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Preparation of Polyclonal Antibodies to *Grapevine fanleaf Virus* Coat Protein Expressed in *Escherichia coli*

¹Nemat Sokhandan Bashir, ²Davood Koolivand and ³Seyed Ali Akbar Behjatnia

¹Department of Plant Protection, Faculty of Agriculture, The University of Tabriz, Tabriz, Iran

²Department of Plant Protection, Faculty of Agriculture, The University of Zanjan, Zanjan, Iran

³Department of Plant Protection, Faculty of Agriculture, The University of Shiraz, Shiraz, Iran

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Corresponding Author:

Nemat Sokhandan Bashir

Department of Plant Protection,

Faculty of Agriculture,

The University of Tabriz, Tabriz, Iran

Tel: +98 4133392036,

Fax: +98 4133356006

ABSTRACT

Full length GFLV Coat Protein (CP) gene of *Grapevine fanleaf Virus* (GFLV) as one of the most destructive viruses of grapevine around the world was amplified from a previously prepared clone Kh4-5-3 with a pair of newly-designed primers. The CP gene was inserted into bacterial expression vector pET-21a (+) and the construct (pET21aGFLV CP) was cloned in *Escherichia coli* strain Rosetta. Optimization of expression was done by induction with 0.25, 0.5, 1, 2 and 6 mM final concentration of IPTG each for 3, 4, 6 and 16 h. Induction with 1 mM IPTG for 4 h proved to be the most efficient. Expression of the CP was verified with SDS-PAGE, Western blotting and Dot Immunobinding Blot Assay (DIBA) by the use of commercially available anti-GFLV antibody. The expressed CP was purified as the denatured or native protein before using as the antigen for raising anti-GFLV CP antiserum in rabbits. Specificity and titration of the antiserum was determined by Plate-Trapped Antigen Enzyme-Linked Immune Sorbent Assay (PTA-ELISA). Efficiency of the anti-GFLV CP IgG purified from the antiserum was demonstrated in Western blotting and Double Antibody Sandwich (DAS)-ELISA. This is the first report on preparation of polyclonal antibodies against recombinant GFLV CP isolate from Iran and its application in the virus detection.

Key words: Antibody, coat protein, expression, GFLV, recombinant

INTRODUCTION

Preparation of antibodies to plant viruses by expressing virus Coat Protein (CP) gene in bacteria has been boosted by the advent of recombinant DNA technology in recent years (Ling *et al.*, 2007; Nickel *et al.*, 2004; Minafra *et al.*, 2000). This method bears advantages as virus purification, a crucial step in conventional antibody preparation is not required. In addition, the cloned CP gene can be preserved indefinitely in deep freezer for future uses.

Grapevine fanleaf Virus (GFLV) is one of the most destructive viruses of grapevine that causes symptoms, such as open petiole sinuses, shark-toothed leaf edges, mosaic, vein yellowing, stem fasciations, zigzag stems, leaf distortion and shortened internodes (Raski *et al.*, 1983) and results in yield losses of up to 80% (Andret-Link *et al.*, 2004). The GFLV is

spread in vineyards all over the world and occurs in Iran (Sokhandan-Bashir *et al.*, 2011). It is transmitted exclusively by the dagger nematode, *Xiphinema index*, which survives in vineyard soils for many years, while retaining GFLV (Vigne *et al.*, 2005). The GFLV belongs to subgroup A of the genus *Nepovirus* in the family Secoviridae (Sanfacon *et al.*, 2009).

The GFLV particles are isometric and 30 nm in diameter. The virus genome is comprised of two single-stranded, positive sense RNA-1 and RNA-2 (Pinck *et al.*, 1988) each containing one large Open Reading Frame (ORF) and coding for a polyprotein (P1 and P2). P1 is proteolytically processed into five proteins including a putative proteinase cofactor, putative helicase, virus genome-linked protein (VPg), cysteine proteinase and a putative RNA-dependent RNA polymerase (RdRp). P2 is cleaved into three proteins including a Homing

Protein (HP), Movement Protein (MP) and Coat Protein (CP) from N- C-terminus, respectively (Andret-Link *et al.*, 2004). The CP has a predicted molecular mass of approximately, 56 kDa.

Molecular methods such as RT-PCR are generally not suitable tests for indexing large numbers of samples due to the costs and relative complexity of execution (Fajardo *et al.*, 2007). Indexing on woody indicators as an alternative, takes a long time for symptoms to appear (Nickel *et al.*, 2004). Therefore, serological tests particularly Enzyme-Linked Immune Sorbent Assay (ELISA) has been used commonly for screening a large number of samples (Zimmermann *et al.*, 1990). Antibody is the main reagent of ELISA and purified from the antiserum, which is raised against highly purified virus. However, virus purification might be a lengthy procedure (3-4 days for nepoviruses) and provide varying and occasionally unsatisfactory results concerning specificity and titer of the prepared antiserum because the virus purity is variable (Fajardo *et al.*, 2007). Besides, equipment such as high speed and ultra-centrifuges may be unavailable in laboratories, particularly in under developed countries. But, advances in recombinant DNA technology has facilitated preparation of antibodies against viral gene products, such as CP. Expression of Foreign genes in *Escherichia coli* is relatively simple, fast and inexpensive for producing high quantities of proteins with minimal post-translational modifications (Hartley, 2006). In addition, fusion of tags to target genes facilitates their expression and improves their solubility, stability and purification (Butt *et al.*, 2005).

Proteins from several plant viruses have been produced in *E. coli* and used for raising virus-specific antibodies for immunodiagnosis (Lee and Chang, 2008; Cerovska *et al.*, 2006; Jain *et al.*, 2005; Abou-Jawdah *et al.*, 2004;

Cerovska *et al.*, 2003; Hourani and Abou-Jawdah, 2003; Korimbocus *et al.*, 2002; Kumari *et al.*, 2001). In this study, we report the use of GFLV CP expressed in *E. coli* as the antigen to produce GFLV-specific polyclonal antibodies, which proved to be efficient, when tested in ELISA and Western blotting.

MATERIALS AND METHODS

PCR: Polymerase chain reaction (PCR) was done with the forward GFLV 2048 (5'ACGGATCCGGATTAGCTGGT AGAGGAG3') and reverse GFLV3559 (5'CCGAAGCTTC TAGACTGGGAAACTGG3') primers on a previously cloned GFLV CP (clone Kh4-5-3, GenBank accession FJ513386) to amplify the CP gene without the stop codon (1512 bp). The *Bam*HI and *Hind*III sites (underlined) were engineered in the primers to facilitate cloning, respectively. A 50 µL PCR reaction contained 1X PCR buffer, 2 mM MgCl₂, 0.2 mM dNTPs, 2.5 pmol each primer, 1 ng plasmid DNA and 2.5 units *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania). The thermo-profile included 1 cycle of 94°C for 2 min; 35 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 100 sec and 1 cycle of 72°C for 5 min.

Construction of expression plasmid: The amplified CP gene was cloned into pGEM-T. Easy cloning vector (Promega, Fitchburg, WI, USA), as described elsewhere (Sokhandan-Bashir *et al.*, 2011) to increase quantity of the fragment for gel purification purpose. Both pGEM GFLV CP and pET21a (+) (Novagen, Madison, WI, USA) were double-digested with *Bam*HI/*Hind*III. Then, the CP DNA and linearized pET vector were purified from agarose gel before ligating to each other to prepare the construct pET21a GFLV CP (Fig. 1). *Escherichia coli* DH5 was transformed with

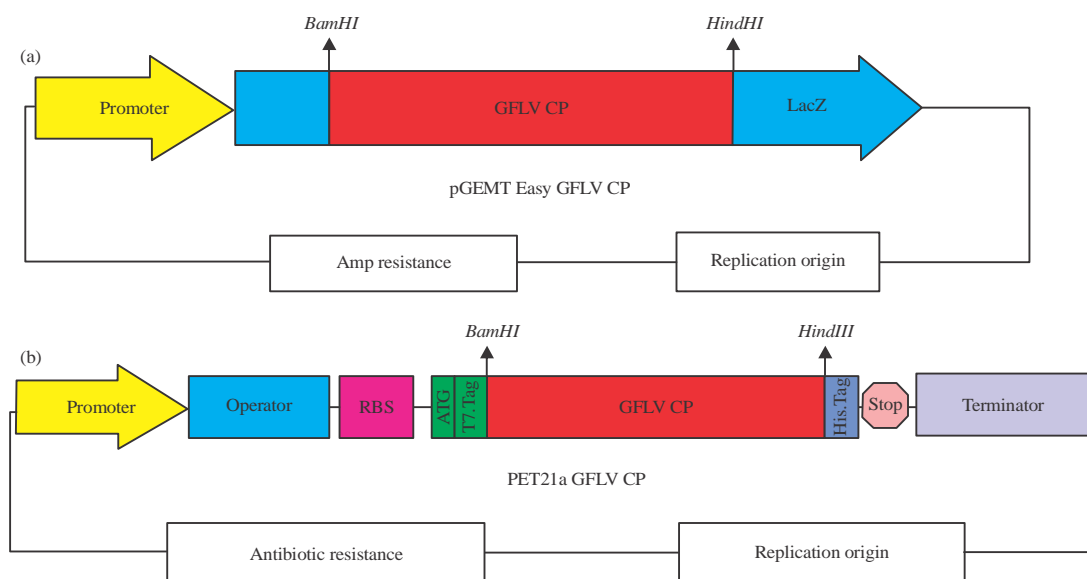


Fig. 1(a-b): A diagram showing *Grapevine fanleaf* virus coat protein gene cloned into the (a) Cloning vector and (b) Expression vector

pET21a GFLV CP by the heat shock method (Chung *et al.*, 1989) and the transformants were selected on ampicillin. Plasmid was extracted using AccuPrep® Nano-Plus Plasmid Mini kit (Bioneer, Alameda, CA, USA) according to the manufacturer's instruction and subjected to *Bam*HI/*Hind*III restriction analysis before sequencing on both strands with the universal T7-promoter and terminator primers in Pasteur Institute (Tehran, Iran).

Expression and analysis of GFLV CP: Competent *E. coli* Rosetta cells were transformed with pET21aGFLV CP. A desired colony carrying the correct construct was grown for about 8 h in 2x YT medium (tryptone 16 g L⁻¹, yeast extract 10 g L⁻¹, NaCl 5 g L⁻¹) containing 100 µg mL⁻¹ ampicillin in a shaker incubator (37°C, 150 rpm). Next, the culture was diluted 50 times in 10 mL of 2x YT medium and grown at 37°C with 200 rpm in a shaking incubator until the optical density (OD_{600 nm}) of 0.4-0.6. Then, 1.5 mL of non-induced culture was sampled before adding isopropyl-β-D thiogalactopyranoside (IPTG) at concentrations of 0.25, 0.5, 1, 2 or 6 mM to induce the T7 promoter. A 1.5 mL samples from the cultures were taken for analyses after 3, 4, 6 and 16 h of the induction and centrifuged at 9000 g for 7-10 min in a Heraeus Megafuge 1.0 R rotor 3041 (Hanau, Germany). The resulting pellet was suspended in 1×phosphate-buffered saline, PBS buffer (8 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 2.7 mM KCl and 136.8 mM NaCl, pH 7.4) and centrifuged at 15000 g for 10 min. The supernatant and pellet were examined to which fraction contained the expressed protein. The pellet was resuspended in Lysis buffer (10 mM Tris/HCl, 100 mM NaH₂PO₄, 8 M urea, pH 8) containing Lysosyme (1 mg mL⁻¹). Then, both pellet and supernatant samples were denatured by boiling for 5 min with an equal volume of 2x sample buffer pH 6.8 (50 mM Tris-HCl pH 6.8, 2% SDS, 10% Glycerol, 1% 2-mercaptoethanol, 12.5 mM EDTA, 0.02% bromophenol blue) before separating (each sample 15-20 µL) on duplicate 12% Polyacrylamide Gels Electrophoresis containing Sodium Dodecyl Sulphate (SDS-PAGE) (Laemmli, 1970) for 4 h at 100 V. One gel was stained with coomassie brilliant blue G-250 and followed by destaining buffer (H₂O 80%, methanol 10%, glacial acetic acid 10%). The unstained gel was blotted onto a nitrocellulose membrane (Amersham Hybound, UK) by a wet electro-transfer apparatus (Akhtarian, Tehran, Iran). The membrane was probed with 1:1000 dilution of commercial anti-GFLV IgG (in PBS) (Agdia, Elkhart, USA) and incubated with rabbit anti-mouse IgG-alkaline phosphatase conjugate. Then, 100 µL of Nitro Blue Tetrazolium (NBT) was added into 15 mL alkaline phosphatase buffer, before mixing with 50 µL of 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) (Agdia, Elkhart, USA) to stain the membrane until the targeted protein bands reached the desired intensity. The reaction was stopped by washing the membrane in deionized water for several minutes, before the membrane was air-dried on a filter paper and photographed. Based on fusion of a His•tag in the C-terminus of the expressed protein, Western blotting was also done with anti-His•tag antibody (Sigma-Aldrich, USA) under the same conditions as described above.

Purification of recombinant GFLV CP: A colony of *E. coli* Rosetta carrying pET21a GFLV CP was grown and 100 mL of the induced culture was centrifuged at 6,000 g for 15 min. The soluble extract from the pellet was mixed with the denaturant urea, loaded onto His•Bind Column Chromatography Kit (Novagen, Madison, WI, USA) and purification proceeded according to the manufacturer's instruction. Finally, urea was removed from the purified preparation by dialyzing against several changes of PBS buffer with constant stirring at 4°C. The recombinant native GFLV CP was purified by the use of T7•Tag Affinity Purification Kit (Novagen, Madison, WI, USA) as per the manufacturer's instruction.

Concentrations of the purified proteins were determined according to Bradford (1976). Then, 10 µg of each purified protein sample was resuspended in 100 µL sample buffer (defined above), boiled for 4 min and 20 µL aliquot was loaded on 12% SDS-PAGE. The protein bands separated by SDS-PAGE were electro-blotted onto a nitro-cellulose membrane in wet system and followed by incubation with appropriate antibody and conjugate as described above.

The purified protein samples were also subjected to Plate Trapped Antigen (PTA)-ELISA (Mowat and Dawson, 1987). Further, the purified proteins were tested by DIBA (dot immunobinding assay) on nitrocellulose membrane as described by Hampton *et al.* (1990).

Injections into rabbits and antibody preparation: Two New-Zealand 6-month old female rabbits were injected with native or refolded-denatured purified proteins. Injections were administered three times with 2-week intervals. A 500 µL (100 µg mL⁻¹) aliquot of the protein was emulsified with an equal volume of Freund's complete adjuvant (Biogen, Mashhad, Iran) for the first injection and Freund's incomplete adjuvant for the subsequent two injections. One milliliter emulsified soluble recombinant coat protein (antigen), which was diluted in PBS and contained Freund's adjuvant was injected subcutaneously. The rabbits were bled 2 weeks after the last injection. Then, the serum fractions were collected and stored at -20°C until required. The immunoglobulin fraction from the antiserum was purified using Protein-A IgG Purification Kit (Thermo Scientific, Waltham, MA, USA). After neutralization of the purified IgG by a gel filtration-based desalting column, conjugate was prepared by the use of EasyLink Alkaline Phosphatase Conjugation Kit (Abcam, Cambridge, UK). Antibody concentration was estimated from the light absorption at 280 nm (A₂₈₀). The polyclonal antibodies developed against the recombinant GFLV CP was evaluated for sensitivity and specificity by ELISA and Western blotting.

Titration of antiserum: The antiserum was titrated against the recombinant CP in a plate-trapped antigen (PTA)-ELISA with the antiserum dilutions in the range of 1:512-1:131072 in PBS buffer. Each washing step was done in three times, each time for 5 min, with ELISA wash buffer (PBST: PBS containing 0.05% Tween-20). First, the expressed CP (1 µg mL⁻¹) was coated on the plate and incubated at 37°C

for 3 h before washing. Then, blocking was done with PBS containing 2% BSA at 37°C for 2 h. After washing, different dilutions of the antiserum in PBS were added and incubated at 37°C for 3 h. Next, the antiserum was collected and the plate was washed, the conjugated antibody (HRP-conjugate), which was diluted 1:2000 in PBS was added and incubated at 37°C for 3 h. Then, the plate was washed, the substrate TMB (3, 3', 5, 5'-Tetramethylbenzidine) was added and kept in the dark room for 30 min. Finally, the stopper buffer (3 M NaOH) was added to the plate and absorbance values were measured at 405 nm by Anthos 2020 plate reader (Anthos, Salzburg, Austria).

Efficiency of anti-GFLV CP IgG in Western blotting and DAS-ELISA: Crude leaf extracts from healthy and GFLV-infected grapevine leaves, and the expressed GFLV CP from induced and non-induced bacteria were tested. Leaf samples were ground in the buffer containing (per liter) 32 g Tris-base, 37.2 g Tris-HCl, 8 g NaCl, 20 g PVP (MW: 25000), 10 g PEG (6000), 0.05% Tween 20, 0.2 g NaN₃, mixed with an equal volume of 2×sample buffer and boiled for 5 min before separating on 12% SDS-PAGE and subjecting to Western blotting as described before. The blotted membrane was first incubated with 1:1000 dilution of the recombinant anti-GFLV CP IgG at 4°C with gentle shaking overnight and after 4 times washing with PBST, was incubated with 1:5000 dilution of mouse anti-rabbit IgG HRP-conjugate (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 2 h. The bands were visualized by reacting with freshly prepared DAB substrate (Roche Applied Science, USA).

In examining efficiency of the anti-GFLV CP IgG by Double Antibody Sandwich (DAS)-ELISA the micro-titer plate was coated with the antibody solution (in coating buffer) for 3 h at 37°C before washing as described previously. Next, crude leaf extracts and the purified expressed protein samples were tested against the anti-GFLV CP. A 100 µL aliquot from each sample was added into the coated plate. After overnight incubation at 4°C, the plate was washed, followed by adding alkaline phosphatase conjugated anti GFLV CP IgG (diluted 1:2000 in conjugate buffer). The plates were incubated for 3 h at 37°C and washed three times with PBST before adding the substrate, P-nitrophenyl phosphate. Absorbance value at 405 nm was measured by Anthos 2020 (Anthos, Salzburg, Austria). A sample was considered positive, if its mean absorbance value was twice greater than of the healthy control.

RESULTS

Cloning and expression of GFLV CP: Nucleotide sequencing data confirmed in-phase insertion of the full length GFLV CP gene in the expression vector. When *E. coli* Rosetta was transformed with pET21a GFLV CP and the resultant desirable colonies were grown in the presence of IPTG, a subsequent SDS-PAGE analysis showed that the CP (~60 kDa) was efficiently expressed under induction with

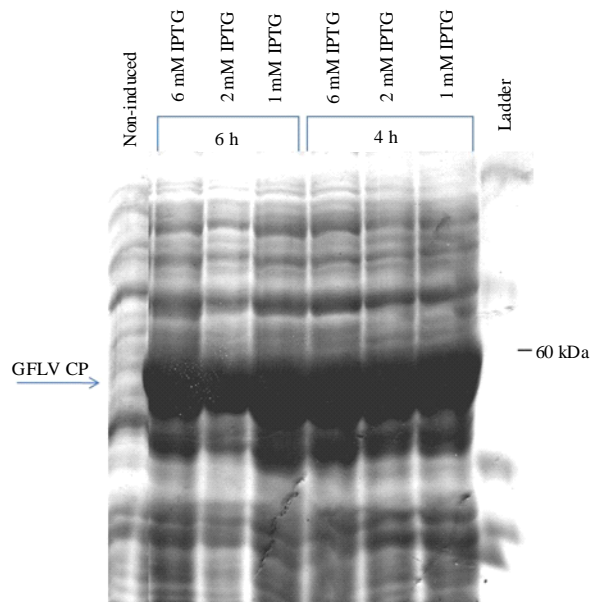


Fig. 2: A 12% SDS-PAGE presenting *Grapevine fanleaf* Virus coat protein gene expressed under different concentrations of IPTG and duration of induction

IPTG, whereas no CP band was observed with the non-induced sample. When a range of IPTG concentrations at 0.25, 0.5, 1, 2 and 6 mM each for duration of 3, 4, 6 and 16 h were applied a subsequent SDS-PAGE analysis revealed that up to 4 h after induction, the CP gene expression was increased as did by the duration of induction by 1mM IPTG (Fig. 2). However, beyond 4 h of induction, there was no increase in the expression level. The identity of the expressed protein was confirmed to be GFLV CP by Western blotting, where a corresponding spot was revealed on the nitrocellulose membrane (Fig. 3).

In addition, SDS-PAGE analysis of soluble and cell debris (pellet) fractions from IPTG-induced bacterial cultures showed that the major part of GFLV CP fusion protein was present in the pellet fraction.

Further, when protein samples from bacteria carrying the CP gene was subjected to DIBA, they reacted with the commercial anti-GFLV antibody and developed strong spots, whereas no signals were produced with the control samples.

Purification and characterization of expressed GFLV CP:

The purified native or denatured recombinant GFLV CP when subjected to SDS-PAGE analysis appeared to have one distinct expected band of about 60 kDa. Also, Western blotting showed presence of one distinct band after adding BCIP/NBT (data not shown). Yield of the native or denatured GFLV CP fusion protein purified from 100 mL of *E. coli* growth was about 0.8 or 1 mg, respectively, as estimated by Bradford assay and spectrophotometer.

Efficiency of the antibody: Anti-GFLV CP serum which was prepared in rabbit had a titer of 1: 8192 as determined by

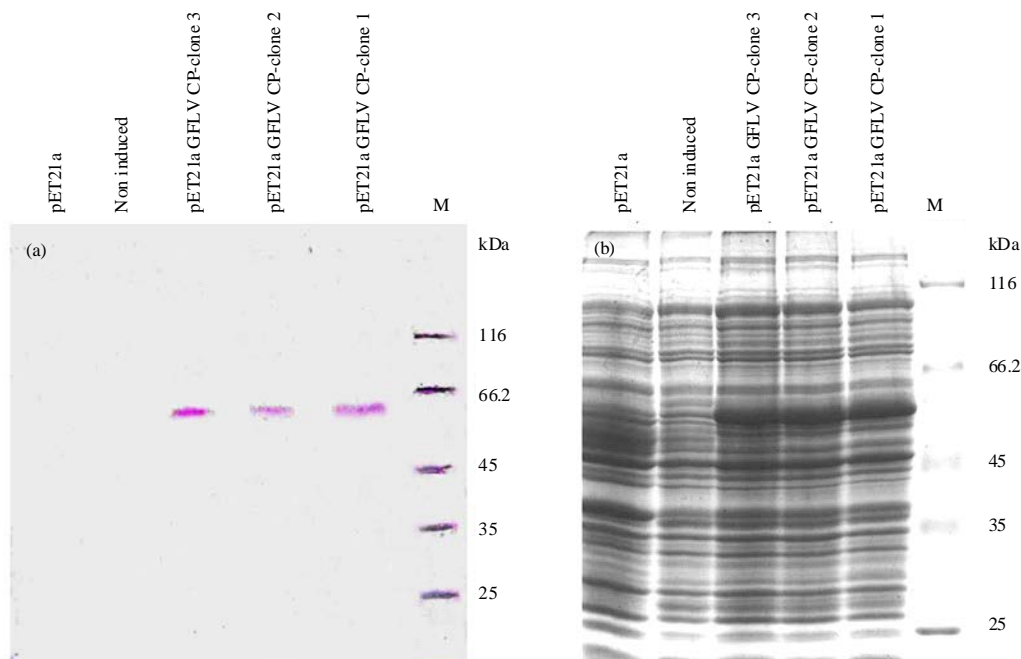


Fig. 3(a-b): Expression analyses of *Grapevine fanleaf virus* coat protein gene by SDS-PAGE (right) and Western blotting (left). Anti-GFLV antibody and the conjugate were used in 1:1000 dilutions. Non induced: Crude protein sampled from culture before induction by IPTG and M: Protein size marker. Samples from three replicate clones of *Escherichia coli* carrying pET21aGFLVCP show expression of the coat protein with a size about 60 kDa

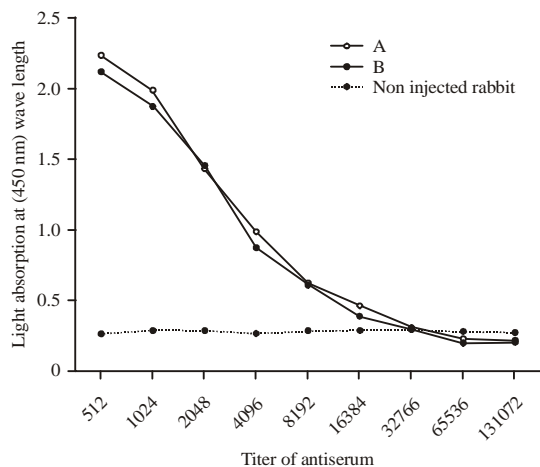


Fig. 4: Titration of antiserum prepared against recombinant coat protein of *Grapevine fanleaf virus* by the use of indirect ELISA. The graph is based on absorbance values of the wells treated with different dilutions of the antiserum. The protein purified through His•Tag column (A) or T7•Tag (B) column were used as the antigens

PTA-ELISA (Fig. 4). This recombinant antiserum reacted with the expressed GFLV CP and detected GFLV in the infected plant tissue.

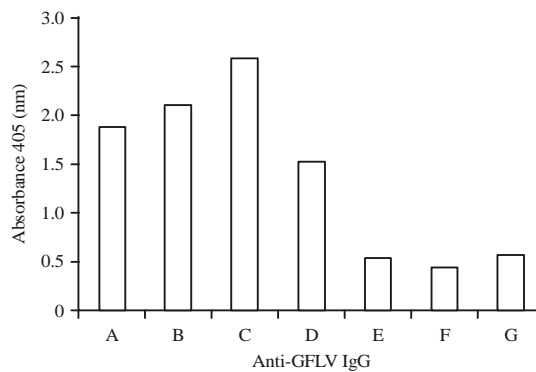


Fig. 5: Efficiency of anti-GFLV IgG prepared against the virus recombinant coat protein in DAS-ELISA. The curve is based on absorbance values at 405 nm

The purified IgG and the alkaline phosphatase-conjugated IgG (both at 1:1000 dilution) detected the expressed GFLV CP and GFLV virions in DAS-ELISA (Fig. 5). Also, the antibody reacted efficiently in Western blotting with the expressed protein as well as extract from the GFLV-infected grapevine leaves (Fig. 6).

DISCUSSION

The possibility of raising antibodies against the recombinant GFLV CP expressed in *E. coli* was studied.

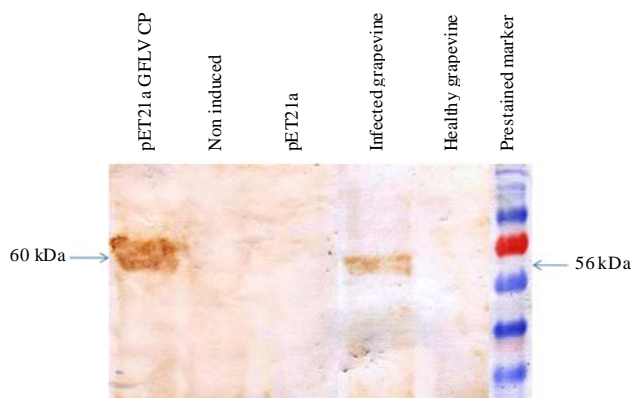


Fig. 6: Western blotting of protein from bacteria 4 h after induction with IPTG. A pre-stained marker included for size determination. Because the recombinant GFLV CP is tagged, its size is slightly bigger than the actual GFLV CP size

Antibodies against recombinant proteins of a number of plant viruses have been prepared successfully (Iracheta-Cardenas *et al.*, 2008; Lee and Chang, 2008; Raikhy *et al.*, 2007; Jain *et al.*, 2005; Abou-Jawdah *et al.*, 2004; Nickel *et al.*, 2004; Cerovska *et al.*, 2003; Hourani and Abou-Jawdah, 2003; Korimbocus *et al.*, 2002; Kumari *et al.*, 2001). By the use of different IPTG concentrations it was concluded that the optimal expression was achieved, when transformed *E. coli* Rosetta cells were induced with 1 mM IPTG at OD = 0.5 and grown at 37°C for 4 h. Different IPTG concentrations including 1 mM (Bragard *et al.*, 2000; Kadkhodayan *et al.*, 2000; Saini and Vrati, 2003; Thomas and Baneyx, 1996) and 0.4 mM (Jacob and Usha, 2002) have been reported as the optimal concentration with various genes of interest for expression of *Cardamom mosaic virus* CP and 0.1 mM (Petrzik *et al.*, 2001) for that of *Prunus necrotic Ring Spot virus* (PNRSV) CP in *E. coli*. Similarly, an optimal duration in expression at a particular IPTG concentration may vary according to the gene of interest or other conditions even though expression for 3-4 h at 37°C has been widely reported as being optimal (Bragard *et al.*, 2000; Jacob and Usha, 2002; Liu *et al.*, 2001; Saini and Vrati, 2003). Like other virus systems such as Faba bean necrotic yellows virus (Kumari *et al.*, 2001), sugarcane yellow leaf virus (Korimbocus *et al.*, 2002), Potato mop-top virus (Cerovska *et al.*, 2003, 2006) and Groundnut bud necrosis virus (Jain *et al.*, 2005) where tagged viral CP was expressed in insoluble fraction, the fusion GFLV CP was detected in the insoluble fraction (pellet) from the transformed *E. coli*. Expression of recombinant fusion protein in the soluble fraction has also been reported elsewhere (Hema *et al.*, 2003; Hourani and Abou-Jawdah, 2003; Lee *et al.*, 2008; Raikhy *et al.*, 2007), but we detected only a small amount of the fusion protein in the soluble fraction.

Possible influence of the T7•Tag and His•Tag, fused in the CP, on antibody specificity might be a point of concern.

Although, there are vectors that possess a cleavage facility to remove the tags from the expressed protein before injecting into animal the tags are not expected to have significant immunogenic properties on the CP expressed in *E. coli* (Gulati-Sakhuja *et al.*, 2009; Kumari *et al.*, 2001; Mutasa-Gottgena *et al.*, 2000). Also, tags at the N- and C-terminus are not expected to have significant immunogenic properties (Kumari *et al.*, 2001; Mutasa-Gottgena *et al.*, 2000). It was found in the present study that affinity-purified fusion GFLV CP as the good antigen to raise specific and efficient polyclonal antiserum.

Because the fusion GFLV CP possessed T7•Tag at the N-terminus T7•Tag affinity chromatography kit was used for the native protein purification. Also, a denaturing buffer containing urea was used to purify recombinant protein in insoluble and soluble fractions of the transformed *E. coli* cells. The purified protein was confirmed by SDS-PAGE, Western blotting, DIBA and ELISA. The CP reacted successfully with GFLV antibodies in DIBA as well as Western blotting. Similarly, Potato virus Y (PVY) CP expressed in *E. coli* has been reported to be detectable in DIBA (Amer *et al.*, 2004). However, ELISA is preferable due to its more sensitivity.

The use of purified expressed fusion protein as the antigen gives consistent results in terms of quantity and quality of the antigen (Raikhy *et al.*, 2007). Thus, the recombinant viral CPs expressed in bacterial cells have great potentials as the alternative sources of antigens for raising specific antibodies to plant viruses. They can be produced in large quantities and manipulated or modified as needed for specific uses. In contrast to the conventional method of antigen preparation, which is associated with very poor yields for GFLV, the purification procedure described above can be completed in 3 days.

A non-specific background reaction was encountered in the Western blotting with anti-GFLV CP which is not unusual because non-specificities have also been reported with recombinant antisera prepared against fusion proteins of other viruses (Gulati-Sakhuja *et al.*, 2009; Cerovska *et al.*, 2003; Hourani and Abou-Jawdah, 2003; Kumari *et al.*, 2001). Such non-specificity may be tackled by optimizing the procedure. Presumably, premature or partial transcripts of target gene are also produced which may be translated into shorter chains of proteins reacting in the subsequent Western blot. This non-specificity seems to be rectified by the use of monoclonal antibodies. As another explanation, some non-target proteins may contain repeats of histidine residues that end up in non-specific reactions.

Antiserum raised against the GFLV CP detected the CP in PTA-ELISA efficiently. A 1:1000 dilution of the IgG purified from the antiserum was efficient to detect the expressed GFLV CP in *E. coli* and the virus in infected plant tissue in both Western blotting and DAS-ELISA. Similar results with 1:1000 dilution of recombinant IgG for Western blotting have been reported in detection of infected plant tissue (Gulati-Sakhuja *et al.*, 2009; Cerovska *et al.*, 2003; Hourani and Abou-Jawdah, 2003). However, there are reports of antibodies prepared against recombinant viral proteins,

which do not detect the related virus in DAS-ELISA (Cеровска *et al.*, 2006; Korimbocus *et al.*, 2002) due to inability of the coated antibodies to react with native viral epitopes even though it was not case in this study.

This study addressed the possibility of using GFLV CP expressed in *E. coli* to produce GFLV-specific antibodies for applications in serological and immunoblotting tests.

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REFERENCES

- Abou-Jawdah, Y., H. Sobh, N. Cordahi, H. Kawtharani, G. Nemer, D.P. Maxwell and M.K. Nakhla, 2004. Immunodiagnosis of *Prune dwarf* virus using antiserum produced to its recombinant coat protein. *J. Virol. Meth.*, 121: 31-38.
- Amer, M.A., M.H. El-Hammady, H.M. Mazyad, A.A. Shalaby and F.M. Abo-El-abbas, 2004. Cloning, expression and nucleotide sequence of coat protein gene of an Egyptian isolate of potato virus Y strain NTN infecting potato plants. *Egypt. J. Virol.*, 1: 39-50.
- Andret-Link, P., C. Laporte, L. Valat, C. Ritzenthaler and G. Demangeat *et al.*, 2004. Grapevine fanleaf virus: Still a major threat to the grapevine industry. *J. Plant Pathol.*, 86: 183-195.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72: 248-254.
- Bragard, C., G.H. Duncan, S.V. Wesley, R.A. Naidu and M.A. Mayo, 2000. Virus-like particles assemble in plants and bacteria expressing the coat protein gene of Indian peanut clump virus. *J. Gen. Virol.*, 81: 267-272.
- Butt, T.R., S.C. Edavettal, J.P. Hall and M.R. Mattern, 2005. SUMO fusion technology for difficult-to-express proteins. *Prot. Expr. Purific.*, 43: 1-9.
- Cеровска, N., T. Moravec, P. Rosecka, P. Dedic and M. Filigarova, 2003. Production of polyclonal antibodies to a recombinant coat protein of potato mop-top virus. *J. Phytopathol.*, 151: 195-200.
- Cеровска, N., M. Filigarova and T. Pecenkova, 2006. Production of polyclonal antibodies to a recombinant potato mop-top virus non-structural triple gene block protein 1. *J. Phytopathol.*, 154: 422-427.
- Chung, C.T., S.L. Neimala and R.H. Miller, 1989. One step preparation of competent *E. coli* transformation and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci. USA.*, 86: 2172-2175.
- Fajardo, T.V.M., D.R. Barros, O. Nickel, G.B. Kuhn and M. Zerbini, 2007. Expression of *Grapevine leafroll*-associated virus 3 coat protein gene in *Escherichia coli* and production of polyclonal antibodies. *Fitopatologia Brasileira*, 32: 496-500.
- Gulati-Sakhuja, A., J.L. Sears, A. Nunez and H.Y. Liu, 2009. Production of polyclonal antibodies against *Pelargonium zonate* spot virus coat protein expressed in *Escherichia coli* and application for immunodiagnosis. *J. Virol. Meth.*, 160: 29-37.
- Hampton, R., E. Ball and S. Beboer, 1990. Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens. American Phytopathological Society, St. Paul, MN., USA., ISBN-10: 0890541159, Pages: 389.
- Hartley, J.L., 2006. Cloning technologies for protein expression and purification. *Curr. Opin. Biotechnol.*, 17: 359-366.
- Hema, M., N. Kirthi, P. Sreenivasulu and H.S. Savithri, 2003. Development of recombinant coat protein antibody based IC-RT-PCR for detection and discrimination of sugarcane streak mosaic virus isolates from Southern India. *Arch. Virol.*, 148: 1185-1193.
- Hourani, H. and Y. Abou-Jawdah, 2003. Immunodiagnosis of Cucurbit yellow stunting disorder virus using polyclonal antibodies developed against recombinant coat protein. *J. Plant Pathol.*, 85: 197-204.
- Iracheta-Cardenas, M., B.D. Sandoval-Alejos, M.E. Roman-Calderon, K.L. Manjunath, R.F. Lee and M.A. Rocha-Pena, 2008. Production of polyclonal antibodies to the recombinant coat protein of *Citrus tristeza* virus and their effectiveness for virus detection. *J. Phytopathol.*, 156: 243-250.
- Jacob, T. and R. Usha, 2002. Expression of *Cardamom mosaic* virus coat protein in *Escherichia coli* and its assembly into filamentous aggregates. *Virus Res.*, 86: 133-141.
- Jain, R.K., A.N. Pandey, M. Krishnareddy and B. Mandal, 2005. Immunodiagnosis of groundnut and watermelon bud necrosis viruses using polyclonal antiserum to recombinant nucleocapsid protein of Groundnut bud necrosis virus. *J. Virol. Meth.*, 130: 162-164.
- Kadkhodayan, S., M.S. Knapp, J.J. Schmidt, S.E. Fabes, B. Rupp and R. Balhorn, 2000. Cloning, expression and one-step purification of the minimal essential domain of the light chain of botulinum neurotoxin type A. *Prot. Exp. Purify.*, 19: 125-125.
- Korimbocus, J., S. Preston, C. Danks, I. Barker, D. Coates and N. Boonham, 2002. Production of monoclonal antibodies to sugarcane yellow leaf virus using recombinant readthrough protein. *J. Phytopathol.*, 150: 488-494.
- Kumari, S.G., K.M. Makkouk, L. Katul and H.J. Vetten, 2001. Polyclonal antibodies to the bacterially expressed coat protein of *Faba bean* necrotic yellows virus. *J. Phytopathol.*, 149: 543-550.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680-685.
- Lee, S.C. and Y.C. Chang, 2008. Performances and application of antisera produced by recombinant capsid proteins of *Cymbidium mosaic* virus and *Odontoglossum* ringspot virus. *Eur. J. Plant Pathol.*, 122: 297-306.

- Ling, K.S., H.Y. Zhu, N. Petrovic and D. Gonsalves, 2007. Serological detection of *Grapevine leafroll virus 2* using an antiserum developed against the recombinant coat protein. *J. Phytopathol.*, 155: 65-65.
- Liu, W., R. He and Z. Xue, 2001. Expression of the n-terminal segment of qbrn-2 in *E. coli* and tips on preparation of a recombinant protein. *Protein Pept. Lett.*, 8: 27-32.
- Minafra, A., P. Casati, V. Elicio, A. Rowhani, P. Saldarelli, V. Savino and G.P. Martelli, 2000. Serological detection of *Grapevine rupestris* stem pitting-associated virus (GRSPaV) by a polyclonal antiserum to recombinant virus coat protein. *Vitis*, 39: 115-118.
- Mowat, W.P. and S. Dawson, 1987. Detection and identification of plant viruses by ELISA using crude sap extracts and unfractionated antisera. *J. Virol. Methods*, 15: 233-247.
- Mutasa-Gottgena, E.S., D.M. Chwarszczynska, K. Halsey and M.J.C. Asher, 2000. Specific polyclonal antibodies for the obligate plant parasite *Polymyxa*: A targeted recombinant DNA approach. *Plant Pathol.*, 49: 276-278.
- Nickel, O., M.L.P.N. Targon, T.V.M. Fajardo, M.A. Machado and A.P. Trivilin, 2004. Polyclonal antibodies to the coat protein of Apple stem grooving virus expressed in *Escherichia coli*: Production and use in immunodiagnosis. *Fitopatol Bras.*, 29: 558-562.
- Petrzik, K., I. Mraz and D. Kubelkova, 2001. Preparation of recombinant coat protein of *Prunus necrotic ringspot virus*. *Acta Virol.*, 45: 61-63.
- Pinck, L., M. Fuchs, M. Pinck, M. Ravelonandro and B. Walter, 1988. A satellite RNA in *grapevine fanleaf virus* strain F13. *J. Gen. Virol.*, 69: 233-239.
- Raikhy, G., V. Hallan, S. Kulshrestha and A. A. Zaidi, 2007. Polyclonal antibodies to the coat protein of *Carnation etched ring virus* expressed in bacterial system: Production and use in immunodiagnosis. *J. Phytopathol.*, 155: 616-622.
- Raski, D.J., A.C. Goheen, L.A. Lider and C.D. Meredith, 1983. Strategies against grapevine fanleaf virus and its nematode vector. *Plant Dis.*, 67: 335-340.
- Saini, M. and S. Vрати, 2003. High-level synthesis of Johnson grass mosaic virus coat protein in *Escherichia coli* and its auto-assembly to form virus-like particles. *Protein Expr. Purif.*, 28: 86-92.
- Sanfacon, H., J. Wellink, O. Le Gall, A. Karasev, R. van der Vlugt and T. Wetzler, 2009. Secoviridae: A proposed family of plant viruses within the order Picornavirales that combines the families Sequiviridae and Comoviridae, the unassigned genera *Cheravirus* and *Sadwavirus* and the proposed genus *Torradovirus*. *Arch. Virol.*, 154: 899-907.
- Sokhandan-Bashir, N., A. Pashae and H. Doulati-Baneh, 2011. Characterization of the full length coat protein gene of Iranian *Grapevine fanleaf virus* isolates, genetic variation and phylogenetic analysis. *Iran. J. Biotech.*, 9: 213-221.
- Thomas, J.G. and F. Baneyx, 1996. Protein misfolding and inclusion body formation in recombinant *Escherichia coli* cells over expressing heat-shock proteins. *J. Biol. Chem.*, 271: 11141-11147.
- Vigne, E., G. Demangeat, V. Komar and M. Fuchs, 2005. Characterization of a naturally occurring recombinant isolate of *Grapevine fanleaf virus*. *Arch. Virol.*, 150: 2241-2255.
- Zimmermann, D., P. Bass, R. Legin and B. Walter, 1990. Characterization and serological detection of four closterovirus-like particles associated with leafroll disease on grapevine. *J. Phytopathol.*, 130: 205-218.