Fusion coat protein of pumpkin yellow vein mosaic virus with maltose binding protein: Applications in immunodiagnosis of begomoviruses

Chigurupati Phaneendra · K. R. S. Sambasiva Rao ·
Reetika Kapoor · R. K. Jain · Bikash Mandal

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Abstract Availability of adequate quantity of purified virus preparation from plant tissue is the major limitation in producing polyclonal antibodies (PAb) to begomovirus. Very few examples show successful utilization of *E. coli* expressed recombinant coat protein (CP) for immuno diagnosis of begomoviruses. In the present study, ~771 bp CP gene (~29.0 kDa) of Pumpkin yellow vein mosaic virus (PYVMV) was expressed as a ~71.0 kDa fusion protein with maltose binding protein (MBP) (~42.0 kDa) in *E. coli*. The MBP–CP was obtained in soluble state. The PAb to the purified fusion protein successfully detected PYVMV and other bipartite and monopartite begomoviruses in the field samples at 1:250 dilution in enzyme linked immunosorbent assay. Our study for the first time showed that MBP-tag fusion CP was suitable to produce diagnostic antibody to begomoviruses.

Keywords Pumpkin yellow vein mosaic virus · Coat protein gene · Recombinant protein · ELISA · Western blot

Begomoviruses (family *Geminiviridae*) are emerging constrain in the production of numerous crops [14]. Both, immune- and nucleo-based techniques have been employed for the detection of begomoviruses [5]. Begomoviruses are phloem limiting, generally not sap transmissible and many

of them do not have suitable propagative hosts. As a result, transmission and purification of begomoviruses are difficult and yield of purified virus is usually low. However, tedious method of virus propagation in large number of host plants through whitefly (*Bemisia tabaci*) inoculation and purification of virus from large batches of infected plant tissues were performed to produce polyclonal antibodies (PAb) against a few begomoviruses such as squash leaf curl virus (SLCV) [3], tomato yellow leaf curl virus (TYLCV) [9] and tomato leaf curl Bangalore virus (ToLCBaV) [4]. The traditional method of begomovirus antigen preparation from infected plant tissue generally results into contamination with host proteins and thus limits their use in serological diagnosis. Begomovirus purification by conventional method further limits the renewable production of PAb. As a result, routine detection and identification using serology has been less commonly used for begomoviruses compared to the other plant viruses. To overcome these limitations, molecular biology techniques are currently being used to express the viral genes of interest in *E. coli* and the recombinant antigen is used for the production of PAb. Recombinant antigens for several RNA plant viruses were successfully produced in *E. coli* [6, 7, 10], whereas, very few DNA plant viruses have been used for successful expression of recombinant antigen in *E. coli* [1].

Pumpkin yellow vein mosaic virus, a bipartite begomovirus is known in India since 1950s [13] and the virus was characterized based on host range and serology [8]. Further, phylogenetic analysis of DNA-A sequence revealed that PYVMV was synonymous to squash leaf curl China virus (SLCCNV) reported from China [11, 12]. There is an increasing incidence of PYVMV and many other begomoviruses in the Indian subcontinent [14], but PAb to begomoviruses for immunodiagnosis is not commonly available. It is necessary to have a simple, robust,

C. Phaneendra · R. Kapoor · R. K. Jain · B. Mandal (✉)
Advanced Centre for Plant Virology, Division of Plant
Pathology, Indian Agricultural Research Institute,
New Delhi 110 012, India
e-mail: banipur@gmail.com; leafcurl@rediffmail.com

C. Phaneendra · K. R. S. Sambasiva Rao
Department of Biotechnology, Acharya Nagarjuna University,
Nagarjuna Nagar, Guntur 522 510, Andhra Pradesh, India

rapid and affordable tool in place for immuno-detection of begomoviruses. In this study, we report the expression of recombinant fusion protein containing coat protein (CP) of PYVMV with maltose binding protein for the production of PAb and its suitability for broad spectrum diagnosis of begomoviruses.

The full length CP gene (~ 771 nt long, encoding 257 aa) of PYVMV was amplified, cloned into pGEMT-Easy vector and confirmed by sequencing. A primer pair with *EcoRI*/*HindIII* sites: BM46F (5'aagaattcatggcgaagcgacca3') and BM 47R (5'ggaagctttaattgttaccca3') was used to clone the CP gene into pMAL-c2X vector. The same primer pair with *NcoI*/*HindIII* sites was used to clone the CP gene into another expression vector, pET-28a(+). The PCR reaction consisted of one cycle at 94 °C for 3 min, 30 cycles at 94 °C for 1 min, 49 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 10 min. The pMal/pET-PY-CP constructs were used to transform *E. coli* strains TB1 and BL21 (DE3), respectively. The clones with correct reading frame was confirmed by sequencing and selected for expression studies. The recombinant clones were grown in LB medium supplemented with ampicillin (100 µg/ml)/kanamycin (50 µg/ml) respectively. The bacterial culture was grown at 37 °C until the OD₆₀₀ value reached 0.5. The expression and purification of the

recombinant protein of pMal/pET-PY-CP was carried as per the manufacturer's instructions. The purified protein was dialyzed using Small Wonder Lyser kit method (Promega, USA) and lyophilized. The concentration of the eluted protein was estimated using Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies, Willington, DE) and analysed by SDS-PAGE using 5 % stacking and 12 % resolving gels. The specificity of the fusion protein was determined using anti-MBP or His tag in Western blotting. PYVMV CP gene (~ 29.0 kDa) expression was induced with three different concentrations of IPTG (0.4, 0.5 and 1.0 mM) for 1 and 3 h at 37 °C. The CP was successfully expressed as a fusion protein with MBP (~ 42.0 kDa). Maximum expression of the fusion protein from pMal-PY-CP construct was achieved in *E. coli* TB1 cells. Higher induction was observed with 0.4 mM IPTG at 37 °C for 3 h. Unlike other vector systems [1, 6], the fusion protein in the present study was found in the soluble fraction. Whereas the pET-PY-CP clone did not show any visible expression under above conditions. The expressed pMal-PY-CP fusion protein was purified using amylose resin and specificity of the purified protein was detected using anti-MBP antiserum [1:10,000, NEB, Ipswich, MA, USA], which revealed expected fusion protein was ~ 71.0 kDa.

Fig. 1 Analysis of polyclonal antibodies developed against fusion coat protein Pumpkin yellow vein mosaic virus with maltose binding protein by DAC-ELISA. **a** Determination of antiserum titer,

b Detection of optimum dilution of PYVMV-infected tissue as antigen at different dilutions, **c** Detection of viral antigen using different concentrations of recombinant purified protein

The purified recombinant CP (500 µg) was emulsified with an equal volume of Freund's incomplete adjuvant and injected intramuscularly into a New Zealand white rabbit five times at an interval of one week. The rabbit was bled one week after the last injection and the crude antiserum was mixed with glycerol (1:1, v/v) and stored at -80°C .

Antibody was evaluated by direct antigen coated enzyme-linked immunosorbent assay (DAC-ELISA) using crude antiserum as described by [2]. Sample was prepared by grinding leaf tissue in coating buffer containing 2 % polyvinyl pyrrolidone (PVP, MW 40,000). Goat-anti-rabbit-IgG-AP conjugate (Sigma-Aldrich, St. Louis, MO, USA) was used at 1:30,000 dilution as a secondary antibody. The ELISA results were recorded at $A_{405\text{ nm}}$ at 1 h after addition of substrate *p*-nitrophenyl phosphate (0.5 mg/ml; Sigma, USA) by an ELISA reader (BIO-TEK instruments Inc., Winooski, VT, USA). A reaction was considered positive when the mean absorbance value for a sample was twice greater than the mean value of healthy control.

DAC-ELISA was used to determine the titer of antiserum at various dilutions [1:10, 1:20, 1:50, 1:100, 1:250, 1:500 and 1:1,000] using PYVMV infected pumpkin leaf tissues. To identify the optimum dilution of infected leaf sap, two fold dilutions of the infected leaf extracts [1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256] were evaluated by ELISA. In order to determine the minimum quantity of viral antigen required for ELISA detection, purified recombinant CP at different concentrations [5, 10, 25, 50, 100, 500 and 1,000 ng] were tested. The antiserum showed titer of 1:250, when tested repeatedly with infected crude extract as antigen. The mean absorbance of the infected sap was decreased slowly ranged from 2.15 to 0.07 at dilution 1:10 to 1:500. However, the absorbance value of infected sample was more than twice of the healthy sample at all the dilutions (Fig. 1a). The optimum crude sap dilution to detect virus from infected tissue was 1:2 (Fig. 1b). Serially diluted purified protein could be detected at concentration as low as 5 ng/200 µl (Fig. 1c).

Table 1 Detection of begomovirus in the different plant samples collected during 2010–2013 from Delhi by DAC-ELISA using polyclonal antiserum to recombinant coat protein of pumpkin yellow vein mosaic virus (PYVMV)

Plant species	Year of collection	Type of sample	Virus associated ^a	Symptoms ^b	No. of ELISA +ve ^c samples/No. tested (OD value range)
Cucurbitaceous plant					
Pumpkin (<i>Cucurbita moschata</i>)	2010	Frozen (-20°C)	PYVMV	YVM	4/5 (0.24–0.51)
	2011	Lyophilized	PYVMV	YVM	7/10 (0.15–0.29)
	2011	Field	PYVMV	YVM	8/8 (0.32–0.88)
	2011	Field	PYVMV	YVM	8/8 (0.32–0.88)
	2012	Field	PYVMV	YVM	30/30 (0.4–1.58)
Ridge gourd (<i>Lufa acutangula</i>)	2012	Field	Begomovirus	YM	4/5 (0.27–0.46)
Bitter gourd (<i>Momordica charantia</i>)	2012	Field	Begomovirus	LC	0/3 (0.09–0.1)
Bottle gourd (<i>Lagenaria siceraria</i>)	2012	Field	Begomovirus	CLM	0/3 (0.1–0.16)
Cucumber (<i>Cucumis sativus</i>)	2012	Field	Begomovirus	YM	6/6 (0.22–1.0)
Muskmelon (<i>C. melo</i>)	2012	Field	Begomovirus	YM	4/6 (0.30–0.72)
Solanaceous plant					
Tomato (<i>Solanum lycopersicum</i>)	2012	Field	ToLCNDV	LC	25/25 (0.17–1.31)
	2012	Whitefly inoculated	ToLCNDV	LC	17/22 (0.31–0.8)
Chilli (<i>Capsicum annum</i>)	2013	Field	ToLCNDV	LC	1/1 (0.73)
<i>N. benthamiana</i>	2012	Agroinoculated	ToLCBaV	LC	25/25 (0.17–1.31)
Fabaceous plant					
Mung bean (<i>Vigna radiata</i>)	2012	Field	MYMIV	YM	0/2 (0.1–0.17)
Cowpea (<i>V. unguiculata</i>)	2012	Field	MYMIV	GM	18/21 (0.41–1.0)

M mosaic, *YM* yellow mosaic, *GM* golden mosaic, *YVM* yellow vein mosaic, *LC* leaf curl, *CLM* curly mottle, *PYVMV* Pumpkin yellow vein mosaic virus, *ToLCNDV* tomato leaf curl New Delhi virus, *ToLCBaV* tomato leaf curl Bangalore virus, *MYMIV* mungbean yellow mosaic India virus

^a Confirmed by PCR

^b The OD values at $A_{405\text{ nm}}$ after 1 h of addition of substrate. All OD values were subtracted from the buffer value (OD 0.13–0.15). The absorbance in the healthy control ranged from 0.01 to 0.09. Samples with absorbance value more than twice of healthy control were considered as ELISA positive

ELISA was also performed to validate the PAb to detect PYVMV and other begomoviruses infecting different plant species collected during 2010–2013 from Delhi. Leaf samples either fresh, lyophilized or frozen (-20°C) were ground in coating buffer 1:2 (w/v) containing 2 % polyvinyl pyrrolidone (PVP, MW 40,000) and coated ELISA plates were incubated for 1 h at 37°C . Subsequently, the ELISA was performed as described in the previous section. Of 53 pumpkin yellow vein samples tested, 49 were positive (OD 0.15–1.58). The antiserum was also effective in detecting other begomovirus from Cucurbitaceous, Solanaceous and Fabaceous hosts collected from field as well as green house (Table 1). Higher OD values of 0.8–1.58 were obtained from tender leaves pumpkin, cowpea, cucumber, tomato and chilli showing fresh infection either in the field or green house. Whereas, the weaker reaction observed in frozen (OD 0.2–0.51) and lyophilized tissue (OD 0.15–0.29) indicated that storing leaf tissues at -20°C or lyophilization of leaf tissues might have caused virus degradation.

These results showed that tender leaf tissues with fresh infection are better source of samples for the detection of begomovirus by ELISA with the PAb developed in the present study.

Our attempts to purify the virus using conventional methods failed to yield adequate quality and quantity of purified viral antigen of PYVMV and hence the recombinant fusion protein approach was carried out to develop PAb for use in serological diagnosis of begomoviruses. The use of rCP as an immunogen to develop antibodies has been shown as of great value particularly for viruses that are difficult to propagate and purify (Lee and Chang, 2008). PAb to geminiviral proteins expressed in *E. coli* using recombinant technology was demonstrated earlier by using expression vectors pETH-3c [1] and pQE-30UA [4]. In both these previous studies the expressed protein was in insoluble state, which was difficult to purify. Whereas, the pMAL-c2X vector system used in the present study expressed soluble recombinant protein, which was easier to purify from the *E. coli* lysate. The PAb generated against the His-tag fusion CP (35.0 kDa) of ToLCBaV, a monopartite begomovirus showed low titer (1:400) [4]. In the present study also, low titer PAb was obtained against MBP-CP (71.0 kDa) of PYVMV, a bipartite begomovirus. However, relatively higher titer (1:10,000) was reported against His-tag fusion CP of cabbage leaf curl virus [1]. Although, the titer of the PAb to PYVMV was low, it was useful in detecting both monopartite and bipartite begomovirus affecting various plant species. This is the first

report of immunodiagnosis of begomovirus with using *E. coli* expressed MBP-CP fusion protein.

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