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Production of Polyclonal Antibodies to a Recombinant Coat Protein of *Potato mop-top virus*

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With 2 figures

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Abstract

The coat protein (CP) coding regions of two Czech *Potato mop-top virus* (PMTV) isolates were sequenced and shown to be identical. One, the Korneta isolate CP gene, was cloned in several expression vectors. The recombinant PMTV-CP was expressed in *Escherichia coli* and the purified recombinant protein was used to produce PMTV-specific polyclonal antibodies. The antiserum had a titre of 1 : 2000 in an indirect enzyme-linked immunosorbent assay (ELISA) and reacted specifically in immunoblotting and IPTA-ELISA (indirect plate-trapped antigen (PTA)-ELISA).

Introduction

Potato mop-top virus (PMTV), the type member of the genus *Pomovirus* of fungus-transmitted soil-borne plant viruses (Torrance and Mayo, 1997), occurs in potato-growing regions of northern and central Europe and also in other parts of the world (Jones, 1988). PMTV was first described in the Czech Republic in 1983 (Novak et al., 1983). PMTV causes a wide range of symptoms in haulms and tubers, which vary depending on the potato cultivar and environmental conditions (Kurppa, 1989). This variation in symptom expression causes difficulties in the identification of the virus disease. The virus in field conditions is transmitted by motile zoospores of the plasmodiophoromycete fungus *Spongospora subterranea* (Wallr.) Lagerh. (Arif et al., 1995), which also causes powdery scab on tubers. Effective and environmentally acceptable chemical control of the fungal vector is not available, and there are no sources of resistance or tolerance to PMTV that have been deliberately used in breeding programmes.

PMTV has tubular, rigid particles, measuring 18–22 × 100–150 nm or 250–300 nm. Discrepancies in reported length are probably due to the fragility of the particles, which readily disintegrate and uncoil from one end (Kassanis et al., 1972). The capsid of the virus consists of a single type of protein subunit encoded by a distinct virus gene (Kashivazaki et al., 1995). The entire genome of PMTV consists of three positive-strand RNA molecules, RNA 1, 2 and 3 of 6.5, 3.2 and 2.4 kb, respectively (Kallender et al., 1990). The complete nucleotide sequence (6043 nucleotides) of RNA 1 was determined by Savenkov et al. (1999).

There are many difficulties involved in the diagnosis of PMTV because this virus infects tubers and haulms erratically and often occurs in concentrations below a detectable limit. Both Mills (1987) and Kurppa (1989) reported difficulties in using enzyme-linked immunosorbent assay (ELISA) for diagnosing PMTV and attributed them to the high background reaction of polyclonal antibodies and the uneven distribution of the virus in potato plants and tubers. Polyclonal antisera and a panel of monoclonal antibodies (MAbs) have been produced against the Scottish isolate PMTV-T by Torrance et al. (1993).

The data obtained by sequencing the coat protein (CP) gene of some other PMTV isolates (Reavy et al., 1997, 1998) showed that some of them seem to be highly conserved in this region. Nevertheless, there is considerable difference in virulence and significant variation among isolates in biological properties (Harrison and Jones, 1970).

The main objective of this study was to evaluate the possibility of using a recombinant PMTV-CP for antiserum production and diagnostics in comparison with commercially produced antibodies raised against PMTV.

Materials and Methods

Isolates

We used two isolates of PMTV from the Czech Republic – Pacov and Korneta, isolated by Dr Dědic at the Potato Research Institute, Havličkův Brod. Viruses were propagated in *Nicotiana debneyi* by mechanical inoculation using the sap extracted from symptomatic leaves.

Immunocapture and RT-PCR

cDNA of PMTV RNA 3 was obtained by immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR). The tubes were coated with 100 μl anti-PMTV immunoglobulin G (IgG) (1 μg/ml) (Adgen, Auchincruive, Scotland, UK) in coating buffer for 3 h at 37°C. The wells were then washed [3 × 150 μl phosphate-buffered saline (PBS) + T] and 100 μl of the homogenate of PMTV-infected leaves in conjugate buffer (1 : 10) was added. The samples were incubated overnight at 4°C and again washed three times in PBS + T. After the last wash, RT and amplification with Superscript II (Gibco, KRD Prague, Czech Republic) and *Taq* polymerase (Fermentas, St Leon-Rot, Germany) were performed as recommended by the manufacturers.

We used PMTV-specific primers MT-CP5A corresponding to nucleotides 313–333 and reverse MT-CP3A nucleotides 822–841 in sequence of RNA 3 (AJ243719). Primers also contained *Eco*RI, *Nco*I (sense) and *Xho*I (antisense) restriction sites as non-template overhangs. The reaction was carried out in 30 cycles of 30-s denaturation at 94°C, 30-s annealing at 55°C and 1-min elongation at 72°C.

Sequencing

The RT-PCR products were directly cloned to pUC57T/A (Fermentas) using 3′-A overhangs generated by *Taq* polymerase and sequenced using an ALFexpressII Sequencer with the AutoRead Sequencing Kit (AP Life Science, Uppsala, Sweden). Plasmid pUC57 was used for subcloning the insert into Gateway (Gibco) pDEST15 and pDEST17, in the first one our protein was expressed as a fusion protein with glutathione-S-transferase (GST) and in the second one in fusion with 6×His tag.

Expression of CP gene

This was performed in Luria–Bertani (LB) medium. To produce the recombinant CP of PMTV, a 5 ml of overnight culture of BL21 DE3 cells containing plasmids with appropriate constructs mentioned above was added to 50 ml of LB and the culture was grown to an optical density at 600 nm (OD_{600}) of 0.6 and then induced with IPTG (Serva, Heidelberg, Germany) in a final concentration of 0.2 mM. The culture was than incubated for 3 h or overnight at 20°C. Bacterial cells were harvested by centrifugation and stored at −80°C until required.

Preparation of CP-enriched fractions

PMTV-CP was partially purified from 125 ml of the bacterial culture (BL 21 DE3) according to the procedure described by Lin and Cheng (1991). The pellet and supernatant from these centrifugations, partially resuspended in a small volume of PBS or of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), electrophoretic buffer were subjected to further testing. The main part of fusion proteins was insoluble and was in the pellets. The pellets were further suspended in a small volume of PBS and purified by high-speed centrifugations through 30% sucrose cushion (2 h; 90 000 ***g***, 27 000 rpm in Beckman Ti 50.2 rotor, Palo Alto, CA, USA). Individual CP fractions from different steps of the purification process were subjected to Western blot analysis and triple antibody sandwitch (TAS) ELISA using Adgen anti-PMTV antibodies. The protein concentration in each fraction was assayed both spectrophotometrically ($A_{280} = 1.0$ for 1 mg/ml) and with Bradford's (1976) method.

Polyacrylamide gel electrophoresis in the presence of SDS

A 1 ml aliquot of cultured bacterial cells was pelleted by centrifugation for 1 min, resuspended in 100 μl of the Laemmli buffer, boiled for 2 min and aliquots were loaded on 12% polyacrylamide gel containing SDS (Laemmli, 1970). Coomassie Brillant Blue R250 was used to visualize the separated proteins.

Western blot analyses

For these purposes, the proteins separated by SDS-PAGE were electroblotted to a nitrocellulose membrane (0.45 μm; Sleicher & Schuell Protran, Dassel, Germany) in semidry system (OMNI-TRANS apparatus, Omnibio Brno, Czech Republic) according to Hirano and Watanabe (1990). After blotting the membrane was stained non-specifically with Ponceau S (Sigma Aldrich, St Louis, MO, USA). The membrane was then incubated for 1 h in 4% bovine serum albumin (BSA) in PBS and then washed four times in PBS. For detection of recombinant PMTV-CP, the commercial (Adgen) and our laboratory antibodies were used. The bands of interest were visualized by reaction with the substrate 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium tablets (BCIP/NBT) (Sigma) according to Sambrook et al. (1989).

Immunoblotting with anti-recombinant PMTV-CP serum showed that it contained antibodies specific for the bacterial host proteins. However, these antibodies were readily removed by cross-absorption with preparation of *Escherichia coli* cells containing the same Gateway pDEST17 vector with cloned CP from *Potato virus A* (PVA) (Moravec, 2001).

Antisera production

The antisera against the bacterially expressed PMTV-CP were prepared in mice given three subcutaneous and one intraperitoneal injections at three weekly intervals, each of 50 μg of recombinant protein or inclusion bodies purified from sucrose cushion and washed in PBS. The purified protein or inclusions were emulsified with an equal volume of Freund's complete adjuvant for the first injection and with Freund's incomplete adjuvant for the two subsequent injections; both recombinant protein and inclusion bodies were soluble in both types of adjuvant. For the last injection the antigen was diluted in PBS. The mice were bled 3 weeks after last injection. The serum fractions were collected and stored at −20°C until required.

Table 1
Detection of *Potato mop-top virus* (PMTV)-infected tissue and bacterially expressed PMTV-coat protein (CP) (BE-CP) in indirect PTA-enzyme-linked immunosorbent assay (ELISA) (IPTA-ELISA) using IgG produced against recombinant PMTV-CP

Material tested	Commercial antibodies (Adgen)	IgG against BE-CP	
		A	B
Buffer	0.20[1]	0.20	0.20
Healthy plants (*Nicotiana debneyi*) (dilution 1 : 10)	0.20	0.20	0.20
Infected leaves (*Nicotiana debneyi*) (dilution 1 : 10)	0.58	0.42	0.56
PMTV-CP (BE-CP) (10 μg/ml)	OVER	0.80	0.98

[1]A_{405} values after incubation for 30 min.
IPTA-ELISA: plates were coated with antigens diluted in standard coating buffer and incubated overnight at 4°C. The ELISA plates were washed four times with phosphate buffered saline with 0.05% Tween 20 (PBST). Commercial antibody (1 μg/ml) or anti BE-CP IgG (1 μg/ml; A = antiserum raised against purified recombinant protein PMTV-CP; B = antiserum raised against purified inclusion bodies containing recombinant PMTV-CP) in conjugate buffer were added and incubated for 2 h at 37°C. Swine anti-mouse IgG conjugated to alkaline phosphatase (SWAM-AP, Sigma Aldrich) diluted 1 : 10 000) was added and incubated for 3 h at 37°C. Finally, the plates were washed and substrate (0.1 mg *p*-nitrophenyl phosphate/ml of 0.1 M diethanolamine buffer, pH 9.8) was added.

The immunoglobulin fraction from the antisera was obtained using caprylic acid fractionation (Steinbuch and Audran, 1969).

ELISA

Individual CP fractions from different steps of the purification process were subjected to various types of ELISA. Double Antibody Sandwich-ELISA (DAS-ELISA) was performed as described by Clark and Adams (1977). The conditions for indirect PTA-ELISA (IPTA-ELISA) were as reported in the legend to Table 1.

Results

Sequencing PMTV-CP gene

Primers for CP amplification were designed with restriction sites *Eco*RI and *Nco*I on forward primer and *Xho*I on the reverse to allow easy in-frame cloning into several bacterial expression vectors. The 547-bp-long PCR product containing then CP coding region was first cloned into pUC57 (Fermentas) using the T/A overhangs generated by *Taq* polymerase. This plasmid was used for sequencing of the PCR products and for subcloning into following plasmids: pMPM4 which allows tightly regulated expression from arabinose-induced pBAD promotor, pET22 (Novagen, Merck, Czech Republic) – T7 polymerase-based system with signal peptide for translocation to periplasmic space, pGEX-T3 (AP) with N-terminally fused GST or to pThioHisB (Invitrogen) with terminally fused bacterial thioredoxin (data not shown). PMTV-CP cloned into Gateway expression vectors were selected for further studies because of high expression levels.

The CP of both isolates (Korneta and Pacov) had the same sequence (100% identity between sequenced parts). The sequences were submitted to GenBank (accession numbers AF393507 and AY208159).

Expression and purification of PMTV-CP

A clone containing the CP gene of isolate Korneta was used for further experiments. *Nco*I and *Xho*I fragment carrying the CP gene was ligated into Gateway expression plasmids and transformed into *E. coli*. After optimization of cultivation (time, temperature, inductor concentration) the cells were harvested and the presence of desired protein was demonstrated by immunoblotting using commercial antibodies against PMTV (Adgen).

Several bacterial clones containing the recombinant vectors coming from the Gateway were isolated and tested by Western blot analysis for PMTV-CP expression. (Fig. 1).

Non-induced clones produced much lower but visible amounts of this protein, while in non-transformed *E. coli* no such protein was detected. SDS-PAGE revealed a strong band at a position corresponding to the M_r either of about 20 kDa, this is expected M_r of the complete PMTV-CP with 6×His tag, or about 50 kDa, what is the expected M_r of the fusion protein PMTV-CP-GST (Fig.1). Immunoblotting experiments with a purified preparation of virus particles did not reveal evidence of the virus CP, because we were not successful in isolating intact PMTV particles. PMTV particles are fragile and some proteins may be lost during virus purification (Torrance et al., 1993).

There are several methods for isolating of desired protein from a bacterial lysate. We choose the fractionation according to Lin and Cheng (1991) for its ease of handling. The main content of recombinant PMTV-CP was found in the non-soluble cytoplasmic fraction, as was shown by comparison of all fractions obtained by this method in Western blot analysis (not shown). Although the GST protein is supposed to increase the fraction of soluble protein, most of the expressed proteins remain in the insoluble form (Fig. 2).

Production of polyclonal antisera to a recombinant PMTV-CP

For antisera production, we used the purified product from the expression of the recombinant vector resulting from the Gateway cloning with 6×His tag in *E. coli* BL21. We used both inclusion bodies and recombinant CP from inclusion bodies solubilized by 6 M urea purified on sucrose cushion. Antisera were obtained from bleeds taken 3 weeks after the fourth injection. The anti-PMTV-CP sera had titres of one of 2048 when tested in an indirect ELISA with sucrose-cushion-purified fused proteins or inclusion-bodies-coated microtitre plates.

ELISA tests

When the polyclonal antibodies from the antiserum produced against recombinant PMTV-CP were used in DAS-ELISA as the coating or as a conjugate, a weak

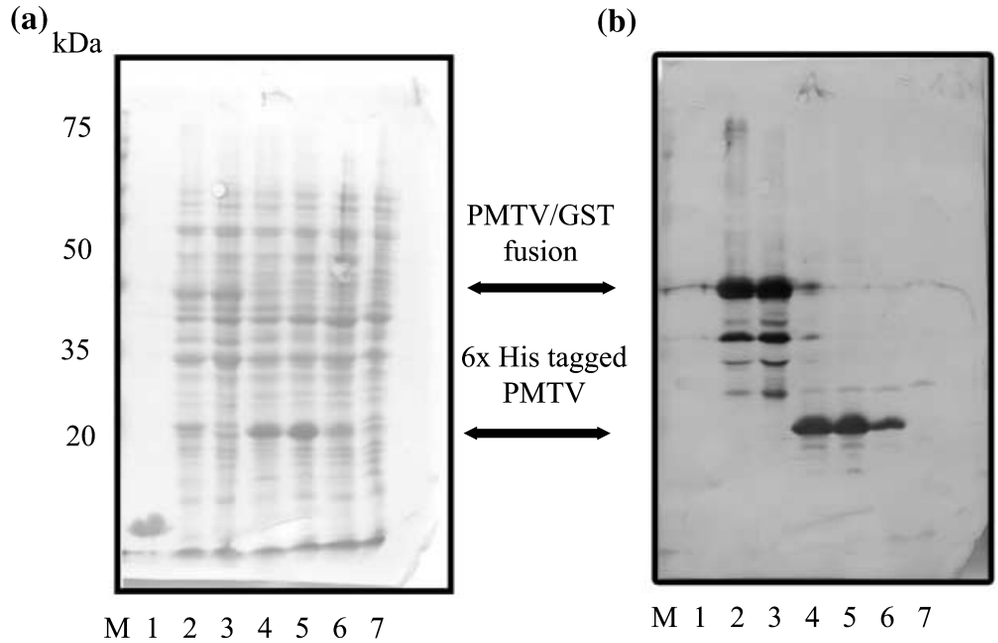

Fig. 1 Expression of fusion proteins containing *Potato mop-top virus* coat protein (PMTV-CP) in *Escherichia coli* (GATEWAY). (a) Nitrocellulose membrane stained with Ponceau S after blotting of sodium dodecyl sulphate (SDS) gel containing lysates of *E. coli* cells. Lane M contains molecular size standards, lane 1 = purified PMTV, lane 2 = fusion protein PMTV/glutathione-S-transferase (GST) – after O/N induction, lane 3 = the same after 3-h induction, lane 4 = fusion protein PMTV/6×His – O/N-induction. (b) Lane 5 = the same after 3-h induction, lane 6 = the same expression system without induction; line NK = BL21 cells + pET22 as a control. (c) Immunoblot analysis of total SDS soluble proteins. The presence of the desired protein was shown with commercial antibodies against PMTV (Adgen)

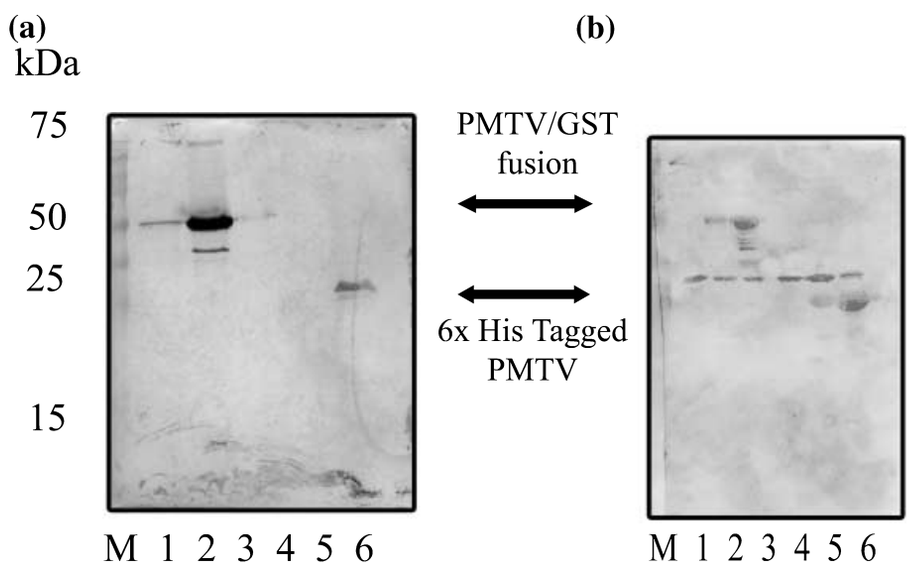

Fig. 2 (a) Localization of expressed *Potato mop-top virus* coat protein (PMTV-CP) proteins after cell fractionation. The presence of fusion proteins was demonstrated by immunoblot analysis on the nitrocellulose membrane by using monoclonal antibodies (MAb) against PMTV (Adgen). Lane M contained molecular size standards, lanes 1, 4 = trichloro acetic acid (TCA) precipitated extract of cultivation media, lanes 2, 5 = insoluble and lanes 3, 6 = soluble whole cell extract fraction. (b) Immunoblot analysis of total sodium dodecyl sulphate soluble proteins. Line M contained molecular size standard, line 1 = negative control, line 2 = fusion protein PMTV/glutathione-S-transferase (GST) – after 3-h induction, line 3 = the same after 3-h induction; line 4 = fusion protein PMTV/6×His after 3-h induction, 5 = the same after 3-h induction, 6 = PMTV-CP (purified inclusion bodies).The presence of the desired protein was demonstrated using polyclonal antibodies prepared against recombinant PMTV-CP

reaction with a high background against healthy controls was obtained (data not shown).

In TAS-ELISA, when plates were coated with IgG produced against PMTV (Adgen) it was possible to detect both recombinant antigens (isolated inclusions and protein) of PMTV-CP. No reaction was obtained in virus-infected tissue and the purified virus (data not shown).

In indirect PTA-ELISA we obtained weaker A_{405} values for antiserum prepared against purified recombinant

protein PMTV-CP in comparison with antiserum raised to inclusion bodies containing the same protein, when they were used at the same concentration. Generally, the reactions with all antigens used were with both antisera prepared against recombinant PMTV-CP weaker then the reactions carried out with commercially produced antibodies (Adgen) (Table 1).

In conclusion, the antisera raised against partially purified recombinant PMTV-CP were superior to the homologous antigen. No reaction was observed with sap from infected leaves, measurable values were only obtained using IPTA-ELISA.

Western blot analysis

In Western blot analysis, all polyclonal antibodies reacted strongly with the recombinant PMTV-CP of 20 kDa. As we have been unsuccessful in purifying PMTV, we could not test the reactivity of our antibody with purified virus. Another SDS-PAGE protein band of *ca*. 30 kDa gave a reaction with both antibodies (Fig. 2). The reaction of the IgG prepared from the mouse serum against recombinant PMTV-CP gave the same pattern as the commercial antibodies (Adgen). Kumari et al. (2001) obtained very similar results with an antiserum prepared against *Faba bean necrotic yellows virus* (FBNYV), assuming that this protein could be a recombinant CP linked to a bacterial protein(s). However, from our experiments we can conclude that this protein is most likely to be an *E. coli* protein that contaminated the preparations used for immunization, because it did not react with the commercial PMTV-MAb (Fig. 2).

Discussion

We determined the nucleotide sequences of two different field isolates of PMTV from the Czech Republic. From the comparison with other known sequences we can conclude that this part of the PMTV genome is highly conserved. These results correlate well with the data obtained by sequencing of different isolates of PMTV-CP genes by Reavy et al. (1997, 1998) and Nielsen and Nicolaisen (2001).

The expression of viral CP in *E. coli*, followed by purification and polyclonal antiserum production, has been reported for a number of plant viruses. These antisera have been used successfully for plant virus detection by Western blot analysis and IPTA-ELISA, but they have failed in DAS-ELISA (Nikolaeva et al., 1995; Jelkmann and Keimkonrad, 1997). There are few recent reports, however, in which antibodies produced against recombinant viral proteins of *Tomato spotted wilt virus* (TSWV) (Vaira et al., 1996) and *Grapevine leafroll-associated closterovirus-3* (GLRaV-3) (Ling et al., 2000) were found to be effective in detecting the viruses using DAS-ELISA. Petrzik et al. (2001) have also demonstrated the successful use of an antiserum against *Prunus necrotic ringspot virus* (PNRSV) recombinant CP for the detection of the virus by DAS-ELISA. Our results confirmed that the antisera produced against recombinant viral proteins were able to detect the viral proteins/antigens of concern by Western blot analysis, indirect PTA-ELISA but not by DAS-ELISA, which suggested preferential detection of denaturated PMTV-CP.

In contrast to the conventional method of antigen preparation, which is for PMTV very demanding with very poor yields, the purification procedure described above can be completed in 3 days. One litre of bacterial culture fluid yielded about 8 mg of PMTV-CP, an amount sufficient for a repeated immunization of laboratory animals for antisera preparation.

We believe that the recombinant viral CPs expressed in bacterial cells have great potential as an alternative source of antigens for raising specific antibodies to plant viruses. They can be produced in large quantities and can be manipulated or modified as need for specific uses.

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