Brief Report

Heterologous Expression of Potato Virus Y Coat Protein, Isolate Pot187

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Background: The advent of recombinant DNA technology has facilitated heterologous expression of proteins from various sources in different host systems including *Escherichia coli*. If a plant virus coat protein is expressed in the bacterium it can be used as the antigen for antibody preparation. Such a recombinant antigen preparation can be particularly useful where equipment such as ultracentrifuge is unavailable to purify virus particles to use as the antigen for conventional antibody preparation.
Objectives: Heterologous protein expression and purification of the full length *Potato virus Y* (PVY) coat protein (CP) from isolate pot187 (an affiliate of strain N) to be used as an antigen was the aim of the study.
Materials and Methods: Reverse transcription Polymerase Chain Reaction (RT-PCR) was carried out to amplify an 801 bp fragment of the CP gene from PVY-infected potato leaves. The amplicon was cloned into pGEM-T Easy. The cloned fragment was restricted by *Bam*HI + *Sac*I and the purified fragment was cloned into the expression vector pET21a(+) which was restricted with the same enzymes. The generated plasmid was introduced into *E. coli* strain Rosetta^{TM}. The expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) and its protein content was subjected to SDS-PAGE and western blotting.
Results: SDS-PAGE analysis of protein from the induced bacteria showed a ~35 KDa protein corresponding to PVY CP. Expression of the recombinant protein was confirmed by anti-His anitibody.
Conclusions: The full-length cDNA of PVY-CP was amplified from the infected potato leaves. The cDNA was heterologously expressed in *E. coli*. The produced recombinant CP can be used as an antigen to generate polyclonal antibody.

Keywords: Cloning; Coat protein; *E. coli*; Expression; PCR; PVY-pot187; Recombinant

1. Background

Potato virus Y (PVY) is the type species of the genus *Potyvirus*, family *Potyviridae*. Potyvirus is accounted for one-third of viral infections in crops (1). PVY has a wide host range in *Solanaceae*, *Amaranthaceae*, *Fabaceae*, *Chenopodiaceae*, and *Asteraceae*. PVY causes significant losses in four main crops, potato, pepper, tomato and tobacco around the world. The most frequently occurring symptoms are mosaic, mottling, deformation, spotting and chlorosis on leaves, and necrotic ring spots on the tubers (2). PVY is transmitted in a non persistent manner by more than 50 aphid species among which *Myzus persicae* is the most efficient vector (3).

PVY strains PVY^{O}, PVY^{N}, PVY^{C}, PVY^{NTN} and PVY^{N} –W are recognized by their distinct host responses (4-6). PVY^{O} causes mosaic and mottling and the tobacco veinal necrosis strain (PVY^{N}) causes necrotic symptoms in *N. tabacum*. Stipple streak strain (PVY^{C}) induces hypersensitivity in many potato cultivars, and both PVY^{NTN} (potato tuber necrotic ring disease) and PVY^{N}-W infect potato cultivars.

PVY particles are flexuous rods of ~750 nm in length and 11-15 nm in diameter, and consist of capsid proteins arranged in a helical symmetry around the RNA genome. The single-stranded, positive-sense genomic RNA (~9.7-10 kb) of PVY has a viral genome-linked protein (VPg) at the 5′ of the genome and poly (A) at 3′ ends. The RNA encodes a single, large polypeptide that is cleaved by three viral-encoded proteases into 10 proteins including P1, HC-Pro, P3, 6K1, CI, 6K2, NIa-Vpg, NIa-Pro, NIb and CP proteins (7). Coat protein (CP) protects the RNA genome, involves in aphid transmission, cell-to-cell movement

and also contributes to the viral genome amplification. The CP NH_2- and COOH-terminal residues are exposed so that mild trypsin treatment removes the termini leaving a core of ~24 kDa.

Expression of foreign genes such as viral CP in *E. coli* is relatively fast and inexpensive for producing high quantities of proteins (8). Besides, fusion of tags to the expressed proteins produced in *E. coli* facilitates their purification. The CP gene of several plant viruses have been expressed in *E. coli* as recombinant antigens for raising virus-specific antibodies (9, 10). As to PVY, expression of the CP gene from Wilga isolate of PVY has been reported (11). In Iran, a report on expression of PVY^N CP gene in potato plants has been published recently (12); however, to the best of our knowledge there is no report on expression in bacteria.

2. Objectives

This study aimed at expressing of PVY CP gene in *E. coli* from a native isolate known as pot187 (13). The recombinant CP can be used as an antigen in preparation of viral-specific antibodies for serological detection of the virus.

3. Materials and Methods

3.1. Virus Source and RT-PCR

PVY-pot187 originally isolated from potato in Ardabil province, Iran (13). PVY-pot187 was used as the source isolate to amplify the CP gene. It was propagated in potato, *Solanum tuberosum* via mechanical inoculation with the use of 0.1 M potassium phosphate pH 7.4. Total RNA was isolated from 100 mg leaves of infected potatos four weeks post-inoculation (14).

Sequences of PVY CP isolates were retrieved from GenBank, aligned and a consensus sequence based on frequencies of substituted nucleotides was determined in GeneDoc program (15) and used as the template for primer design. The forward PVY CP-F 5′CACGGATCCGGAAATGACACAATTGATGC3′ and reverse PVY CP-R 5′CACGAGCTCTCATGTTCTTAACTCCAAGTAG3′ primers were designed corresponding to nucleotides 8566-8585 and 9347-9366 of PVY isolate Iung-2 (GenBank accession JF927750) using Oligo 5 (16). A fragment (819 bp) encoding CP protein with engineered restriction sites *BamH*I and *Sac*I sites (underlined) was amplified. The native stop codon was removed from PVY CP-R to allow the reading frame continue through the C-terminal His*Tag of pET-21a (+) (Novagen, USA). This vector provides T7*Tag and His*Tag which are fused to the expressed protein and, therefore, facilitate solubility and purification of the expressed protein. All the reagents were purchased from (Fermentas, Lithuania). Reverse transcription was performed in 10 μL reaction containing 2 μL (out of 20 μL purified total RNA) of leaf RNA, 0.4 μL of PVY CP-R (5 pmol.μL^{-1}) and 1.1 μL RNase-free sterile distilled water. The mixture was incubated at 70°C for 5 min, immediately placed on ice and 7.5 μL of reverse transcription mix containing 2 μL of reverse transcriptase buffer, 10 mM dNTPs, 20 Units RNAsin and 80 Units M-MuLV reverse transcriptase was added into each reaction to a final volume of 10 μL. The reaction mixtures were incubated at 25°C for 10 min, 4°C for 60 min and 70°C for 10 min in a CG1-960 thermo cycler (Corbett Research, Australia). PCR reaction was performed in 25 μL reaction containing 2 μL of the synthesized cDNA, 1× PCR buffer, 2 mM $MgCl_2$, 2 pmol each primer, 5 mM dNTPs and 1 Unit *Taq* DNA polymerase. The PCR was started at 94°C for 2 min, followed by 35 cycles of 94°C for 45 s, 50°C for 45 s and 72°C for 45 s. A final extension at 72°C for 10 min was also applied. The products were run on 1.2% (w/v) agarose in 0.5× TBE buffer, stained with ethidium bromide and photographed by Gel- Digi doc (Kiagen, Tehran, Iran).

3.2. Cloning

The PCR product was first ligated into the cloning vector pGEM-T Easy (Promega, USA). The amplicon (20 ng) was added into a 10 μL (final volume) ligation mix containing 1× ligation buffer, 50 ng pGEM-T Easy and 1 unit T_4 DNA ligase. The reaction was kept at 22°C for 90 min before placing at 4°C for 16 h. The ligation mix (1 μL) was used to transform *E. coli* DH5α competent cells (13). Both the recombinant pGEM-T Easy and pET21a (+) vectors were digested with *Bam*HI + *Sac*I. The released CP fragment and linearized pET21a (+) were purified from agarose gel by the use of QIAquick Spin kit (Qiagen, Australia). The purified fragments were ligated before introducing to *E. coli* strain Rosseta. The resultant recombinant pET21aPVYCP was sequenced with the T7- promoter and Terminator primers in Bioneer (Germany) and submitted to BLAST analysis (17) to ascertain identity of the amplified fragment as PVY CP.

3.3. Expression of the PVY-CP Gene in E. coli

Overnight culture of *E. coli* (200 μL) carrying pET21aPVYCP or pET-21a (+) (control) was inoculated into 10 mL fresh LB containing 0.1 mg.mL^{-1} ampicillin and grown to an optical density of 0.6 before

induction with 0.1 mM final concentration of IPTG (isopropyl-β-D-thiogalactopyranoside). The culture was further grown for 3 h at 37°C and 1.5 µL of the bacteria was pelleted by centrifugation at 15000 g for 1 min. The pelleted cells were resuspended in 100 µL of Laemmli buffer (18) and stored at -24°C for further analysis.

3.4. SDS-PAGE and Western Blotting

The pelleted bacteria were boiled for 2 min and 15 µL aliquots were run on 12% polyacrylamide gel containing SDS and stained with Coomassie Brillant Blue. The separated proteins (unstained) were electro-blotted onto a nitrocellulose membrane in a wet system (Akhtarian, Tabriz, Iran). After blocking with 2% BSA, the membrane was incubated for 2 h with mouse anti- His*Tag antibody (1:1000). The secondary antibody and universal alkaline phosphatase-conjugated rabbit anti mouse (1:1000), were added and incubated at 22°C for 2 h. After each step, the membranes were washed three times with PBS-Tween 20 (PBS-T). The membrane was incubated in the freshly prepared substrate, nitro blue tetrazolium (NBT,100 µL) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 50 µL) in 100 mM Tris buffer, pH 9.5 containing 100 mM NaCl and 0.1 $MgCl_2$.

4. Results

4.1. Isolation, Amplification and Cloning

Leaves of *S. tuberosum* inoculated with PVY-pot187 showed mosaic, rugose and yellowing 10-15 days post inoculation. RT-PCR on total RNA isolated from the infected leaves resulted in a DNA fragment of a ~801 bp corresponding to PVY CP (Figure 1A). BLAST analysis of the obtained nucleotide sequence for PVYCP revealed 97% similarity with PVY strain NTN (GenBank accession AY840082). The PVY-pot187 CP sequence was submitted to GenBank under the accession number KJ865800.

When the *CP* cDNA and linearized pET21(+) were purified from gel (Figure 1A), ligated and transformed into *E. coli* strain Rosetta, subsequent *BamH*I + *Sac*I digestion of the extracted plasmids confirmed presence of CP gene (Figure 1B). Sequencing of the recombinant pET21aPVYCP reconfirmed correct insertion of the CP fragment into the vector.

4.2. Expression of the PVY-CP Gene in E. coli

SDS-PAGE revealed heterologous expression of an about 35 kDa protein corresponding to PVY CP. The maximum expression was obtained 3 h post induction (Figure 2A). Western blot indicated presence of a 35 kDa recombinant protein using anti-His*Tag antibody (Figure 2B).

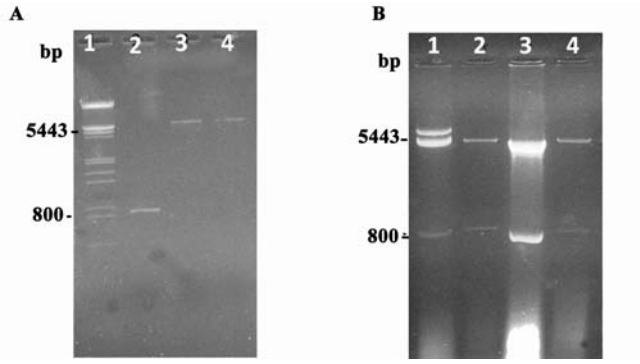

Figure 1. Analyses on 1.2% agarose of gel-purified Potato virus Y coat protein cDNA and linearized pET21a (+)(A) and restriction analysis of the cloned fragment (B). (A) lane 1: lambda DNA *Eco*RI + *Hind*III, lane 2: purified PVY CP cDNA, lanes 3-4: linearized vector (replicate samples). (B) restriction analysis of recombinant pET21a(+) carrying the insert after cloning in *Escherichia coli* DH5α. Lane 1: A previously identified pET-21aPVYCP used as a size marker, lanes 2-4: recombinant plasmids from three colonies transformed with pET-21aPVYCP

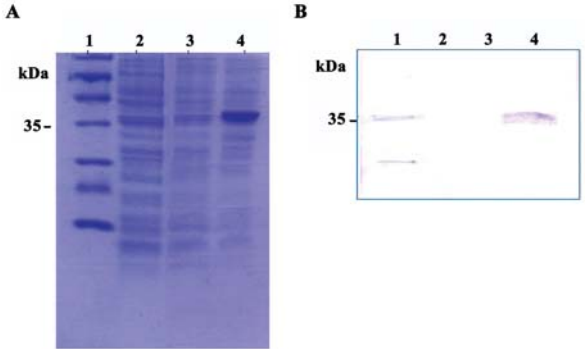

Figure 2. A: SDS-PAGE and B: western blot analyses of Potato virus Y coat protein expressed in *Escherichia coli*. (A), lane 1: protein size marker (Fermentas, Lithuania), lane 2: sample from bacteria containing pET21a (+) with no CP insert, lane 3: *E. coli* containing pET21aPVYCP non-induced, lane 4: the same as lane 3 but induced with 1 mM M IPTG and grown for 4 h afterwards. (B) The lanes correspond to that of (A) after subjecting to western blotting with anti-His•Tag

5. Discussion

Because the main control measure against plant viruses is rather prevention, timely application of detection techniques is crucial. PVY CP specific

primers were designed and full-length PVY CP gene (801 bp) was efficiently amplified in this study for the gene expression purpose whereas others have used degenerate primers (19) because degenerate primers are sufficient for detection purposes only and not for the expression purpose as pursued in this study (16).

Sequence of the amplified fragment revealed a close relatedness of PVY-pot187 to PVY isolate Iung-2 (PVY^{NTN} strain group) (5) which also agreed with the report (31) which suggests the studied Iranian PVY isolates might be relatives of PVY^{NTN}. This is a derivative of PVY^{N} strain, which induces vein necrosis in tobacco leaves and provokes PTNRD and severe chlorotic mosaic in potato leaves (5).

A few studies have dealt with PVY from Iran (20), but to the best of our knowledge there is no report concerning expression of the virus CP gene in *E. coli*. Analysis showed that the CP can be efficiently expressed via pET21a (+) in *E. coli* (Figure 2A). Induction with 0.1 M IPTG before growing for 3 h resulted in a ~35 KDa (including the Tag sequences) intense protein band corresponding to PVY CP, which was absent in non-induced bacteria or in bacteria that had no *CP* gene. Fusion of His•Tag allowed us to use anti-His•Tag antibody to confirm the expression of a recombinant protein in western blot.

6. Conclusions

This study demonstrated usefulness of biotechniques in precise isolation of PVY CP gene from isolate PVY-pot187. The expressed CP can be used as the antigen for antiserum preparation. It may also facilitate further research on PVY and establishment of resistance against the virus.

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Authors' contribution

Nemat Sokhandan Bashir acted as the chief investigator and prepared the manuscript, Mahin Poorsmaile performed the lab work and Mohammad Hajizadeh was the associate supervisor.

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