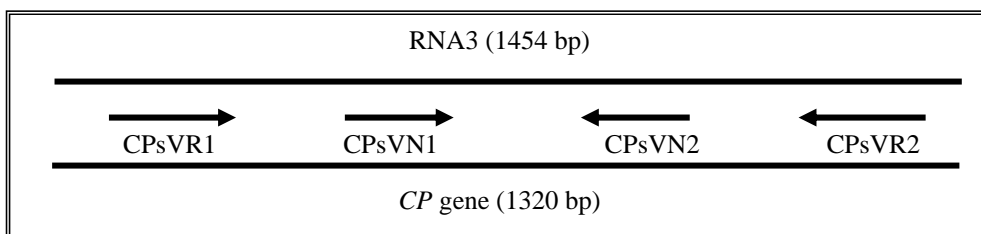


Fig. (1): RNA3 in CPsV genome which illustrates the location of the designed primer sets.

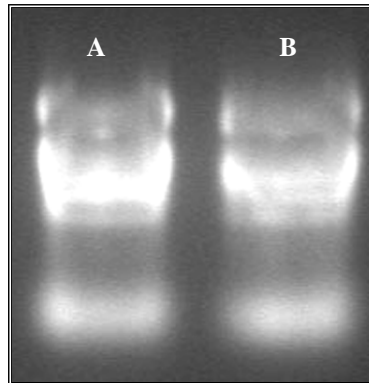


Fig. (2): Infected citrus tree cv. Navel orange exhibited bark scalling upper grafting zone above the bud union.



Fig. (3): Citrus Dweet tangor seedlings inoculated with CPsV-EG by grafting transmission showing oak leaf pattern (OLP).

Fig. (4): Total RNA extracted from +ve ELISA citrus tree (A) and CPsV-free citrus tree (B).



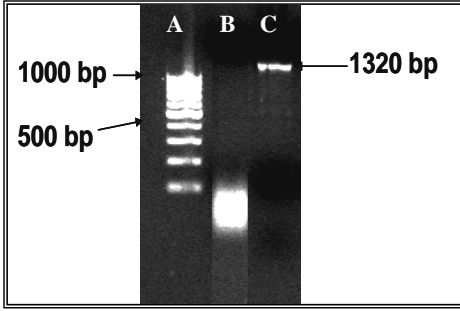


Fig. (5): RT-PCR for amplification of full length of CPsV-*cp* gene, by the primers CPsVR1 and CPsVR2 and fragment of 1320 bp was amplified from the +ve ELISA citrus tree sample, but no DNA band was amplified from the CPsV-free citrus tree sample. A: 100 bp DNA standard marker (Fermentas). B: RT-PCR for the negative sample, C: RT-PCR for the positive sample.

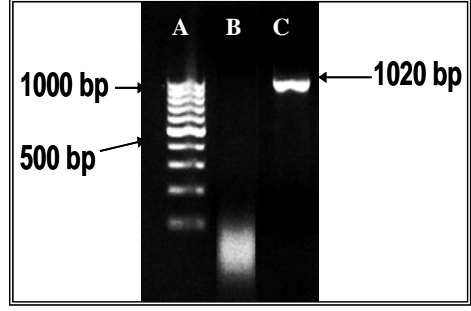


Fig. (6): RT-PCR for amplification of partial length of CPsV-*cp* gene, using CPsVN1 and CPsVR2 primers producing the expected fragment of 1020 bp in positive sample. A: 100 bp DNA standard marker (Fermentas). B: RT-PCR for the negative sample, C: RT-PCR for the positive sample.

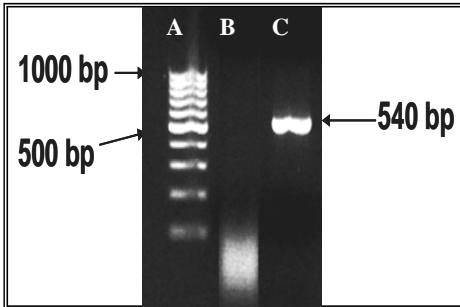


Fig. (7): RT-PCR for partial length of CPsV-*cp* gene using the CPsVR1 and CPsVN2 primers and 540 bp was obtained with +ve-ELISA citrus tree but not with CPsV-free citrus tree. A: 100 bp DNA standard marker (Fermentas). B: RT-PCR for the negative sample, C: RT-PCR for the positive sample.

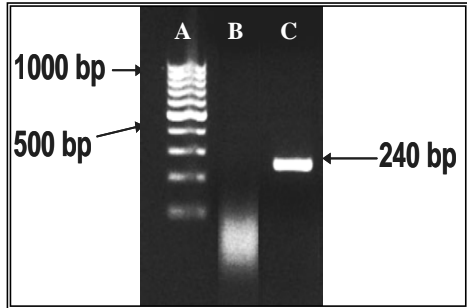


Fig. (8): RT-PCR for the core region of the *cp*-gene using the primers CPsVN1 and CPsVN2 to amplify a fragment of 240 bp from the +ve-ELISA citrus tree. A: 100 bp DNA standard marker (Fermentas). B: RT-PCR for the negative sample, C: RT-PCR for the positive sample.