POLYCLONAL ANTIBODIES TO A RECOMBINANT COAT PROTEIN OF POTATO VIRUS A

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Summary. – Specific mouse antibodies against a recombinant coat protein (CP) of Potato virus A (PVA) were produced. The PVA CP gene was cloned in an expression vector pMPM4Ω. After expression in *Escherichia coli* the presence of the expressed CP was proved by Western blot analysis using polyclonal and monoclonal antibodies (MAbs). The expressed CP was purified by centrifugation in CsCl density gradient or on a sucrose cushion. The production of virus-like particles (VLPs) was proved by electron microscopy. The purified CP was used for preparation of a mouse antiserum which had a titer of 1:1024 in ELISA and reacted specifically in Western blot analysis and indirect plate-trapped antigen ELISA (PTA-ELISA).

Key words: Potato virus A; recombinant coat protein; Escherichia coli

Introduction

Aphid-transmitted potyviruses (members of the genus *Potyvirus*) are the largest group of plant viruses, which is economically one of the most important. PVA (species *Potato virus A*) is one of the earliest discovered potyviruses, it occurs worldwide, infects many potato cultivars, and can cause yield losses of about 40% (Bartels, 1971). The PVA genome consists of single stranded positive-sense RNA of approximately 10 kb. Potyviruses belong to the picorna-like supergroup of viruses. Their genome is expressed as a large polyprotein, which is subsequently cleaved by proteases to yield several functional proteins including a conserved ordered gene set of non-structural proteins that are involved in RNA replication. Only two proteins (VPg and CP) have been detected in virions, while several others in infected plants but not in virions The region coding for CP is located at the 3'-end of genomic RNA. It has been shown that CP monomers of potyviruses when expressed in heterologous hosts (e.g. *E. coli*), self-polymerize to produce VLPs (Jagadish *et al.*, 1996).

The main objective of this study was to assess the use of a recombinant PVA CP expressed in *E. coli* for antiserum production and diagnostics in comparison with polyclonal and MAbs raised against PVA particles.

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Abbreviations: BSA = bovine serum albumin; CP = coat protein; DAS-ELISA = double-antibody sandwich ELISA; GLRaV-3 = Grapevine leafroll-associated virus 3; MAb = monoclonal antibody; PBST = phosphate-buffered saline with 0.05% Tween 20; PNRSV = Prunus necrotic ringspot virus; PTA-ELISA = plate-trapped antigen ELISA; PVA = Potato virus A; PCR = polymerase chain reaction; RT = reverse transcription; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TSWV = Tomato spotted wilt virus; VLP = virus-like particle

Materials and Methods

Virus. PVA isolate Lichte Industrie was kindly provided by Dr. Dědič, Potato Research Institute, Havlíčkův Brod, Czech Republic.

Cloning of CP gene. The immunocapture was performed as follows. The plates were coated with 100 µl/well of anti PVA IgG (1 µg/ml) in a coating buffer for 3 hrs at 37°C. The wells were then washed 3 times with 150 µl/well of phosphate-buffered saline with 0.05% Tween 20 (PBST) and the homogenate of PVA-infected leaves in a conjugate buffer (PBST, 0.2% bovine serum albumin, and 2% polyvinylpyrrolidone, 1:10, 100 µl/well) was added (Čeřovská *et al.*, 1998). The plates were incubated overnight at 4°C and washed again 3 times in PBS+T. Then reverse

transcription (RT) and polymerase chain reaction (PCR) using the Access Reverse Transcription Kit (Promega) under manufacturer's conditions was done. The primers for PCR amplification of the CP coding region were designed according to the sequence of the PVA isolate LIN (Acc. No. Z21670, Puurand *et al.*, 1994). The upstream primer PVA-CP-5A (5'-AAAA**CCATGG**AAGCCGGA ACTCTTGATGC-3') with the *NCoI* restriction site at its 5'-end contained also the initiation codon ATG. The downstream primer PVA-CP-3D (3'-AAAAA**AGATCT**TTTCCCACTCAAAC TCACTGTTG-5') contained the *BglII* restriction site (the restrictions sites in the primers, used in the cloning procedure, are underlined). The same primers were used both in RT and PCR. The PCR product of 964 bp was cleaved with appropriate enzymes and inserted into the pMPM4W expression vector (Meyer *et al.*, 1994). The *E. coli* MC1061 cells transformed with the recombinant plasmid were identified by ampicillin selection and restriction analysis.

Expression of CP gene was performed in transformed *E. coli* cells grown in LB medium. To produce the recombinant PVA CP, 5 ml of an overnight culture was added to 500 ml of fresh LB, the culture was grown to the A_{600} of 0.6 and then induced with arabinose (Sigma-Aldrich) in final concentration of 0.5% overnight at 20°C. Bacterial cells were then harvested by centrifugation and stored at -80°C until used.

Preparation of CP-enriched fractions. PVA CP was partially purified from one liter of the grown bacterial culture. The cells were centrifuged at 15,000 x g for 15 mins. The pellet obtained from the cell culture was resuspended in 100 ml of 20 mmol/l Tris-HCl pH 7.5 with 0.2 mg/ml lysozyme (Sigma) and 100 µg/ml DNAse I (Roche), and incubated at 37°C for 10 mins. After disintegration in glass homogenizer on ice the bacterial lysate was centrifuged. The pellet containing main part of CP was further purified by centrifugation in CsCl density gradient or on sucrose cushion (Čeřovská *et al.*, 1997).

Protein concentration in each fraction was assayed both spectrophotometrically (A_{280} = 1.0 for 1 mg/ml) and by Bradford's method (Bradford, 1976).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). The grown bacterial cells (1 ml) were pelleted by centrifugation, resuspended in the Laemmli buffer (100 µl), boiled for 2 mins and aliquots were loaded on 12% polyacrylamide gel containing SDS (Laemmli, 1970). For visualization of the separated proteins Coomassie Brilliant Blue R250 was employed.

Western blot analysis. The proteins separated by SDS-PAGE were electroblotted to a nitrocellulose membrane (0.45 µm, Protran, Sleicher&Schuell) in semidry system (OMNI-TRANS apparatus, Omnibio Brno, Czech Republic) according to Hirano and Watanabe (1990). The blot was stained non-specifically by Ponceau S (Sigma Aldrich), incubated for 1 hr in 4% bovine serum albumin (BSA) in PBS and washed four times in PBS. For recombinant PVA CP detection the polyclonal antibodies or a MAb prepared in our laboratory (Šubr *et al.*, 1996) were used. The bands were visualized with BCIP/NBT (Sigma) according to Sambrook *et al.* (1989).

ELISA. Individual CP fractions from different steps of the purification process were subjected to various types of ELISA (Filigarová *et al.*, 1994; Clark and Adams, 1977), using the antibodies prepared in our laboratory.

Electron microscopy. A carbon film (approximately 100 mµ thick) loaded onto copper grids (Bradley, 1967) was used as a support. Purified recombinant PVA CP proteins, prepared by centrifugation in CsCl density gradient (Čeřovská *et al.*, 1997), were examined. The samples were applied directly on the carbonated grids and negatively stained with uranyl acetate (Tandler, 1990).

Antisera production and antibodies used. The antisera against the recombinant PVA CP were prepared in mice by three subcutaneous and one intraperitoneal injections of 50 µg of the purified CP in three-week intervals. The purified CP was emulsified with an equal volume of the Freund's complete adjuvant for the first injection and with the Freund's incomplete adjuvant for subsequent two injections. For the last injection the antigen was diluted in PBS. The mice were bled three weeks after the last injection. The serum fractions were collected and stored at -20°C until used. Our polyclonal antibodies and a MAb prepared against PVA particles (Filigarová *et al.*, 1994) were used for comparison. To reduce non-specific background reactions, the mice antisera were cross-absorbed with healthy tobacco plant extracts diluted 1:20. The polyclonal antibodies were used at an IgG concentration of 1–5 µg/ml. The concentration of the MAb was 1–5 µg/ml. Primary and secondary antibodies were diluted in a conjugate buffer (PBS containing 2% polyvinylpyrrolidone and 0.2% BSA).

Results

Expression and purification of PVA CP

Several bacterial clones containing the recombinant vector were isolated and tested by Western blot analysis for PVA CP expression (Fig. 1). The non-induced clones produced much lower but visible amounts of the protein, while in non-transformed *E. coli* no such protein was detected. SDS-PAGE revealed one strong band at a position corresponding to M_r of about 32 K. This was the expected M_r of PVA CP.

From several methods of isolation of a specific protein from bacterial lysate we chose the simple fractionation for its easy performance. The highest content of recombinant PVA CP was found in the insoluble cytoplasmic fraction. That was proved by comparison of all fractions obtained by this method by Western blot analysis (data not shown).

Electron microscopy and antisera production

Electron microscopy of purified recombinant PVA CP preparations obtained by centrifugation in CsCl density gradient or on sucrose cushion revealed VLPs (Fig. 2). This was the evidence that CP monomers of PVA, when expressed in a heterologous host (*E. coli*), self-polymerized to produce VLPs as it has been proved for the Johnsongrass mosaic virus (Jagadish *et al.*, 1996). The N- and C-terminal regions of VLPs are surface-exposed and not required for virion

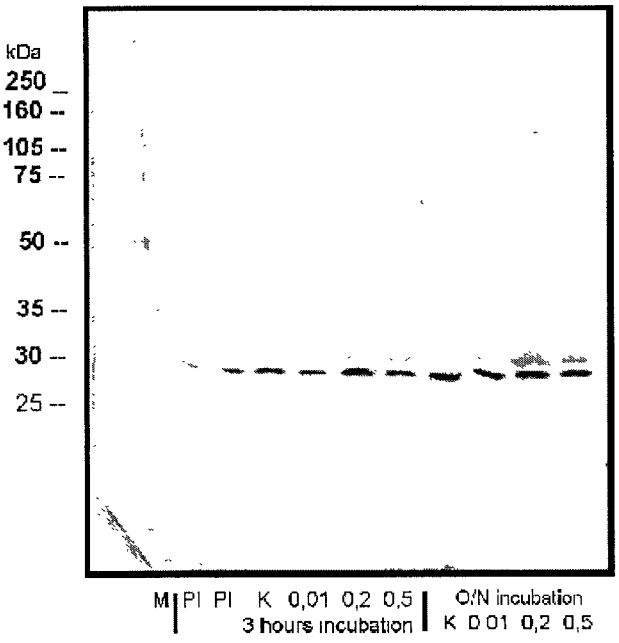

Fig. 1

Western blot analysis of expression of recombinant PVA CP in *E. coli*

Total proteins obtained from bacterial cells were subjected to Western blot analysis. Molecular size standards (lane M); lysates of non-induced bacterial cells harboring the recombinant vector (lanes PI); a lysate of bacterial cells harboring the original plasmid (pMPM4) without the inserted PVA CP gene, a negative control (lane K); For other samples the concentration of the inductor as well as the time of induction are indicated. The blot was probed with MAb 151 raised against PVA CP.

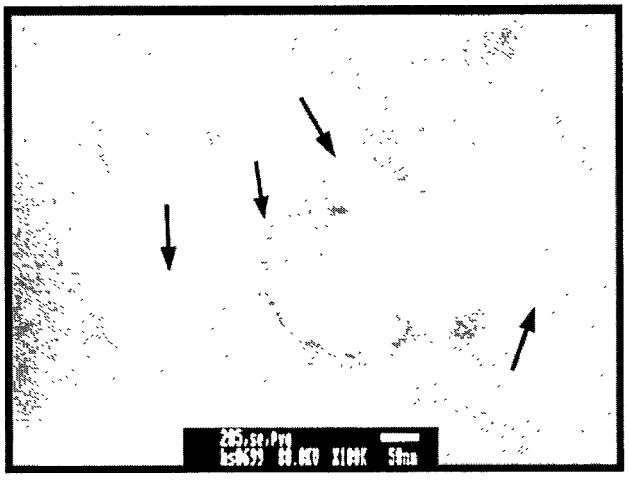

Fig. 2

Micrograph of chimeric PVA VLPs

PVA VLPs were obtained by purification of recombinant PVA CP by centrifugation in CsCl density gradient. The arrows indicate VLPs.

assembly. Such chimeric particles are highly immunogenic even in the absence of adjuvant (Jagadish *et al.*, 1996). However, in our experiments we had to use four doses of the recombinant antigen in comparison to only three doses of virus purificate of the same concentration to obtain a similar titer of antiserum. The titer of the prepared mouse PVA CP antiserum was 1:1024 in indirect PTA-ELISA.

ELISA

When the polyclonal antibodies from the antiserum produced against recombinant PVA CP were used in a double-antibody sandwich ELISA (DAS-ELISA) as the coating or conjugate component a weak reaction with a high background (control reaction with "healthy" non-infected samples) was obtained (data not shown).

In DAS ELISA, when plates were coated with IgG produced against purified PVA (Filigarová *et al.*, 1994), it was possible to detect the recombinant PVA CP and the purified virus but no reaction was obtained with PVA-infected tissue (data not shown).

In indirect PTA-ELISA we obtained weaker A_{405} values for the antiserum prepared against recombinant PVA CP in comparison to MAb PVA-151, when they were used at the same concentration. On the other hand, in the reaction with PVA-infected leaves, the values with the antiserum against the recombinant PVA CP were higher (Table 1). With other MAbs prepared in our laboratory we obtained similar results.

Summing up, in DAS-ELISA, the antiserum raised against the partially purified recombinant PVA CP reacted most strongly with the homologous antigen, less strongly with the PVA purificate and not at all with PVA-infected leaves. Measurable values for PVA-infected leaves were only in PTA-ELISA.

Western blot analysis

In Western blot analysis, all polyclonal antibodies and the MAb reacted strongly with the recombinant PVA CP of approximately 32 K and PVA purificate corresponding to the 32 K protein band in Fig. 3. Moreover, another SDS-PAGE protein band of approximately 30 K gave a reaction with polyclonal antibodies and MAb 151 (Figs. 1 and 3). The reaction of the IgG prepared from the mouse serum against recombinant PVA CP gave the same pattern as the polyclonal antibodies prepared in our laboratory against the PVA purificate from plants. Kumari *et al.* (2001) have obtained very similar results with an antiserum prepared against Faba bean necrotic yellows virus, supposing that this protein could be a recombinat CP linked to a bacterial protein(s). In our experiments performed with the *E. coli*

Table 1. Detection of PVA-infected tissue, PVA purificate and recombinant PVA CP by indirect PTA-ELISA

	A_{405}	
	Detection with MAb 151	Detection with IgG against recombinant PVA CP
Buffer	0.20	0.20
Healthy plants (dilution 1:10)	0.20	0.20
PVA-infected leaves (dilution 1:10)	1.00	1.35
PVA purificate (30 µg/ml)	> 3.00	1.65
Recombinant PVA CP (10 µg/ml)	> 3.00	0.80

MAb 151 was obtained by immunization of mice with PVA-LI isolate (Filigarová *et al.*, 1994). Indirect PTA-ELISA: plates were coated with antigens diluted in standard coating buffer overnight at 4°C. The plates were washed with PBST four times. MAb 151 (1 µg/ml) or IgG against recombinant PVA CP (1 µg/ml) in conjugate buffer were added and incubated for 2 hrs 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 hrs at 37°C. Finally, the plates were washed and the substrate (0.1 mg/ml p-nitrophenyl phosphate in 0.1 mol/l diethanolamine buffer, pH 9.8) was added. A_{405} was read after 30 mins.

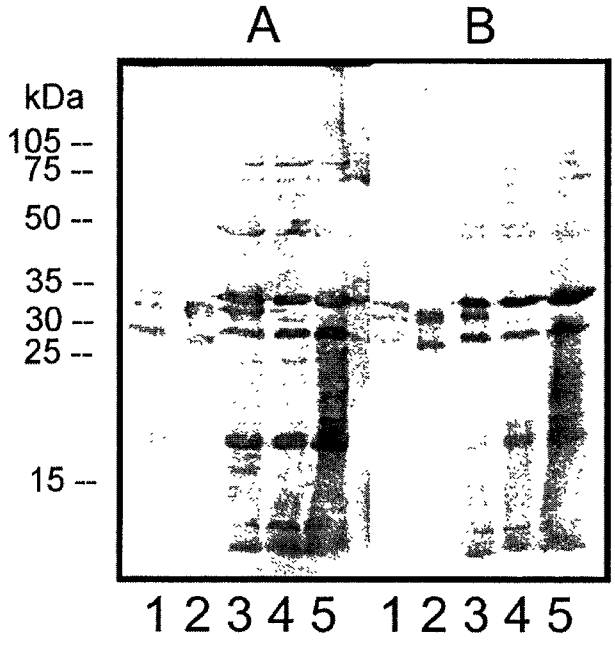

Fig. 3

Western blot analysis of expression of recombinant PVA CP in *E. coli* using polyclonal antibodies against recombinant PVA CP (A) and polyclonal antibodies against PVA from PVA-infected tobacco plants (B)

The recombinant PVA CP purified by centrifugation in CsCl density gradient (lanes 1); PVA purified from PVA-infected tobacco plants (lanes 2); a lysate of induced bacterial cells expressing the recombinant PVA CP (lanes 3); a lysate of non-induced bacterial cells harboring the recombinant vector, a negative control (lanes 4); a lysate of bacterial cells harboring the original vector (pMPM4) without the inserted PVA CP gene (lanes 5).

carrying the pMPM4 Ω plasmid without the inserted PVA CP gene we got the same reaction scheme with the 30 K band with all polyclonal antibodies and the MAb (data not shown). We can conclude that this protein is a part either of bacteria or the vector but not a part of the expressed protein linked to bacterial proteins.

Discussion

The expression of viral CP in *E. coli*, followed by purification and polyclonal antiserum production, has been reported for a number of different plant viruses. These antisera have been used successfully for plant viruses detection by Western blot analysis and indirect PTA-ELISA, but they have failed in double-antibody sandwich ELISA (DAS-ELISA, Nikolaeva *et al.*, 1995; Jelkman and Keim-Konrad, 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 by DAS-ELISA. Also the work of Petrzik *et al.* (2001) have demonstrated the successful use of an antiserum against Prunus necrotic ringspot virus (PNRSV) recombinant CP for detection of this virus by DAS-ELISA. Similarly to the abovementioned facts the results obtained in this study 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 DAS-ELISA. In contrast to the conventional method of antigen preparation, which can take 2 or more months, the purification procedure described above can be completed in 3 days. One liter of bacterial culture fluid yielded about 10.5 mg of PVA CP, an amount sufficient for a repeated immunization of laboratory animals for antisera preparation.

Here it is demonstrated that in contrast to the conventional method of antigen preparation we developed a self-polymerizing expression system using a potyvirus CP and host (*E. coli*) cells for production of VLPs devoid of viral RNA. These particles are non-replicating and non-

pathogenic and can present host-protective antigens in a particulate and multimeric form (Jagadish *et al.*, 1996).

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