Expression of *Cucumber mosaic cucumovirus* coat protein in *Escherichia coli* and production of specific polyclonal antiserum

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Received: November 22 2013
Accepted: December 22 2013

**ABSTRACT**

*Cucumber mosaic cucumovirus* (CMV) was isolated from naturally infected cucumber plants serologically depending on indirect enzyme-linked immunosorbant assay (I-ELISA) and biologically by mechanical inoculated on *Chenopodium amaranticolor* as a local lesions host and maintained on *Nicotiana tabacum* cv. (White Burley). Infected tobacco plant tissue used to perform immunocapture reverse transcriptase polymerase chain reaction (IC-RT-PCR) to isolate the viral coat protein gene (*cp*). The agarose gel analysis of the PCR product indicated a single band with 657 bp length, which is the expected size for the CMV *cp*. The isolated CMV *cp* gene has been ligated into the PinPoint™ Xa-1 T-Vector depending on the T-A cloning principle. The new plasmids were transformed into competent *Escherichia coli* cells, further the gene integration success and orientation were screened by isolation of plasmids from transformed bacteria and treatment with *Bgl*II and *Bam*HI restriction enzymes. It was found that total plasmid length was 3331 bp without insert, while fragment separated after restriction digestion was approximately 660 bp. The PCR product of the minipreparation indicated a single band with 657bp which is the expected size for the CMV *cp* gene. After induction of CMV coat protein (CP) expression using Isopropyl β-D-1-thiogalactopyranoside (IPTG), transformed bacteria lysate analyzed using SDS-PAGE revealed a band with a molecular weight of 25KDa (the expected of CMV CP molecular weight). Western blotting analysis was carried out and confirmed that all the bands expected for viral CP react positively with CMV antiserum. The induced viral CP was purified from *E. coli* transformed cells using regenerated SoftLink™ Soft Release Avidin Resin yielding about 11 mg per 1 liter culture. Polyclonal antiserum produced in a New Zealand white rabbit by injecting of the produced fusion protein and Immunoglobulins G (IgGs) were purified from the collected antiserum. IgGs titration was performed using I-ELISA, the highest titeres were 1:512 & 1:256 for antisera produced using fusion protein and purified virus, respectively. It was clearly observed that purified antiserum of *E. coli* viral fusion protein was more reactive and specific as it did not have any cross reactions with healthy tobacco plant sap.

**KEYWORDS:** *Cucumber mosaic cucumovirus*, Isolation, Coat protein gene, Cloning, Protein expression, Coat protein purification, Antiserum production, Immunoglobulins G purification, Evaluation.

**INTRODUCTION**

*Cucumber Mosaic virus* (CMV, genus: *Cucumovirus*, family: *Bromoviridae*) is geographically wide spread and has been reported in Australia, North America, New Zealand, Europe and Africa (Roossinck and Sokhandan-Bashir et al., 2012). It has four functional pieces of single stranded RNAs, packaged in icosahedral protein particles with molecular weight of 25 KDa, about 30 nm in diameter (Nouri et al., 2012). The virus is transmitted by numerous species of aphid in a non-persistent manner (Brunt et al., 1996 and Gildow et al., 2008). The virus has an extremely wide host range and it induces severe symptoms. Douine et al. (1979) listed 775 plant species representing 365 genera and 85 families that are susceptible to CMV infection. In Egypt CMV infects cucurbit and solanaceous crops, banana and sugar beet causing remarkable yield loss (El-Afifi et al., 2007).

Serological methods especially the enzyme linked immunosorbant assay (ELISA) are widely used in the detection of viral infections (Portsmann and Kiessig, 1992). They are relatively sensitive, inexpensive, simple and suitable for the testing of many samples simultaneously (Clark, 1981). Additionally, more specific antibodies are a prerequisite for the application of the extremely sensitive technique immunocapture reverse transcriptase polymerase chain reaction (IC-RT-PCR) (Wetzel et al., 1992; Komorowska and Malinowski,2009).
Generally, molecular methods such as RT-PCR are not suitable as routine tests for indexing large numbers of samples due to costs and the relative complexity of execution. As a consequence, serology has traditionally been the most used method of plant virus diagnosis in a large number of samples, using ELISA as the method of choice (Zimmermann et al., 1990; Ling et al., 2000; Fajardo et al., 2007).

Recently, advances in recombinant DNA technology, coupled with its ease to manipulate and its rapid growing rate in a less expensive media had established E. coli as a leading host organism to produce high protein quantities of scientific interest (Ashoub et al., 2009). Many of the expression systems have advantages and limitations but E. coli as a prokaryotic system was used widely because of its high expression levels of many heterologous proteins, low cost, efficient generation time, and fast high density cultivation (Sokhandan-Bashir et al., 2012).

Virus purification is usually a labor-intensive procedure with varying, occasionally unsatisfactory results concerning purity and concentration of the final preparation (Ling et al., 2007). The production of high quality virus-specific antiserum suitable for large scale of virus detection and based on virus purification procedures faces substantial drawbacks, i.e., including complex virus infections, low yields of virus particles, contamination of antigens with plant proteins, presence of inhibitory compounds (Radaelli et al., 2008; Khan et al., 2012). On the other hand, it was found that the production of virus-specific antiserum using recombinant virus genes expressed in E. coli “which can overcome such difficulties” has been applied to produce a number of polyclonal antiserum against coat protein (CP) of several plant viruses, i.e., Bean yellow mosaic potyvirus (Hammond and Hammond, 1989), Plum pox potyvirus (Mattanovich et al., 1989), Soybean mosaic potyvirus (Liu et al., 1993), Cucumber mosaic cucumovirus (Khan et al., 2011; Sokhandan-Bashir et al., 2012).

The aim of this study is to express CMV coat protein in E. coli, which was then used for the production of specific polyclonal antibodies. As antibodies with higher specificity are a crucial reagents in carrying out serological tests for screening against viruses in plant material such as seeds and other propagation material and also for field plants testing.

MATERIAL AND METHODS

Virus isolation:
Fifty samples of naturally infected cucumber plants (Collected from the open field of Faculty of Agriculture, Ain Shams University, Cairo, Egypt) showing mosaic, mottling and malformation were used for virus isolation. According to Koenig (1981) I-ELISA was performed on samples using polyclonal antibodies specific for CMV (Agdia Inc., USA). Samples giving CMV positive results were mechanically inoculated on Chenopodium amaranticolor. Local lesions produced were used as a source of single lesion isolation, and finally the virus was maintained in Nicotiana tabacum cv. White Burley under greenhouse conditions (28°C ±2).

Isolation CMVcp gene using IC-RT-PCR:
Immunocapturing, the isolation of CMV RNA and cDNA synthesis using IC-RT-PCR was performed using infected tobacco leaf collected 15 days post CMV inoculation according to Minafera and Haddi (1994) as follows: the wells of an ELISA plate were coated by adding 200 µl of IgGs (diluted to 10-1 using coating carbonate buffer (pH 9.6)) and incubated 4 hrs at 37°C, after that, plate was washed 3 times with PBST, 5 min each. Plant tissues were ground in sample buffer (1 gm : 5 ml), then centrifuged at 3000 rpm/10 min at 4°C, 200 µl of the supernatant were added to each pre-coated wells and incubated overnight at 4°C, then plate was washed 3 times, 5 min each with PBST.

Twenty five of pre-heated transfer buffer (10 mM Tris-HCl (pH 8.0) containing 1% Triton X-100) were added to each well and kept at 65°C for 5 min. Aliquots of 5 µl from the resulting released RNA solutions were immediately transferred to microcentrifuge tube. To each tube the following components were added and incubated at 42°C/2 h for cDNA synthesis: 1 µg of reverse coat protein gene primer, 4 µl of 5X first strand buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl2), 3 µl of 0.1M dithiothreitol (DTT), 5 µl of 0.3M 2-ME, 2.5 µl of 10 mM of each deoxynucleotide triphosphate (dNTPs), 1 µl RNasin (40 unit) and 1 µl of Molny Murine Leukemia Virus (M-MLV) (200 unit). Final product was used immediately for PCR reaction.

The following primers (Invitrogen Corp., USA) designed depending on CMV cp gene sequences collected from GenBank web site (http://www.ncbi.nlm.nih.gov) were used for the amplification of CMV cp gene were:

5’ATGGACAAATCTGAATCAAC3’ (Sense) 5’TCAAACTGGGAGCACCCAG3’ (Antisense).
PCR was performed as described by Ghosh et al. (2002) using PerkinElmer Cetus Thermal Cycler PE 9700 (PerkinElmer Inc., USA). Five µl from resulting cDNA were transferred to tube containing 45 µl PCR reaction mixture. PCR program was 94°C initial melting for 3 min followed by 35 cycles of 94°C/1 min, 55°C/1 min, 72°C/2 min and 72°C/10 min final extension.

For PCR product analysis, 1.5% agarose gel was used and electrophoresis was carried out in Sub-Cell DNA apparatus (Bio-Rad® Lab., USA) at 80 V. The amplified gene band was visualized on an UV Transilluminator and photographed by Gel Documentation System (AlphaImager® TM1220, Documentation and Analysis system, Canada).

Cloning of CMV cp gene:

The virus cp gene (PCR product) was ligated with the PinPoint™ Xa-1 T-Vector (Promega Corp., USA) depending on the T-A cloning principle (Holton and Graham, 1991) and according to manufacturer instructions manual. The gene was cloned with the vector in-frame with the fragment of biotin-binding protein (BBP) as mentioned by Abou Zeid et al. (2002).

The recombinant plasmids were transferred into competent E.coli strain BL21 cells and successfully transformed bacteria were selected depending on blue/white colonies screening. Transformation and selection were carried out as described by Hanahan (1985).

Minipreparation and restriction enzyme digestion:

The recombinant plasmid was isolated from E. coli transformed cells according to the miniprep procedure described by Sambrook et al. (1989). The preparation was subjected to restriction analysis to confirm cp gene orientation prior to protein expression. As described by Sambrook et al. (1989) with some modifications, 15 µl of the recombinant plasmid was mixed with 2 µl from both BglII and BamHI restriction enzymes (Promega Corp., USA), the total volume was raised to 30 µl by deionized water. The reaction was incubated at 37°C for 2 h, and then 20 µl of the digested product was mixed with 6 µl agarose gel loading buffer and electrophorised on 1% agarose gel.

PinPoint™ Xa-1 original plasmids (without the CMV cp gene) and recombinant plasmids with the CMV cp gene in addition to PCR product of the minipreparation using viral cp gene specific primers were loaded to the same gel as controls.

Induction of cp gene expression and detection of the expressed protein:

As recommended by manufacture (Promega) instructions, culture of E. coli carrying the PinPoint™ Xa-1 expression fusion was started by inoculating 5 ml of LB medium containing 100 µg/ml ampicillin with a 24 h colony and culture was incubated overnight at 37 °C on shaker. Overnight culture was diluted to 1:100 by adding 50 µl of culture to 5 ml of LB medium containing 2 µM biotin, 100 µg/ml ampicillin and incubated for 1 h at 37 °C on shaker. For protein expression induction, 100 µM isopropyl β-D-thiogalacto-pyranoside (IPTG) was added to culture, incubated for 4 h at 37 °C with shaking. Bacterial cells were then collected by centrifugation and further subjected to subsequent freezing/thawing, sonication and centrifugation treatments for protein extraction (Sambrook et al., 1989). The proteins were then separated by 12 % SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970), electro-botted to nitrocellulose membranes and assayed by Western blotting immunoassay using polyclonal CMV coat protein specific antibodies (Iracheta-Cardenas et al., 2002).

Protein purification:

For the purification of large protein quantities, one liter of transformed bacteria culture in LB broth containing 2 µM biotin and 100 µg/ml ampicillin was prepared. The protein expression was induced by adding 100 µM IPTG to the bacterial culture and incubated 4 h at 37 °C on shaker. The cells were disrupted by sonication according to Sambrook et al. (1989) and centrifuged at 8000 rpm for 10 min at 4 °C and the fusion protein was then purified using 3 ml regenerated Soft Link™ Soft Release Avidin Resin according to Promega instructions. The eluted fusion protein was quantified by the Bradford assay (Bradford, 1976) at A595 nm using spectrophotometer. The protein was then analyzed and separated by electrophoresis in 12 % SDS-PAGE and detected using Western blotting immunoassay. As a control a purified CMV preparation was also loaded to gel.

Antisera production and evaluation:
Depending on the method described by Madhubala et al. (2005) polyclonal antiserum produced in a New Zealand white rabbit by injecting of the produced fusion protein and a purified CMV preparation as a control (purified as described by Lot et al., 1972) (1 mg of virus with Freund’s incomplete adjuvant, 1 : 1) intramuscularly six times at 10 days interval. The animal was bled 15 days after the last injection and the antiserum was collected.

Immunoglobulins G (IgGs) purification was carried out as mentioned by Clark and Adams (1977). Final dialyzed proteins were loaded on 10 ml of diethylaminoethyl (DEAE) cellulose column (Whatman Inc., USA), eluted using half strength phosphate buffer saline (PBS) and collected in microtube. Absorption of fractions was measured at 280 nm and concentration of IgGs was adjusted to 1 mg/ml (OD\textsubscript{280}=1.46) using half strength PBS, then stored at 4°C. IgGs dilution end points were determined using I-ELISA against clarified infected tobacco sap and healthy tobacco sap was used as a control.

RESULTS

Isolation of CMV virus:

CMV was isolated from naturally infected cucumber plants showing mosaic, mottling and malformation. Samples gave positive I-ELISA results ranging from 0.897 to 1.095 were mechanically inoculated on Ch. Amaranticolor leaves and gave chlorotic local lesions. Lesions were extracted and inoculated on N. tabacum cv. White Burley for maintenance.

Isolation of CMV cp gene using IC-RT-PCR technique:

Infected tobacco plant tissues were used to perform IC-RT-PCR to isolate the viral cp gene. The agarose gel electrophoresis of the PCR product indicated a single band sized 657bp, which is the expected size for the CMV cp gene (Figure 1).

![Figure 1. IC-RT-PCR results for the isolation of CMV cp gene (Lane 1-2). M: DNA Ladder (Promega, USA).](image)

Cloning of CMV cp gene:

The PCR isolated CMV cp gene has been ligated into the PinPoint\textsuperscript{TM} Xa-1 T-Vector depending on the T-A cloning principle. The new recombinant plasmid vector was transformed into competent E. coli cells, further the gene integration success and orientation were proved by isolating the recombinant PinPoint\textsuperscript{TM} Xa-1 T-Vector from transformed bacteria and treating it with BglII and BamHI restriction enzymes, which restricted to certain sites through the polylinker region of the plasmid vector. It was found that total plasmid length was 3331 bp without the insert, while the fragment separated after restriction digestion was approximately 660bp insert length (gene length plus some added nucleotides after the enzymes cutting sites) compared to the PCR product using the recombinant PinPoint\textsuperscript{TM} Xa-1 T-Vecor as a template, which gives a single band with 657bp length, the expected size for the CMV cp gene (Figure 2).
Expression, detection and purification of the CMV coat protein transformed E. coli cells:

Transformed bacteria lysate analyzed using SDS-PAGE revealed a band with a molecular weight of 25KDa (Figure 3A) after induction of protein expression using IPTG.

To produce large amount of the fusion protein, large scale culture from transformed E. coli was prepared and protein was purified depending on affinity purification using SoftLink™ Soft Release Avidin Resin. About 11 mg of expressed protein was purified from 1 liter of bacterial culture. The purified protein gave a band with molecular weight of 25KDa, which is expected to CMV coat protein (Figure 3A).

Result was confirmed by transferring gel bands to nitrocellulose membrane for Western blotting analysis, and all the bands expected for viral CP (25 KDa) react strongly with CMV specific antiserum (Figure 4B).
Antiseras production and evaluation:
IgGs titration results were illustrated in Table (1), the highest titers were 1:512 & 1:256 for antisera produced using fusion protein or purified virus preparation, respectively. It was clearly observed that purified antiserum of viral fusion protein produced from bacterial cells was more reactive as it gave higher I-ELISA values and more specific for not giving any cross reactions with healthy tobacco plant sap.

**Table 1. Titration and evaluation of CMV IgGs produced from fusion protein and purified virus preparation against infected and healthy tobacco sap**

<table>
<thead>
<tr>
<th>Dilution</th>
<th>I-ELISA values at 405 nm*</th>
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<tr>
<td></td>
<td>IgG1</td>
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<td></td>
<td>I</td>
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<tr>
<td>Crude</td>
<td>0.914</td>
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<tr>
<td>1:1</td>
<td>0.825</td>
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<tr>
<td>1:2</td>
<td>0.810</td>
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<tr>
<td>1:4</td>
<td>0.805</td>
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<tr>
<td>1:8</td>
<td>0.787</td>
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<tr>
<td>1:16</td>
<td>0.731</td>
</tr>
<tr>
<td>1:32</td>
<td>0.711</td>
</tr>
<tr>
<td>1:64</td>
<td>0.677</td>
</tr>
<tr>
<td>1:128</td>
<td>0.649</td>
</tr>
<tr>
<td>1:256</td>
<td>0.550</td>
</tr>
<tr>
<td>1:512</td>
<td>0.504</td>
</tr>
<tr>
<td>1:1024</td>
<td>0.200</td>
</tr>
<tr>
<td>Purified*</td>
<td>1.353</td>
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<td></td>
<td>1.042</td>
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</table>

*Each ELISA result (R) was the average of three readings.
**Reaction of CP fusion protein (1) and crude purified virus (2) with crude antiserum.

Note: Reaction of infected (I) and healthy (H) tobacco leaves sap with antisera produced by rabbit immunization with CP fusion protein (IgGs1) and from purified virus preparation (IgGs2).

**DISCUSSION**

* Cucumber mosaic cucumovirus (CMV), one of the most common virus infecting some economical plants in Egypt, the virus infects important crops like sugar beet, banana and cucurbits causing significant loss in yield quality and quantity (El-Affifi et al., 2007; El-Borollosy and Oraby, 2012).

Such virus is difficult to diagnose due to the periodic changes in symptoms and also for the need of high quantity of antiserum with higher specificity to perform routine serological tests for indexing large numbers of samples in field (Sokhandan-Bashir et al., 2012).

The CMV cp was isolated depending on IC-RT-PCR technique and then this gene has been ligated into the PinPoint™ Xa-1 T-Vector depending on the T-A cloning principle. The obtained recombinant PinPoint™ Xa-1 T-Vector was transformed into competent E. coli cells, further the cp gene integration was proved by restriction enzymes analysis. The PCR product of the isolated recombinant PinPoint™ Xa-1 T-Vector from the transformed E. coli cells produced a single band with 657bp, which is the expected size for the CMV cp gene. There are numerous reports of the expression of plant viruses CP in E. coli (Hammond and Hammond, 1989; Mattanovichet al., 1989; Liu et al., 1993; Khan et al., 2012; Sokhandan-Bashir et al., 2012).

Transformed bacteria lysate analyzed using SDS-PAGE revealed a band of CMV CP with a molecular weight of 25KDa. Western blotting analysis was carried out and confirmed that all the bands expected for viral CP react positively with CMV antiserum. Such work agreed with that performed by Khan et al. (2012) and Sokhandan-Bashir et al. (2012).

The induced viral CP was purified from E. coli transformed cells using regenerated SoftLink™ Soft Release Avidin Resin yielding About 11 mg of protein from 1 liter of bacterial culture. This result is a promising result when compared with that obtained by Khan et al. (2012) who produced a total yield of about 8 mg CMV CP per 1 liter of cell culture.

Polyclonal antiserum produced by rabbit immunization and IgGs were purified from the collected antiserum. Depending on I-ELISA it was clearly observed that purified antiserum of E. coli viral fusion protein has higher dilution end point, having more reactivity for giving higher I-ELISA values, and
specificity as it did not give any cross reactions with healthy tobacco plant sap which can improve the performance of the antiserum.

Barbieri et al. (2004) and Sokhandan-Bashir et al. (2012) concluded, based on observations with Watermelon mosaic potyvirus and Cucumber mosaic cucumovirus, respectively that antibodies produced against expressed CPs in E. coli tend to be more specific, reducing the occurrence of unexpected heterologous reactions.

The use of fusion CPs as an immunogen to develop antibodies has been of great value particularly for viruses that are difficult to purify (Petrovicet al., 2003) and for those that are subject to degradation during the purification process (Barbieri et al., 2003).

REFERENCES


