Molecular characterization of Indian isolate of peanut mottle virus and immunodiagnosis using bacterial expressed core capsid protein

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Abstract Peanut mottle virus (PeMoV), a seed borne potyvirus was recorded in India in 1978, however the virus was not characterized at molecular level. In the present study, an isolate of PeMoV infecting peanut in southern India was characterized based on host reactions and coat protein (CP) gene sequence, which revealed that the Indian isolate was very close to a peanut isolate reported from Israel and distinct from pea isolate reported from USA. The core region of CP gene that contained majority of the predicted epitopes was successfully expressed (1.75 mg/l) in *Escherichia coli* as a 22 kDa protein. A high titer polyclonal antibody (PAb) to the expressed core CP was produced, which efficiently detected PeMoV. The antiserum was useful in specific detection of PeMoV as it showed negligible cross reactivity with the other potyviruses e.g., peanut stripe virus, potato virus Y, papaya ringspot virus and onion yellow dwarf virus. The PAb was validated in ELISA using 1,169 field and greenhouse samples of peanut which showed 1.85–26.3 % incidence of PeMoV in peanut seed multiplication field during 2011–2012. This is the first report of immunodiagnosis of PeMoV with a PAb to recombinant core CP of PeMoV.

Keywords Peanut · PeMoV · ELISA · Recombinant antigen

Introduction

Peanut mottle virus (PeMoV; genus *Potyvirus*, family *Potyviridae*) was first described from USA in 1965 [14] and since then it has been reported from many peanut growing areas worldwide [2, 4]. The virus generally induces greenish mottle symptoms in peanuts. Other than peanut, PeMoV is known to infect soybean (*Glycine max*), French bean (*Phaseolus vulgaris*) and *Cassia sp* [14]. PeMoV spreads naturally through infected seeds and aphids. PeMoV is of quarantine significance as it is a true seed borne virus in peanut [1]. Seed transmission of PeMoV can result in 25 % yield reduction [14].

In India, PeMoV was first noticed in 1976–1977 in the intercepted peanut germplasm imported from USA [25]. Incidentally, in the similar time (1977), natural occurrence of the virus was recorded on peanut, soybean and pea in Punjab [26]. It has been reported to occur on winter/summer peanut crop mainly in the states of Andhra Pradesh, Maharashtra and Gujarat, where losses have been reported to vary from 5 to 30 % (http://www.ikisan.com/Crop%20specific/Eng/links/ap_groundnutDisease%20Management.shtml). Although, PeMoV is known to occur in India, the virus so far has not been characterized at molecular level.

For the diagnosis of plant viruses, polyclonal antibodies (PAbs) are traditionally generated by immunizing animal with the purified virus. The quality of PAbs depends on the purity of the virus preparation used for immunization. Often, PAbs raised against the purified virus cross reacts with host protein limiting their use in serological assays. Bacterial (*Escherichia coli*) expression of protein is simple,

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fast and inexpensive for generating high amount of purified proteins with minimal post translational modifications [9]. Purification of PeMoV is often difficult because of aggregation of virus particles, which limits in the routine production of highly specific PAb [28]. The methods of PeMoV purification described by Paguio and Kuhn [22] was not found ideal to purify PeMoV, as there was considerable loss of infectivity after initial clarification or yield was low [24]. The bacterial expressed plant viral protein has been used as a recombinant antigen for raising antibodies for immunodiagnosis of several plant viruses [5, 6, 11–13, 15, 17, 18, 20, 21, 30]. However, recombinant antigen of PeMoV has not been utilized for immunodiagnosis. An Indian isolate of PeMoV is first characterized in the current study based on host reactions, CP gene sequence and expression of core CP gene in *E. coli*. The PAb produced using the recombinant core CP was successfully utilized for immunodiagnosis of PeMoV in the field and greenhouse samples of peanut.

Materials and methods

Virus isolate and sap inoculation

An isolate of PeMoV (Gn-Hyd-1) was obtained from peanut leaves collected from a field near Hyderabad, Andhra Pradesh in 2010. The virus isolate was maintained on peanut through sap inoculation in the greenhouse. In order to study the host reaction of the virus isolate, sap inoculation was conducted to various plant species.

Molecular cloning of CP gene

A RNeasy plant mini kit (Qiagen, Chatsworth, CA, USA) was used to isolate total RNA from 100 mg of infected peanut leaves. A pair of primers BM463F tcaggtgawaayaa-gagtaaa and BM564R tacatttgacgcatacctaacaga, were designed based on the CP gene sequence of PeMoV in the database. The RT-PCR was conducted in two steps: The first-strand cDNA was synthesized in 20 µl mixture containing 14 µl 5 × First-Strand buffer, 1 µl 10 mM of dNTP mix, 1 µl 20 mM DTT, 2 µl 10 µM of each forward and reverse primer, 1 µl (100 units/µl) SMARTScribe™ III reverse transcriptase enzyme (Clontech, CA, USA), 10 µl RNA template (400–500 ng) and the final volume was adjusted with nuclease free water. The mixture was subjected to 42 °C for 90 min followed by inactivation at 70 °C for 15 min using a thermal cycler (Biometra T Personal, Germany). PCR was conducted in 50 µl reaction mixture containing 2 µl cDNA, 5 µl 10× Ex Taq PCR buffer, 4 µl 2.5 mM dNTPs, 2 µl 10 µM of each primer, 1.25 unit of Ex Taq DNA polymerase (Takara Bio. Inc., Japan) and nuclease free water

to make up the volume. The temperature cycles consisted of 1 cycle at 94 °C for 3 min, 30 cycles at 94 °C for 45 s, 54 °C for 45 s, and 72 °C for 1 min and final extension at 72 °C for 10 min. The amplified DNA fragment was cloned in T&A vector (RBC, New Taipei city, Taiwan) and sequenced. The sequence was compared with the other isolates of PeMoV and other potyviruses. Sequence identity was analysed using BioEdit software (<http://www.mbio.ncsu.edu/BioEdit/BioEdit.html>). For comparison of the isolates color graph was constructed based on the pair wise percent sequence identity of the CP using Microsoft Excel (version 2007). The Phylogenetic relationship was analyzed using MEGA5 software (www.megasoftware.net/mega5). Epitope analysis on the CP gene sequence was carried out by using IEDB software (http://tools.immuneepitope.org/tools/bcell/iedb_input).

Preparation of expression construct

The core CP was amplified using a primer pair, BM211F *gaatccatgagaatgccactggtggc* and BM212R *gtcgaccaaatgtcttaaatagtcacgttat*, cloned in T&A vector and sequenced. The core CP gene clone was restricted with *Bam*HI and *Sal*I and recloned in pET28a(+) expression vector (Novagen, Darmstadt, Germany), which was used to transform *E. coli* BL21(DE3) strain. The transformed colonies were screened by colony PCR with gene specific primers and restriction digestion with site-specific enzymes, which were finally confirmed by sequencing.

Expression and purification of core CP

The overnight grown culture containing the construct of core CP of PeMoV was grown in 5 ml of Luria broth at 37 °C and induced for expression by adding 0.5 mM IPTG for 1–4 h with vigorous shaking at 200 rpm. The bacterial cells were collected by centrifuging at 10,000×g at 4 °C for 10 min and resuspended in 20 µl 1× SDS-PAGE sample buffer. Protein was analysed by polyacrylamide gel electrophoresis in 12 % resolving gel and 5 % stacking gel using discontinuous Tris-Glycin SDS-PAGE. After electrophoresis, the gel was stained with 0.25 % Coomassie brilliant blue 250R.

Purification of the recombinant protein was carried out using 1,000 ml of induced culture using 0.5 mM IPTG for 4 h at 37 °C at 200 rpm. Cells were harvested by centrifugation at 10,000×g for 10 min and samples were sonicated (Misonix sonicators, Newtown, CT, USA) at power level between 2 and 3, at 20–30 % duty for 12 bursts. Sonication was repeated twice to release more trapped protein. Both the soluble and insoluble fraction were analysed by SDS-PAGE and insoluble fraction was dissolved in 6 M Urea. The protein was further purified using Ni-NTA column chromatography (Novagen, Darmstadt,

Germany) as per manufacturer's protocol. The purified protein was dialyzed using dialysis membrane (Small Wonder Lyser, Punjab) to remove the salt impurities with continuous stirring for 6 h in PBS (pH 8.0) then examined in 12 % SDS-PAGE.

Production of polyclonal antibodies (PAb)

The purified recombinant core CP of PeMoV (300 µg) was emulsified with an equal volume of Freund's complete adjuvant and injected intramuscularly into New Zealand white rabbit. Two weeks after, two booster injections were administered with 200 µg of protein at 10 days interval. Three more booster injections were continued with 100 µg of protein at 10 days interval. The antiserum was isolated from rabbit blood and stored at -80°C mixing with glycerol (1:1, v/v). The reactivity of antiserum was determined by Western blot. The expressed PeMoV protein was resolved in SDS-PAGE and electro blotted on to nitrocellulose membrane using semi dry blotting apparatus (Atto, Taito-ku, Tokyo) for 45 min at 96 mA at room temperature. The membrane was blocked with 5 % non-fat powdered milk overnight at 4°C and then incubated with antiserum (1:5,000 dilutions) for 1 h at 37°C . After washing, the membrane was incubated with 1:20,000 dilution of goat anti-rabbit IgG alkaline phosphates conjugate (Sigma Aldrich, St. Louis, USA) at 37°C for 1 h. The band of interest was visualized after adding BCIP/NBT (Genei, Bangalore). The titer and cross reactivity of antiserum was examined by direct antigen coated ELISA [19].

Evaluation of PAb by ELISA

To determine titer, different dilutions of antiserum (1:2,000, 1:5,000, 1:10,000, 1:20,000) were tested with both healthy and infected peanut plant sap at a dilution of 1:2 (w:v). The plates were coated with leaf extracts in coating buffer containing 2 % Polyvinylpyrrolidone (PVP) and incubated overnight at 4°C . After three washings with PBS-T (1 × PBS buffer and 0.05 % Tween-20), the wells were blocked with 0.5 % bovine serum albumin solution in PBS-T at room temperature for 1 h. After one gentle washing with PBS-T, antiserum diluted with sample buffer (PBS-T with 0.2 % PVP and 0.02 % ovalbumin) was added to the plates and incubated at 37°C for 1 h followed by three washings with PBS-T. Goat anti-rabbit IgG-AP conjugate (1:20,000) was added and incubated for 1 h at 37°C . Finally, the plates were washed three times with PBS-T and *p*-nitrophenyl phosphate substrate (0.5 mg/ml) was added. ELISA was carried out in three replicates of each 100 µl of test sample in each well. The absorbance was measured by the ELX 800 Universal Microplate Reader (BIO-TEK Instruments Inc., Winooski, VT, USA) at OD 405 nm after 1 h of adding substrate.

To determine optimum amount of antigen in crude plant sap, different dilutions of sap 1:2, 1:5, 1:10, 1:20 were tested with fixed antiserum dilution of 1:2,000. Cross reactivity of PeMoV antiserum with the other potyviruses peanut stripe virus (PStV), papaya ringspot virus (PRSV), onion yellow dwarf virus (OYDV) and potato virus Y (PVY) was examined by ELISA. For this, PeMoV antiserum (1:2,000) was used to detect the respective viruses in extract (1:5 dilution) prepared from 200 mg of leaf tissues infected by the respective potyviruses.

Detection in peanut field samples by ELISA

Field samples of peanuts originating from different places near Hyderabad (Table 1) were collected during 2011–12 and tested for the incidence of PeMoV by ELISA as described in the previous sections using 1:5,000 dilution of PAb with 1:5 dilution of sap.

Results

Characterization of the PeMoV isolate

The PeMoV Gn-Hyd-1 isolate induced dark and light green patches on the newly developed leaves (Fig. 1a). Sap inoculation in three independent batches of peanut plants cv. JL-24 resulted in average rate of transmission of the isolate as 46.43 % at 9–12 days post inoculation (dpi) as judged by ELISA (Table 1). French bean cv. Top Crop developed only small dark brown spots (Fig. 1b) on the inoculated leaves by 4–5 dpi in all the inoculated plants but it did not develop any systemic symptoms. The cowpea plants produced neither local nor systemic symptoms and were tested negative by ELISA. RT-PCR from symptomatic peanut leaf resulted in amplification of 913 bp band (Fig. 1c). Molecular cloning and sequencing (JX088125) confirmed that the amplified product contained full length of CP gene of PeMoV, which showed the closest sequence identity of 99.6 % with another peanut isolate reported from Israel (DQ868539). The amino acid sequence comparison with the Israel isolate showed a single amino acid change in the residue 3. The sequence identities among the nine PeMoV isolates, PStV and PVY was summarized as colored graph based on CP (Fig. 1d). The present isolate, however showed 93.5–97.8 % sequence identity with the other PeMoV isolates reported from pea in the USA and shared 59.3 % sequence identity with Peanut strip virus, another peanut infecting potyvirus. Phylogenetic analysis showed that the present isolate was distinct from pea isolates from USA and it clustered with the peanut isolate from Israel (Fig. 1e).

Table 1 Detection of peanut mottle virus in peanut field and greenhouse samples of peanut by ELISA using antiserum to core capsid protein during 2011–2012

Samples/source ^a	Period of collection	No of samples tested	No of positive samples ^b	Percentage of incidence	Positive sample OD range at 405 nm
Experimental fields, CRIDA	October, 2011	62	4	6.45	0.276–0.369
Experimental fields, CRIDA	Jan–Feb, 2012	240	0	0	0
Seed multiplication field-1, ICRISAT	December, 2011	175	46	26.3	0.242–1.239
Seed multiplication field-2, ICRISAT	December, 2011	30	2	6.7	0.381–0.540
Seed multiplication field-3, ICRISAT	Jan, 2012	90	2	2.2	0.451–0.517
Seed multiplication field-4, ICRISAT	Feb, 2012	162	3	1.85	0.254–0.698
Exotic accession from Senegal, greenhouse, ICRISAT	April, 2012	223	2	0.9	0.419–0.665
Sap transmission of PeMoV isolate (Gn-Hyd-1) to peanut cv. JL-24	Jan, 2012	65	42	64.6	0.267–1.425
	March, 2012	90	11	12.2	0.860–2.120
	May–June, 2012	32	20	62.5	0.440–0.615

^a CRIDA Central Research Institute for Dryland Agriculture, ICRISAT International Crops Research Institute for the Semi-Arid Tropics

^b A reaction was considered positive if sample was twice or more than twice of healthy control value

Antiserum dilution 1:5,000, Sap dilution 1:2, O.D. was recorded 1 h after adding substrate. Average healthy O.D. 0.052–0.103

Fig. 1 Peanut mottle virus (PeMoV) Gn-Hyd-1 isolate showing dark green patches on **a** peanut and local necrotic spots on **b** French bean **c** RT-PCR amplification of the full length coat protein (CP) gene from symptomatic peanut leaf (Lane 1) **d** Sequence identity matrix of CP **e** Phylogenetic relationships of Gn-Hyd-1 isolate with the other

Expression and purification of PeMoV core CP

Epitope analysis of the complete CP gene of PeMoV showed that epitopes were located within 65–246 amino acid positions. The core CP coding region of 543 nt that contained six of the seven predicted epitopes were selected

PeMoV isolates and potyviruses, peanut strip virus (PStV) and potato virus Y (PVY) based on amino acid sequence of CP. The neighbour-joining tree was constructed in MEGA 5 programme. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches

for expression. The sequence analysis of the expression construct showed that the core CP gene was inserted in frame with his-tag at both the ends. The preliminary SDS-PAGE analysis showed that the protein of about 22 kDa was present in pellet (insoluble fraction). The protein expression was observed in *E. coli* at 1 h post induction. The level of

Fig. 2 Expression of core coat protein (CP) of peanut mottle virus in *E. coli*. **a** SDS-PAGE gel showing the protein in soluble (SL) and insoluble fractions (IN) at 1, 2.5 and 4 h post induction with 0.5 mM IPTG. **b** Western blot of the samples in figure a using PeMoV antisera

(1:5,000 dilution), Lane UN: Total protein from uninduced cell culture. **c** 12 % SDS-PAGE showing Ni-NTA affinity purified recombinant core CP. E1 & E2: first & second elution fractions, Lane M: Pre-stained protein marker (Fermentas)

expression was increased with the increase of induction time and at 4 h post induction, there was higher level of expression (Fig. 2). Purification resulted in elimination of other major protein as judged by SDS-PAGE. About 1.75 mg protein was purified from a liter of induced culture.

Properties of PAb

The PAb to PeMoV could recognize 22 kDa *E. coli* expressed protein as demonstrated in Western blot (Fig. 2b). The PAb showed high ELISA readings (>2.0 O.D.) with the expressed protein (5 µg/ml) at 1:5,000 dilution. The titer of PAb however, with 1:2 dilution of crude plant sap of peanut leaf showed as < 1:20,000 (Fig. 3a). At 1:2,000 dilution of antiserum, the detection level in 1:2 dilution of sap was appreciable (~S/H ratio 5) (Fig. 3b). When different dilutions of plant sap (1:2, 1:5, 1:10 and 1:20) were used to determine the optimum sap dilution, the healthy plant sap showed no significant difference in ELISA readings (0.2–0.3) with the increase of dilution of sap. Whereas, at 1:2 dilution of diseased sap, the ELISA reading was 1.65, which was 4.5 times higher than the corresponding healthy reading. With the increase of diseased sap dilution, the ELISA reading declined and at 1:20 dilution of sap the reading was equal to that of healthy control (Fig. 3b). The PeMoV antiserum showed low ELISA readings (0.3–0.45) with PSTv, PRSV, OYDV and PVY, whereas all four viruses PSTv, PRSV, OYDV, PVY showed higher ELISA readings of 1.328, 1.494, 0.924, 0.918 respectively with their respective homologous PAb (Fig. 4).

Detection of PeMoV in peanut samples

During 2011–2012, a total of 1,169 peanut samples from field and greenhouse were tested to validate the PeMoV antiserum by ELISA (Table 1). These included leaf

samples from experimental fields of Central Research Institute for Dry land Agriculture (CRIDA), Hyderabad (302 samples), seed multiplication fields at ICRISAT (457 samples) and exotic peanut plants from Senegal grown in the glasshouse of ICRISAT (223 samples). The ELISA results revealed that the incidence of PeMoV in the seed multiplication fields, ICRISAT varied from field to field and ranging between 1.85 to 26.3 % during 2011–2012. In the experimental field of CRIDA, Hyderabad 6.45 % incidence of PeMoV was detected during Kharif season of 2011 in 90 days old crop of peanut cv Narayani at post sowing whereas, no incidence of PeMoV was detected during Rabi season in 2012 in 68 and 120 days old crop of cv Narayani, grown at CRIDA. Overall, the detection of PeMoV varied from 0 to 26.3 % in the field samples. When the exotic peanut accessions from Senegal grown in the greenhouse were tested, PeMoV was detected in only two samples (EC733860 and EC733891) out of 78 accessions.

Discussion

PeMoV spreads to new geographical location primarily through infected seeds [7]. In India, PeMoV was not known until 1977 and probably, the virus was introduced to India through infected seeds during germplasm import from USA [25]. PeMoV isolate from India was previously characterized based on host range, transmission, serology, particle morphology and physico-chemical properties [24–26] and revealed that the Indian isolate was similar to those reported from USA [14], Africa [4] and Israel [29]. The present study in CP gene sequence analysis revealed that the Indian isolate was near identical to a peanut isolate reported from Israel [29], whereas it was different from the USA isolates reported from pea (Fig. 1d).

Fig. 3 DAC ELISA using **a** different dilutions of peanut mottle virus antiserum with the infected peanut plant sap at dilution of 1:2. **b** Different dilutions of peanut leaf sap with PeMoV antiserum at dilution of 1:2,000. OD at 405 nm was recorded 1 h after adding

substrate, *p*-nitrophenyl phosphate. S/H ratio represents the absorbance value of sample OD₄₀₅/Healthy OD₄₀₅ measured 1 h after adding substrate

Fig. 4 DAC ELISA showing cross reactivity of different potyviruses with the PeMoV antiserum at a dilution of 1:2,000 (1:2 sap dilution). OD at 405 nm was recorded 1 h after adding substrate, *p*-nitrophenyl phosphate. *PSiV* Peanut stripe virus, *PRSV* Papaya ringspot virus, *OYDV* Onion yellow dwarf virus, *PVY* Potato virus Y. Reactivity of the respective virus with their homologous antisera was shown

Under field conditions PeMoV is often overlooked due to lack of prominent symptoms. Serological assays have been widely used for the detection of occurrence of PeMoV in field as well as in seeds [3, 24]. The antiserum to PeMoV was previously produced against the virus purified from infected plant tissues. Purification of plant virus involves cumbersome process such as maintenance of virus isolate, propagation to a large number of host plant and isolation of virus particle to a reasonable level of purity from the infected plant tissues. These constraints in preparation of PeMoV antigen were overcome in the present study by successfully over-expressing core capsid protein of PeMoV in *E. coli* as immunogenic antigen. The construct is potential for scale up preparation of PeMoV antigen for renewable production of PAb. The core CP gene was stably overexpressed in *E. coli*. The major expressed protein appeared in the insoluble fraction, which could be

successfully purified. Similar results of expression of recombinant His-tagged CP in the insoluble fraction have been reported for several plant viruses, pelargonium zonate spot virus [27], faba bean necrotic yellows virus [15], sugarcane yellow leaf virus [13], potato mop-top virus [5, 6] and groundnut bud necrosis virus [12]. However, for some plant viruses such as sugarcane streak mosaic virus, cucurbit yellow stunting disorder virus, cymbidium mosaic virus, the His-tagged viral CP was expressed in soluble fraction [10, 11, 16, 23]. The PAb against core CP successfully detected the expressed protein as well as the virus in infected peanut leaf samples. The peanut leaf sap at 1:2 dilution and antiserum at 1:2,000–1:5,000 dilution were suitable parameters for highly satisfactory detection of the virus in field samples as it showed about 4–6 S/H ratio. The PAb to the core CP showed no cross reactivity to PSiV, another peanut infecting potyvirus [8]. Therefore, the PAb

generated against the core CP is useful for specific detection of PeMoV in the field samples of peanut.

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