THE ATTEMPTS TO PRODUCE ANTISERUM AGAINST APPLE STEM PITTING VIRUS COAT PROTEIN (ASPV CP) OBTAINED IN PROKARYOTIC AND EUKARYOTIC EXPRESSION SYSTEMS

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A B S T R A C T

Cloning and expression of the coat protein (CP) gene of *Apple stem pitting virus* (ASPV; Family: *Flexiviride*, Genus: *Foveavirus*) using *in vivo Escherichia coli* and *Pichia pastoris* as well as *in vitro* wheat germ extract gene expression system are described. The coat protein gene was amplified for five ASPV isolates representing three groups characterized by a different size of CPs. The amplified RT-PCR products were cloned into bacteria and yeast expression vectors. Plasmid containing the CP gene of the isolate MT32 was used as a template for expression in wheat germ extract.

Proteins of expected sizes, reacting with ASPV antiserum in Western blotting experiments, were obtained in three expression systems for the different virus isolates used in this study. The purified proteins were used for immunization and preparation of rabbit polyclonal antisera. The obtained antibodies could be used for immunocapture RT-PCR detection of ASPV in woody tissue, but ELISA results were not satisfactory.

Key words: ASPV, coat protein, differentiation, expression, antibody

INTRODUCTION

Serological techniques based on utilization of specific antisera are the most popular of the various phytodiagnostic methods. They are relatively sensitive, inexpensive, simple and suitable for the testing of many samples simultaneously (Clark, 1981). Additionally, specific antibodies are a prerequisite for the application of the extremely sensitive technique – immunocapture (IC)-RT-PCR (Wetzel et al., 1992). A limited amount of

antisera specific for *Apple stem pitting virus* (ASPV) were prepared in few laboratories. Either purified virus particles (Koganezawa and Yanase, 1990; Kalashjan, unpublished data) or fusion protein expressed in a bacterial system (Jelkmann et al., 1992) were used. All these antisera were suitable for detection of ASPV using IC-RT-PCR and Western blotting (Jelkmann and Keim-Konrad, 1997; Nemchinov et al., 1998, Komorowska, 2002). However, the suitability of ELISA for detection of ASPV in fruit trees was hampered by low sensitivity and high background (Malinowski et al., 1996; Jelkmann and Keim-Konrad, 1997).

Properties of antiserum depend strongly on the amino acid composition and conformation of the protein used for immunization. Therefore, the study was undertaken to check the influence of the virus isolate and the expression system used for protein preparation and immunization on the quality of polyclonal antibodies and their ability to react with native virus particles.

In this paper, we describe the preparation of the ASPV coat protein in *Escherichia coli*, *Pichia pastoris* and wheat germ extract systems. We also report the attempts to raise polyclonal virus-specific antisera for immunodiagnostics.

MATERIAL AND METHODS

Virus sources

Five isolates of ASPV which were biologically characterized and differing in number of deletions in the N-terminal part of CP were used in this study (Tab. 1). Isolate PSA-H was kindly provided by Prof. Jelkmann (Dossenheim, Germany). The other isolates were from the collection of plant viruses at the Research Institute of Pomology and Floriculture, Skierniewice, Poland.

cDNA synthesis

A coat protein gene was amplified directly from leaf extracts of infected trees using SC-RT-PCR method as described previously (Komorowska et al., 1999). The only modification was using primers ASPV7956-ASPV9263 (Schwarz and Jelkmann, 1998) with the sequences necessary for cloning added at 5' ends (underlined): *att*B1 (5'-ggggacaagtttgtacaaaaaagcaggctca atgacttccaatggatccca-3') and *attB2* (5'-ggggaccactttgtacaagaaagctgggtca tagccgccccggttaggtt-3')

Cloning and expression of the CP gene and purification of the expressed protein

Three different expression systems based on: bacteria (Gateway Cloning System, Invitrogen), yeast (EasySelect *Pichia* Expression System, Invitrogen) and wheat germ extract (TNT Coupled Wheat Germ Extract System, Promega) were used to obtain the coat protein of ASPV isolates. The product of RT-PCR was cloned into the prokaryotic gene fusion vector pDEST17 *via* pDONR201 (Invitrogen). The plasmid pDEST17 contained an expression cassette enabling the production of CP with N-terminal affinity tag of six histidines

Table 1. Characteristics of a coat protein gene of ASPV isolates used for expression

ASPV isolate	Host species	The length of CP gene [bp]	Number of deletions	Position of deletion (s)*	GenBank accession number
PSA-H	*Pyrus communis*	1245	0	-	D21828
ST54	*Pyrus communis*	1191	1	8006-8059	AF345892
ST113	*Pyrus communis*	1125	2	8006-8059 8233-8298	AF345894
MT32	*Malus x domestica*	1191	1	8006-8059	AF438521
J335	*Malus x domestica*	1191	1	8006-8059	AF491930

*The numbers correspond to nucleotide positions in a reference isolate PSA-H (accession no. D21828)

(6-His tag). The recombinant plasmids were isolated from the transformed *Escherichia coli* BL21 SI (Invitrogen) and sequenced to check the integrity, proper position and orientation of the cloned gene. The expression of the hybrid protein and its purification from 1 ml or 1 l of liquid medium was performed under non-denaturing conditions on NTA-resin (Qiagen) according to the manufacturer`s instructions.

Simultaneously, the gene encoding ASPV CP was cloned into pPICZ A vector (EasySelect *Pichia* Expression Kit, Invitrogen, USA) in frame with the C-terminal tag of histidines. The construct was used to transform *Pichia pastoris* GS115 electrocompetent cells. Zeocin-resistant clones were obtained and one of them was selected for expression. Cells were grown in 25 ml of a Buffered Glycerol-complex Medium (BMGY) until OD_{600} reached 2.0. Then cells were pelleted by low speed centrifugation and resuspended in 100 ml of Buffered Methanol-complex Medium (BMMY). They were induced by adding 100% methanol to a final concentration of 0.5% every 24 hours for four days. Yeast cells were harvested (by centrifugation) and lysed in 5 ml of Breaking Buffer. The lysate was mixed with 2 ml of the 50% Ni-NTA resin (Qiagen, USA) and loaded into a 5 ml column (Qiagen, USA). After two washes of the column with 4 ml of wash buffer the protein was eluted with increasing concentrations of imidazole.

Wheat germ transcription/translation reaction was performed according to the manufacturer's instructions in 50 µl total volume. About 1 µg of pD17ASP32 – recombinant pDEST17 plasmid containing the CP gene of isolate MT32 was used. Twenty five µl aliquot of post-reaction mixture was diluted in a lysis buffer (50 mM sodium phosphate buffer, pH 8.0 containing 1.3 M NaCl and 10 mM imidazole) before purification using the same protocol as for bacteria.

Samples obtained in all the expression systems were analyzed by running 4/12% discontinuous SDS-PAGE. Duplicates of resolved proteins were either visualized by Coomassie blue-staining or transferred onto a PVDF membrane (using semi-dry blotter, Pharmacia LKB, Sweden). The membranes were probed with ASPV antiserum S-Ka (kindly provided by Dr. Kalashian, Moldova).

Production of antiserum

The rabbits were immunized with 1 mg of chimeric protein in a solution emulsified with an equal volume of Freund's complete adjuvant and administered in 2-3 subcutaneous injections. After two weeks, two booster injections were given at weekly intervals, using 0.5 mg of protein and incomplete adjuvant.

IC-RT-PCR

IC-RT-PCR was carried out according to the procedure of Candresse et al. (1995) as modified by Malinowski (2005) using primers ASPV9019-ASPV9263 published by Schwarz and Jelkmann (1998).

Enzyme linked immunosorbent assay (ELISA)

All recombinant protein antisera were tested in $F(ab')_2$ELISA (Barbara and Clark, 1982) and in a plate-trapped antigen (PTA) ELISA (Jelkmann and Keim-Konrad, 1997). The polyclonal antisera were used at dilutions of 1:200, 1:400 and 1:800 in PBS-TPO (PBS: 0.01 M sodium/potassium phosphate, pH 7.4, 0.14 M NaCl; TPO: 0.05% Tween 20, 20% polyvinylpyrrolidone 40000, 2% ovalbumine). The $F(ab')_2$ fragments used for comparison have been prepared from antisera kindly provided by Dr. Kalashian (Moldova) and Dr. Yanase (Japan).

RESULTS

Cloning and expression of the CP gene of ASPV isolates

The coat protein gene of five selected ASPV isolates was amplified and cloned into *E. coli* and *P. pastoris* expression vectors. The presence of virus-specific inserts was confirmed by PCR. The orientation and 'in frame' position of the CP gene was checked by sequencing. Small scale expression of recombinant *E. coli* clones was carried out. The protein of the expected size was detected for five isolates in Coomassie blue-stained SDS-poly-acrylamide gel. The molecular weight of expressed His-tagged proteins, calculated on the basis of the known sequence of the CP gene, was 46 kDa for reference isolate PSA-H, 45 kDa for isolates MT32, ST54 and J335, and 42 kDa for isolate ST113 (Fig. 1a). The proteins obtained for all studied isolates reacted with ASPV antiserum, as checked by Western blot analysis. The results obtained for the isolates representing three phylogenetic groups are shown in Figure 1b.

The expressed CP of isolates PSA-H and MT32 was obtained also from transformed *P. pastoris* induced by methanol. Fusion protein of the expected size could be detected on

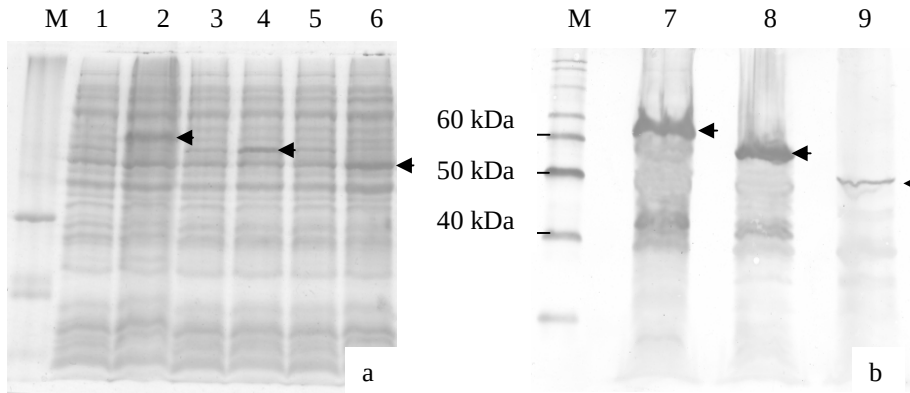

Figure 1. Expression of the ASPV CP from pDEST17
Expressed proteins were resolved by SDS-PAGE and visualized by Coomassie blue staining (a) or analyzed by W. blotting with ASPV antiserum (b). Lanes: 1, 2, 7-isolate PSA-H; 3, 4, 8-isolate MT32; 5, 6, 9-isolate ST113. Lanes: 1, 3, 5-noninduced cells; 2, 4, 6, 7, 8, 9-induced cells, M-SIGMA Low Range MW Protein marker. The arrows indicate the position of the predicted expression product

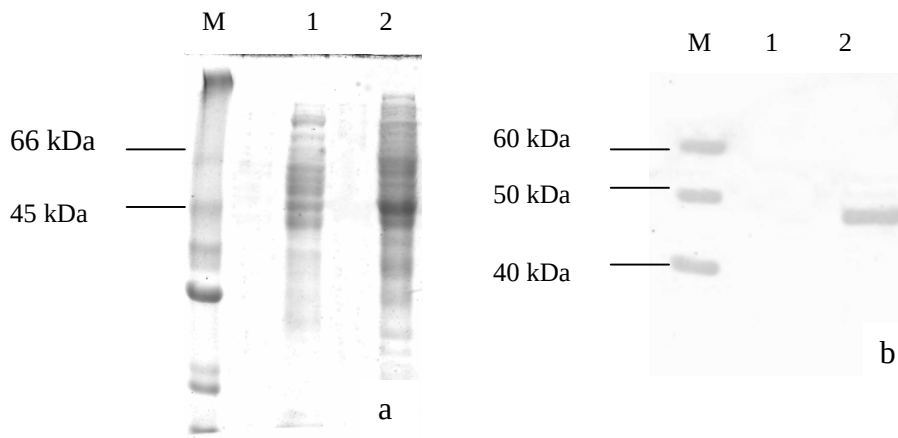

Figure 2. Expression of the ASPV CP (isolate MT 32) from pPICZ A
The proteins were visualized by Coomassie staining (a) or analyzed by Western blotting (b). M-marker, 1-noninduced cells, 2-induced cells. The arrows indicate the position of the predicted expression product.

the membranes using specific antiserum. Results for the isolate MT32 are shown in Figure 2a (Coomassie-stained) and Figure 2b (W. blotting).

Plasmid pDEST17J containing the CP gene of ASPV isolate MT32 was used to initiate a production of protein in wheat germ extract. The electrophoretically resolved products after 60 minutes reaction are shown in Figure 3 (lane 10).

Purification of expressed proteins and production of antisera

The coat protein of isolates MT32, ST113 and PSA-H expressed in *E. coli* as well as CP of isolates MT32 and PSA-H produced in *Pichia pastoris* was purified using Ni-NTA column. Final fractions eluted with the highest concentration of imidazole from the column contained a single protein of expected size (Fig. 3a, lanes 7-9) reacting with ASPV specific antiserum (Fig. 3b). An amount of from 0.3 mg to 1.5 mg of purified protein was obtained from either a one-litre culture of bacteria or a 100 ml culture of yeast.

Application of the same protein purification protocol to wheat germ extract diluted in a suitable buffer resulted in the preparation of the desired protein, accompanied by two other proteins. One of the two other proteins was larger than 70 kDa. The other protein was smaller than 20 kDa, as evaluated by SDS-PAGE (Fig. 3a, lane 11). Only protein of the size expected for ASPV CP was detected on Western blot developed with polyclonal antibodies S-Ka (Fig. 3b, lane 11).

Six antisera were produced against the ASPV coat protein expressed in bacteria (isolates PSA-H, MT32, ST113), yeast (isolates PSA-H, ST113) and wheat germ extract system (isolate MT32). Suitability of polyclonal antibodies isolated from all produced antisera for the IC-RT-PCR method was verified. A specific-sized band of 264 bp was observed for nine tested isolates. However, RT-PCR performed in non-coated tubes resulted in a band of the expected size for all samples though it was of a weaker intensity. The reaction of the prepared antisera was observed on Western blots with expressed recombinant protein but not with the extracts from ASPV infected plants. Similarly, the positive PTA-ELISA results were observed only for purified protein used for immunisation and lysate from non-induced bacterial cells. The reaction of newly prepared antisera with the infected plants was not observed (data not shown).

DISCUSSION

Several attempts to obtain specific antiserum using ASPV particles purified from herbaceous host were only partially successful. The antisera were suitable for ISEM and IC-RT-PCR but not for ELISA. Polyclonal antibodies were also prepared against the coat protein antigen expressed in *E. coli* as a fusion protein (Jelkmann et al., 1992; Jelkmann and Keim-Konrad, 1997).

The aim of this study was the production of ASPV-specific antisera to coat protein antigen expressed in bacteria, yeast and wheat germ extract. Although fusion protein-specific antisera have been widely used in serological assays (Hema et al., 2003; Petrovic, 2003), there are only few reports about their use in ELISA (Vaira et al., 1996; Jelkmann and Keim-Konrad, 1997).

The properties of antiserum strongly depend on the strain of the virus and the conformation of the

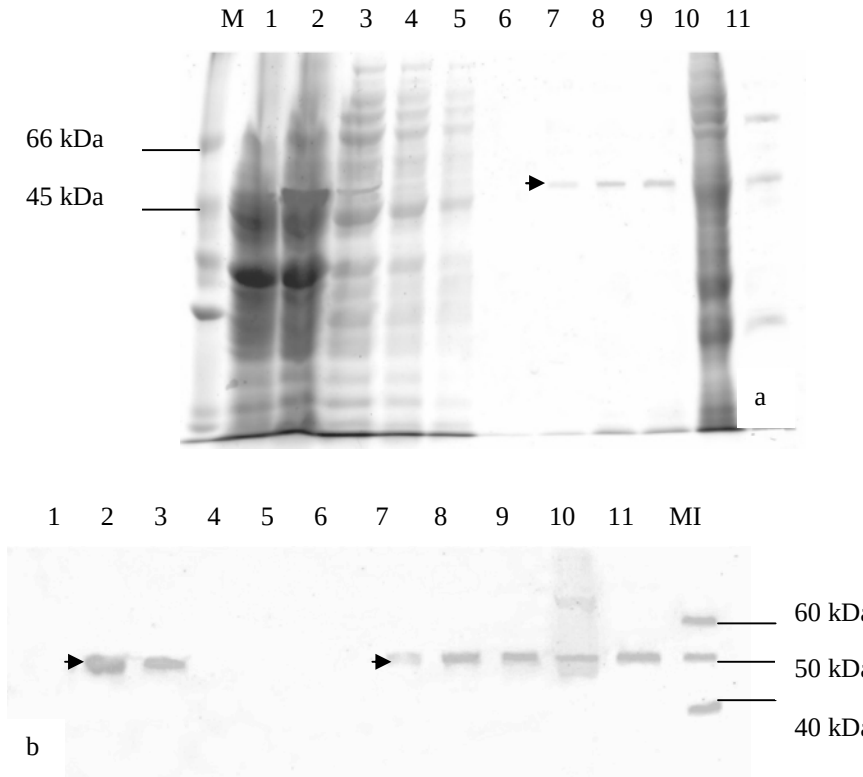

Figure 3. Expression of the ASPV-MT32 CP in bacteria and wheat germ extract and purification of fusion protein on Ni-NTA column
SDS-PAGE with Coomassie blue staining (a) and Western blotting analysis with ASPV antiserum (b). Lanes: 1-non induced cells, 2-induced cells, 3-cell lysate, 4-flow-through, 5-wash, 6-9 eluates for bacteria. Lanes: 10-post-translational crude extract (wheat germ), 11-eluate for wheat germ extract. M-SIGMA Low Range MW Protein marker, MI-MagicMark Western Standard (Invitrogen). The arrows indicate the position of the predicted expression product

coat protein. Three types of ASPV isolates were identified based on the number of deletions in the coat protein gene (Schwarz and Jelkmann, 1998). Three different isolates characterised with CP gene sizes: 1245, 1191 and 1122 nts, one procariotic and two eucariotic expression systems, were used in our experiments.

The proteins obtained in this study were diversified not only by the number of deletions in the CP but also by the place of His tag fusion. The tag was attached either to N'–end (pDEST17 vector) or to C'–end (pPICZ A vector) of ASPV CP. Since it was not removed before immunization, it might have also influenced the serological properties of the fusion protein.

The expression was obtained in all systems. The observed mobility

rate of proteins obtained for different isolates was in agreement with the molecular mass values predicted by a computer assisted translation of corresponding nucleotide sequences.

The purification of proteins expressed in bacteria and yeast was successful. However, there was a problem with purification of fusion protein from wheat germ extract while following the same protocol. Two additional proteins, non-reacting with specific antiserum, probably exogenous ones containing some His residues, were co-purified with a specific fusion protein. Therefore, optimization of imidazole concentration used for elution or adding an extra final separation step (Sephadex column) may be necessary.

Non-denaturing conditions were applied for purification of fusion protein in this study. Jelkmann and Keim-Konrad (1997) reported that much better antiserum was obtained when C'-end His-tagged protein was purified under non-denaturing conditions. This was in comparison to the ones prepared against ASPV CP protein fused with ß-galactosidase and purified by electroelution from polyacrylamide gel (denaturing conditions).

There are potential advantages of using a protein expressed in the eukaryotic systems for immunization. Protein processing, folding and post-translational modifications could be more similar to the corresponding ones, happening in natural plant hosts. It was hoped that expression of ASPV coat protein in eukaryotic host organisms will allow us to produce a purified protein which is structurally more similar to the native protein. This would make it more suitable for antiserum production.

Unfortunately, none of the antisera obtained in this study represented a real improvement over the ones produced earlier in other labs, using either purified virions or fusion proteins expressed in prokaryotic systems. However, all proteins obtained in this study: for three isolates in bacteria, for two isolates in yeast and for one isolate in wheat germ extract reacted in Western blots with S-Ka antiserum prepared against purified virions of ASPV. It means that at least some epitopes were expressed properly in all systems and that their presence was not disturbed too much by electrophoresis under denaturing conditions. Therefore, it is still possible that with some modifications of immunization protocols or further processing the antisera to isolate specific antibodies (Olmsted, 1981; Rybicki, 1986), progress may be reached.

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PRÓBA UZYSKANIA SUROWICY NA BIAŁKO PŁASZCZA WIRUSA JAMKOWATOŚCI PNIA JABŁONI (ASPV), PRZYGOTOWANE W PROKARIOTYCZNYM I EUKARIOTYCZNYCH SYSTEMACH EKSPRESYJNYCH

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S T R E S Z C Z E N I E

W pracy opisano klonowanie i ekspresję genu białka płaszcza wirusa jamkowatości pnia jabłoni (ASPV; Rodzina: *Flexiviride* Rodzaj: *Foveavirus*) z użyciem dwóch systemów ekspresyjnych *in vivo*: bakteryjnego (*Escherichia coli*) i drożdżowego (*Pichia pastoris*) oraz systemu *in vitro* z wykorzystaniem ekstraktu z kiełków pszenicy. Gen białka płaszcza zamplifikowano dla pięciu izolatów ASPV reprezentujących trzy grupy charakteryzujące się różną wielkością białka płaszcza. Produkty RT-PCR wklonowano do bakteryjnego i drożdżowego wektora ekspresyjnego. Plazmid zawierający gen białka płaszcza izolatu MT32 był wykorzystany jako matryca do ekspresji w ekstrakcie z kiełków pszenicy.

Białka o oczekiwanej wielkości, reagujące w metodzie Western blotting z surowicą uczuloną na ASPV, uzyskano w trzech systemach ekspresyjnych dla różnych izolatów wirusa. Oczyszczone białko było użyte do immunizacji i przygotowania surowic. Uzyskane przeciwciała umożliwiały wykrywanie ASPV w jabłoni i gruszy metodą IC-RT-PCR. Wyniki testów ELISA nie były zadowalające.

Słowa kluczowe: ASPV, białko płaszcza, zróżnicowanie, ekspresja, przeciwciała