# IMMUNODETECTION OF BEET NECROTIC YELLOW VEIN VIRUS RNA3-ENCODED PROTEIN IN DIFFERENT HOST PLANTS AND TISSUES

Y. LI[1,3], C. WEI[1], P. TIEN[2], N. PAN[1], Z. CHEN[1,3]

[1]The National Laboratory of Protein Engineering and Plant Genetic Engineering, Peking University, Beijing 100871; [2]Institute of Microbiology, Chinese Academy of Sciences, Beijing, P.R. China; [3]De Montfort University, The Gateway Leicester, LE19BH, UK

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**Summary.** – The protein p25 open reading frame (ORF) of beet necrotic yellow vein virus – BNYVV RNA3 was cloned into bacterial expression vector downstream of the 5-'terminus part of ß-galactosidase ORF and the expressed p25 fusion protein was used to produce an antiserum. The latter was employed to detect the subcellular location of p25 in mechanically inoculated *Tetragonia expansa, Chenopodium quinoa* and sugarbeet leaves by Western blot assay. The results showed that p25 was present as a soluble protein only in the S30 fraction of *T. expansa, C. quinoa* and sugarbeet leaves infected with BNYVV.

**Key words:** beet necrotic yellow vein virus; p25 protein; subcellular localization; Western blot assay

## Introduction

BNYVV, the causative agent of sugar beet rhizomania (Tamada, 1975; Brant and Richards, 1989), a plus-stranded RNA virus with a multicomponent genome (Bouzoubaa *et al.*, 1987; Tamada *et al.*, 1989) is tentatively classified as a member of *Furovirus* genus. The five RNA components (RNAs 1 to 5) of BNYVV have length of 6.8, 4.7, 1.74, 1.5 and 1.4 kb (Kiguchi, T., personal communication). The RNAs are 5'-capped and 3'-polyadenylated and all of them have similarities at the 5'- and 3'-extremities (Bouzoubaa *et al.*, 1987; Quillet *et al.*, 1983). RNAs 1 to 4 have been sequenced, and their biologically active synthetic RNA transcripts, produced *in vitro* by bacteriophage T7 or SP6 RNA polymerase-directed run-off transcription are available (Bouzoubaa *et al.*, 1985, 1986, 1987; Ziegler *et al.*, 1988; Quillet *et al.*, 1989; Li *et al.*, 1995a,b). RNAs 1, 3, 4 and 5 are each capable of coding for a single polypeptide, whereas RNA2 contains six overlapping ORFs with the peculiarity that the first one, located at the 5'-extremity of RNA2 and encoding the 22 K viral coat protein (p22), ends with an amber stop codon which can undergo a suppression and give a protein of 85 K (p85) both *in vivo* and *in vitro* at a rate of about one out of every ten times. The p85 with the coat protein sequence at its N-terminus may be involved in virus assembly and virus-vector interactions (Ziegler *et al.*, 1985; Schmitt *et al.*, 1992).

The viral coat protein ORF readthrough extension into the adjacent ORF has also been reported for soil-borne wheat mosaic virus (SBWMV), type member of the *Furovirus* genus, and for luteoviruses (Waterhouse *et al.*, 1989; Bahner *et al.*, 1990). On RNA2 downstream of the p85 readthrough protein, there exist three ORFs, namely ORF III, IV and V, encoding the proteins p42, p13 and p15, respectively. The member genes of this "triple-gene-block" have homologous counterparts in genomes of barley stripe mosaic virus (BSMV), potexviruses and carlaviruses (Morozov *et al.*, 1989; Mamelink *et al.*, 1990; Koonon *et al.*, 1991). It has been found that the triple-gene-block is responsible for virus cell-to-cell movement (Gilmer *et al.*, 1992). The ORF V encodes a 14 K cysteine-rich protein which binds zinc ions in an *in vitro* test (Niesbach-Klosgen, U., personal communication) and regulates accumulation of RNA2 *in cis* and coat protein *in trans* (Hehn *et al.*, 1995).

**Abbreviations:** BNYVV = beet necrotic yellow vein virus; BSMV = barley strip mosaic virus; ORF = open reading frame; PBS = phosphate buffered saline; SDS = sodium dodecyl sulphate; ssDNA = single-stranded DNA; Tris = tris-(hydroxymethyl)-aminomethane

The 237 K protein (p237) encoded by RNA1 contains a nucleotide triphosphate binding site and associated sequences thought to be characteristic of helicase and a core polymerase consensus sequence (Gorbalenya *et al.*, 1988; Hodgman *et al.*, 1988; Poch *et al.*, 1989) found in all known or presumed plus-strand viral RNA replicases. That RNA1 alone carries all the necessary information for viral replication has been proved by protoplast infection experiments with various combinations of BNYVV RNAs provided as synthetic *in vitro* transcripts (Bouzoubaa, S., personal communication).

RNAs 3 and 4, and the recently discovered RNA5, carry ORFs for proteins p25, p31 and p19, respectively. A 600-nucleotide-long subgenomic RNA3 (a 3'-terminal portion of RNA3) is produced in the course of virus infection and is readily detected in total RNA extracts of infected leaves and roots but it is not encapsidated into virus particles (Bouzoubaa *et al.*, 1991). A 4.6 K ORF (p4.6) is located near the 5'-terminus of subgenomic RNA3 (Jupin *et al.*, 1992). In the case of RNA4, a second small ORF (p6.5) is situated on RNA4 upstream of p31. Whether the ORFs of RNAs 3 and 4, and the 19 K polypeptide encoded by RNA5 are expressed *in vivo* is unclear (Jupin *et al.*, 1991).

Whereas BNYVV RNAs 1 and 2 are sufficient to maintain the virus after mechanical inoculation of *C. quinoa* and *T. expansa* leaves (Li *et al.*, 1995b), the presence of four or all five RNAs seems to be obligatory for natural infection of sugar beet roots involving transmission by fungus (Tamada *et al.*, 1989). Therefore, RNAs 1 and 2 represent apparently the essential minimum for virus replication, while RNAs 3, 4 and 5 may be involved in the symptoms production, virus translocation, and vector transmission. In mechanical infection of *C. quinoa* and *T. expansa* leaves, the presence of RNA3 in the virus inoculum changes the lesion phenotype from a pale green to bright yellow colour (Li *et al.*, 1995b). However, in the mechanically infected sugarbeet plants, the presence of RNA3 in the virus inoculum damages very much the sugarbeet roots and leaves (Li *et al.*, 1995b).

In this study we have used the RNA3-encoded p25 fusion protein expressed in *E. coli* to raise a polyclonal antibody. The latter was used to investigate the subcellular localization of the p25 in BNYVV-infected *T. expansa*, *C. quinoa* and sugarbeet plants by Western blot assay.

## Materials and Methods

*Virus.* Plants were inoculated with a mixture of BNYVV Rg1 isolate (containing only RNAs 1 and 2) and infectious *in vitro* transcripts of RNAs 3 and 4 as described by Li *et al.* (1995b). The virus concentration in strongly BNYVV-infected sugarbeet leaves was determined by ELISA. Leaves showing high virus concentration were used for protein extraction. In the case of *T. expansa* and *C. quinoa*, heavily infected leaves showed about 140 local lesions per leaf. The local lesions were excised for protein extraction.

*Cloning and bacterial transformation* were carried out basically as described by Sambrook *et al.* (1989).

*Antiserum preparation.* A 100 µg dose of the fusion protein was emulsified with an equal volume of complete Freund's adjuvant and injected subcutaneously in the rabbit's hind leg. In order to increase the reaction three booster injections of 100 µg of the fusion protein in incomplete Fruend's adjuvant were given after 1, 2 and 3 weeks. The rabbit was bled 10 days after the last injection. The antiserum was stored in 0.001% sodium azide. Its reactivity was tested by Western blot analysis using a preimmune serum as negative control.

*Preparation of samples for immuno-detection of infected materials.* Virus inoculum or 0.05 mol/l sodium phosphate pH 7.5 (control) was mechanically inoculated on sugarbeet. After transplantation of the infected sugarbeet seedlings for 4 weeks, the leaves from plants expressing systemic symptoms and controls were excised for extraction and subjected to ELISA.

Sugarbeet leaves with systemic infection were used to inoculate *T. expansa* and *C. quinoa*. Controls were inoculated with healthy sugarbeet tissue extract. Ten days after inoculation the local lesions on *T. expansa* and *C. quinoa* leaves were excised for extraction.

*Western blot assay.* Fifty µg of protein per sample was used for SDS-PAGE. Electrophoresis, transfer of proteins to nitrocellulose membrane, and immunological detection of p25 protein with antiserum raised against the fusion protein were performed as described by Burgermeister and Koening (1984).

*Proteins* were extracted from mechanically infected leaves of sugarbeet, *T. expansa* and *C. quinoa* by homogenizing 100 mg (fresh weight) of tissue with 1500 µl of the Laemmli sample buffer and by heating the suspension at 100°C for 5 mins and centrifuging at 13,000 rpm for 10 mins in a minicentrifuge. The protein concentration in the extracts was estimated by dye-binding dot assay.

*Isolation of subcellular components.* Fractions enriched in different subcellular constituents were obtained essentially as described by Godefroy-Coolburn *et al.* (1986). Mechanically infected leaves of sugarbeet, *C. quinoa* or *T. expansa* (4 g) were harvested 10 days after inoculation and cut into small pieces in ice-cold extraction buffer (100 mmol/l Tris, 10 mmol/l KCl, 5 mmol/l $MgCl_2$, 0.4 mol/l sucrose, 10% glycerol, 10 mmol/l ß-mercaptoethanol pH 7.5). The extraction was carried out for 15 mins at 4°C. The cell debris was removed by centrifugation at 2,500 rpm for 5 mins through a nylon screen of 100 µm pore size.

Fractions P1 (nuclei and chloroplast proteins), P30 (membrane proteins), S30 (soluble proteins), ST (residual membrane proteins) and CW (cell wall proteins) were collected basically according to Godefroy-Coolburn *et al.* (1989).

## Results and Discussion

### *Cloning and expression of p25 in E. coli*

The BNYVV RNA3 p25 was expressed as a fusion protein with ß-galactosidase in vector pEX3. Since an *Eco*RV restriction site was absent but required upstream

of the AUG in the cloned insert of p25 ORF, the site-directed *in vitro* mutagenesis (McClary *et al.*, 1989) was applied. The used mutagenic oligodeoxynucleotide primer 5' GCG CCT AAG ATA TCA CCG ATG GCG GTC AC-3' was complementary to RNA3 nt 437-466 except one nucleotide (underlined). The *Eco*RV site was located three triplets upstream of the AUG in this primer. The primer was synthesized with a DNA synthesizer (Biosearch Inc.) and phosphorylated by T4 DNA polynucleotide kinase.

In the case of plasmid pGB533, containing full length DNA sequence of BNYVV RNA3 (Li *et al.*, 1995a), very low yields or sometimes even none of ssDNA were obtained after complementary DNA synthesis, extraction, precipitation and analysis by agarose gel electrophoresis, in contrast to plasmid pGBCP containing the BNYVV coat protein gene. Therefore the *Acc*I/*Xba*I-deleted pGB533 plasmid, lacking 750 bases of the 3'-end of the viral cDNA, was prepared. This *Acc*I/*Xba*I shortened pGB533 was named pgBΔA/X533. After transfection of pGBΔA/X533 into CJ236 cells and infection of the latter with helper phage M13K07, the ssDNA was produced in high yields (Fig. 1). To understand the reason why pGB533 produced so little or sometimes no ssDNA, we analyzed the secondary structure of RNA3. The results showed (Fig. 2) that its 3'-terminal region (nt 800-1300) can fold into a very stable secondary structure.

The second strand synthesis was performed by annealing the phosphorylated mutagenic primer to the single-stranded U-DNA template (pGBΔA/X533) and adding T4 DNA polymerase, T4 DNA ligase and all four dNTPs. The produced heteroduplex

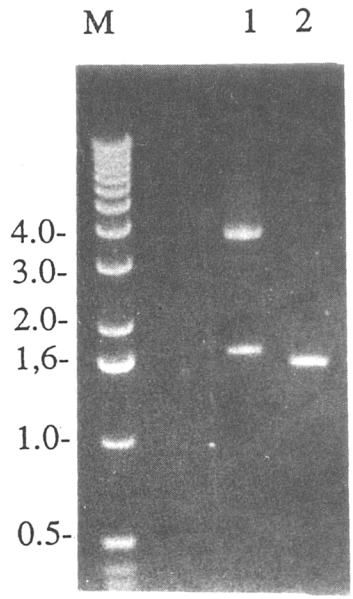


**Fig. 1**
**Agarose electrophoresis of ssDNA synthesized from plasmids pGBΔA/X533 and pGBCP in *E. coli***
ssDNA of pGBΔA/X533 (lane 1) and pGBCP (lane 2). Size marker 1 kb DNA ladder (lane M).

was transfected into *E. coli* strain MV1190 containing an active uracil N-glycosylase which excised the uracils and created apyrimidinic sites. This inactivated the parental strand and the ma-

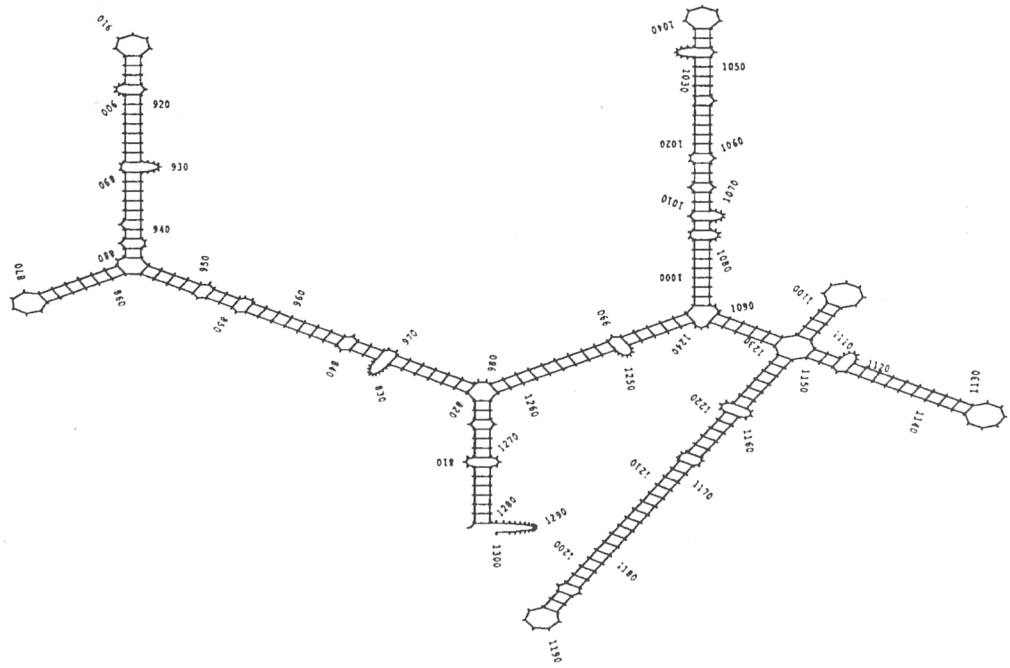

**Fig. 2**
**Predicted secondary structure of a portion (nt 888-1300) of BNYVV RNA3**

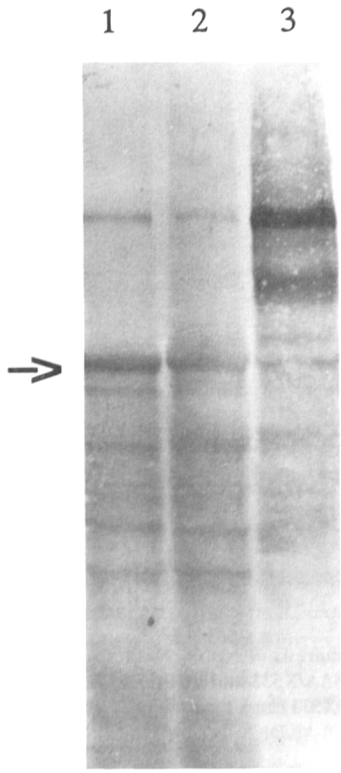


**Fig. 3**
**Western blot assay of p25-ß galactosidase fusion protein**
Antiserum against ß-galactosidase was used for detection. Lysates containing the fusion protein (arrow, lanes 1 and 2). Lysate of bacterial cells transformed with pEX3 (control, lane 3).

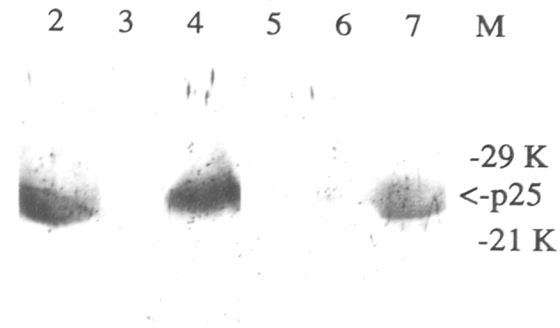


**Fig. 4**
**Western blot assay of p25 in the S30 fraction of BNYVV-infected sugarbeet leaves and local lesions on *T. expansa* and *C. quinoa* leaves**
Antiserum against fusion protein was used for detection. At least 40 µg of protein was applied to each lane. Mock-inoculated *C. quinoa* (lane 1), BNYVV-inoculated *C. quinoa* (lane 2), mock-inoculated *T. expansa* (lane 3), BNYVV-inoculated *T. expansa* (lane 4), mock-inoculated sugarbeet (lane 5), BNYVV-inoculated sugarbeet, samples of lower (lane 6) and higher (lane 7) concentration.

jority of progeny phagemids contained the desired *Eco*RV site (vector pGBM533).

An *Eco*RV/*Bam*HI fragment containing RNA3 p25 ORF was cloned into the *Eco*RV/*Bam*HI-shortened pEX3 vector. Competent cells of *E. coli* POP 2136 strain were transformed with this recombinant plasmid. Positive transformants were grown for 16 hrs at 28°C in medium containing 100 µg/ml ampicillin. The expression promoter was then activated by growing cultures at 42°C. After 2 hrs the amount of fusion protein reached its maximum. An extension of the culturing time did not increase but reduced the amount of the fusion protein. Thus, the fusion protein might have a cytotoxic effect on the bacterial cells. After lysing the bacterial cells by boiling in the Laemmli sample buffer for 1 min the proteins were applied to 10% polyacrylamide gels containing 0.1% SDS.

The concentration of the expreseed fusion protein obtained from 8 ml of bacterial culture ranged from 3 to 4 µg per ml. The protein concentration was estimated by a dye-binding dot assay.

*Isolation of the fusion protein p25-ß-galactosidase*

After SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the gels were either stained with Coomassie Blue R 250 or electroblotted to nitrocellulose membrane for immunostaining with ß-galactosidase antiserum (Fig. 3). For identifying the fusion protein in the gel, its $M_r$ was calculated. Since the RNA3 ORF protein and the *Eco*RV/*Bam*HI-shortened ß-galactosidase have $M_r$ of about 25 K and 45 K, respectively, $M_r$ of the fusion protein should be about 70 K. A protein band with $M_r$ of 75 K was immuno-stained with ß-galactosidase antiserum indeed. To isolate the fusion protein, the bacterial lysate was subjected to a preparative 0.1% SDS-10% PAGE. After electrophoresis, the fusion protein was identified by staining the gel strip containing the molecular mass marker proteins and two gel strips cut a both sides of the preparative gel. The fusion protein band was cut out from the gel, dispersed into small pieces by passing it twice through a 10 ml syringe, and dissolved in PBS by ultrasonic treatment for 3 x 1 min and overnight shaking. The protein was recovered in the supernatant after centrifugation at 13,000 rpm for 10 mins. It was reexamined by SDS-PAGE and/or Western blot assay.

*Western blot assay of p25 in BNYVV-infected plants*

The RNA3 p25 was not detected with antiserum raised against the fusion protein in samples extracted from differ-

ent plant materials. However, the coat protein of BNYVV was found in the same samples with an antiserum raised against BNYVV virions (data not shown).

When individual subcellular fractions of the same plant tissues were analyzed by Western blot using the same antiserum, the p25 protein was detected only in the S30 fraction of systemically infected sugarbeet and local lesions of *T. expansa* and *C. quinoa* leaves, but not in other fractions. These results agree with those of Niesbach-Klosgen *et al.* (1990) obtained with BNYVV-infected *C. quinoa.*

Since the concentration of p25 protein in the sugarbeet was much lower than that in the *T. expansa* (Fig. 4), more concentrated samples were needed for a visible reaction in the case of sugarbeet.

The p25 protein encoded by RNA3 always appeared on Western blots as a very diffuse band. This could be caused by incomplete reduction of disulfide bonds present in this protein, which has a very high cysteine content (8 cysteine residues in 280 amino acids). The same phenomenon was reported also by Niesbach-Klosgen *et al.* (1990) for the BNYVV RNA2 14 K protein, which has a very high cysteine content, too.

Jupin *et al.* (1992) have proved that p25 can bind zinc ions in the form of fusion protein, and they predicted that the region of amino acids 73 to 90 contains "zinc-finger" motif, which probably regulates the transcription of virus genome.

The RNA3 ORF-encoded p25 is a soluble protein localized in the cytosolic S30 fraction of infected leaves of *C. quinoa, T. expansa* and sugarbeet. We do not know yet, if the cytosolic localization of p25 in the leaves reflects the situation in infected sugarbeet roots. It is possible that p25 can interact with subcellular structures specific for the root tissue of sugarbeet resulting in lateral root symptom formation. Recently, Haeberle *et al.* (1995) studied the subcellular localization of p25 and coat protein of BNYVV in infected *Chenopodium murale* and *C. quinoa* by immuno-electron microscopy and detected p25 in both the cytoplasm and nuclei of infected leaf cells. But they failed to label p25 with immunogold in root cells of *C. murale* even though the protein was easily detectable by Western blot assay of root extracts. These results do not correspond to those of Niesbach-Klosgen *et al.* (1990) and ours. This discrepancy may have two possible explanations: (1) epitopes on SDS-denatured and glutaraldehyde-fixed proteins may be different, (2) in the earlier study, p25 associated with the nuclei was released or degraded in the course of preparation of the crude nucleus-enriched fraction (Haeberle and Stussi-Garaud, 1995). Anyhow, further work is needed to elucidate the function of p25 and to solve the question of its possible origin.

Although the amount of p25 is much lower than that of the coat protein in the sugarbeet plants, it apparently can cause great damage to them (Li *et al.*, 1995b). Monoclonal antibodies against p25 expressed in transgenic sugarbeet plants could perhaps block the activity of the trace of p25 so that the virus replication, translocation and/or transcription would be interrupted.

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