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Original article

# Polyclonal antibodies against the recombinantly expressed coat protein of the *Citrus psorosis virus*

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ABSTRACT

Psorosis is a damaging disease of citrus that is widespread in many parts of the world. Citrus psorosis virus (CPsV), the type species of the genus Ophiovirus, is the putative causal agent of psorosis. Detection of CPsV by laboratory methods, serology in particular is a primary requirement for large-scale surveys but their production has been impaired by the difficulty of obtaining sufficient clean antigen for immunization. Specific PAbs against coat protein were produced in *E. coli* using recombinant DNA approach. The full length CP gene fragment was amplified by RT-PCR using total RNA extracted from CPsV infected citrus leaves and CP specific primers. The obtained product (1320bp) was cloned, sequenced and sub-cloned into $p^{ET\text{-}30(+)}$ expression vector. Expression was induced and screened in different bacterial clones by the presence of the expressed protein (48kDa) and optimized in one clone. Expressed CP was purified using batch chromatography under denaturing conditions. Specificity of expressed protein was demonstrated by ELISA before used as antigen for raising PAbs in mice. Specificity of the raised PAbs to CPsV was verified by ELISA and western blotting. The raised PAbs were showed highly effectiveness in screening by ELISA comparing with the commercial antibodies purchased from Agritest, Valanzano, Italy.

The expression of CPsV CP gene in *E. coli*, production of PAbs using recombinant protein as an antigen, the suitability of these antibodies for use in immunodiagnostics against the CPsV Egyptian isolate have been accomplished in this work.



## 1. Introduction

Citrus is a very important crop in many tropical and sub-tropical countries throughout the world, however certain virus and virus-like diseases together with fungal and bacterial diseases can limit production and in some cases are destroying and ravaging citrus as an industry or eliminating backyard trees (Martin et al., 2002). Psorosis is a damaging disease of citrus that is widespread in many parts of the world and is the first of the citrus virus diseases described and the oldest researched citrus virus disease (Garcia et al., 1997). Disease development is slow; it may take several years to manifest symptoms, which include bark scaling of the trunk, and chlorotic flecks and spots on young leaves. Gum may accumulate below the bark scales and may impregnate the xylem producing wood staining and vessel occlusion; these symptoms have been used for field diagnosis of Psorosis (Sertkaya, 2014). Citrus psorosis virus (CPsV), the type species of the genus Ophiovirus, is the putative causal agent of psorosis (Garcia et al., 1997). CPsV is a multi-components ssRNA virus with a coat protein of approximately 48 kDa (Barthe et al.,1998; Petrzik et al., 2001; Martín et al., 2006). CPsV genome consists of three ssRNAs of negative polarity, RNA1 contains 8184nt 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

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1644nt and its complementary strand encodes a 54 kDa protein (Martín et al., 2005), and RNA3 contains 1454nt and its complementary strand encodes the CP (Garcia et al., 1997; Barthe et al., 1998). For many years, laborious and costly indexing on citrus indicators was the only available diagnostic method (Alioto et al., 1999). Nowadays, ELISA and RT-PCR are the most reliable and rapid methods for detecting the presence of the virus in presumed infected-trees in the field (Martin et al., 2002). The RNA3 of CPsV which encodes for CP of 48KDa protein has been used for detection of the virus (Garcia et al., 1997). Detection of CPsV by laboratory methods serology in particular, is a primary requirement for large-scale surveys such as those carried out for sanitary selection in the framework of certification programs (Onghia et al., 2001). Virus particles are normally used as an antigen for polyclonal and monoclonal antibody production to be used subsequently for serological diagnosis (Elgaied et al., 2017). Their production has been impaired by the difficulty of obtaining sufficient clean antigen for immunization, due to the erratic transmission and low concentration of the virus in herbaceous hosts (Martin et al., 2002). In this paper, the expression of CPsV-CP gene of Egyptian viral isolate in *E. coli*, production of polyclonal antibodies using recombinant protein as an antigen, and the suitability of these antibodies for use in ELISA and western blotting immunodiagnostics have been reported.

## 2. Materials and methods

### 2.1. Virus source and CP gene isolation

Twenty-seven (27) samples showing psorosis like symptoms (mottling, ringspot, chlorosis, vein enation, crinkly), bark (bark scaling, gummies, concavities) and fruit symptoms (acorn shaped and ringspot), were collected from citrus trees (cv. navel orange) grown in Qanater area, Qaluobia governorate, Egypt and subjected for detection against CPsV antibodies by DAS-ELISA as described by Clark and Adams, 1977 using monoclonal antibodies (MAbs) specific to CPsV, purchased from Agritest, Valanzano, Italy.

Total RNA was extracted from 50 mg leave tissues of CPsV-infected citrus plants according to the procedure described in tripure isolation reagent manual, Roche, IN, USA. Coat protein gene specific primers were designed according to CPsV sequences available in the genbank database using lasergene (megAlign version 4.0) and primer select version 4.0; CPf GGCG*GGATCC*TCGATTCCTATTAAAGTGT and CPr CGA*AAGCTT*TTACATAGTCGCAGCCA, the consensus nucleotides are underlined, while the italic ones refer to *Bam* H1 and *Hind* III restriction sites in CPf and CPr, respectively.

RT-PCR was carried out using one-step RT-PCR protocol (Qiagen), 2 μg of total extracted RNA, 10 μl of 5× buffer, 2 μl of dNTP mix (containing 10 mM of each dNTP), 2 μl of enzyme mix, and 0.6 μM of each primer in total reaction volume 50 μl adjusted by RNase free $dH_2O$. RT-PCR cycling was performed in the following conditions: 50°C for 30 min, 94 °C for 15 min, 30 cycles 94 °C, 55 °C and 72 °C for 45 sec, and a final extension at 72 °C for 10 min. Amplified PCR products were analyzed on a 1.4% agarose gel.

### 2.2. Cloning, sequencing and expression the coat protein gene

The purified RT-PCR product was ligated into the pGEM®-T easy vector system I according to manufacturer's instructions and transformed *E. coli* (JM 109) cells. Recombinant plasmid was miniprepared from overnight grown *E. coli* in LB containing 100 μg/ml Amp. Recombinant and non-recombinant colonies were screened by blue-white selection. Plasmid recombination was confirmed by restriction endonuclease enzymes analysis. Isolated DNA was subjected for cycle sequencing using the fluorescent dideoxy chain terminator technology, big-dye terminator kit and an applied biosystem 373A sequencer. The determined nucleotide sequence was compared with other sequences deposited in the genbank.

The full length CP gene was sub-cloned into the expression vector $p^{ET\text{-}30(+)}$ (Novagen) in N-terminal tag 6xHis using *Bam* H1 and *Hind* III restriction enzymes and transformed into *E. coli* BL21 competent cells.

Expression of the coat protein gene was induced using 1mM IPTG. The presence of expressed CPsV-CP was verified in four randomly selected clones through rapid screening by SDS-PAGE analysis. Expression level was optimized *via* time course, by harvested cells in different times post induction with IPTG and analyzed by SDS-PAGE 12%.

### 2.3. Purification, specificity assaying and mice injection of the expressed protein

Purification of the expressed coat protein was carried out using Ni-NTA batch chromatography under denaturing conditions. Bacterial cells grown in 200 ml LB media were harvested after 16 h post induction with IPTG and gently resuspended in 4 ml lysis buffer (8 M urea; 0.1 M $NaH_2PO_4$; 0.01 M Tris-Cl, pH 8.0) to recovering the insoluble fusion proteins under denaturing conditions. Cell debris was removed by centrifugation at 10,000 rpm for 30 min at 4 °C. The supernatant was passed through Ni-NTA resin column (Qiagen) after incubation with 50% Ni-NTA slurry by gently shaking for 50 min at room temperature. 6xHis proteins were bound to the column in high pH of 8 M urea. The matrix washed out twice and 6xHis proteins were eluted against a pH gradient down to pH4.5, dialyzed against several changes of phosphate-buffered saline (PBS) solution at 4 °C and analyzed by SDS-PAGE. The concentration of purified protein was estimated using Bradford method. Specificity of the purified expressed proteins was assayed as antigen by DAS-ELISA against CPsV specific MAbs purchased from Agritest, Valanzano, Italy.

Antiserum was raised against expressed 6Xhis-tagged CPsV-CP by immunize Balb-C mice upon the approval of AGERI internal biosafety and bioethics committee. Six weeks old mice were administered by 100 μg expressed coat protein in a primary response; followed by four weekly intraperitoneal injections of 200 μg without remove of 6xhis-tag, where cleavage of the N-terminal fusion protein was not necessary as this not expected to have significant immunogenic properties (Mutasa-Gottgens et al., 2000). Mice were emulsified with an equal volume of freund's complete adjuvant for the first injection and incomplete adjuvents for the four subsequent intramuscular injections at weekly intervals. The blood was collected after 5 injections and incubated at 37 °C for 1h then centrifuged at 4000 rpm.

### 2.4. Evaluation the raised antibodies comparing with the commercial

Specificity of the raised antiserum was evaluated as trapped antibody by ELISA in a dilution 1:5,000 against bacterial cell extracts from transformed bacteria with recombinant and non-recombinant plasmids, total extracted proteins from healthy and CPsV-infected citrus plants as well as the purified fusion proteins. Similarly, western blotting was used to evaluate the raised antiserum against the same mentioned antigens. 12% SDS-PAGE was prepared and the polypeptides were transferred onto a membrane (immobilon® PVDF membrane, millipore cooperation, Bed ford, MA 01730) using a trans-blot apparatus (Bio-Rad). The membrane was blocked in TBS containing 5% BSA, and then the blocking buffer was replaced with the TBS containing raised antibodies (1:5,000). After incubation, the membrane was washed with TBST, and incubated in TBS containing anti-mouse universal antibodies and detection was developed by NBT/BCIP in alkaline phosphatase buffer.

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