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Original Article

Expression of Prunus Necrotic Ringspot Virus Coat Protein in *E. coli*

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Abstract

Background and Aims: Serological assay is considered as one of the best choices for conducting large number of infection tests. Recombinant DNA technology has been used for expression of virus coat protein (CP) gene in prokaryotic bacterial cells such as Escherichia coli and the recombinant CP (rCP) is used as immunogen in antibody production. Heterologous CP protein expression and purification of the full length Prunus necrotic ringspot virus-PNRSV, Ilarvirus genus, from an Iranian isolate as an antigen was the aim of the study.

Materials and Methods: A predominant Iranian PNRSV isolate (PK5) was selected and its CP gene was amplified using specific primers and the nucleotide sequence has been determined. The amplicon was cloned into pET28a(+) expression vector. The amplified CP gene and linearized pET-28a(+) were purified from gel, ligated and transformed into BL21 strain of E. coli. Expression of rCP in transformed BL21 competent cells was tested using SDS-PAGE and Western Blot assays.

Results: RT-PCR on total RNA extracted from the infected leaves resulted in a DNA fragment of approximately 688 bp corresponding to full PNRSV/CP. BLAST analysis of the obtained nucleotide sequence for PNRSV/CP revealed 97% identity to JW isolate (accession no. DQ983491). The size of pET-PNRSV/CP was about 6000 bp. The E. coli BL21 cells harboring recombinant pET-PNRSV/CP successfully expressed the recombinant CP after IPTG induction.

Conclusions: In this study, the recombinant CP gene of a predominant Iranian PNRSV isolates expressed in E. coli. The recombinant CP can be used for producing high quality antibodies against PNRSV.

Keywords: Prunus necrotic ringspot virus, Recombinant coat protein, Expression

Introduction

Prunus necrotic ringspot virus (PNRSV) (*Bromoviridae* family, *Illarvirus* genus) is among a large number of viruses which infect many stone fruit trees like peach, nectarine, cherry, apricot, almond and plum trees, beside apple, hop and rose. PNRSV has a worldwide distribution and causing important losses in stone fruit (1, 2, 3). This virus induces different symptoms including necrotic and chlorotic ring spots, mosaic, deformations in leaves, delayed maturation, reduce growth and reducing productivity, although, the virus may be present latently without symptoms or damage. It was indicated that PNRSV reduced vigor up to 30%, yield reduction 20 to 56%, and decrease fruit quality (1, 2, 4). This virus is transmitted by pollen, seed and vegetative propagation methods (3, 5, 6).

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Seed and vegetative transmission may play an important role in distribution of PNRSV, especially as a result of breeding programs, the international exchange of germplasm and the use of seedlings as rootstocks (7).

The genome consist of three positive-sense RNA with RNAs 1 and 2 encode proteins involved in viral replication, whilst RNA3 encodes a 5' movement protein (MP) and a 3' coat protein (CP).

Stone fruits orchards are widely distributed in almost all geographical regions of Iran, covering a total area of 4,417,500 hectares with an annual production of 201,759.000 (tones) and 4,560 kg/Ha yield (8). Certification demonstrates the first step of protection against fruit tree viruses. Serological assays like Enzyme Linked Immunosorbent Assay (ELISA) is still considered as the best method and routinely used for screening of samples for different tree viruses in large scale as it is relatively cheap. But it requires a persistence stock of large quantity of antiserum for indexing of tree and tissue culture raised plants. Furthermore, the validity of the results depends on the quality of antiserum. Production of polyclonal antibodies requests maintenance, propagation and purification of virus which is time-consuming and needs uniform steps. Recent advances of recombinant DNA technology have led to the use of recombinant proteins expressed in *Escherchia coli* as antigens. The technology based on recombinant DNA was considered for production of antiserum to avoid labile virus purification and virus preservation problems. In this study, the full length of *CP* gene of Iranian PNRSV-PK5 isolate was amplified by polymerase chain reaction (PCR) and cloned in the expression vector pET-28a(+). It was transformed into *E. coli* BL21 competent cells. The expression of recombinant PNRSV/CP gene was induced by IPTG and confirmed by SDS-PAGE and Western Blot, and can be used as a source of antigen for producing high quality antibodies against PNRSV.

Methods

Virus isolates, bacteria strain and plasmid An Iranian PNRSV/PK5 isolate, which collected from Karaj (Alborz, province), was used for amplification of *PNRSV/CP* gene. The complete *CP* gene sequence of PK5 was used to design primers with restriction sites (underlined), *NdeI* site in forward (5′-GGCATATGGTTTGCCGAATTTGCAAT-3′) and *BamHI* site in reverse primers (5′-GGGGATCCTCAGATCTCAAGCAGGTCT T-3′) for directional cloning. The plasmid pET-28a(+) (Novagen, USA) was used as a expression vector, which provides T7 and His Tag, that fused to the expressed protein and, facilitate solubility and purification of the expressed protein. The *E. coli* strain BL21 harboring the recombinant plasmids were used for transformation.

RT-PCR and Cloning Reverse transcription was performed in 20 µL reaction containing 4 µl of total RNA extracted using RNeasy Plant Mini Kit (QIAGEN, USA) according to the manufacturers' instructions. Complete *PNRSV/ CP* gene was amplified using specific primers. For first strand c-DNA synthesis, an RT mix consisting of 2 µl of 10X RT buffer (Sinaclon, Iran), 2 µl of 10 mM dNTP mix, 1 µl (200 U.) of M-MuLV reverse transcriptase (Sinaclon, Iran), 0.5 µl (20 U) Ribonuclease inhibitor (Fermentas, Litvania), 1 µl of the reverse primer (20 pmol/µl), 8.5 µl nuclease free water, was added. The reaction mix was then incubated at 42°C for 1 h followed by treatment at 70°C for 10 min to inactivate the enzyme. Viral cDNA was amplified in 50 µl PCR reactions containing 5 µl of 10X PCR buffer (CinnaGen, Iran), 1 µl of 50 mM $MgCl_2$, 2 µl of 10 mM dNTP mix, 1 µl (20 pmol) of each forward and reverse primers, 1 µl (5 U) of *Pfu* DNA polymerase (CinnaGen, Iran), 4 µl of RT product and 35 µl of nuclease-free water. PCR was performed under the following conditions: 95°C for 5 min; 35 cycles of 95°C for 30 s, 50°C for 30s, and 72°C for 30 s; and 72°C for 10 min. PCR amplification was assessed by electrophoresis in a 1% agarose gel in TBE buffer containing ethidium bromide (final concentration 1 µg/ml). The RT-PCR product was isolated by excising the band from

an agarose gel and purified using Wizard PCR DNA purification system (Promega, USA) according to the manufacturer's recommendations.
The purified PCR product using Wizard Gel and PCR clean-up System (Promega, USA) was ligated into the cloning vector pET-28a(+).

Production of rCP The released CP fragment and linearized pET-28a(+) were purified from agarose gel by the use of QIAquick Spin kit (QIAGEN, USA). Firstly, the expression vector pET-28a(+), digested in two digestion sites *NedI* in 5' end and *BamH1* in 3' end. The reaction mixture was prepared in a final volume of 80 µl and included 25 µl (about 4 µg) pET-28a(+);6 µl, 10X buffer; 2.5 µl from each restriction enzyme and 44 µl deionizer water. The reaction mixture was kept at 37°C, for 4 hr. In other hand, the concentration of the *PNRSV/CP* gene DNA fragment, using a spectrophotometric method, after being driven over agarose gel and extraction from the gel, was determined. Given that in primers, two regions were designed for *NdeI* shear enzymes at the 5' end and *BamHI* at the end of the 3', so this DNA fragment initially entered the enzymatic digestive reaction. This reaction was carried out in a volume of 50 µl. Including 20 µl (about 2.7 µg) PCR cleaned product; 5 µl, 10X buffer; 2 µl from each restriction enzyme and 21 µl deionizer water. The reaction mixture was kept at 37°C, for 4 hr. After digestion and clean-up the vector and PNRSV/CP product using (Wizard Gel and PCR clean-up System, Promega, USA), the insertion of DNA PNRSV-CP gene, into the vector was carried out using T4 ligase and reacted with the following composition. Three µl from the amplicon (50 ng) was added into a 20 µl (final volume) ligation mix containing 2X ligation buffer, 4 µl [(120 ng pET-28a(+))] and 1 unit T4 DNA ligase. The reaction was kept at 22°C for 3 hr before placing at 4°C for 16 hr. The ligation mix (1 µl) was used to transform *E. coli* BL21 competent cells.

Expression of the recombinant CP (rCP) gene in *E. coli* Three recombinant colonies carry off the rCP [(pET28a(+)-PNRSV/CP)], which already confirmed by PCR, were selected for expression of rCP in *E. coli*. In addition, these three plasmids of colonies were extracted and, the nucleotide sequence of the additive portion was determined using available commercial services, in order to ensure the integrity of the sequence of the complementary part in the plasmid. Overnight culture of BL21 (200 µl) carrying pET-PNRSV/CP was inoculated into 5 ml fresh LB containing kanamicin (50 $\mu g/ml^{-1}$) and grown to an optical density of 0.6 to 0.7 before induction with 0.5 mM final concentration of IPTG (isopropyl-β-D-thiogalactopyranoside).
The concentrations of IPTG, growth temperatures (28°C) and induction time (overnight) were optimized for the protein induction conditions.
The bacterial cells containing the recombinant (rCP) were plated by centrifugation. The rCP was purified using Ni-NTA His-tag purification Kit QIAGEN, Valencia, Ca, USA) under non-denatured condition and tested using SDS-PAGE, Western Blotting and ELISA.

Results

Isolation, amplification and cloning PNRSV isolate PK5 was chosen from leaves of *Prunus persicae* with mottling, shot hole and yellowing symptoms. This isolate was inocu-lated and kept on cucumber in greenhouse condition.
RT-PCR on total RNA isolated from the infected leaves resulted in a DNA fragment of approximately 688 bp corresponding to full PNRSV/CP. BLAST analysis of the obtained nucleotide sequence for PNRSV/CP revealed 97% identity to JW isolate (accession no. DQ983491).
The amplified CP and linearized pET-28a(+) were purified from gel, ligated and transformed into BL21 strain. The size of pET-PNRSV/CP was about 6000 bp.

Expression of the PNRSV-CP gene in *E. coli*
The insert of recombinant pET-PNRSV/CP plasmid was sequenced and analysis showed that the CP gene was inserted in the correct orientation, and it was in frame with intact N-terminal 6X histidine tags. The *E. coli* BL21

cells harboring recombinant pET-PNRSV/CP successfully expressed the recombinant CP after IPTG induction. The rCP migrated as approximately 27 kDa in SDS-PAGE analysis (Figures 1A and 1B), but the theoretical molecular weight of PNRSV-CP is approximately 25 kDa. This might be due to the expression of the recombinant protein as fusion protein with histidine tags. In Western blot antiserum against recombinant PNRSV/ rCP, strongly reacted with PNRSV/rCP.

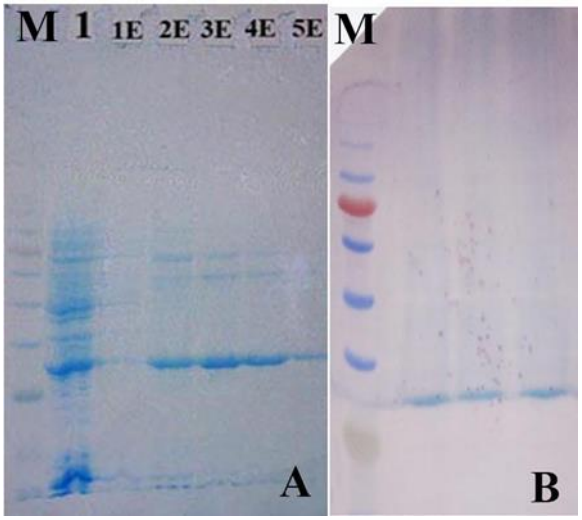

Fig 1. (A) SDS-PAGE of PNRSV/rCP in 12% polyacrylamide gel, stained with Coomassie brilliant blue. M: Broad range protein marker (CinaGene-Iran). Lanes: 1 and 1E to 5E: elution of PNRSV/rCP purified using Ni-NTA agarose. (B) Western blot analysis following electrophoresis in SDS-PAGE, electroblotting on nitrocellulose, and detection PNRSV/rCP.

Conclusions

The occurrence of PNRSV infection has been reported in most of the *Prunus* cultivation areas in Iran (9, 10). In this study, an isolate of PNRSV form peach trees (PK5) in Alborz province, was isolated and the CP of this isolate was amplified using specific primers. The full sequence of PNRSV/PK5 was 675 nucleotides, which was consistence with previously reported (7, 11).

Phylogenetic analysis of PNRSV isolates indicated that the most of isolates grouped in PV96 strain (12, 13, 14, 15, 16). This study showed that the PK5 PNRSV isolate belonged to PV96 strain. The high nucleotide identity (98%) among Iranian isolates may show a dominant strain of this virus in Iran (10, 17).

Until now, different serological methods of ELISA, which all based on the antiserum, were used for viral detection (18, 19, 20). ELISA is routinely for virus detection in large scale assays. Therefore, it needs a large quantity of good quality antiserum. These methods were used for virus detection in high number samples and therefore they need high volume of specific antibodies. For preparation of specific and good antiserum, it needs to access the suitable and especially pure antigen.

Antigens preparation against viruses using traditional methods, which are related to purification of viruses are difficult due to several factors, including contamination with other viruses, low viral concentrations the presence of host inhibitors materials, limited host range, lack of suitable replication host or difficulty of viral transmission to suitable propagation hosts, non-specific response to host proteins, and in some cases the low level of immunization of the viruses (21, 22, 23, 24, 25). Production of specific antibodies using recombinant coat proteins that cloned in *E. coli* system is considered for the overcome the mentioned above problems. The expression of viral proteins in prokaryote system, it leads to high production of target viral proteins, which are enough for production of antiserum. In spite of usefulness of this method, it has some worries about the variation in structural productions (denatured).

Expression and production of polyclonal antibodies against recombinant CP of some viruses have been reported. For example, against recombinant CP of *Potato virus Y* – strain NTN (26); PVY- strain ordinary (27), PVY isolate Pot187 (28), *Potato mop-top virus* (29), and *Potato virus X* (30) could detected the viral proteins either by Western blot or IPTA-ELISA and DAS-ELISA.

Furthermore, Petrzik et al. (31) demonstrated the detection of PNRSV using recombinant CP-based antiserum in DAS-ELISA.

Considering the high cultivation area of stone fruit trees in Iran, it is necessary to replace older and non-economic trees, as well as to develop cultivation and construction of new gardens. Therefore every year a large number of young seedlings are needed. One of the most important methods, in order to decrease the damage of viruses, including PNRSV, is using of healthy, virus free and certified seedlings.

In this respect we used pET-28a(+) vector, in order to expression of PNRSV/CP gene. For increasing the production of soluble protein and facility extraction of protein after adding IPTG (stimulator of protein expression), the LB media were incubated in the low temperature 30°C, instead of 37°C.

The production of PNRSV/rCP in BL21 was confirmed using PCR, and Westren-blot. The recombinant CP can be used for producing high quality antibodies against PNRSV.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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