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Large Scale Production of *Potato Virus* Y Necrotic Strain (PVY^N) Coat Protein through Expression the CP Gene in *E. coli.*

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A fragment with a size of 801 bp from the CP gene was amplified from *Potato virus* YN isolated from potato plants cultivated at Monofyia Governorate. RNA extracted from virus-infected tissues of *D. metel* leaves by the use of degenerate primers through polymerase chain reaction (PCR). Large-scale amount of PVY (N-Egypt) coat protein produced by gene expression technique in *E. coli* through: (1) Insertion of CP gene isolated by RT-PCR into PinPoint™ Xa-1 Vector by ligation and propagation after transformation process in *E. coli*. (2) Isolation of plasmid DNA, then used restriction enzymes *Bam HI* and *Bgl II* to identify clones containing inserts. To confirm the fragment inserted of CP gene sequence, PCR was performed using specific primers for PVY^N CP gene. (3) The gene expression was performed using 100 µM IPTG & 2 µM biotin and incubation for 4h / 37 C° to produce biotinylated protein for *E. coli* with Mr 22.5 KDa and fusion protein with Mr ~48 KDa consists of biotin tag and CP product of PVY^N. (4) The protein was purified by soft link™ soft release avidin resin. About 2.85 mg/ml of the expressed protein was purified from 1 L of bacterial culture.

INTRODUCTION

The *in vivo* expression of recombinant protein is vital to many areas of molecular biology researches. The ability to express and purify cloned gene products in prokaryotic and eukaryotic systems are frequently used for studying protein structure and function, protein-protein and protein-DNA interactions, antibody production and mutagenesis.

Prokaryotic systems have been historically used to express large quantities of proteins for studying the protein structure and for antibody production. The expression of proteins in *E. coli* offers a number of advantages: the methods are relatively inexpensive; proteins can be over-expressed with high yield and expression can be easily regulated. However, the expression of eukaryotic gene products in prokaryoteic systems may pose some problems, particularly if the protein requires post-translational modifications for activity. Another common problem with over-expression in *E. coli* is that the protein produced may be present in insoluble form, resulting in difficulties during purification of active protein.

The technique of gene expression is used by several investigators around the world with different viruses for antiserum production and molecular studies, i.e., *Bean yellow mosaic Potyvirus* (Hammond and Hammond, 1989), *Plum pox Potyvirus* (Mattanovich *et al.*, 1989), *Zucchini yellow mosaic Potyvirus* (Gal-On *et al.*, 1990), *Soybean mosaic Potyvirus* (Liu *et al.*, 1993), *Potato leaf roll*

Luteovirus (Lopeź *et al.*, 1994), *Maize dwarf mosaic Potyvirus* (Sai *et al.*, 1995), *Beet necrotic yellow vein Lurovirus* (Yu *et al.*, 1995). *Tobacco mosaic Tobamovirus* (Hinrichs *et al.*, 1997), and *Tuberose mild mosaic Potyvirus* (Chin-Chin *et al.*, 2002).

The gene expression was also performed for several genes correlated to PVY by many researchers. Hataya *et al.* (1990) worked with cDNA of PVY-TH CP gene by PCR amplification and subcloned into *E. coli* expression vector, pkk223-3. The CP PVY-TH produced in *E. coli* colonies had electrophorotic mobility identical to that of native PVY-TH CP. The maximum expression of the CP in *E. coli* was approximately 7% of the total soluble proteins and this result indicates that the CP gene was functional.

The viral CP gene was expressed into bacteria through cloning the cDNA of PVY in a lambda-DASH vector under the control of a T7 promoter that introduced into *E. coli* carrying the T7-RNA-polymerase gene on a plasmid. The immuno-electron-microscopy of transfected cell extracts revealed virus-like particles, indicating that the proteins involved in its processing and the viral CP retained their native activity (Stram *et al.*, 1993a).

As previously reported for the use of gene expression technique for immunodiagnosis researches with PVY CP gene, this was performed with cytoplasmic cylindrical inclusion (CCI) gene (Ohshima *et al.*, 1993), helper component (*hc*) gene (Stram *et al.*, 1993b), and first protein (P1) gene (Hinrichs *et al.*, 1997 and Jelena *et al.*, 1998).

Therefore, the present study aimed for preparation of PVY^N coat protein through cloning and expression of CP gene in *E. coli*.

MATERIALS AND METHODS

Virus nucleic acid isolation and reverse transcription- polymerase chain reaction (RT-PCR)

Total RNA was extracted by SV total RNA isolation system by spin protocol as recommend by the manufacturer instructions of Promega from 30 mg of PVY^N (previously isolated by Abd El-Mohsen, Nashwa 2004) infected *D. metel* leaves (21 days post virus inoculation) based on the method of Kobs (1998). The synthesis of the first strand c-DNA was carried out using reverse primer (OC1) (5`- TCA CAT GTT YTT SAC TCC AAG YAG -3`) using cDNA synthesis kit (Promega corp. Madison, USA), followed by PCR amplification for coat protein gene of PVY by OC1, foreward primer (OS1) (5`- ATG GSA AAT GAC ACA ATY GAT GCA -3`) according to Abd El-Mohsen, Nashwa (2004). The High Performance Liquid Chromatography (HPLC) purified primer pairs were designed by Thermo Hybaid GmbH, Germany, derived from PVY sequences in GenBank Accession # (D00441, X97895, U09509) as a degenery primers for *cp* gene using DNASTAR Lasergene (DNASTAR Inc, MD).

Ligation of the PinPoint ™ Xa-1 T-Vector and PCR product

The ligation reaction was set up according to manufacturer instructions of Promega by adding 2 µl of PCR product. The PinPoint ™ Xa-1 T-Vector circle map is shown in Fig. (1). Transformation of ligated PCR in *E. coli* (BL 21) based on Abd El-Mohsen, Nashwa (2004). The recombinant plasmid of DNA was isolated by Miniprep protocol according to Sambrook *et al* (1989).

Restriction enzyme digestion for plasmid DNA

The DNA isolated by miniprep procedure was used for screening the clones (to determine the fragment orientation prior to protein expression) by adding the following: 15 µl DNA, 2 µl *Bgl II*, 2 µl *Bam HI* (Promega) and the total volume raised to 30 µl by adding deionized water. The reaction was incubated at 37°C for 3 h. The DNA was tested by PCR using specific primers (OC1, OS1) for confirmation the presence of *cp* gene. The products were analyzed by gel electrophoresis on 1% agarose gel prepared in 1X TBE buffer (Sambrook *et al.*, 1989). The gel was stained with ethidium bromide and examined using UV Transilluminater.

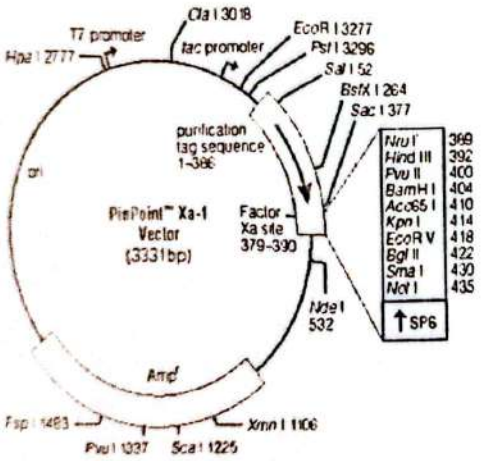

Fig. (1). The PinPoint ™ Xa-1 Vector circle map. Base 1 is the translation start for the purification tag sequence.

Screen of expression of coat protein gene for PVY^N

The following protocol was used to screen bacterial colonies expressing the fusion protein as recommended by manufacturer instructions of Promega. Culture of the *E. coli* carrying the PinPoint™ expression fusions was started by inoculating 5 ml of LB medium plus 100 µg/ml ampicillin with a freshly isolated colony, incubated overnight at 37°C with shaking. The overnight cultures were diluted 1:100 by adding 50 µl to 5 ml of LB containing 2 µM biotin plus 100 µg/ml, incubated for 1 h at 37°C with shaking. The inducer isopropyl ß-D-thiogalacto-pyranoside (IPTG) was added to all cultures in concentration of 100 µM, incubated for 4 h at 37°C with shaking. The tubes were stored at -20°C until detection.

Analysis and detection of the fusion protein from *E.coli*

(1)- 100 µl of each biotinylated cell lysates was transferred to separate 1.5 ml microcentrifuge tubes and spun at 12,000 rpm for 5 min. 50 µl of sample 1X buffer (2.5 ml stacking gel 4X buffer [0.5 M Tris-base (pH 6.8): 0.4% SDS]. 2 ml 10% SDS. 0.5 ml ß-mercaptoethanol. 2 ml glycerol. 0.25 mg bromophenol blue) were added to each tube and vortex. The resuspended cells were heated at 95°C for 5 min with occasional vortexing for lysis the cells. coated the proteins with SDS. The heat-treated samples (5 µl) were loaded onto a 12% SDS-PAGE minigel along with molecular weight markers according to Laemmli (1970). The samples were run as recommended by the supplier of the electrophoresis unit for 4 h. Following electrophoresis, the gel was transferred to nitrocellulose membrane using transfer buffer in semidry western transfer system for 2 h. The expression of viral CP was tested by western blotting immunoassay. The membrane was blocked in 1% nonfat dry milk in TBST buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20) for 30-60 min at room temperature. The membrane was rinsed with TBST three times 5 min for each. The

specific antiserum (Sanofi. In) for PVY diluted 1/ 1000 in TBS (10 mM Tris-HCl (pH 8.0), 150 mM NaCl) was added overnight at room temperature. The membrane was incubated with goat antirabbit-alkaline phosphatase conjugate diluted 1/ 7000 in conjugate buffer (TBST, 2% polvinyl pyrrolidone, 0.2% egg albumin) and incubated for 1 h at room temperature. The substrate solution (NBT/ BCIP prepared in 10 ml H2O) was added, incubated at room temperature with gentle agitation until purple bands appeared.

(2)- The biotinylated cell lysates of the fusion protein was located by dot-blot technique according to Smith and Banttari (1987) on nitrocellulose membranes by adding 5 µl of samples on membrane, then blocked and rinsed as previously described. The membrane was poured with 3 µl of streptavidin alkaline phosphatase in 15 ml of TBST and incubated for 30 min at room temperature with gentle agitation. The membrane was washed 3 times for 5 min each with TBST and rinsed very briefly with deionized water to remove any residual of Tween-20. The freshly prepared NBT/BCIP solution was added; incubated at room temperature with gentle agitation until purple dots appear.

Protein purification system of the fusion protein from *E.coli*

The purification of fusion protein depending on softlink™ soft release avidin resin by column capture using the following steps. For isolating large quantities of the fusion proteins, one liter of LB medium containing biotin (2 µM final concentration) and ampicillin (100 µg/ml), incubated for additional hour at 37°C with shaking. The protein expressed by adding the inducer IPTG (100 µM final concentration) to bacterial culture, incubated 4 h at 37°C with shaking. The cells were harvested by centrifugation at 8,000 rpm for 10 min. The pellets were resuspended by stirring in 10 volumes of cell lysis buffer (g/ml) [50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5% glycerol]) kept on ice. The bacterial cells suspensions transferred to a container and placed on ice, were further disrupted by a sonicator using 15 s pulses with a 15 s pause between each for two min, followed by centrifuging the crude lysate at 10,000 rpm for 15 min at 4°C to remove cell debris. The supernatent was mixed with resin (< 1 ml /min) to allow efficient capture, this passing repeating over the resin several times. The column was washed with at least five column-volumes with lysis buffer. To elute the protein, cell lysis buffer containing 5 mM biotin was added. Fractions were collected immediately of 0.5 mls when the volume of elution buffer equal to one-half the volume of resin. The flow from the column was stopped, wait 15 min to allow for release of the fusion protein. The eluted proteins were tested by measuring the concentration by Bradford assay (Bradford, 1976) at OD_{595} nm and using the streptavidin-alkaline phosphatase assay. The purified protein was analyzed in 12% SDS-PAGE and tested by western-blotting immunoassay. Dot-blot technique as previously described was also applied.

RESULTS AND DISCUSSION

Production of PVY^N CP by gene expression in *E. coli*

Total RNA extraction and RT-PCR:

In the present study, specific nucleotide sequence of PVY^N from the

CP gene was investigated to confirm the identity and detection of the virus isolate by use degenerated primers and DNA polymerase through PCR technique. The degenerated primers were based on the amino acid sequence of conserved regions that were used to search for members of a gene family, homologous genes from different species (Kopin *et al.*, 1990). or related viruses (Mack and Sninsky, 1988 and Manos *et al.*, 1989).

A DNA fragment of 801 bp in size was amplified as PCR product from PVY RNA as the expected size from PVY infected tissue using primer pair with RT-PCR. The isolated DNA fragment corresponding to the CP region is shown in Fig (2).

The protocols for total RNA (TNA) isolation are mainly based on the separation of RNA from other cellular macromolecules through differences in solubility or sedimentation. The purity and integrity of isolated RNA are critical for its effective use in procedures such as RT –PCR, Northern blotting, oligo (dT) selection of poly $(A)^+$ RNA, cDNA synthesis, and translation *in vitro*. The successful isolation of intact RNA by any procedure requires four important steps: 1) effective disruption of cells or tissue; 2) denaturation of nucleoprotein complexes; 3) inactivation of endogenous ribonuclease (RNase) activity; 4) purification of RNA away from contaminating DNA and protein. The most important of these is the immediate inactivation of endogenous RNase activity which is released from membrane – bound organelles upon cell disruption (Rappolee *et al.*, 1989; Baker *et al.*, 1990; Adams, 1992; and all methods of RNA isolation use strong denaturants to inhibit endogenous RNase for isolation of intact RNA.

Cloning PCR product and transformation

Amplified c-DNA for PVY-CP gene which adenine added to the 3́ ends in PCR technique by *Taq* DNA polymerase that has a terminal transferase activity. PCR products with such single 3́ adenylate extension can be cloned into a PinPoint™ Xa-1 T-Vector which containing complementary 3́ thymidine overhangs (TA cloning), generating compatible ends for direct ligation with PCR product without further enzymatic modifications. The cloning of DNA requires the production of large quantities of the target DNA in ligation reaction, so the following process accomplished by transformation in comptent bacteria cells *E. coli* (BL 21) to replicate the target DNA many times with their DNA in a relatively short time-frame. Selection of clones in bacterial cells carrying the recombinant plasmid was further screened through isolation of plasmid DNA by standard miniprep procedure. Then used restriction enzymes *Bam HI* and *Bgl II* to easily identify clones containing inserts, which restricted into polylinker region for the vector. The PinPoint™ Xa-1 T-Vector is approximately 3.35 kb in length, whereas target DNA was 801 bp in length (Fig. 3). This indicates that clones containing DNA of CP gene were in correct orientation prior to protein expression. To confirm the fragment inserted that CP gene sequence was applied by PCR using specific primers for PVY CP gene (Fig. 3).

Detection of the expressed fusion protein

The PCR product 801 bp of DNA was constructed in the expression vector PinPoint™ Xa-1 by direct cloning and transformation in *E. coli* strain BL 21 for protein expression. The results show that

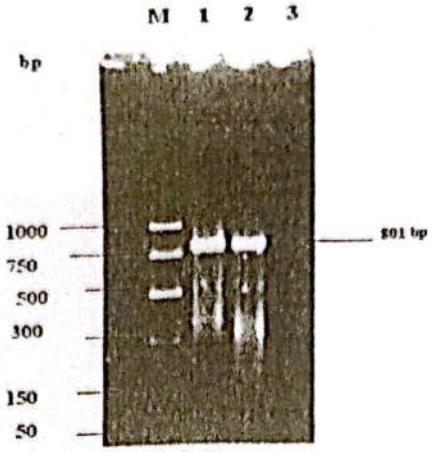

Fig. (2): Detection of CP genes of PVY by RT-PCR. PCR products with size of about 801bp was amplified. Lanes 1, 2: PVY isolated from infected *D. metel* leaves, Lane 3: *D. metel* healthy control leaves, Lane M: marker (50, 150, 300, 500, 750, 1000 bp).

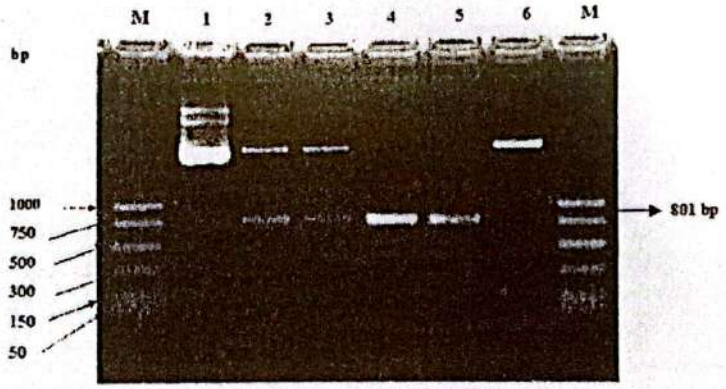

Fig. (3): PinPoint™ Xa-1 Vector restriction analysis; the recombinant plasmid containing the PVY^{N}-CP gene (lane 1); the plasmid DNA restricted by two enzymes (*Bam HI, Bgl II*) (lanes 2, 3); PCR performed by specific primers for PVY-CP gene from plasmid miniprep (lanes 4, 5); the PinPoint™ Xa-1 Vector with no insert (lane, 6).

protein expression of PVY CP gene in fusion with biotin-tag sequence have a Mr about 48 KDa, in size when detected by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in bacterial lysates after induction of proteins for 4 h using 100 µM IPTG and in fusion protein purified using soft-link avidine resin (Fig. 4A). These data were confirmed by transferring the SDS-PAGE to nitrocellulose membrane for western blotting analysis by specific PVY antiserum. It was found that the approximately 48 KDa protein in bacterial lysates as well as the PVY CP

purified by avidine resin both strongly reacted with PVY antiserum (Fig. 4B). Another technique depends on reaction biotinylated protein with streptavidin alkaline phosphatase that conjugated with biotin in a very strong reaction detected by addition of BCIP and NBT as substrate (Fig. 5).

In order to produce large amount for the fusion protein, a high expression clone was selected for large-scale culture and the protein was purified by affinity purification with avidin-resin. About 2.85 mg/ml of expressed protein was purified from 1 L of bacterial culture. It was shown that plant virus antiserum could be prepared by immunizing the viral CP expressed in bacteria (Li *et al.*, 1998; Nagel and Hiebert, 1985 and Nikolaeva *et al.*, 1995).

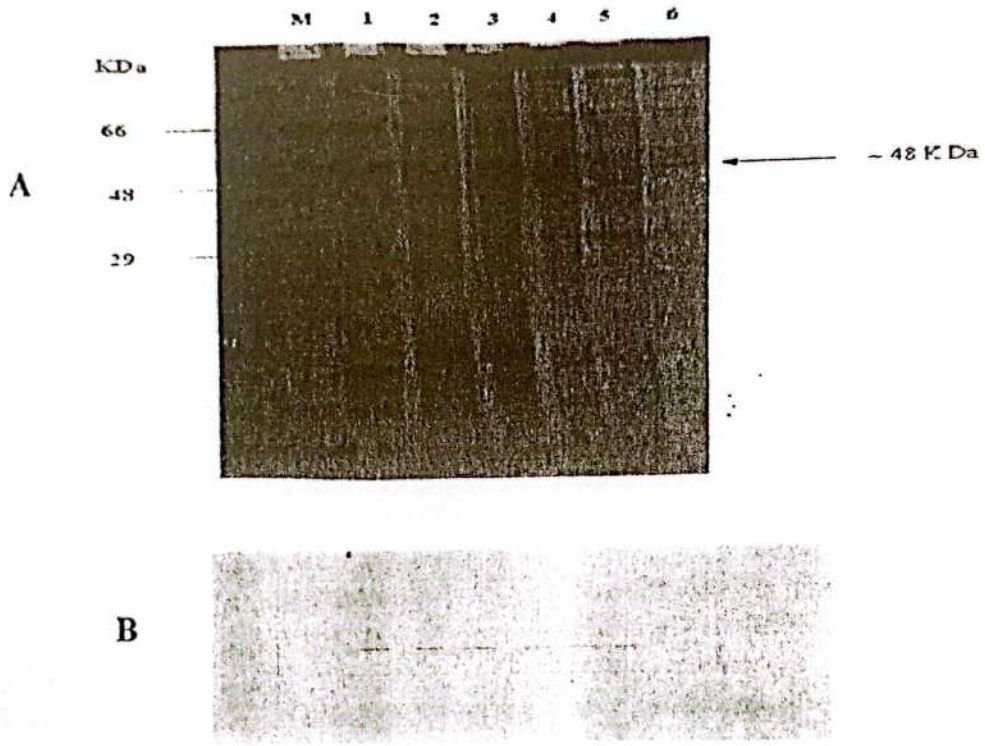

Fig. (4A): 12.5% SDS-PAGE showing the protein expression of PVY/CP gene in fusion with biotin-tag sequence. Lane M= protein marker (29, 48, 66 KDa), Lanes 1-5= IPTG–induced bacteria lysate after 4 h containing CP insert, Lane 6= fusion protein purified using soft-link avidine resin. (B) Western blot showing the reactions of the transferred fusion proteins with specific antibodies stained with BCIP/ NBT substrate.

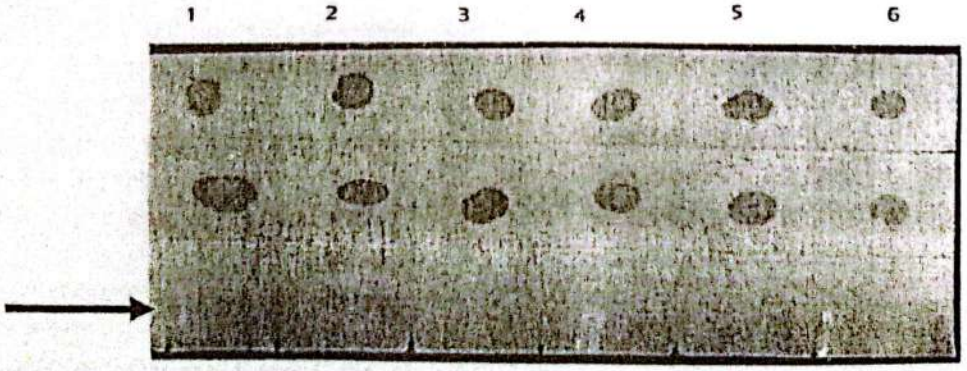

Fig. (5): Dot blot on nitrocellulose membrane for detection of the biotinylated fusion protein for PVY^N bacteria lysate before purification (1-5) ; after purification (6) ; the arrow refere to negative control of *E.coli* lysate.

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