Serological Methods to Confirm Expression of Coat Protein Gene From an Iranian Isolate of Cucumber Mosaic Virus in Escherichia coli

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Background: Cucumber mosaic virus (CMV) has isometric particles with a diameter of about 28 - 29 nm. Detection and prevention are the critical steps in the control of plant viruses. Detection in a large number of samples is still done by serological methods due to their robustness and perhaps low cost.

Objectives: To this end, our aim was to express the CMV CP gene in E. coli to be used as the antigen for antibody production in the future.

Materials and Methods: Coat Protein (CP) gene cDNA from an isolate (B13) of Cucumber Mosaic Virus (CMV) was subcloned from pTZ57RCMVCP to pET21a expression vector and transformed to E. coli strain Rosetta. Expression of CMV CP was successful and confirmed by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), wherein a ~30- kDa protein band was revealed. Induction by Isopropyl-Thiogalactoside (IPTG) at final concentrations of 0.5 to 2 mM appeared to produce similar results as to the amount of the expressed protein, which was judged by intensity of the band on SDS-PAGE.

Results: The identity of the expressed protein was confirmed by immunoassays such as western blot, Dot-Immunobinding Assay (DIBA) and Enzyme-Linked Immunosorbent Assay (ELISA) by the use of anti-CMV antibody.

Conclusions: This is the first report of expression of CMV CP gene in Iran, which is important for the preparation of anti-CMV antibody and paving the way for the use of the virus coat protein as a nanomaterial.

Keywords: Methods; Cloning; Expression; Gene; Escherichia coli

1. Background
Cucumber Mosaic Virus (CMV) is a worldwide-spread plant virus with an extensive host range infecting about 1200 species in almost 85 families of plants including cereals, fruits, vegetables and ornamentals (1). It belongs to the genus cucumovirus in the family Bromoviridae. This virus causes symptoms such as yellow mosaic, leaf distortion and stunting on Nicotiana glutinosa. Cucumber Mosaic Virus particles are isometric with a diameter of about 28 - 29 nm (2, 3) and the virions consist of 180 identical copies of the virus-encoded coat protein (CP) (4). Cucumber Mosaic Virus is a multi-component virus consisting of three single-stranded genomic RNAs and two subgenomic RNAs known as sgRNA4 and sgRNA4A (2, 5, 6). Proteins encoded by RNAs 1 and 2 are needed for viral genome replication while RNA3 encodes the Movement Protein (MP) and Coat Protein (CP), both of which are involved in cell-to-cell and long-distance movement of the virus (2). The CMV CP is translated from subgenomic (sg) RNA 4 corresponding to the 3’ proximity of the viral RNA 3 (4). The sgRNA4A has been recognized as the viral repressor of host RNA silencing (VSR). The CMV is transmitted mechanically by sap and by aphids in a non-persistent, stylet-borne manner (7). Detection and prevention are the critical steps in the control of plant viruses. Detection in a large number of samples is still done by serological methods due to their robustness and perhaps low cost (8, 9). After the advent of recombinant DNA techniques, expression of viral genes in E. coli has been an important strategy for obtaining large-scale recombinant proteins that can be used for production of virus-specific antibodies (10-13).

2. Objectives
To this end, our aim was to express CMV CP gene in E. coli to be used as the antigen for antibody production in the future.

3. Materials and Methods

3.1. Cloning and Sequencing
The CMVCP gene was previously amplified from an Iranian CMV isolate (B13) by PCR, cloned in pTZ57R/T and sequenced with the following accession number AY670070 (13). Here, the CMV CP gene was subcloned from pTZ57C
MVCP to pET2a (+) (Novagen, USA) by the use of BamHI and SaeI sites (Figure 1). The recombinant plasmid pET2aCMVCP was used to transform the E. coli strain Rosetta by a heat shock procedure (14). Then, the plasmid was subjected to sequencing in order to ascertain the correct sequence before subjecting to expression (Macrogen, South Korea).

3.2. Expression

A transformed colony of E. coli strain Rosetta carrying pET2aCMVCP was cultured overnight at 37°C in 5 mL of Luria Bertani (1% peptone, 0.5% yeast extract, 1% NaCl) containing 50 µg/mL ampicillin. Then, 1 mL of the overnight culture was added to 50 mL of fresh LB-ampicillin and growth was continued until the culture density reached an optical density of 0.6. Then, CP expression was induced by a heat shock procedure (14). Then, the plasmid was subjected to sequencing in order to ascertain the correct sequence before subjecting to expression (Macrogen, South Korea).

3.3. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis and Western Blotting

Proteins from the harvested cells were extracted with 160 µL of Laemmli buffer (125 mM Tris/HCl, pH 6.8, 2% sodium dodecyl sulfate, 9% glycerol, 0.7 M 2-mercaptoethanol, and 0.002% bromophenol blue) and boiled for five minutes. Proteins present in the supernatant and pellet fractions of the cell lysate were separated by 12% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) for four hours at 120 V (16) and visualized by 0.2% Coomassie Blue R staining solution. Then, the protein bands were electroblotted onto a HybondTM-N nitrocellulose membrane (Amersham, UK) using a wet transfer system (Akhtarian, Iran) at 100 mA during one hour for western blot analysis. Blocking was done by the use of 5% (w/v) Bovine serum albumin (BSA) powder in the buffer (8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 2.7mM KCl, 137 mM NaCl, pH 7.3) for one hour. After washing twice with Phosphate Buffered Saline-Tween 20 (PBST) membranes were incubated in 1:1000 dilution of polyclonal anti-CMV antibodies (Agdia, USA) for two hours at room temperature, followed by washing as above and incubating in alkaline phosphatase-conjugated anti-rabbit IgG for two hours at room temperature. After washing three times, the CMV CP specific band was visualized by incubating the membrane in the substrate Nitroblue Tetrazolium Chloride 5-Bromo-4-Chloro-3-Indoly-Phosphate (NBT/BCIP), toluidine salt, Roche solution.

3.4. Dot Immunobinding Assay (DIBA)

The recombinant CMV CP extracted from E. coli was blotted onto a HybondTM-N nitrocellulose membrane. The membrane was dried at least for five minutes, immersed in PBST containing 1% BSA, and shaken and incubated for one hour at room temperature. Then, the membrane was washed three times with PBST (0.2 g KH₂PO₄, 2.9 g Na₂HPO₄·12H₂O, 8g NaCl, 0.2 g KCl, 0.2 g NaN₃, 1 litre dH₂O and 0.5 mL Tween 20) with agitation before incubation with the primary CMV-specific polyclonal antibody at a dilution of 1:1000 in PBST for two hours at 37°C. This was followed by washing of the membrane three times (as above), before incubating in conjugate anti-body (Alkaline Phosphatase-Labeled Goat Anti-Rabbit Globulin (APL-GAR)) diluted at 1:10000 in PBST for two hours at room temperature. Then, it was subjected to four washes as above. Finally, the membrane was incubated in NBT/BCIP substrate solution and then washed by distilled water to stop the reaction.

3.5. Enzyme-Linked Immunosorbent Assay

Enzyme-Linked Immunosorbent Assay (ELISA) was performed with the use of anti-CMV polyclonal antibody (Agdia, USA). Briefly, plates were coated with 100 µL (5 µg/mL) of antibody, incubated for three hours at 37°C, and washed three times with PBST. After blocking with PBST containing 1% BSA, 100 µL of the recombinant CMV CP extracted from 1.5 mL of bacterial culture was added, incubated for two hours at 37°C and washed by PBST (as above). Sap of CMV-infected cucumber leaves and bacterial cells carrying pET2a (no insert) were added as the positive and negative samples, respectively. Then, 1:2000 dilution of goat anti-rabbit IgG conjugate (Agdia, USA), was added and incubated for four hours at 37°C before washing five times with PBST. Finally, 150 µL of p-nitrophenyl phosphate solution (1 mg/mL) was
added to each well and the yellow color development was monitored by the use of the Anthos 2020 (Austria) micro-titre ELISA reader by measuring absorbance values at 405 nm every 15 minutes for 90 minutes after the addition of the substrate.

4. Results

4.1. Preparation of Construct

Primers for the CMV CP cDNA amplification, which had been designed with restriction sites BamHI and SacI on the forward and reverse primer, respectively, facilitated in-frame cloning in the bacterial expression vector. After ligation of the CP cDNA into pET2la, subsequent sequencing confirmed correct insertion. As expected, additional nucleotides were inserted into the CP cDNA because of the restriction sites used for cloning; however, the insertion was in frame and no stop codon was created.

4.2. Expression of Coat Protein in Escherichia coli

Analysis by SDS-PAGE of total protein from bacterial cells that carried pET21aCMVCP and were induced by IPTG in the range of 0.5 to 2 mM (final concentration) for four hours showed a higher level of expression of the expected protein, approximately 25 kDa in size, corresponding to the CMV CP (17) (Figure 2 A). Four independent transformants (clones) were examined for the expression, among which two clones with a desired level of the expression were selected for further analysis.

4.3. Detection by Western Blotting, Dot Immuno-binding Assay and Enzyme-Linked Immunosorbent Assay

Western blotting and DIBA with a rabbit anti-CMV polyclonal antibody confirmed the identity of the recombinant protein as CMV CP. Accordingly, a protein band with 26 KDa molecular weight, which was used as a positive control, produced a signal after 15 to 30 minutes of incubation with the substrate (Figure 3 A). While no reaction occurred with the extract from bacterial cells containing pET21a, only a weak reaction appeared with the non-induced bacteria (negative controls). Subjection to ELISA of sap from CMV-infected cucumber leaf, which used as a positive control, resulted in production of yellow color 30 minutes after addition of the substrate whereas no signal was visually detected with the protein from bacterial cells containing pET21a(+) (no insert), which were used as the negative control (Figure 3A, 3B).

5. Discussion

All of the expression systems have advantages and limitations. *E. coli* as a prokaryotic system because of its high expression capacities of many heterologous proteins, low cost, efficient generation time and fast high-density cultivation is known as a successful host. Accordingly, expression of CP gene from numerous plant viruses has been successfully done in *E. coli* (18-21). Apart from the pET vector system, a number of other vector systems such as pGEX (22, 23), pTrchis (24) and pTBG (H) (25) have also been used for expression of foreign genes in *E. coli*. With all the vector systems available, *E. coli* strain Rosetta has been the cell line of choice for expression of genes of interest (26, 27). Strain Rosetta belongs to the so-called “B strains” that are deficient in lon protease and lack the ompT outer membrane protease. The strains having these proteases can degrade proteins during purification (28). Therefore, some target proteins may be more stable in these strains than in host strains containing these pro
fraction of the cell lysate. In most cases of heterologous expression of genes in \textit{E. coli}, it has been reported that the expressed protein is present as an insoluble fraction at 37°C; however, when large-sized proteins are expressed in \textit{E. coli} or when small-sized proteins are over-expressed, insoluble difficult-to-hydrolyze protein complexes are formed (33). This research showed that CMV CP open reading frame placed under the control of T7 promoter is expressed in \textit{E. coli}. After induction of expression, the levels of CP were monitored over a four to five-hour period by SDS-PAGE and expression was confirmed by immunoblot tests including western blot, DIBA and ELISA.

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### Authors’ Contributions

Afsin Rostami and Nemat Sokhandan Bashir developed the original idea and the protocol, abstracted and analyzed data, and wrote the manuscript. Mohammad Hajizadeh and Davood Koolivand contributed to the development of the protocol and abstracted the data.

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