

# BACTERIAL EXPRESSION OF RECOMBINANT PLANT VIRAL PROTEINS FOR RAISING ANTIBODIES

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## Introduction:

Our laboratory is concerned with raising antibodies against structural and nonstructural plant viral proteins, mainly against potato viruses. For recombinant antigens preparation different experimental techniques have been developed. Antigenic proteins which are non-toxic for bacteria were prepared by conventional cloning procedures followed by bacterial expression. By these methods we prepared antibodies against coat proteins of potato virus A (PVA) and potato mop-top virus (PMTV), and against the part of PMTV RNA-dependent RNA polymerase.

Some proteins are hard to express in bacteria for many reasons - proteins could be toxic for bacteria or they can be without modification improperly folded, thus being more unstable and degraded. The example of hard-to-express proteins are triple gene block proteins (TGBp) of plant viruses, which enable the spread of plant virus infection from infected cell to a neighboring non-infected cell. The first one, coding for TGBp1, contains RNA helicase consensus sequence motifs and displays *in vitro* nucleic acid binding activity. In order to obtain strong bacterial expression of PMTV movement proteins - triple gene block protein 1 (TGBp1) that seemed to be hard to express in bacteria, we have shortened the original cloned gene in two ways: by standard digestion with suitable restriction enzymes, and by exonucleaseIII/S1 digestion. The first approach was limited by the restriction map of the cloned fragment, and only few possibilities were exploited, while the second approach has given rise to the palette of similar constructs, differing only by several nucleotides, gradually deleted from the one end of full-length clone. The transformed bacteria were plated on the agar plate and screening of the transformants was performed with colony-blot procedure using anti-His antibodies after induction with IPTG from the inducible promoter. The screening revealed not only transformants with the correct reading frame of TGBp1, but also indicated expression levels of individual variants. Colonies giving the strongest signals were then selected for large culture expression.

## Materials and Methods

### Virus source and IC-RT-PCR

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

cDNA of PMTV RNAs was obtained by immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR). The reverse transcription and subsequent polymerase chain reaction (RT-PCR) was done using PMTV specific primers (aaccatggaagcggattcaacggaagt as a sense primer and atagatctccggaccatacctgtctgttt 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 restriction sites that will be relevant for further recloning, *NcoI* and *BglII*, respectively (underlined). The PCR was carried out in 30 cycles: 30 s denaturation at 94 °C, 30s 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) using 3'-A overhangs generated by Taq polymerase. Then it was recloned into plasmid pQE32 (equipping the construct with 6xHis tag) using *BamHI* and *BglII* sites (from which the two variants with deleted *KpnI* and *PstI* fragments from the 3' end were obtained, and into plasmids pQE30 and pQE31 using *SacI* and *HindIII* sites (QIAGEN GmbH, Germany). The sense primer introduced *NcoI* site to the start position of TGBp1; to make this site unique for further experiments, we destroyed pQE30/31 *NcoI* site prior to cloning by treatment with *NcoI*, *Klenow* fragment and *T4ligase* (MBI Fermentas). The *NcoI*- plasmids were named pQE30/31NK. After inserting the fragment into *SacI* site of pQE30/31NK, the *NcoI* site at 5' end of amplified gene was unique.

### Exonuclease digestion

The plasmids were then digested with *SacI* making ends resistant to exonucleaseIII and *NcoI* giving ends sensitive to exonuclease III. The digestion with exonucleaseIII and nuclease S1 were performed according to the manual obtained with ExoIII/S1 Deletion Kit (MBI Fermentas). To obtain deletion variants differing by only a few nucleotides, we used frequent aliquoting and tried different temperatures and salt concentration to slow down the reaction. The typical course of reaction is shown in Figure 2. Then, all aliquotes were mixed in one tube and the volume containing approximately 1 mg of DNA was loaded on the 1% SeaPlaque agarose gel (Cambrex Bio Science Rockland, Inc.). Fragments in the range of 4,7 to 4,2 kb was isolated, religated and used to transform competent cells.

Colony blotting, sequencing, SDS PAGE and Western blot analysis were done according Pečenková *et al.*, 2005.

### Antisera production

The antisera against the bacterially expressed TGBp1 were prepared in rabbits by three subcutaneous and one intramuscular injections of 350 µg of electroforetically purified protein in the three weeks intervals. The purified 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 three weeks after the last injection. The serum fractions were collected and stored at -20 °C until required.

Fig. 1. (A) The schematic drawing of TGBp1 gene cloned into pQE30/31NK vectors (pQE30/31 previously treated with *NcoI* and *Klenow* fragment) with marked restriction sites relevant for cloning and exonucleaseIII digestion. The *SacI* and *HindIII* sites were used for recloning of TGBp1 gene from pTZR into pQE30/31NK plasmids. (B) The schematic drawing of construction of TGBp1 deletion variants. The *SacI* site was used to create a DNA end resistant to exonucleaseIII digestion while the digestion with the *NcoI* produced ends sensitive to exonucleaseIII digestion.

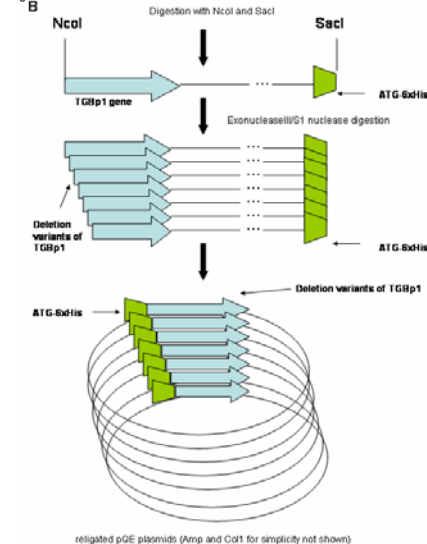


Fig 2: pQE30/31NK carrying TGBp1 gene linearized with *SacI* and *NcoI* restriction enzymes. 2. The example of progressive exonucleaseIII/S1 nuclease digestion of and subjected to exonucleaseIII/S1 nuclease digestion at 37°C, with 100mM NaCl. Samples were taken in intervals of 35".

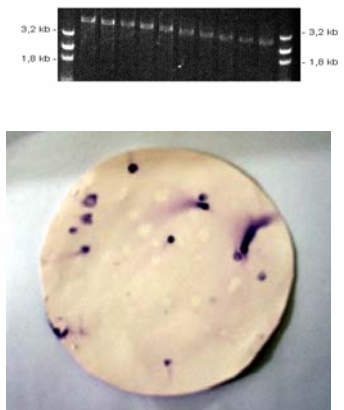


Fig. 3. Detection of clones with high-level expression of TGBp1 deletion variants by anti-His antibodies after colony blotting.

Fig. 4. Comparison of expression levels in selected clones carrying TGBp1 deletion variants. (A) Coomassie stained SDS-PAGE gel of expressed full-length TGBp1 and its deletion variants. Lane 1: pre-stained protein molecular weight marker (Amersham Life Science); lane 2: empty pQE32; lane 3: non-induced apparently full-length TGBp1 in pQE32; lane 4: induced apparently full-length TGBp1 in pQE32; lane 5-12: TGBp1 deletion variants - colonies exo1, exo3, exo5, exo11, exo13, exo14, exo15 and exo16, respectively; in all cases the whole cell extracts from transformed *E. coli* TG1 were used; lane 13: soluble fraction of whole cell extract of colony exo13; lane 14: insoluble fraction of whole cell extract of colony exo13; lane 15: insoluble fraction after centrifugation through 30% sucrose cushion. (B) Western blot analysis. The arrangement of samples in lanes is as in (A).

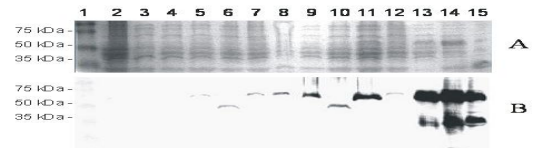


Fig 5. Western blot analysis of anti-TGBp1 polyclonal antibodies. Lanes 1 and 2: leaf extracts from *N. benthamiana* infected with Czech type PMTV; lanes 3 and 4: leaf extracts from *N. benthamiana* infected with Danish type PMTV; lane 5: healthy *N. benthamiana*; lane 6: non-soluble fraction of whole cell extract from the colony transformed with plasmid with cloned insert from TGBp1 gene that was used for polyclonal antibodies production; lanes 7 and 8: expression of the same construct after and prior to induction, respectively; lanes 9 and 10: expression of pQE32 plasmid after and prior to IPTG induction; lane 11: molecular weight marker (Amersham, Life Science); lane 12: leaf extracts from healthy *N. benthamiana*; lane 13: PMTV-infected leaf extract; lane 14: healthy *Solanum tuberosum* shoot extract; lane 15: healthy *S. tuberosum* tuber extract; lanes 16 and 17: PMTV infected *S. tuberosum* tuber extracts. Since the protein used for polyclonal anti-body raising was prepared from the gel, some reactivity with culture transformed with empty pQE32 was observed.



## Conclusions:

In this work, we describe a method of producing antisera against bacterially expressed proteins which were hard to express in its original full length. The cloned TGBp1 gene from PMTV was digested by conventional restriction digestion, however, this approach was limited to the 3' end of the gene where two suitable restriction enzymes sites were located. Additionally, the vector with cloned TGBp1 gene was deleted on its 5' end employing exonucleaseIII digestion. This way we obtained the sets of recombinant molecules which code for proteins differing only in several amino acids on the N-terminus of expressed protein, just behind the 6xHis tag. Beside the randomness as its main benefit, the approach involving exonucleaseIII digestion achieves results in the short period of time. The exonucleaseIII reactions under different conditions could be performed in half-a-day. If the standard cloning procedures go smoothly (one prior to exonucleaseIII digestion and the other one after exonucleaseIII digestion), it is possible to obtain the collection of clones in one week with the expectation that some of them will give the high yield protein expression. Additional one to two days are needed for the screening of the whole collection of clones by colony blotting procedure. We used one of our constructs for polyclonal antibodies raising. The obtained antibodies showed reactivity with the homologous recombinant antigen as well as with PMTV infected material.

## Reference

Pečenková, T., Filgarova, M., Cerovska, N. (2005) Protein expression and Purification, 41: 128-135