Expression and Production of Polyclonal Antibodies against Recombinant Coat Protein of Peanut bud necrosis virus

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Abstract

In vitro gene expression strategy was used for the production of polyclonal antiserum to the coat protein (CP) of Peanut bud necrosis virus (PBNV). The GBNV CP gene from peanut isolate was cloned into pQE-30UA expression vector and transformed into Escherichia coli (M15) cells. Expression of the CP gene of GBNV was induced in vitro and recombinant protein (~34 KDa) was purified and used for immunization of rabbits to produce the GBNV-specific polyclonal antiserum. The antiserum had a titre of 1:5000 in an indirect Enzyme Linked Immunosorbent Assay (ELISA) and reacted specifically in Western blot. The resulting antiserum was used to develop an Immunocapture Reverse Transcription-Polymerase Chain Reaction (IC-RT-PCR) assay and compared its sensitivity levels with ELISA for detection of GBNV isolates. The recombinant antiserum successfully detected natural infection of GBNV in economically important crops and weed hosts from South India.

Keywords: ELISA; IC-RT-PCR; Peanut bud necrosis virus; Polyclonal antiserum; Western blotting

Introduction

Peanut bud necrosis virus (PBNV) is one of the re-emerging viral diseases on several economically important crops such as peanut, tomato, chilli, potato, sunflower, black gram, green gram, cowpea, soybean, jute, taro, cotton, carrot, onion, etc., in India. Groundnut bud necrosis virus (GBNV) also called Peanut bud necrosis virus (PBNV) is the type member of the genus Tospovirus (family Bunyaviridae) [1]. The bud necrosis disease was distributed in South and South-East Asia [2], and its cause was first identified as Tomato spotted wilt virus (TSWV) in India [3]. After characterization, the virus was identified as distinct Tospovirus and named as Groundnut bud necrosis virus (GBNV), which is placed in serogroup IV [1,4]. The virus particle is enveloped, quasi-spherical of 80–120 nm diameter and has a tripartite, single-stranded, ambisense RNA genome. The RNAs are designated L (large), M (medium) and S (small) and have a size of calculated 8.9, 4.8 and 2.9 kb, respectively. They are bounded by nucleocapsid (N) protein [5]. The L-RNA codes for the RNA-dependent RNA polymerase and is translated from the viral complementary sense RNA (vc). The m-RNA encodes a non-structural (NSm) protein in the viral (v) sense and the precursor for the glycoprotein’s G1 and G2 in the VC sense. The S-RNA encodes NSs protein in the V sense and N protein in the VC sense.

The bud necrosis disease on groundnut under field conditions recorded yield losses up to 50 percent depending upon plant growth at the time of infection [6]. The GBNV incidence is highly variable from field to field in a season and year to year, ranging from <1% to >50% [7]. The GBNV cause an estimated annual loss of US$89 million in Asia [8] and 70-90% of groundnut in India [9]. The out -breaks of bud necrosis disease caused by GBNV in tomato result yield loss up to 100% in India [10]. The GBNV is transmitted by thrips (Thrips palmi) in a persistent manner [11]. In recent times, thrips and tospoviruses have become a serious problem in various Leguminosae, Solanaceae and Cucurbitaceae crops. The bud necrosis disease is characterized by mosaic and chlorotic spots on leaves, severe necrosis of buds and petioles and affected plants are stunted. Double Antibody Sandwich (DAS)-ELISA using polyclonal antibodies to the whole virion was used for detection of TSWV isolates in plant extracts [12]. Immunocapture PCR for the detection of GBNV was reliable for the amplification of target virus sequences from peanut and other crops [13,14]. The RT-PCR was developed to detect several tospovirus species, and genome reassortants were analysed to unveil the genetic factors responsible for symptoms of Watermelon silver mottle virus (WSMoV) [15]. Degenerative primers designed from conserved region of CP gene sequence from GBNV and Watermelon bud necrosis virus (WBNV) are used in diagnosis of GBNV and WBNV isolates in India [16]. The print capture RT-PCR was developed for detection of potato stem necrosis disease caused by GBNV [17]. The genetic diversity of Peanut bud necrosis virus infecting different economically important crops in South India detected by RT-PCR method [18].

The main objective of this study was to evaluate the possibility of utilizing a recombinant GBNV-CP for the production of antiserum and its diagnosis.

Materials and Methods

Virus isolates and maintenance

The GBNV infected peanut plants were collected from different places in the Indian states of Andhra Pradesh, Tamil Nadu and Karnataka. In naturally infected GBNV groundnut samples, chlorotic and necrotic ring spots on leaves and necrosis of stem and bud in peanut were observed. The bud necrosis virus infected samples were sap inoculated on cowpea (cv-C-152, a diagnostic assay host) plants using chilled 0.05 M phosphate buffer (pH 7.0) containing 0.1% 2-mercaptoethanol.

Isolation of total RNA and Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from 100 mg of healthy and GBNV infected peanut plants and reverse transcribed using M-MLV reverse transcriptase (Promega). The resulting cDNA was used as a template for PCR using degenerative primers designed from conserved region of CP gene sequences from GBNV and WBNV for detection of tospovirus species in India [19].
infected groundnut (GBNV-GN) leaf samples using RNeasy Plant MiniKit according to the manufacturer’s instructions (Qiagen, USA). The resulting total RNA was incubated with GBNV-C-P gene-specific reverse primer at 65°C for 5 min and snap-chilled on ice for 2 min. Complementary DNA (cDNA) was synthesized using *Moloney Marine Leukemia Virus* (M-MuLV) reverse transcriptase (Fermentas, Canada) at 42°C for 1 h. The CP genome forward primer, 5’-ATGTCTAACGT(C/T)AAGCA(A/G)CTC-3’, and reverse primer, 5’-TTACAATTCCAGCGAAGGACC-3’, were used to amplify the complete N gene of GBNV [4]. Two µl of cDNA was amplified in a 25 µl reaction volume containing 2.5 U of Taq DNA Polymerase (Fermentas, USA), 10 pmol of GBNV-C-P forward and reverse primer, 2.5 mM Magnesium Chloride (MgCl2) and 10 mM each deoxynucleotide triphosphates (dNTP’s). PCR amplification conditions included an initial denaturation cycle of 5 min at 94°C, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 1 min at 56°C and extension for 1 min at 72°C with final extension for 2 min at 72°C. Amplified products (800 bp) were resolved following electrophoresis through 1% agarose gel containing ethidium bromide (10 mg/ml).

**Cloning and transformation**

The PCR product (800 bp) was eluted by QIAquick gel extraction, cloned in to vector pTZ57R/T (Fermentas, Canada) and sub-cloned into 6x His tagged pQE30 UA expression vector (Qiagen, USA) according to manufacturer’s instructions. Recombinant clones were used to transform *E. coli* DH5α cells containing pREP4 repressor plasmid by following standard molecular biology protocols [19]. The recombinant clones were maintained on Luria Agar (Genei, Bangalore) according to the standard molecular biology protocols [19]. The CP gene of GBNV was PCR amplified using specific primers. In a 25 µl PCR vials (200 µl) were loaded initially PCR vials (200 µl) were loaded with 50 µl of coating buffer having homologous antiserum (polyclonal antisera) to CP at 1:500 dilutions (v/v). Then the tubes were incubated overnight at 4°C. After incubation the antibody coated PCR vials were washed four times with PBS-T and the vials were loaded with crude leaf extracts prepared in extraction buffer (PBS-T containing 2% PVP) ranging from 1:50, 1:100, 1:500, 1:1000, 1:5000, 1:7000 and 1:10000 dilutions (v/v) (Table 1) and then incubated for overnight at 4°C. After incubation, the vials were washed thrice with PBS-T and once with PBS. Similar to RT-PCR, first strand cDNA synthesis was carried involving GBNV CP-R primer and Mu-MLV RT (Fermentas, USA) following the manufacturer’s protocol. The prepared cDNA was then amplified using the GBNV-C-P primers by taking 2 µl of cDNA. The CP gene of GBNV was PCR amplified using specific primers. In the same manner, the sensitivity of IC-RT-PCR at various dilutions of antibodies (1: 50, 1: 100, 1:500, 1: 1000, 1: 5000, 1:7000 and 1: 10000) was also evaluated (Table 2).

**Expression of CP gene and preparation of CP fractions**

To produce the recombinant CP of GBNV, 5 ml of overnight culture of M15 pREP4 cells containing plasmid with GBNV-C-P/pQE-30 construct was added to 50 ml of LB (Luria-Bertani) broth and the culture was grown to an optimal density at 600 nm (OD600) of 0.6 and then incubated overnight at 4°C. After incubation the antibody coated PCR vials were washed thrice with PBS-T and the vials were loaded with crude leaf extracts prepared in extraction buffer (PBS-T containing 2% PVP) ranging from 1:50, 1:100, 1:500, 1:1000, 1:5000, 1:7000 and 1:10000 dilutions (v/v) (Table 1) and then incubated for overnight at 4°C. After incubation, the vials were washed thrice with PBS-T and once with PBS. Similar to RT-PCR, first strand cDNA synthesis was carried involving GBNV CP-R primer and Mu-MLV RT (Fermentas, USA) following the manufacturer’s protocol. The prepared cDNA was then amplified using the GBNV-C-P primers by taking 2 µl of cDNA. The CP gene of GBNV was PCR amplified using specific primers. In the same manner, the sensitivity of IC-RT-PCR at various dilutions of antibodies (1: 50, 1: 100, 1:500, 1: 1000, 1: 5000, 1:7000 and 1: 10000) was also evaluated (Table 2).

**Results**

**Virus isolates and maintenance**

The GBNV symptoms were first appear in the young leaflets as mosaic, chlorotic spots and mild mottle, which later develop into chlorotic and necrotic rings, streaks and extend to petioles to terminal buds. Infection at a young age results in the death of the plant due to severe necrosis. The bud necrosis disease samples were
Production of polyclonal antiserum to a recombinant GBNV-CP

In antisera production, the purified product was used from the expression of recombinant vector resulting from the gateway cloning with 6x His tag in E. coli M15. Used both inclusion bodies and recombinant CP from inclusion bodies, solubilised by 8 M urea, purified on sucrose cushion. Antiserum was obtained from bleeds taken 3 weeks after the fourth injection.

ELISA test

The polyclonal antiserum from the antiserum produced against recombinant GBNV-CP was used in DAC-ELISA. The anti-GBNV-CP sera had titres of 1:5000 when tested by indirect ELISA (Table: 2). The resulting antiserum was used to detect GBNV infecting different crops (Peanut, Tomato, Chilli, Black gram, Jute, Calotropis, Taro, Onion, Cotton, Sunflower, etc). The GBNV-GN leaf antigens were tested against 1:1000 dilutions in indirect ELISA (Table: 1).

Expression and purification of GBNV-CP

The GBNV CP gene of peanut Tirupati isolate was used for further experiments. The GBNV-CP/pQE-30 construct was used to transform E. coli (M15) cells. After initial analysis of rCP expression by SDS-PAGE, the expression levels were again checked for the feasibility of overexpression of rCP in E. coli system at different intervals of time, a mini volume (10 ml) expression was carried out for the feasibility of its purification by Ni-NTA columns. After that the cells were harvested and the presence of GBNV coat protein band on Coomassie brilliant blue stained in SDS-PAGE with an approximate molecular mass of 34 kDa (Figure 2). The fusion protein was confirmed using polyclonal antiserum to GBNV-CP in Western blotting as described by O’Donell et al. [22]. The non-induced clones produced much lower but visible amount of this protein, while in non-transformed E. coli no such protein was detected. The most of recombinant GBNV-CP was found in the insoluble cytoplasmic fraction, it was proved by comparison of all fractions by Western blot analysis.

Table 2: Titration of antiserum from different bleeds against healthy and PBNV-GN leaf antigens by DAC-ELISA. *A405 OD values are average values of duplicate wells.

| Antibody Dilutions (v/v) | Antibody dilutions | | | | |
|--------------------------|--------------------|---|---|---|---|
| 1:50 | 0.10 | 0.70 | 2.20 | 2.33 | 2.19 | 2.18 |
| 1:100 | 0.10 | 0.37 | 2.60 | 2.02 | 2.00 | 2.00 |
| 1:500 | 0.08 | 0.33 | 2.44 | 2.54 | 2.23 | 2.20 |
| 1:1000 | 0.08 | 0.25 | 2.30 | 2.50 | 2.19 | 2.10 |
| 1:5000 | 0.07 | 0.15 | 2.00 | 2.58 | 2.74 | 2.35 |
| 1:7000 | 0.07 | 0.10 | 0.95 | 2.34 | 2.40 | 1.98 |
| 1:10000 | 0.07 | 0.10 | 0.86 | 1.10 | 1.0 | 0.98 |

Table 1: Detection of PBNV-GN leaf antigens against 1:1000 dilutions (v/v) of IV bleed antiserum by DAC-ELISA. *A405 OD values are average values of duplicate wells.

| Antigen dilutions | Antibody dilutions |
|-------------------|--------------------|
| 1:50 | 0.35 | 2.67 | 0.33 | 2.77 | 0.22 | 2.43 | 1.19 | 0.88 | 1.18 | 0.19 | 0.57 | 0.17 | 0.3 |

RT-PCR, cloning and sequencing

In RT-PCR, a single band of expected size (800 bp) (Figure 1) corresponding to the GBNV CP gene was observed when total RNA extracted from infected tissue was used. The identity of the 831 bp product was confirmed by cloning (pTZ57R/T vector) and sequencing. In RT-PCR, a single band of expected size (800 bp) (Figure 1) corresponding to the GBNV CP gene was observed when total RNA extracted from infected tissue was used. The identity of the 831 bp product was confirmed by cloning (pTZ57R/T vector) and sequencing.

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Western blot (Electro-Blot Immunoassay)

In Western blot analysis, the polyclonal antiserum strongly reacted with the recombinant GBNV-CP. Western blot revealed a strong band at position corresponding to ~34 kDa, which is the expected molar mass of GBNV-CP (Figure 3).

Purification of expressed protein

The recombinant poly-histidine-tagged expressed protein (GBNV-CP) was purified by Ni-NTA affinity column chromatography and these fractions were analysed by SDS-PAGE and Western blot analysis. The expressed recombinant protein and purified protein have approximately molecular mass of 34 kDa (Figure 4).

IC-RT-PCR

The recombinant coat protein antibody based IC-RT-PCR was successfully developed and tested for detection of GBNV isolates. The PCR product (800 bp) was amplified from all samples tested, but no such amplification was observed in extracts from healthy peanut plants (Figure 5a). At 1:500 dilution of rGBNV-CP based antiserum for capturing of virions, GBNV-CP was successfully amplified up to 1:5000 antigen dilutions (Figure 5a and 5b). Thus rGBNV-CP based polyclonal antiserum were proved to capture the virus particles even up to 1:5000 leaf antigen dilutions (v/v) as the GBNV-CP amplification by IC-RT-PCR was clearly evident upon said antibody dilution. In the same way the polyclonal antiserum produced against rGBNV-CP captured GBNV virions in crude leaf extracts up to 1:10,000 v/v antibody dilutions (Figure 6a and 6b).

Discussion

Peanut is one of the most important oil and food legume crop, grown in many countries of the world. It is an essential crop for small-hold farmers who grow peanut for food, oil, feed and confectionery purposes. The GBNV is easily sap-transmissible to the members of the Leguminosae, Solanaceae, Cucurbitaceae and Fabaceae was observed in localized and systemic infection.

The CP gene of GBNV isolates was initially targeted for the genetic characterization of GBNV associated with mosaic and necrosis disease of groundnut and other crops in India. Sequence analysis of the CP gene helps to exploit it for the production of recombinant GBNV-CP and thereby facilitates the production of rGBNV-CP based polyclonal antiserum for the sensitive detection of GBNV. The GBNV-CP specific primers was amplified ~800 bp of CP gene of GBNV-groundnut isolate. As the initial BLAST analysis of the obtained sequence clearly showed that the present virus isolate sequence closely related to GBNV isolates. The CP gene of GBNV revealed that sequenced region contained a single open reading frame (ORF) of 831 nucleotides that could potentially code for a protein of 276 amino acids. The sequence of Peanut-Tirupati isolate was deposited in the NCBI GenBank (Accession number EF179100). The complete sequence of GBNV-CP shared 99.6% identity at nucleotide and amino acid levels respectively with other reported GBNV isolates.

The recombinant DNA technology is an useful approach for high level expression of GBNV-CP in prokaryotic expression systems (like E. coli). Prokaryotic expression system is valuable for the production of good quality recombinant antigen based polyclonal antiserum and their
Further exploitation in the development of sensitive antibody-based serological and molecular diagnostics for detection of GBNV. Hence this approach was followed in the present study for production of rCP of BNV-CP based antibodies and their further use in the development of diagnostic tests for GBNV.

The conditions (IPTG conc. and temperature) were optimized for the over expression of rCP of GBNV-GN in E. coli system. The expressed rCP was purified under denaturing conditions even though the initial expressed protein was found in soluble form, because the His-tag of rCP under native conditions was noticed to be inaccessible for binding in Ni-NTA column purification. Hence the rCP of GBNV was purified under denaturing conditions using urea. The authenticity of the rCP was checked by EBLA using heterologous antibodies. The mean molecular weight of rCP was calculated as ~34 kDa by EBLA of crude peanut leaf extracts and amino acid sequence of CP gene. The urea was further removed by dialysis and the removal of urea can actually facilitate the folding of GBNV-peatnut- rCP to its near original conformation. The protein purified under denaturing conditions does not actually affect if the purpose is antibody production [19].

However, there are several reports, the polyclonal antibodies produced against the viral proteins of Tomato spotted wilt virus [23], Grapevine leaf roll-associated closterovirus-3 is detected in ELISA [24], Prunus necrotic ring spot virus antiserum was successful used to detect virus by DAS-ELISA in prunus [25]. Gulati-Sakhuja et al. [26] have also successfully used an antiserum produced against Pelargonium zonate spot virus recombinant CP for the detection of the virus by ELISA. Banana streak MY virus (BSMYV) antiserum (1:2000) was used in antigen coated plate–enzyme linked immunosorbent assay (ACP–ELISA) for the detection of virus in banana plants [27]. Alfalfa mosaic virus (AMV) polyclonal antiserum was used for the detection of virus in soybean plants in USA [28]. Our results with CP are in agreement with the results obtained for antibodies raised against recombinant Watermelon bud necrosis virus [29], Potato mop-top virus [30], Potato virus Y [31], Potato virus X [31].

In DAC-ELISA, the rCP based polyclonal antiserum produced against GBNV-peanut-Tirupati isolate successfully detected the GBNV-peanut isolates in crude leaf antigen up to 1:5000 (v/v) antibody dilutions (4th bleed) and able to detect the GBNV in groundnut leaf extracts diluted up to 1:1000 dilution v/v with 1:5000 antibody dilution.

In IC-RT-PCR, the polyclonal antiserum (1:500 dilution v/v of 4th bleed) produced against rCP captured the GBNV virions even up to 1:5000 v/v dilutions of crude leaf extracts. The produced antibodies were found to be highly specific in the detection of GBNV-GN isolates in crude leaf extracts (1:10 dilution w/v) by IC-RT-PCR as the virus was detected even at 1:10,000 dilution of 4th bleed antiserum. This shows that the produced antiserum can economically be used for detection of GBNV associated with mosaic and necrosis disease of peanut and other crops also.

The GBNV in crude leaf extracts using polyclonal antiserum produced against purified GBNV up to 1:4000 v/v antibody dilutions [29]. Similarly polyclonal antiserum was used to detect GBNV and WBNV infection in plant samples based on CP gene sequence [32-35]. GBNV infection was detected from cowpea, mungbean, soybean, tomato and urdbean [16].

The antiserum produced against GBNV- rCP was applied successfully for the sensitive detection of GBNV in both plant and vector hosts.

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