Directional cloning of *Citrus* psorosis coat protein gene and protein expression

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Abstract

The full length of the coat protein gene of two Turkish isolates of Citrus psorosis virus was amplified by RT-PCR. The product of the CP-gene was cloned into the TA vector for sequencing in both directions, sense, and antisense. The full length of ORF CP-gene (1318 bp) of CPsV was cloned into pET101/D-TOPO with blunt end ligation. The 20 positive colonies for CPsV-CP gene were checked by PCR and sequenced for both ends. The correct direction insertion of the colonies was identified and a few of them were selected for protein expression. The Western blot was run for the CPsV-CP gene expression. The 48.6 kDa protein was expressed by induced cells with IPTG including CPsV-CP in 4 selected bacterial cells. The expressed coat protein of CPsV was shown with western blotting

Keywords: CPsV, open reading frame, TOPO cloning, Western blot

INTRODUCTION

Citrus psorosis virus (CPsV) is a member of the genus *Ophiovirus*, was first shown to be graft transmissible in citrus in the World (Fawcett and Bitancourt, 1943; Derrick et al., 1993; Milne et al., 2000). This pathogen is common worldwide and has two races "A" and "B". The CPsV shows different symptoms such as oak leaf patterns on the leaves of oranges, mandarins, lemons, and grapefruits. It shows also scaly trunks of oranges and mandarins. The shock symptom of the shoots on 'Pineapple' and 'Madam Vinous' indicator oranges, and flecking chlorosis on the leaves of rough lemon ocur when inoculated with the CPsV infected budwood (Wallace, 1957; Roistacher, 1991; Kayim, 2010). CPsV is filamentous particles with a 3-4 nm diameter consisting of three single-stranded negative sense RNAs and a coat protein of 48 kDa (Barthe et al., 1998; Martin et al., 2004). Serological tests like enzyme linked ımmuno sorbent assay (ELISA) and direct tissue blot ımmunoassay (DTBI) using polyclonal and monoclonal antibodies can be used to detect virus infection in citrus large scale. Serological detection has an advantage in time, cost, and labor when compared to molecular detection and biological indexing. To detect all isolates of CPsV in the field condition, it needs specific antibodies to react with specific isolates as CPsV has more diversity. The development of recombinant polyclonal antiserum specific to CPsV isolates in Turkey will provide a major advantage to detect CPsV-infected trees on a large scale. Therefore, Turkish isolates of CPsV were used to construct the pET101/D-TOPO-CPsV-CPG and induce protein expression in the bacterial cell.

MATERIALS AND METHODS

The RT-PCR and cloning of the full length of ORF of CPsV-CPG into pET101/D-TOPO

Total RNA was extracted from infected leaves of *Citrus sinensis* collected from a citrus orchard in Adana using an RNeasy Plant mini kit according to the protocol provided by the manufacturer's handbook (Qiagen, Germany). The RT-PCR was performed to amplify the full length of the coat protein gene (CPG) of Citrus psorosis virus (CPsV) using a standard protocol and cloned into a TA vector for sequencing (Barthe et al., 1998; Kayim et al., 2005) and amino acid translation of the nucleotides of the full length of ORF was shown in Figure 1. The full length of the ORF of the CPVs-CPG was inserted into pET101/D-TOPO with a blunt end by adding cacc at the 5' end of the ORF and deleting the stop codon from the 3' end (Figure 1).

Turkish isolates 2 of CPV-CPG 438aa

MSNPIKVSQLIDAKTKWESEKEKSPAEILTLLKTYGVLRDTGALSSKSIAFLSGLSKDKR
VILTDEGLAEFKSVENPHDQGGKPAFTLASSSNIKITDIEVIKQKMEQETFQIDQLKLKE
QIENFIKTVTLDDESYTEGEIIIKHFGNPDAELNMLITAGTKILDGLVYVSMKGDTKSLN
LFKMEQVDGVCDSDIIKNIRVAKRAIQAAFVLVFTQGSLPGKADDKRKVPEFVKSKLYDG
DVSLSQISEELSHAPTKKFPARVFLKIDIDNLPSAVCSRCKLNIAGNRSVRYAGFASSFQ
TKQKLSPAVGATPESLMPLLETNQKIEKSIAIRDFLKTMEGQWKNQKRLHPLSGEKPTIK
NFTLKLTCAIIYSLTPDGRIDMAERIITDKNKGFQNDRNFFGDGEGPTRTWSVLTKPEAD
FSNITVDGLKGIFGVASN

Figure 1. The full length of The amino acid sequence of Citrus psorosis virus coat protein gene of Turkish isolates.

The product of the coat protein gene was 1400 bp. This product included some more nucleotides before start codon at the 5 prime end some stop codons at the 3 prime end. These nucleotides were trimmed and CACC nucleotides were added at the 5 prime ends for TOPO cloning (Figure 2).

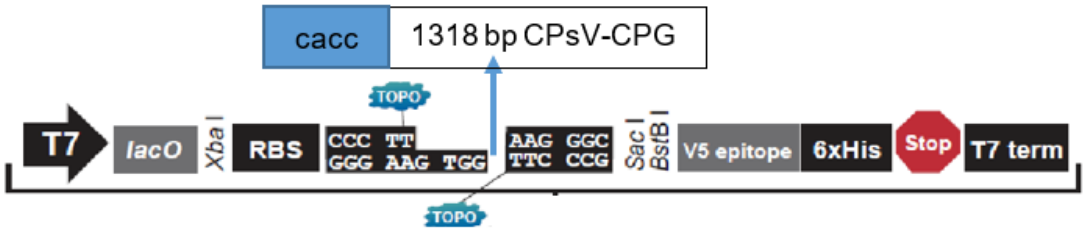


Figure 2. Cloning of the CPsV-CPG (1322 bp) into pET101/D-TOPO (5753 bp).

RESULTS AND DISCUSSION

For bacterial expression of the coat protein gene of the CPsV was inserted into the pET-101/D-TOPO (Invitrogen, ThermoFisher, Scientific, TR), successfully. The 20 colonies were selected on the antibiotic plates for PCR amplification. The 20 of them produced the full length of the coat protein gene product. From positive PCR amplification, 5 of them were selected for sequencing. All selected PCR amplification was correct for the full-length gene insertion, but only one was in the correct direction. This is-directional cloning is due to the resemblance of CACC nucleotides in front of the forward primer to reverse primer nucleotides. If there is similarity at the 5 prime end of the forward primer and the 5 prime end of the reverse primers, wrong directional cloning is occurring. This problem was repeated several times. Therefore we deleted these similar nucleotides from 5 prime end of the antisense primer. Then the correct direction of the cloned gene was obtained (Figure 3). The pET101/D-TOPO is 5753bp length. The expected size of the cloned gene 1322 bp was on the pET101/D-TOPO expression vector. The resulting plasmid was 7075 bp length.

All cloning protocols were used according to Invitro gen manufacturer's instructions. The correct direction of CPG insertion was proved by sequencing of cloned vector (pET101/D-TOPO-CPsV-CPG) and transformed into competent *E. coli* BL21 by electroporation according to manufacturer's instructions (Invitrogen, ThermoFisher Scientific).

Expression of recombinant CPsV coat protein Western blot analysis and protein purification

Colonies of *Escherichia coli* BL21 containing the CP from CPsV isolates, 2 and 5 were cultured in LB for 4 h at 37°C, and expression was induced by adding iso-propyl-β-D-thiogalactopyranoside (IPTG). Bacterial cells were then collected by centrifugation at 14000 rpm for 5 min.

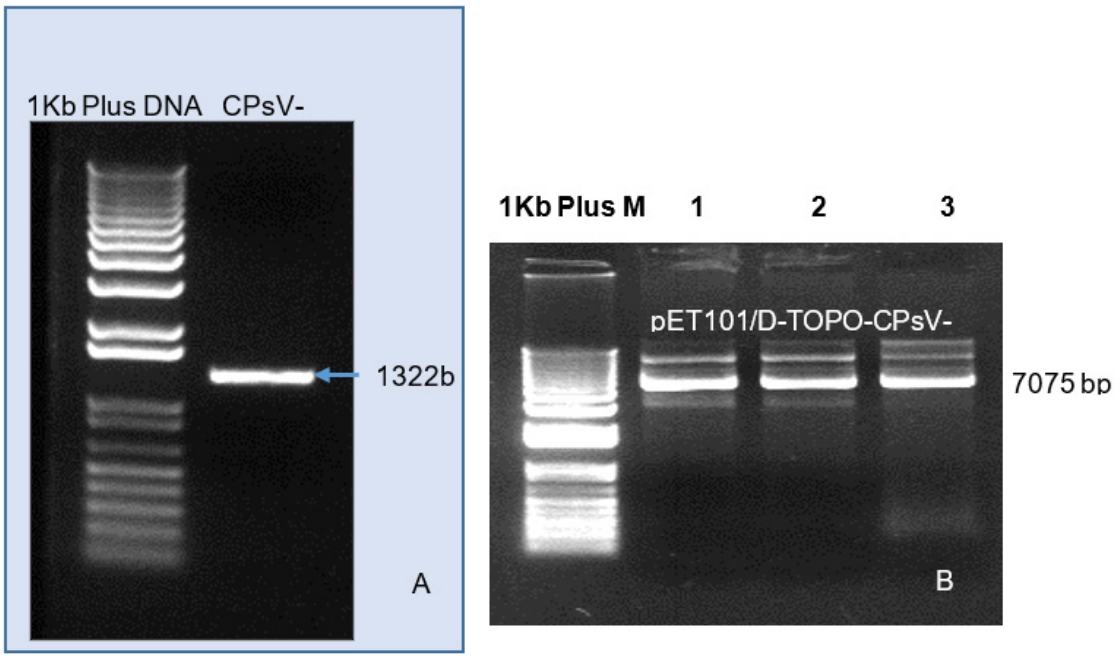


Figure 3. RT-PCR product of CPG on agarose gel (A). Selection of three clonies including the full length of CPVs-CPG on pET101/D-TOPO CPVs-CPG on 1% agarose gel (B).

SDS-gel electrophoresis was run at 100 V for 1 h after loading 10 µL of induced clone sample into wells. Western blot wet transfer was run at 80 V for an hour. Washing steps and development were done according to the protocol explained by the manufacturer and Kayim et al. (2004) (Figure 4). The protein expression was induced with 1mM (IPTG) in BL21 cells and the protein size was correct according to the expected size of 48.6 kDa (Figure 4).

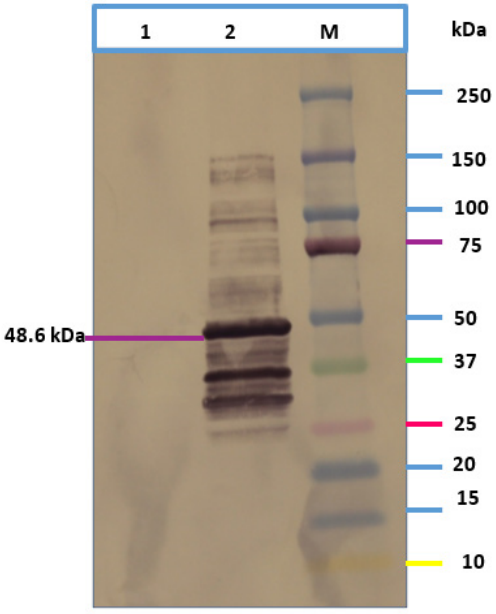


Figure 4. Western blot of uninduced (1) and induced (2) samples of clones (Turkish isolate number 2).

The expected size of CPG of CPsV was 48 kDa. But the product was 48.6 kDa. Since the vector is including some more amino acids after ORF of the CPG of CPsV before stop codons, the protein size is increasing. This does not create any problems for antisera production and disease detection. Protein purification was done using the NI-NTA column denaturing method with some modifications by the company. Amicon Ultra centrifugal filters were used at 4°C for the concentration of dialyzed proteins. Nikolaeva et al. (1995) were first to produce polyclonal antisera to the coat protein of Citrus tristeza virus (CTV) expressed in *E. coli*. Then serological detection of CTV with antibodies developed to the recombinant coat protein has been done successfully by Iracheta-Cárdenas et al. (2009). In this work CPsV-CP has been cloned to bacterial expression vector and protein expression has been induced in *E. coli* and protein was strongly visualized by Western-blotting (Figure 4).

This protein needs to be extracted from the gel and injected into mammal animals to produce antiserum. Then several antisera should be tested for CPsV detection with several dilution tests.

Turkish isolates of CPsV were different than Florida isolate CPsV-4, Italian, and Spanish isolates since their antisera (kindly provided by Moreno) did not react with Turkish isolates 2 and 5 antigens by ELISA test. This recombinant protein product is the first step to developing recombinant polyclonal antisera for large scale field test for future.

CONCLUSIONS

The full length of the coat protein gene of the Turkish isolate of Citrus psorosis virus was RT-PCR amplified. First, the CPG of CPsV was cloned into TA to get the full length of the gene sequence result. Then cloned into pET101/D-TOPO for bacterial expression of the protein in component cells by inducing with IPTG. The blunt end ligation on the TOPO cloning kit was very easy but several insertions came out in the wrong direction. We modified the reverse primer at the 5 prime ends if there is any similarity with CACC nucleotides at the 5 prime ends of the forward primer. High Protein expression was shown by SDS-PAGE gel, then transferred to nitroselulose membrane by Western blotting.

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