In Vitro Expression and Production of Antibody Against *Cymbidium mosaic virus* Coat Protein

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Abstract Polyclonal rabbit antisera were produced using coat protein of *Cymbidium mosaic virus* (CymMV) Indian isolate expressed in *E. coli* as GST fusion. The expressed protein was purified by GST-fusion protein purification kit for use as an immunogen in rabbits. Antisera prepared in this manner reacted in double antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA) with extract from CymMV-infected tissue. The results indicate that polyclonal antisera prepared from expressed CymMV coat proteins were useful for the detection of CymMV in an array of assays. The detection system developed is highly effective for detection of Indian strain of the virus in comparison to kits available in the international market.

Keywords CymMV · ELISA · In vitro · Expression · Orchidaceae · CP

The Orchidaceae is the largest, widely distributed and most diverse plant family, consisting of more than 800 natural genera and over 35,000 species found in almost every region of the world [3]. Most of the orchids are grown for cut flowers and they have been reported to be infected with more than 50 viruses [13]. *Cymbidium mosaic virus*

(CymMV) (Genus: *Potexvirus*, family flexiviridae) and *Odontoglossum ringspot virus* (ORSV) (Genus: *Tobamovirus*, family: virgaviridae) have been reported to be the two most prevalent and important viruses infecting orchids that have attained worldwide distribution [9, 10]. Their high incidence in cultivated orchids has been attributed to the stability and ease of transmission of these two viruses through cultural practices [7] and has attracted many concerns among the growers.

Here we show the expression and purification of a recombinant CymMV coat protein (CP) and use it to raise polyclonal antibodies. Similarly, a number of expression vectors viz. pGEX [6], pET [6], pTBG(H) [12], pT7. This [1], pProEX HTb [5] and pVEX [8] have been used for the respective gene study. *E. coli* BL 21 has been widely used for expression of gene of interest [5, 8].

Sequence of complete CP of CymMV isolated from *Phaius tankervilleae* (AJ564562) was used for the primer designing. Specific primer pair was designed containing *Bam*HI and *Eco*RI sites in upstream (5'-CGGGATCCATGGGAGAGTCCACTCCA-3') and downstream primers (5'-GGAATTCTCAGTAGGGGTCCAGGC-3'), respectively.

CymMV CP gene was amplified using above primers and cloned in pGEM-T easy vector. For PCR amplification, advantage HF 2 PCR system was used (BD Biosciences USA). The PCR reaction was performed for 30 cycles at 94 °C for 45 s, 57.5 °C for 1 min and 72 °C for 1 min with final extension at 72 °C for 10 min after 5 min initial denaturation at 94 °C. Amplified fragment was separated on 1.0 % agarose gel, specific band was excised from the gel and eluted using gel extraction kit (Sigma). The *Bam*HI and *Eco*RI-digested PCR product and pGEX-2TK were ligated and transformed in *E. coli* BL21 (DE3). After transformation, recombinant clone screening and sequencing (to ensure inframe cloning) one clone was selected and used for expression.

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The selected colony was inoculated in 5 ml of LB supplemented with ampicillin and grown overnight at 37 °C at 200 rpm in an incubator shaker. Yeast trypton (YT) broth (250 ml, supplemented with ampicillin) was then inoculated with 1 ml of overnight grown culture and kept for incubation at 37 °C at 200 rpm till the OD₆₀₀ reaches 0.5. After that, 500 µl of IPTG (100 mM) was added to the culture medium and incubated in a shaker. Culture (5 ml) was pelleted after different incubation time intervals (1, 2, 3 and 4 h) and the pellet was kept for checking of expression level. Different IPTG concentrations (ranging from 0.5 to 1.5 mM) and different temperature combinations (ranging from 20 to 37 °C) were used to standardize the concentration of IPTG and the temperature of induction for optima; expression of the CymMV CP as soluble fraction.

Bacterial pellet (above step) was suspended in 800 µl of 1× PBS and sonicated till it becomes clear. It was then centrifuged at 12,500 rpm for 10 min at 4 °C, pellet and supernatant were collected in separate tubes. Pellet was again suspended in 800 µl 1× PBS. Supernatant and resuspended pellets (5–10 µl) from each of the tubes were run on 12 % SDS-PAGE and silver stained.

After standardization of conditions for optimum expression, separation of IgG from whole serum was carried out by Protein-A Sepharose affinity chromatography (Pierce, Belgium). CymMV specific antibodies were then separated from the antibody mixture (containing antibodies against CymMV and GST) using GST orientation kit (Pierce, Belgium) as per manufacturer's instructions. The best expression level of soluble CymMV CP was observed when the protein was expressed and sampled after 3 h of IPTG (1 mM) addition (Fig. 1a). The purified protein was analyzed on SDS-PAGE for integrity and purity (Fig. 1b). Concentration of the purified protein was determined by UV absorption and by Bradford [2] method. The expressed

protein of approximate size 25 kDa fused with GST 28 kDa was obtained in the supernatant. Purity of the purified protein was checked by electrophoresis on 12 % SDS-PAGE gel that revealed distinct band of approximate ~53 Kda as expected (Fig. 1).

The purified protein was used as antigen for immunization of rabbits and antisera were raised using procedures as described by Hampton et al. [4]. Antisera against the CymMV CP were raised in healthy New Zealand white male albino rabbits by injecting the purified *E. coli*-expressed CymMV CP (100 µg) along with complete adjuvant through subcutaneous and intramuscular routes, separately. Serum was collected after the booster injection (intravenously with incomplete adjuvant) separately and stored in aliquots at –20 °C along with 0.02 % sodium azide. Antibodies produced were used as coating antibodies in various dilutions in DAS-ELISA tests along with the conjugated antibodies procured from commercial sources to identify the titer of the antibodies. Antibodies reacted specifically in DAS-ELISA with known positive and negative samples and they were also titrated. IgG from whole serum was separated by Protein-A Sepharose affinity chromatography and by ammonium sulphate precipitation. Concentration of IgG was determined to be 12 mg/ml. The antibodies were diluted to final concentration of 1 mg/ml IgG with 1× PBS buffer. IgG specific for CymMV was separated from the mixture of antibodies (containing antibodies against CymMV and GST) by GST orientation kit (Pierce, Belgium) as per manufacturer's instructions. Concentration of IgG was again determined and finally the antibodies were diluted to the concentration of 1 mg/ml IgG and stored at 4 °C after adding sodium azide to a concentration of 0.02 % (w/v).

Separated CymMV antibodies 2 ml (1 mg/ml concentration) were conjugated with alkaline phosphatase (2 mg).

Fig. 1 Gel photographs of expression of CymMV CP. **a** SDS PAGE (12 %) of CymMV coat protein gene expressed in pGEX-2TK and transformed in *E. coli* BL 21 as GST fusion protein induced with IPTG. Lane M Broad range protein markers (Bio-Rad, USA), lane 1–4 induced fusion protein with different IPTG concentrations (0.2, 0.5,

1.0 and 1.5 mM conc. of IPTG respectively) and lane 5 control without induction arrow showing fusion protein. **b** SDS-PAGE of expressed and purified fusion protein (Lane M Broad range protein marker, lane 1–2 fusion protein eluted through affinity chromatography column, lane 3 induced crude supernatant)

Activity of conjugate was checked by DAS-ELISA using antibodies produced in rabbit as coating antibodies at optimum dilution. It reacted well with the known positive sample up to the dilution of 1:1000, whereas negative in the negative control samples.

Antibodies were tagged with alkaline phosphatase using standard protocol [4]. Specificity and performance of antibodies and enzyme conjugated antibodies was checked by standard DAS-ELISA using known positive and negative samples. For reference and serological testing, antibodies for CymMV were procured from commercial sources (Agdia, Elkhart, USA).

To check the reliability of antibodies, leaves from different orchid cultivars were checked by DAS-ELISA using the purified antibodies and enzyme conjugate. Results were also compared with those obtained using standard kit. To check stability of the IgG-AP conjugate, DAS-ELISA was conducted using known positive and negative samples during 2004–2005. A part of antibodies and conjugate was kept at room temperature whereas remaining was stored in refrigerator (4 °C). They were used for the studies later on. Results were found positive every time in first part, whereas using other part, results were positive only up to 2 months. This indicates that activity of the kit remains as such for 2 months and whole year from 2004 to 2005 (as mentioned above) at room temperature and in refrigerator, respectively.

To check efficacy of the kit, different orchid samples (leaves) were analysed for presence or absence of CymMV using DAS-ELISA as earlier described. At the same time they were also checked by standard commercial kit (Adgia Elkhart, IN) as shown (Fig. 2) as per the manufacturer's instructions.

It was found that in some cases such as *C. tracyanum* and *P. tankervilleae*, the test kit works better than the compared commercial kit, that is test kit gives very strong positive signal in DAS-ELISA for some cultivars infected with CymMV while the standard kit gave somewhat lesser values (Fig. 2). Thus kit prepared in the present study was sensitive for the detection of Indian strain of CymMV as standard commercial kit. It was observed that the test kit works well and gave optimum results when kept at 4 °C for one year. Keeping the kit reagents at room temperature for longer period degrades their quality and often gave false results. At room temperature, the kit components were stable for two months. Therefore, it was recommended to keep the reagents in a refrigerator with temperature of 4 °C.

In the present study, two routes of subcutaneous and intramuscular immunizations were used. Injections containing 100 µg purified coat protein was administered at weekly intervals. First four injections were given with Freund's complete adjuvant while the final injection was

given intravenously with Freund's incomplete adjuvant. The reactivity of antibodies raised in present study was evaluated by DAS-ELISA and it revealed high titer (1:1000) raised through intramuscular in comparison to subcutaneously raised antiserum (1:200–500). In the present study, IgG was separated from whole serum by ammonium sulfate precipitation and by affinity chromatography using NAb antibody purification kit. It contains protein A bound to Sepharose column, that specifically binds to Fc region of antibodies from the whole serum which was then eluted in low pH buffer. IgG specific for CymMV were separated from the mixture of antibodies (against CymMV and GST). Purified CymMV IgG were conjugated with alkaline phosphatase. The titer of conjugate was found to be 1:1000.

Using test kit and standard commercial kit for reference, field trials were conducted. Different genera and species of orchids were checked by DAS-ELISA. Kit prepared in present study was found to be as sensitive as commercially available standard kit for the detection of Indian isolates of CymMV. ELISA based kit was preferred more because it is a sensitive, cost effective, consistent (in results), easy to perform and time saving test. The reason may be that we have used Indian strain for raising antisera. The Indian CymMV isolates shares 87–97 % and 66–98 % sequence homology with the other available sequences at nucleotide level and amino acid level, respectively, and 94–99 % and 91–99 % with each other [11], some of the crucial amino acids might be different resulting in a better detection of Indian strains. The method we developed was found useful, less time consuming and economical than the routinely used methods of developing antibodies. These antibodies

Fig. 2 Sensitivity test for indigenously made and commercial antisera against CymMV. DAS-ELISA was performed to compare the detection limit of antibodies against CymMV. Infected orchid leave samples were ground in extraction buffer and was diluted into fivefold. The home made antisera were diluted into 10^5 time dilution as the commercial one and the values of OD405 of each sample were measured after 60 min incubation. *C. tigrinum* was taken as a healthy plant. A reaction was considered positive when the OD405 value was three times higher than the healthy plants

are shown to be good reagents for the detection of CymMV infection. Due to strict quarantine laws and increasing demand of virus tested clean planting material, the indigenously developed kit can found enormous use in routine diagnostic tests.

CymMV is a major virus prevalent in Indian orchids and has widely spread in orchids wherever orchids are grown on large scale [7]. The viral coat protein expressed in *E. coli* BL 21 can be used for raising antisera against the virus. The raised antisera will find various applications in different serological procedures including certification programmes.

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