

EXPRESSION OF SWEET POTATO FEATHERY MOTTLE VIRUS COAT PROTEIN AND PRODUCTION OF SPECIFIC ANTISERUM FOR DIAGNOSTIC ASPECTS

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Sweetpotato (*Ipomoea batatas* L.) has a diverse range of positive characteristics including high yield per unit area, nutritional value, and resistance to several production stresses (Kays, 2005). However, yield of sweetpotato is affected by several pathogens (Clark and Moyer, 1988; Moyer and Salazar, 1989). One of these is the sweet potato feathery mottle virus (SPFMV), a major component of the sweet potato virus disease, SPVD (Gibson *et al.*, 1998; Gibson and Aritua, 2000) combined with the sweet potato chlorotic stunt virus, SPCSV (Brunt *et al.*, 1996). SPFMV has long been recognized to occur wherever sweetpotatoes are grown. SPFMV, a type member of the potyviruses, is transmitted by several genera of aphids including the cotton aphid (*Aphis gossypii*) and the green peach aphid (*Myzus persicae*) in a non-persistent manner (Brunt *et al.*, 1996). Symptoms vary with regard to the cultivated variety and environmental factors that often makes identification difficult (Brunt *et al.*, 1990). Serological methods are widely used in the detection of viral infection (Portsmann and Kiessig, 1992). Recently, advances in recombinant DNA technology, coupled with its ease to manipulate and its rapid growing rate in a

less expensive media had established *Escherichia coli* (*E. coli*) as a leading host organism to produce high protein quantities of scientific interest. One of these interests is the expression of proteins as an antigen for antibody production. Several approaches were used for the expression of antigens. One of the aspects to express proteins in *E. coli* is to clone genes of interest into an expression vector coding for an amino terminus of a highly expressed protein, carrier protein. The carrier sequence is often an *E. coli* gene, but it can be a gene from any other organism that is strongly expressed in *E. coli*. The carrier sequence provides the necessary signals for high expression. In such vectors, the portion of the fusion protein encoded by the carrier can be as small as one amino acid (Amann and Brosius, 1985). The carrier sequence can also code for an entire functional protein. β -galactosidase (Rüther and Müller-Hill, 1983), *trpE* (Koerner *et al.*, 1991), maltose-binding protein (Guan *et al.*, 1987), glutathione-S transferase (GST, Smith and Johnson, 1988), and thioredoxin (LaVallie *et al.*, 1993) are widely used as carrier regions. They also facilitate the purification of the fusion protein by affinity chromatography. The

pGEX vectors are designed for foreign polypeptides expression as fusions to the C terminus of GST, a common 26-kDa cytoplasmic protein of eukaryotes. GST gene used in the pGEX vectors was originally cloned from *Schistosoma japonicum* (Smith *et al.*, 1986). The fusion proteins remain soluble and can be purified from lysed cells *via* the GST affinity to glutathione immobilized on agarose beads. Recovery of the fusion proteins is achieved by elution with reduced glutathione at neutral pH. The main advantage of this system for expression and recovery of foreign proteins from *E. coli* is that most fusion proteins remain soluble. Denaturing conditions are not required at any stage during purification. Consequently, foreign polypeptides may retain their functional activities and antigenicity.

In this study, SPFMV coat protein gene was expressed in *E. coli* as a fusion to GST of pGEX4-1 vector. The purified antigen was used to immunize mice and antiserum production. The produced antiserum was evaluated for its utility of SPFMV detection using dot-ELISA.

MATERIALS AND METHODS

Cloning of SPFMV coat protein

Primers used in this study to recover the coat protein gene were designed based on the sequence of the Egyptian SPFMV isolate (Ashoub and El-Far, 2008a). Total RNA was extracted from SPFMV-infected sweetpotato plants using Tri-Pure kit (Roche, USA)

according to the manufacturer's standard protocol. One µg of RNA was denatured at 70°C for 5 minutes in the presence of 100 pmol of primer P-SPFMV-CP-R (5'-TTTCTCGAGGTGCACACCCCTCATT CCTAAGAG-3', a newly introduced *Xho*I is underlined). Samples were chilled on ice for 2 minutes. RNA was reverse-transcribed for 1 hour at 42°C in the presence of 1 x reverse transcriptase buffer, 20 mM dNTPs, and 200 U of M-MuLV reverse transcriptase (Promega, USA). After incubation, PCR was carried out using 5 µl of cDNA in 1 x PCR buffer, 10 pmol of primer P-SPFMV-R, 10 pmol of primer P-SPFMV-CP-F (5'-AAAGAATTCTCTGGTAACCCCTG AATTT-3', a newly introduced *Eco*RI site is underlined), 1.5 mM MgCl₂, 10 mM dNTPs, and 2 U of *Taq* DNA polymerase (Fermentas, USA). Samples were subjected to 30 cycles of PCR with 30 seconds of denaturing at 94°C, 30 seconds of annealing at 60°C, and one minute of extension at 72°C. The series of cycles was preceded by 3 minutes initial denaturing at 94°C. Five µl of the PCR products were analysed on a 1% agarose gel in TAE buffer and visualized by ethidium bromide staining (Ausubel *et al.*, 1995). Amplified DNA was purified using PCR purification kit (Promega, USA) according to the manufacturer's standard protocol. After *Xho*I and *Eco*RI digest, DNA was cloned into *Xho*I/ *Eco*RI sites of pGEX4-1 (Amersham, USA) and transformed into *E. coli* JM109 (Promega, USA). Bacteria were allowed to grow overnight on 5 ml LB media supplemented with 100 µg/ml ampicillin.

Plasmid was purified using alkaline lyses method (Ausubel *et al.*, 1995) and introduced into *E. coli* BL21 (DES) (Amersham, USA). Individual colonies were obtained on LB agar plates supplemented with 100 µg/ml ampicillin.

Protein expression

Single colonies were allowed to grow overnight on 5 ml LB ampicillin media supplemented with 100 µg/ml ampicillin. Bacterial cultures were diluted 1:10 with LB media and allowed to grow further for one hour before IPTG was added to concentrations ranged from 0.1 to 1 mM. Cultures were left to grow further for 4 hours at 30°C. One ml of bacteria was collected by centrifugation at 14,000 rpm for 1 minute at 4°C. Bacterial pellets were resuspended in 100 µl protein sample buffer and boiled for 5 minutes at 100°C. An aliquot of 15 µl was analysed on 13% SDS-PAGE (Ausubel *et al.*, 1995). Bacteria found expressing the protein was subjected to preparative scale and SPFMV-CP/GST fusion was affinity-purified using glutathione agarose beads (Amersham, USA) following the protocol described by Frangioni and Neel (1993).

Antiserum production

Three hundred µg SPFMV-CP/GST proteins were injected in mouse according to the following regime. Fifty µg of protein were emulsified with and equal volume of complete adjuvant for the first injection followed by two of 75 µg and a final injection of 100 µg in an incomplete adjuvant. Injections were

carried out subcutaneously on weekly intervals. Blood was collected from heart one week following last injection. Antiserum was collected by incubation of blood at 37°C for 15 minutes and centrifuged at 4000 rpm for 10 minutes. Antiserum was kept at 4°C till use.

Evaluation of antiserum

Produced antiserum was evaluated for its efficacy to distinguish between healthy and SPFMV-infected sweetpotato plants following dot-ELISA technique described by Ashoub *et al.* (2008b). Dilutions of 1:1000 up to 1: 15,000 of the produced antiserum were used as the primary antibody.

RESULTS AND DISCUSSION

In a previous study, (Ashoub *et al.*, 2008b) came to the conclusion that serological methods were very efficient to identify SPFMV infection. The work was carried out using antiserum obtained from the International Potato Center (CIP). In a continuation to this approach, it has found necessary to produce more reliable antiserum for the detection purposes. In this study, experiments were extended to make use of recombinant DNA technology to establish *E. coli* for the production of high quantity of SPFMV coat protein as an antigen with the interest to produce specific antiserum for diagnostic purposes. SPFMV coat protein gene was amplified as RT-PCR product and cloned into pGEX4-1 vector to express SPFMV coat protein as a fusion to GST. The main advantage of pGEX

system for expressing and recovering foreign proteins from *E. coli* is that most fusion proteins remain soluble; meanwhile denaturing conditions are not required at any stage during purification (Smith and Johnson, 1988). Additional features include the efficiency and rapidity of purification and the high level of inducible expression are achieved with the strong *tac* promoter. Furthermore, the GST carrier is small compared to other carriers which make GST not interfering with specific antibody-antigen interactions. To establish the optimal conditions for protein expression, different concentrations ranged from 0.1 to 1 mM of IPTG were applied to induce the 61 kDa SPFMV-CP/GST fusion protein. The results indicated no difference in the level of protein expression with regard to increasing the IPTG concentration (Fig. 1). On the other hand, the lower IPTG concentrations are preferred for protein induction because higher concentrations have retardant effect on bacterial growth rate (Xing-Guo *et al.*, 2006). Figure (2) indicates the evaluation of the SPFMV-CP/GST fusion protein purification steps. On the large scale of protein purification, it is critical that the fusion protein remains in soluble form. To facilitate this target, 2% Sarcosyl prior to sonication and 2% Triton X-100 prior to binding to glutathione agarose beads were added. Frangioni and Neel (1993) indicated the enhancement of Sarcosyl in concentrations 1-2% and 2-4% Triton X-100 on the solubilisation of GST fusion proteins. The SPFMV-CP/GST fusion remained in the supernatant post sonication and sample

clarification by centrifugation (Fig. 2, SN). The protein was efficiently bound to the glutathione agarose beads as indicated by the reduction in the amount of the SPFMV-CP/GST remained in the supernatant post incubation and collection of the beads (Fig. 2F). The washing processes used resulted in no SPFMV-CP/GST release from the beads (Fig. 2, W) while the target protein was eluted in a pure form from the beads by the addition of reduced glutathione at pH 7.5-8 (Fig. 2E). Some additional faint bands were also recovered. These might be degradation fragments of the fusion protein that bound to glutathione agarose beads. Such fragments are very difficult to eliminate, except by increasing the stability of the fusion protein through the addition of protease inhibitors. One mM phenylmethylsulfonyl fluoride (PMSF) was added to the lysis buffer combined with 5 mM of DTT and 1 mM EDTA. However, the additional bands could not be removed from the SPFMV-CP/GST preparation.

To evaluate the efficacy of SPFMV-CP/GST fusion as an antigen to raise efficient antiserum for viral detection, antiserum was produced in mice because it requires only ~300 µg of fusion protein for the production of antiserum and the immunisation regime requires one month.

Moreover, because GST is not interfering with the specific antibody-antigen interaction, the SPFMV-CP/GST fusion was used for immunisation without a prior enzymatic cleavage to the fusion

and further purification of the SPFMV-CP. The raised antiserum was evaluated for its efficiency to distinguish between healthy and SPFMV infected sweetpotato via dot-ELISA as an easy qualitative method for viral detection (Salazar and Fuentes, 2000). Figure (3) shows that the antiserum raised against SPFMV-CP/GST fusion was efficient to distinguish viral infected samples in higher dilutions (1/15,000) without any background of the healthy plants or the nylon filters. On the other hand, the lower dilutions (1/1000-1/5000) were efficient to distinguish infected samples. However, the background on the nylon filters was much higher. The antiserum with the dilution of 1/8000 to 1/10,000 is recommended for viral detection through dot-ELISA. For a larger scale of antiserum production for commercial scale utilities, the fusion protein will be used to immunize rabbits.

SUMMARY

The pGEX glutathions S-transferase (GST) fusion protein system was used to express the coat protein of the sweet potato feathery mottle virus in *E. coli*. The fusion protein was affinity-purified from the crude extract of total bacterial protein in a soluble native form and used as an antigen for mice immunisation without the need to cleave the GST carrier. The resulted antiserum was implemented for viral diagnostic purposes using dot-ELISA technique as a reliable qualitative method. Raised antiserum was efficient to detect the viral

infection in sweetpotato plants when dilution of up to 1/15,000 was used for detection. The recommended antiserum dilution ranged from 1/8000 to 1/10,000 in which no background from the antiserum in either the healthy samples or the filters was observed.

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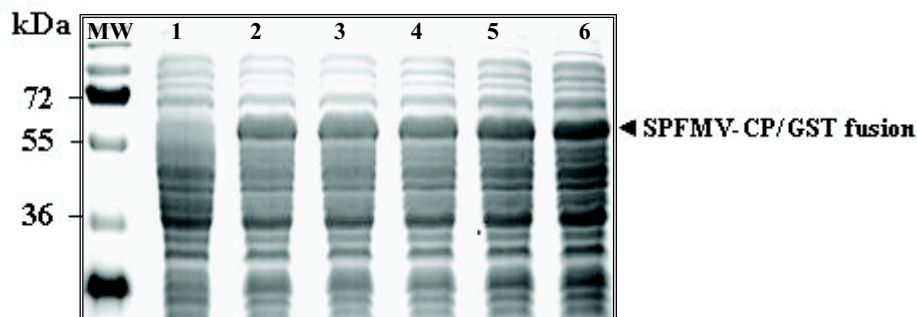


Fig. (1): Effect of IPTG concentration on the expression of SPFMV-CP/GST fusion. IPTG-free medium (lane 1). Different concentrations of IPTG in mM ranged from 0.1 (lane, 2), 0.2 (lane 3) 0.3 (lane 4) 0.5 (lane 5) and 1 mM (lane 6) were used for fusion protein induction. The 61 kDa SPFMV-CP/GST is indicated by an arrow. Prestained molecular weight marker (MW) is indicated.

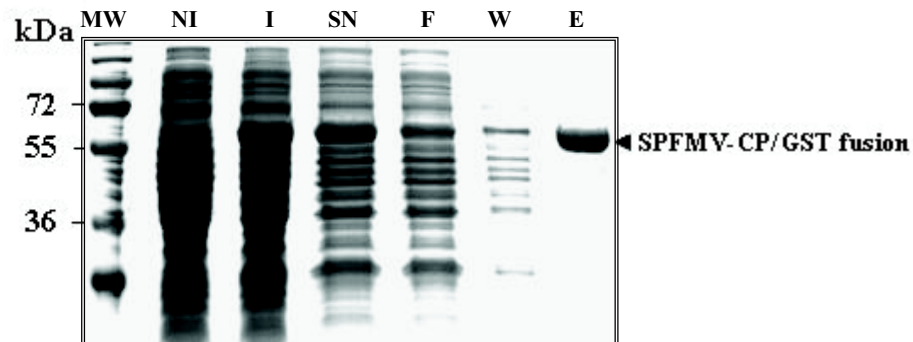


Fig. (2): Steps involved in the expression and purification of SPFMV-CP/GST fusion. (NI) represents the non-induced protein, (N) protein after induction, (SN) supernatant after protein sonication and clarification, (F) flow after protein binding to glutathione agarose beads, (W) washing of glutathione agarose beads and (E) elution of SPFMV-CP/GST fusion. The 61 kDa SPFMV-CP/GST is indicated by an arrow. Prestained molecular weight marker (MW) is indicated.

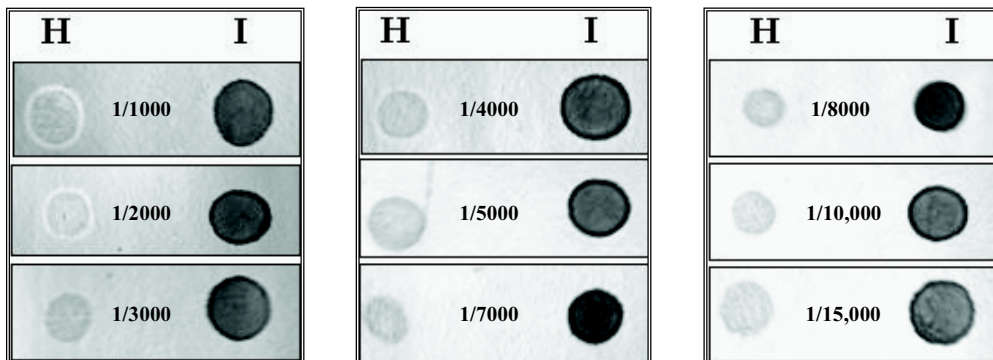


Fig. (3): Dot ELISA of healthy (H) and SPFMV-infected sweetpotato plants (I). Antiserum dilutions used are indicated on the right-hand side.