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Production of Polyclonal Antibodies to a Recombinant *Potato Mop-top Virus* Non-structural Triple Gene Block Protein 1

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Abstract

The gene encoding the triple gene block protein 1 (TGBp1) of *Potato mop-top virus* (PMTV) was cloned into expression vector pQE32 tagging the protein with 6xHis on the N-terminus. When the gene was enshortened on its 3'-end by two different restriction digestions, efficient and high yield bacterial expression was achieved in both cases, as shown by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. One of these two constructs was used for raising rabbit polyclonal antibodies. The obtained sera and antibodies were tested for the detection of PMTV in laboratory host *Nicotiana benthamiana* and natural host *Solanum tuberosum* by enzyme-linked immunosorbent assay (ELISA) as well as by Western blots. The obtained antisera are more suitable for Western blot analysis of infected plants than for ELISA.

Introduction

The *Potato mop-top virus* (PMTV) is a fungus-transmitted soil-borne virus and the type member of the genus *Pomovirus* (Torrance and Mayo, 1997). PMTV occurs in potato-growing regions in Europe, North and South America and Asia in cool wet climate causing a wide range of symptoms in haulms and tubers, which vary depending on the potato cultivar and environmental conditions, thus complicating the identification of the virus disease (Jones, 1988).

PMTV has tubular and rigid particles, measuring 18–22 × 100–150 or 250–300 nm (White et al., 1972) encapsidating three RNA components, namely RNA 1, RNA 2 and RNA 3 (Kallender et al., 1990; Kashiwazaki et al., 1995; Savenkov et al., 1999). The third RNA, 2.9 kb long, contains a triple gene block (TGB) encoding three proteins involved in cell-to-cell movement, and an additional open-reading frame (ORF) for a predicted 'cystein-rich' protein (CRP) with unknown function (Mayo et al., 1996; Fig. 1).

The analysis of nucleotide sequences showed that PMTV genomes from different parts of the world are

highly conserved which is the fact that usually makes the diagnosing of viral disease more simple (Mayo et al., 1996; Sandgren et al., 2001; Nielsen and Nicolaisen, 2003; Čeřovská et al., 2003a; Pečenková et al., 2004). However, the detection based solely on the presence of PMTV coat protein (CP) seems to be insufficient as it was recently shown that the distribution of PMTV RNAs varies in different parts of infected plants and that the multipartite virus PMTV is capable of establishing infection without the CP-encoding RNA, and also without the putative CRP (Savenkov et al., 2003). Thus, detection based on some other non-structural protein could be advantageous when combined with CP detection methods and polymerase chain reaction (PCR) methods (Arif et al., 1994; Rantanen et al., 1999; Mumford et al., 2000). Antisera raised against triple gene block protein 1 (TGBp1), for example, could be used for such a purpose as well as for studying the fate of this protein in infected plant and its role in the viral movement through plant tissue.

TGB proteins are found in viruses belonging to several different genera. The TGB1 proteins could be generally divided into two groups: *Hordeivirus*-like TGBp1 and *Potexvirus*-like TGBp1 (Morozov and Solovyev, 2003). In both cases TGBp1s contain a NTP/helicase sequence domain and belong to helicases of superfamily I (SF-I) with the typical presence of seven conserved motifs (Gorbalenya et al., 1989). Both hordei-like and potex-like TGBp1, like many known helicases, can form homodimers or oligomers and are capable of self-interaction (Gorbalenya and Koonin, 1993; Cowan et al., 2002). TGBp1 of rod-shaped *Pomovirus* and *Hordeivirus* are larger comparing with potex-like TGBp1s – 39–63 kDa and contain additional long N-terminal domains (Solovyev et al., 1999). The presence of arginine/lysine-rich clusters was found in these extensions as well as the region of sequence similarity upstream of the helicase domain (Solovyev et al., 1999).

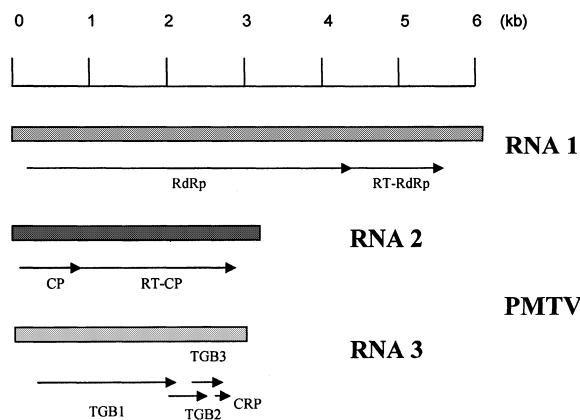


Fig. 1 The tripartite genome of *Potato mop-top virus* (PMTV). The translation products are represented by arrows. Description of abbreviations used in the Fig. 1 are: RdRp, RNA-dependent RNA polymerase; RT, read-through protein; CP, coat protein; TGB, triple gene block; CRP, cystein-rich protein

Unfortunately, PMTV full-length TGBp1 (463 amino acid, 51 kDa) was hard to express in its full-length in bacterial system (Pečenková et al., 2005). Therefore, we decided to examine bacterial expression of TGBp1 gene shortened on its 3'-end by two different restriction digestions and to use one of these two constructs for raising rabbit polyclonal antibodies.

Materials and Methods

Virus source and IC-RT-PCR

The PMTV isolate 54-10 was kindly provided by Dr Steen Lykke Nielsen from the Danish Institute of Agricultural Sciences, Flakkebjerg, Denmark.

cDNA of PMTV RNAs was obtained by immunocapture reverse transcription (IC-RT) PCR as follows. 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 washed again three times with PBS + T. After the final wash the RT and amplification with Superscript II (Gibco, Gaithersburg, MD, USA) and *Taq* polymerase following manufacturer's recommendations were carried out. The RT and subsequent PCR was performed using PMTV-specific primers (aaccatggaaa-gcggattcaacggaagt as a sense primer and atagat-cttccggaccatacctgtctgttt as an antisense primer), based on the sequence of Sw isolate available in GenBank database under the accession number AJ277556. The sense and antisense primers have introduced restrictions sites that will be relevant for further experiments, *Nco*I and *Bgl*II respectively (underlined). The PCR was carried out in 30 cycles: 30 s denaturation at 94°C, 30 s annealing at 55°C and 1 min elongation at 72°C.

Cloning procedure

The 1.3 kb fragment of interest was cloned to pTZR7T/A (Fermentas, St. Leon-Rot, Germany) using

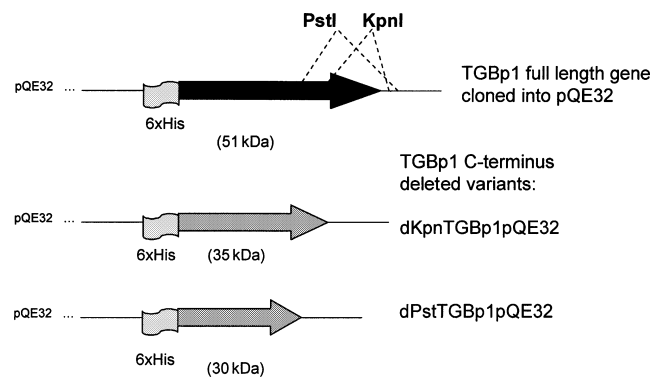


Fig. 2 The two *Potato mop-top virus* (PMTV) triple gene block protein 1 (TGBp1)-deleted variants that were used for protein production in bacteria. The C-terminus deletions were obtained using *Pst*I and *Kpn*I restriction sites located close to 3'-end of TGBp1 gene and in pQE32 polylinker

3'-A overhangs generated by *Taq* polymerase and then re-cloned into plasmid pQE32 (Qiagen GmbH, Hilden, Germany) using *Bam*HI and *Bgl*II sites, thus equipping the construct with 6xHis tag. The plasmid was then subjected to two different digestions: *Pst*I and *Kpn*I digestion, for which the suitable restriction sites were present in the second half of the TGBp1 gene as well as in the polylinker of pQE32. In both cases, the C-terminal fragment of several hundred base pairs was cut out (Fig. 2). All molecular cloning procedures were performed according to Sambrook et al. (1989).

Sequencing

The cloned fragments were sequenced in order to check the integrity of ORF, the presence of 6xHis tag on the N-end of expressed protein and the extent of TGBp1 deletion. The sequencing was performed using an ALF-expressII Sequencer with the AutoRead Sequencing Kit (AP Life Science, Goeteborg, Sweden), using QIAexpress pQE sequencing primers (Qiagen GmbH). The sequence analyses were carried out using programs available on EXPASY server (Gasteiger et al., 2003). The deduced amino acid sequence was aligned with the TGBp1 sequence available in GenBank under accession number AY426745 by CLUSTALW (Thompson et al., 1994).

Bacterial expression

The proteins were expressed and prepared in either standard (10 ml) or large (500 ml) culture of *Escherichia coli* according to the QIAexpressionist handbook (Qiagen GmbH). The protein expression was induced by 0.5 mM isopropyl-beta-thiogalactopyranosid (IPTG) for 3 h. For expression we used TG1 strain of *E. coli*, which were found to be the most suitable for our experiments.

Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE)

Deleted variant of TGBp1 was partially purified from 500 ml of the bacterial culture of selected colony. The pellet from 2 ml of bacterial culture was resuspended in 100 µl of the Laemmli buffer, boiled for 2 min and

aliquots were loaded on 12% polyacrylamide gel-containing sodium dodecyl sulphate (SDS; Laemmli, 1970). For visualization of the separated proteins Coomassie Brilliant Blue R250 was employed. Both the soluble and insoluble fractions prepared according to Lin and Cheng (1991) were subjected to SDS polyacrylamide gel electrophoresis (PAGE).

Chelating chromatography

The chromatography was performed on chelating Sepharose Fast Flow (Amersham Biosciences, Piscataway, NJ, USA) charged with Ni^{2+} ions according to manufacturer's protocol.

Western blot analysis

The proteins separated by SDS-PAGE were electroblotted to a nitrocellulose membrane (0.45 μm , Schleicher and Schuell Protran) in semidry system (OMNI-TRANS apparatus, Omnibio Brno, Czech Republic) according to Hirano and Watanabe (1990). The membrane was then incubated for 1 h in 4% bovine serum albumin (BSA) with PBS and then washed four times with PBS. The recombinant tagged proteins were detected with anti-His antibodies (Amersham Bioscience). The bands of interest were visualized by reaction with a substrate BCIP/NBT (Sigma Aldrich, St. Louis, Missouri, USA) according to Sambrook et al. (1989).

ELISA

Individual TGBp1 fractions from different steps of the purification process and plant material infected with PMTV were subjected to various types of enzyme-linked immunosorbent assay (ELISA); the double-antibody sandwich (DAS)-ELISA followed that of Clark and Adams (1977). The conditions for indirect plate-trapped antigen (IPTA) ELISA were performed according to Čeřovská et al. (2003b), in brief.

Plates were coated with antigens diluted in standard coating buffer and incubated overnight at 4°C. The ELISA plates were washed with PBS + T for four times. Commercial antibody (1 $\mu\text{g}/\text{ml}$) or anti-TGBp1 IgG (5 $\mu\text{g}/\text{ml}$) in conjugate buffer were added and incubated for 2 h at 37°C. Swine antirabbit IgG conjugated to alkaline phosphatase (SWAR-AP, Sigma Aldrich, St. Louis, Missouri, USA; 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 per ml of 0.1 M diethanolamine buffer, pH 9.8) was added.

Antisera production

The antisera against the bacterially expressed TGBp1 were prepared in New Zealand rabbits by three subcutaneous and one intramuscular injections of 350 μg of electrophoretically purified protein, which was directly cut out from the SDS gel, followed by its comminuting in liquid nitrogen and resuspension in PBS for 3-week interval. The protein was emulsified with an equal volume of Freund's complete adjuvant for the first injection and with Freund's incomplete adjuvant for subsequent

two injections. For the last injection the antigen was diluted in PBS. The rabbits were bled 2 weeks after the last injection. The serum fractions were collected and stored at -20°C until required. The immunoglobulin fraction from the antisera was obtained using caprylic acid fractionation (Steinbuch and Audran, 1969) and conjugated to alkaline phosphatase (Avrameas, 1969).

Results

Designing of C-deleted constructs of TGBp1

The PCR fragment comprising TGBp1 gene was cloned into pQE32 (Qiagen GmbH) using *Bam*HI and *Bg*II sites, thus being equipped with 6xHis tag. The plasmid was then digested with *Pst*I restriction enzyme that cutting out 520 bp from the 3'-end and with *Kpn*I, cutting out 428 bp from the 3'-end (Fig. 2). After religation and transformation two colonies, namely dKpnTGBp1pQE32 and dPstTGBp1pQE32 were sequenced. The alignment of obtained sequences with the full-length TGBp1 gene for the same isolate is illustrated in Fig. 3. Three mutations that appeared during the experimenting are marked in bold as illustrated in Fig. 3. However, according to protein structure prediction we did not expect these mutations to interfere with immunogenic features of antigen.

Expression and purification of PMTV-TGBp1

Both above-described constructs were used to transform *E. coli* TG1 and contrary to the full-length TGBp1, the strong protein expression for C-deleted TGBp1 variants was obtained. We selected one of these two constructs, namely dPstTGBp1pQE32 to produce the recombinant protein in large bacterial culture and to purify it (Fig. 4). We tried to purify the protein by means of chelating chromatography using its His-tag. The purified protein was not proved to have the homogenic pattern in analysis. For that reason we decided to use for antibodies raising the electrophoretically purified antigen.

Production of polyclonal antisera to a recombinant PMTV-TGBp1

For antisera production we used the protein product from the expression vector dPstTGBp1pQE32 transformed to *E. coli* TG1 clone. Antisera were obtained from bleeds taken 2 weeks after the fourth injection. The anti-PMTV-TGBp1 sera had titres of 1/2000 when tested in an indirect ELISA with purified fused protein. Obtained antisera gave strong reaction with homologous recombinant antigen in IPTA-ELISA. No reactions was obtained using DAS-ELISA (data not shown). In DAS-ELISA we were not able to detect the reactions with plant materials, in IPTA-ELISA we obtained significant differences in absorbances between the healthy and infective materials after overnight incubation with substrate (Table 1).

Western blot analysis

The obtained polyclonal antibodies reacted strongly with the homologous recombinant TGBp1 antigen for

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1                                                    50
dPstTGBp1pQE32 MRGSHHHHHH GIRLTMESGF NGSRPHRVKK DILDRVNPVN TQGSSGTTGN
dKpnTGBp1pQE32 MRGSHHHHHH GIRLTMESGF NGSRPHRVKK DILDRVNPVN TQGSSGTTGN
TGB1p1          .....MESGF NGSRPHRVKK DILDRVNPVN TQGSSGTTGN

51                                                    100
dPstTGBp1pQE32 AFRTNSNKKT QNWKPRSGPG NRNEGDQTKN IKSDLQQPSE VYPEEQVRPE
dKpnTGBp1pQE32 AFRTNSNKKT QNWKPRSGPG NRNEGDQTKN IKSDLQQPSE VYPEEQVRPE
TGB1p1          AFRTNSNKKT QNWKPRSGPG NRNEGDQTKN IKSDLQQPSE VYPEEQVRPE

101                                                    150
dPstTGBp1pQE32 SSTGESVKQQ SEPHRVLEDK KQSGKTAGSS VRIPEEGGGG LGSANYLGKR
dKpnTGBp1pQE32 SSTGESVKQQ SEPHRVLEDK KQSGKTAGSS VRIPEEGGGG LGSANYLGKR
TGB1p1          SSTGESVKQQ SEPHRVLEDK KQSGKTAGSS VRIPEEGGGG LGSANYLGKR

151                                                    200
dPstTGBp1pQE32 QLDFVAKLCV ESGFKSTGKP LKRYPAEFFK SSGLLEKFDK YLSSRLDKGC
dKpnTGBp1pQE32 QLDFVAKLCV ESGFKSTGKP LKRYPAEFFK SSGLLEKFDK YLSSRLDKGC
TGB1p1          QLDFVAKLCV ESGFKSTGKP LKRYPAEFFK SSGLLEKFDK YLSSRLDKGC

201                                                    250
dPstTGBp1pQE32 NLSQRESEVV LKNLRSKRAE QSFLAGAVTG VPGSGKTTLL RKVQCEGGFN
dKpnTGBp1pQE32 NLSQRESEVV LKNLRSKRAE QSFLAGAVTG VPGSGKTTLL RKVQCEGGFN
TGB1p1          NLSQRESEVV LKNLRSKRAE QSFLAGAVTG VPGSGKTTLL RKVQCEGGFN

251                                                    300
dPstTGBp1pQE32 SIVILGNPRS KTEFSNLPSC YTAKEILLG IAIKCEVLLI DEYTLTTSGE
dKpnTGBp1pQE32 SIVILGNPRS KTEFSNLPSC YTAKEILLG IAIKCEVLLI DEYTLTTSGE
TGB1p1          SIVILGNPRS KTEFSNLPSC YTAKEILLG IAIKCEVLLI DEYTLTTSGE

301                                                    350
dPstTGBp1pQE32 ILLLQPSLTS .....
dKpnTGBp1pQE32 ILLLQKITNS RIVILFGDRA QGSSNTLCSP EWLQVPRVDL QPSLTS....
TGB1p1          ILLLQKITNS RIVILFGDRA QGSSNTLCSP EWLQVQVIFQ SLTSRRRFGKA

351                                                    400
dPstTGBp1pQE32 .....
dKpnTGBp1pQE32 .....
TGB1p1          TANLCRRQGF DFEQSEHEDK VVESPYEGSS PPTDINIVFS ESTREDLLEC

401                                                    450
dPstTGBp1pQE32 .....
dKpnTGBp1pQE32 .....
TGB1p1          GIESTLVSDV QGKEYNTVTL FIPDEDEEYL TNAHLRSVAF SRHKFVLEIR

451                                                    478
dPstTGBp1pQE32 .....
dKpnTGBp1pQE32 .....
TGB1p1          CNPELFMQLI NGELASKQQP QTDRYGP
    
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Fig. 3 The alignment of the obtained *Pst*I and *Kpn*I-deleted triple gene block protein 1 (TGBp1) with the sequence of the full-length TGBp1 available in GenBank (accession number: AY426745, the first 12 amino acids were introduced with primer sequence). The 6xHis tag and amino acids that differ as a consequence of restriction enzyme digestion are introduced in boxes. Additionally, three mutations that appeared during experiments are marked in boxes as well

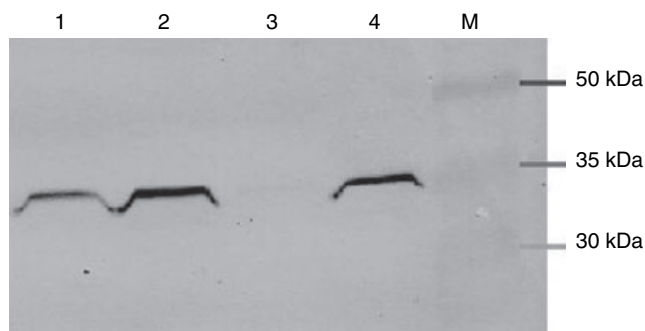


Fig. 4 The bacterial expression of triple gene block protein 1 (TGBp1) construct used for polyclonal antibodies production visualized using anti-His antibodies. Lane 1: bacterial extract from dPstTGBp1pQE32 transformed colony before IPTG induction; lane 2: bacterial extract from dPstTGBp1pQE32 transformed colony after IPTG induction; lane 3: expression of empty pQE32 plasmid after IPTG induction; line 4: non-soluble fraction of whole cell extract from the colony transformed with dPstTGBp1pQE32; lane M: molecular weight marker (Amersham Life Science, Little Chalfont, UK)

Western blot analysis (Fig. 5). The strong band at a position of M_r of approximately 32 kDa was revealed corresponding to the deduced molecular weight of the

Table 1
Detection of bacterially expressed PMTV-TGBp1 (BE TGBp1) and TGBp1 in infected tissue by means of IPTA-ELISA using IgG produced against recombinant TGBp1

Material tested	Commercial antibodies against PMTV-CP (Adgen)	IgG against BE-CP	
Buffer	0.20 ^a	0.20	
Healthy plants <i>Nicotiana benthamiana</i> (dilution 1 : 10)	0.20	0.20	0.30 ^b
PMTV-infected leaves <i>Nicotiana benthamiana</i> (dilution 1 : 10)	0.58	0.42	1.60 ^b
PMTV (BE TGBp1; 10 µg/ml)	0	1.10	1.90 ^b

^aA₄₀₅ values following 60 min incubation.
^bA₄₀₅ values following overnight incubation.
TGBp1, triple gene block protein 1; PMTV, Potato mop-top virus; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; IPTA, indirect plate-trapped antigen.

shortened TGBp1 which was used as an antigen. We did not detect specific reaction with the cells from the culture transformed with the empty pQE32. Our

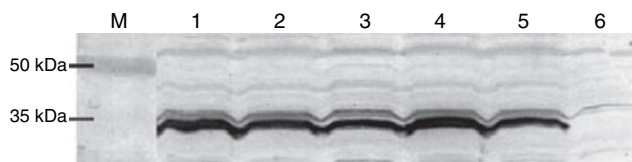


Fig. 5 Western blot analysis of reactivity of anti-triple gene block protein 1 (TGBp1) polyclonal antibodies with homologous antigen. Lanes 1 and 2: bacterial extract from the expression of the colony transformed with dPstTGBp1pQE32 construct prior and after induction; lanes 3 and 4: extract from another colony transformed with the same construct; lane 5: non-soluble fraction of whole cell extract from the colony transformed with plasmid with cloned insert from TGBp1 gene that was used for polyclonal antibodies production; lane 6: expression of empty pQE32 plasmid after IPTG induction; lane M: molecular weight marker (Amersham Life Science)

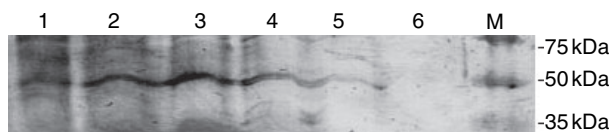


Fig. 6 Western blot of leaf and tuber extracts from *Potato mop-top virus* (PMTV)-infected *Nicotiana benthamiana* and *Solanum tuberosum* visualized using anti-triple gene block protein 1 (TGBp1) antibodies. Lanes 1 and 2: extracts from tubers of two different cultivars of *S. tuberosum* infected with Czech PMTV isolate Korneta-Nemilkov; lanes 3 and 4: leaf extracts from the same samples as in lanes 1 and 2; lane 5: leaf extract from *N. benthamiana* plant infected with PMTV Czech isolate Korneta-Nemilkov; lane 6: healthy *N. benthamiana* control plant; M: molecular weight marker (Amersham Life Science)

antibodies revealed that IPTG non-induced cells produced visible amounts of the antigen.

No reaction was seen with extracts from the leaves of healthy *Nicotiana benthamiana* plants. The slight reactions were apparent with the leaves of *N. benthamiana* infected with the Danish PMTV isolate 54-10 (faint band at a position corresponding to the $M_r = 50$ K of the complete TGBp1). The stronger reactions were detected with the leaves of *N. benthamiana* infected with the Czech PMTV isolates. The stronger reaction, which was obtained with the heterologous Czech isolate than with the homologous Danish one could be explained with the higher titre of the virus in the Czech isolate. Bands of the same size were found for extracts from PMTV-infected tubers and shoots of *S. tuberosum* (Fig. 6).

Discussion

We cloned the gene encoding the TGBp1 of PMTV into expression vector pQE32. In order to obtain strong bacterial expression of TGBp1 protein that seemed to be hard to express in bacteria or was toxic for bacteria, we tried to shorten this gene on its 3'-end by two different restriction digestions. After that efficient and high yield bacterial expression was achieved, as confirmed by SDS-PAGE and Western blot of high range bacterial culture lysate exploiting the presence of 6xHis tag on the N-terminus of these constructs.

One of these two constructs was used for raising rabbit polyclonal antibodies. The obtained sera and

antibodies were tested for the detection of PMTV in laboratory host *N. benthamiana* and natural host *Solanum tuberosum* by ELISA as well as by Western blots. The obtained antisera have been used successfully for plant virus detection by Western blot analysis and IPTA-ELISA, but they have failed in DAS-ELISA.

According to our expectation the failure to get positive results when the obtained antibodies were used for coating ELISA plates (DAS-ELISA) suggests that our antibodies not only recognize native epitopes, but also epitopes which are affected by some denaturation steps (e.g. binding to the surface of ELISA plates in binding buffer with high pH or SDS-PAGE in case of Western blot analysis), as this binding may affect their conformation (Korimbocus et al., 2002).

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 study of Petrzik et al. (2001) have 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 and IPTA-ELISA but not by DAS-ELISA, what suggested preferential detection of denatured PMTV proteins. These results obtained for TGBp1 were consonant with the former results obtained for antibodies raised against recombinant PMTV-CP (Čeřovská et al., 2003b), and Helias et al. (2003), who obtained only one serum reacting in DAS-ELISA (which gave in comparison with commercial polyclonal antiserum 10 times lower results).

The use of recombinant proteins is an attractive strategy for the production of antibodies against viruses, which are present in low concentrations in infected plants, or are difficult to purify. On the other hand, the use of antibodies against recombinant structural proteins in diagnostic tests seems to be impeded by their inefficiency in recognizing native epitopes. Alternative detection techniques, such as tissue blot immunoassay or DIBA-ELISA, may need to be developed to overcome these difficulties (Korimbocus et al., 2002).

The recombinant viral non-structural proteins expressed in bacterial cells have great potential as a source of antigens for raising specific antibodies. In the case of PMTV this way obtained antibodies could be used as an option for diagnostic purposes because as it was proved previously this virus is able to establish infection without the CP-encoding RNA (Savenkov et al., 2003). Thus, the detection based on some other non-structural proteins could be profitable when combined with other detection methods. Additionally, the benefit of obtaining anti-TGBp1 antibodies for us was their possible application in functional studies of

the fate of this protein in infected plant and its role in the viral movement through plant tissue.

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