

## Detection of potato mop-top virus capsid readthrough protein in virus particles

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**Potato mop-top furovirus (PMTV) RNA 3 encodes the 20 kDa coat protein and a larger readthrough protein of 67 kDa. The readthrough protein is expressed by suppression of the amber stop codon which terminates the coat protein gene. A 21 kDa C-terminal fragment of the readthrough protein was cloned, fused to glutathione S-transferase and expressed in *E. coli*. An antiserum prepared against purified fusion protein was used in ELISA to detect the readthrough protein in extracts of PMTV-infected leaves. Immunogold labelling studies showed that the readthrough protein was located near one extremity of some of the virus particles.**

Potato-mop top furovirus (PMTV) is transmitted by the soil-borne powdery scab fungus *Spongospora subterranea* (Jones & Harrison, 1969; Arif *et al.*, 1995). PMTV particles contain three species of single-stranded RNA (Scott *et al.*, 1994). Nucleotide sequence analysis of RNA 3 of the Scottish isolate T of PMTV (PMTV-T) shows that it contains an open reading frame encoding the coat protein (20 kDa) which is terminated by an amber stop codon and followed in-phase by a second open reading frame encoding a readthrough domain of 47 kDa (Kashiwazaki *et al.*, 1995). The genomes of two other furoviruses, soil-borne wheat mosaic virus (SBWMV; Shirako & Wilson, 1993) and beet necrotic yellow vein virus (BNYVV; Bouzoubaa *et al.*, 1986) also encode capsid proteins with stop codons that can be suppressed to produce readthrough proteins.

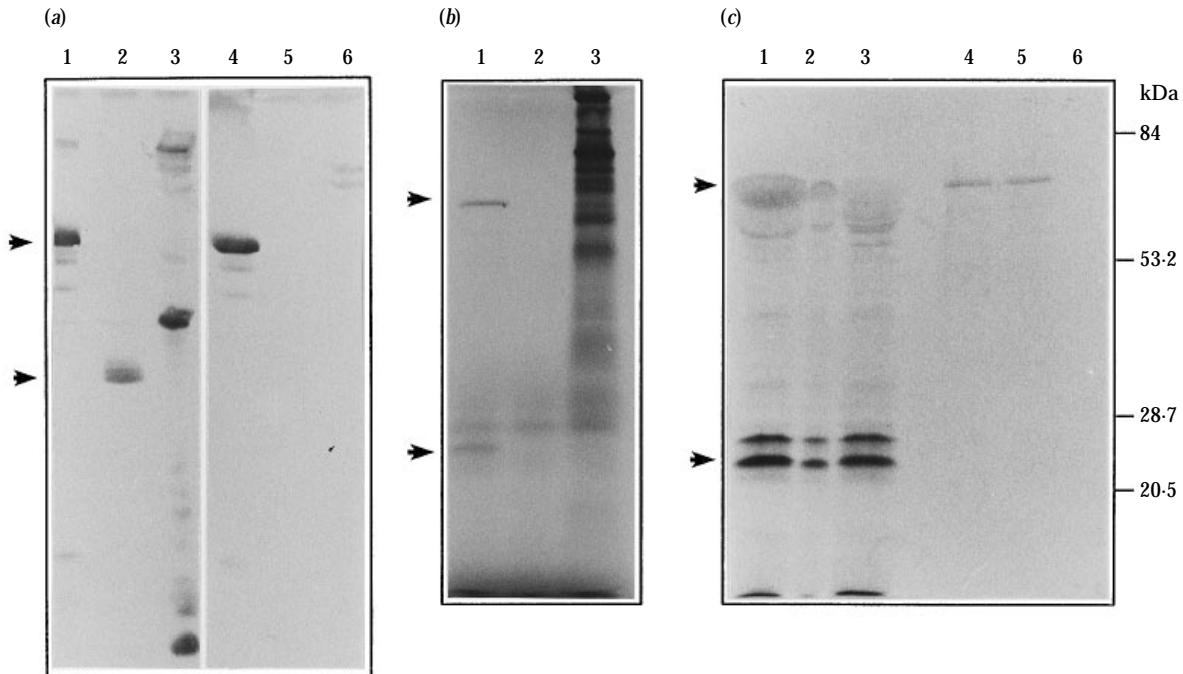
Immunogold labelling experiments have shown that the BNYVV capsid readthrough protein, p75, is incorporated into virions and is predominantly located at one end of the particles (Haerberle *et al.*, 1994). The readthrough domain of BNYVV plays a role in virus assembly (Schmitt *et al.*, 1992) and transmission of the virus by its fungal vector, *Polymyxa betae* (Tamada & Kusume, 1991). Spontaneous deletions which occur in the C-terminal part of the readthrough domain during manual passage have been shown to abolish transmission by *P. betae* (Tamada & Kusume, 1991; Tamada *et al.*, 1996). The role

of the putative PMTV capsid readthrough protein is unknown but by analogy with BNYVV it may also play a role in virus transmission by the fungal vector. This paper reports the production of a PMTV readthrough-specific antiserum and detection of the readthrough domain in virus particles. The results provide the first evidence that the readthrough domain is expressed and remains attached near one extremity of a few of the virus particles.

Preliminary attempts to clone the sequence which encodes the complete readthrough domain were unsuccessful (data not shown). Therefore, a 575 bp *Bam*HI- and *Eco*RI-digested fragment from an RNA 3 clone (pPMTV-21) of PMTV-T (Kashiwazaki *et al.*, 1995) that encodes the C-terminal portion (551 bp sequence) of the readthrough protein was ligated into the expression vector pGEX-3X (Pharmacia). *E. coli* strain TG1 cells were transformed for expression of a glutathione S-transferase protein C-terminally fused to the cloned readthrough protein fragment (GST-RT; approximate molecular mass 49 kDa). Preliminary experiments indicated that following induction by 0.05 mM IPTG for 4 h at 30 °C the GST-RT was expressed in an insoluble form. Experiments to promote soluble expression by altering both the induction temperature and concentration of IPTG proved unsuccessful (data not shown). However, soluble GST-RT was obtained by adding 6 M urea to *E. coli* lysates (prepared as described by Frangioni & Neel, 1993) followed by extensive dialysis against PBS (0.01 M, pH 7.4). Insoluble proteins were removed from the lysate by centrifugation (8000 g for 5 min). Purified preparations of GST-RT were obtained by affinity chromatography on glutathione-Sepharose 4B (GS4B) resin (Pharmacia) following the manufacturer's instructions. The purified fusion protein was treated with Factor Xa protease (Promega) to remove GST but the free readthrough protein fragment became insoluble after cleavage, and the complete fusion protein was therefore used as immunogen.

An anti-GST-RT serum (code name anti-RT) was prepared in a New Zealand White rabbit. Approximately 70 µg of purified GST-RT fusion protein was emulsified in Freund's incomplete adjuvant and injected subcutaneously at four sites on the back on days 0 and 10. Antiserum was obtained from bleeds taken 2 weeks after the second injection and at 2 week intervals thereafter. The anti-RT serum had a titre of 1/2000 when tested in ELISA with affinity-purified GST-RT-coated

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**Fig. 1.** (a) Reaction in immunoblots of GST-RT antiserum with *E. coli*-expressed proteins. Lanes 1, 2 and 3, untreated antiserum; lanes 4, 5 and 6, antiserum cross-absorbed with lysate of *E. coli* cells expressing GST. Lanes 1 and 4, purified GST-RT; lanes 2 and 5, purified GST; lanes 3 and 6, control *E. coli* lysate. Arrows indicate purified GST-RT (49 kDa) and purified GST (26 kDa). (b) Autoradiogram of SDS-PAGE of *in vitro* translation products of PMTV-T RNA 3. Lane 1, translation products of PMTV RNA 3; lane 2, negative control (translation mix minus template); lane 3, positive control (TMV template RNA). Autoradiogram developed after overnight (ca. 16 h) exposure to gel. (c) Autoradiogram of translation products after immunoprecipitation with anti-RT antiserum. Lanes 1 and 2, supernatant or unprecipitated fractions, and lanes 3 and 4, precipitated fractions (anti-RT serum used at dilutions of 1/50, 1 and 4 or 1/250, 2 and 5); lane 5, supernatant and lane 6, precipitate after incubation with control anti-CMV antiserum. Arrows indicate PMTV readthrough protein and coat protein. Positions of molecular mass markers (Bio-Rad prestained low range) are shown on the right of (c). Autoradiogram developed after 1 week exposure to gel.

microtitre plates. The immunoglobulin (Ig) fraction was obtained by ammonium sulphate precipitation.

Immunoblotting with the anti-RT serum showed that it contained antibodies specific for the GST-RT fusion protein, the carrier GST and bacterial host proteins. However, the antibodies against bacterial host proteins and GST were readily removed following cross-absorption with a preparation of *E. coli* cells expressing GST (Fig. 1a).

*In vitro* transcription, translation and immunoprecipitation were done essentially as described by Ziegler *et al.* (1985). Briefly, the pPMTV-21 clone was digested with *SpeI*, purified by phenol-chloroform, and transcript was prepared using an RNA transcription kit (Stratagene) following the manufacturer's instructions. *In vitro* translation of transcript RNA was by a rabbit reticulocyte lysate system (Boehringer). The [<sup>35</sup>S]methionine-labelled products of translation were visualized by autoradiography after SDS-PAGE (Laemmli, 1970).

Three bands were evident following SDS-PAGE of *in vitro* translation products, with estimated molecular masses of 66.6, 24.9 and 22.4 kDa (Fig. 1b). The 24.9 kDa product is probably an artefact of the system as it was also visible in the controls. The ratio of readthrough to coat protein was estimated by

densitometry in two independent experiments. After adjustment to allow for the difference in methionine residues (the coat protein and readthrough contain two and seven methionine residues respectively), the result indicated that suppression of the amber stop codon occurred with a frequency of 17%.

The 66.6 kDa (readthrough) and the 22.4 kDa (coat protein) translation products were immunoprecipitated with the anti-CP Ig (data not shown), and the 66.6 kDa translation product was immunoprecipitated with the anti-RT Ig (Fig. 1c). None of the translation products were precipitated by the control anticucumber mosaic virus (CMV) Ig.

Previously, immunoblotting experiments with purified preparations of virus particles did not reveal evidence of readthrough protein (Torrance *et al.*, 1993). Since PMTV particles are fragile it is possible that the readthrough protein may be lost during virus purification. Therefore, the following experiments were done with extracts from infected plants. The anti-RT antiserum was used in ELISA to detect readthrough protein in extracts from leaves infected with PMTV-S (a Scottish isolate; Arif *et al.*, 1995) and M18 (a Peruvian isolate; Mayo *et al.*, 1996). Tests using anti-PMTV coat protein MAb

**Table 1.** Detection of readthrough protein in PMTV-infected leaf extracts by ELISA and immunosorbent electron microscopy

(a) ELISA using anti-PMTV MAb SCR 69-coated microtitre plates

PMTV isolate*	Detecting antibody†	
	Anti-RT	Anti-CP
PMTV-M18	0.54 (1.81)‡	2.20 (2.20)
PMTV-S	0.26 (0.84)	2.12 (2.56)
Control extract	0.10 (0.22)	0.09 (0.19)

(b) Numbers of virus particles per standard area trapped on antiserum-coated electron microscope grids

Virus	Coating antibody†			
	Anti-CP	Anti-RT	Anti-TRV	None
PMTV-M18	490	219	44	0
PMTV-S	298	116	19	6
TRV	7	14	938	19

\* PMTV-infected (or control non-infected) leaf extracts were used at 1/10 dilution.

† Anti-RT, anti-readthrough Ig; anti-CP, anti-potato mop-top virus coat protein Ig; anti-TRV, anti-tobacco rattle strain PRN Ig preparation.

‡  $A_{405}$  recorded after 1 h, and 16 h in parentheses.

SCR 69 or polyclonal Ig (anti-CP or anti-RT) to coat ELISA plates (Torrance, 1992; Torrance *et al.*, 1993) showed that readthrough protein was detected in the extracts, and the results from a typical experiment using MAb SCR 69 to coat plates are given in Table 1(a). These tests show that readthrough protein is produced in plants, but cannot determine whether it is attached to some virus particles or exists as free readthrough protein which is trapped or detected in ELISA via the CP domain.

In five independent immunosorbent electron microscopy (ISEM) experiments (done essentially as described by Roberts, 1986), the anti-RT Ig preparation specifically trapped PMTV particles on coated grids. We consistently observed that fewer particles were trapped (from the same leaf extracts) by the anti-RT than by the anti-CP Ig preparation. The average number of virus particles per standard grid aperture at 10000 $\times$  magnification was recorded to compare numbers of particles trapped by the different antisera, and particle counts from one experiment are given in Table 1(b). The numbers of particles of the unrelated tobacco rattle virus strain PRN (TRV) trapped on anti-RT or anti-CP grids were similar to control uncoated grids.

Immunogold electron microscopy experiments were done to try to determine the location of the readthrough protein in

the virus particles. An anti-RT Ig preparation (0.5 mg) was dialysed against 2 mM sodium tetraborate buffer, pH 9.0, and then conjugated to colloidal gold (Aurobeads G10 RPN476, Amersham) following the manufacturer's instructions. The methods for antibody coating and labelling were those of Pereira *et al.* (1994). Grids were stained with 2% sodium phosphotungstate, pH 6.5. The experiments were done by trapping particles with anti-CP Ig because larger numbers of particles were trapped by anti-CP than by anti-RT Ig, and preliminary experiments showed that few particles were gold-labelled irrespective of the trapping Ig. In two experiments, only eight out of 2200 virus particles trapped on grids by anti-CP Ig were labelled with gold after incubation with anti-RT-gold conjugate (Fig. 2a), and in a total of six experiments, only 24 virus particles were found to be associated with gold. Of the 24 gold-labelled particles, 21 were labelled at one extremity and more than one gold particle was seen associated with approximately half of them. In a few instances, gold particles were seen close to the ends of virus particles which seemed to be uncoiling (Fig. 2b). This is the same extremity that is labelled with SCR 68-gold conjugate, and is thought to be the concave end of the particle (Pereira *et al.*, 1994). There was almost no gold found elsewhere on the grids (Fig. 2c).

Therefore, the electron microscopy data show that anti-RT antibodies can trap virus particles, and anti-RT-gold was located at one extremity of the particles. Although only a few particles were labelled with gold, the labelling was very specific, and there was almost no background labelling. Also, where virus particles were associated with gold it was mostly at one extremity indicating that this was not a chance occurrence. The low background labelling also supports the hypothesis that little free readthrough occurs in plants. Far fewer particles were labelled with gold in our experiments compared with the 4–20% labelled in experiments with BNYVV (Haeberle *et al.*, 1994). However, some of the PMTV particles were labelled with immunogold at the extremity at which particles uncoil, and it is possible that the readthrough protein may be lost during uncoiling, which seems characteristic of PMTV (Pereira *et al.*, 1994; Harrison, 1974).

It was shown that the N terminus of the coat protein is exposed at the surface along the sides of the particles (Pereira *et al.*, 1994). It is unlikely that readthrough protein (or smaller fragments of it undetected by our antiserum) remains attached to the sides of the particles because MAb SCR 69 (which is specific for the N-terminal residues) coats virus particles evenly along the entire length (Pereira *et al.*, 1994); the presence of the readthrough domain would probably cause steric hindrance and prevent binding of the MAb. Our results are consistent with the view that the complete readthrough domain remains attached only to the coat protein subunits at one extremity of the particles.

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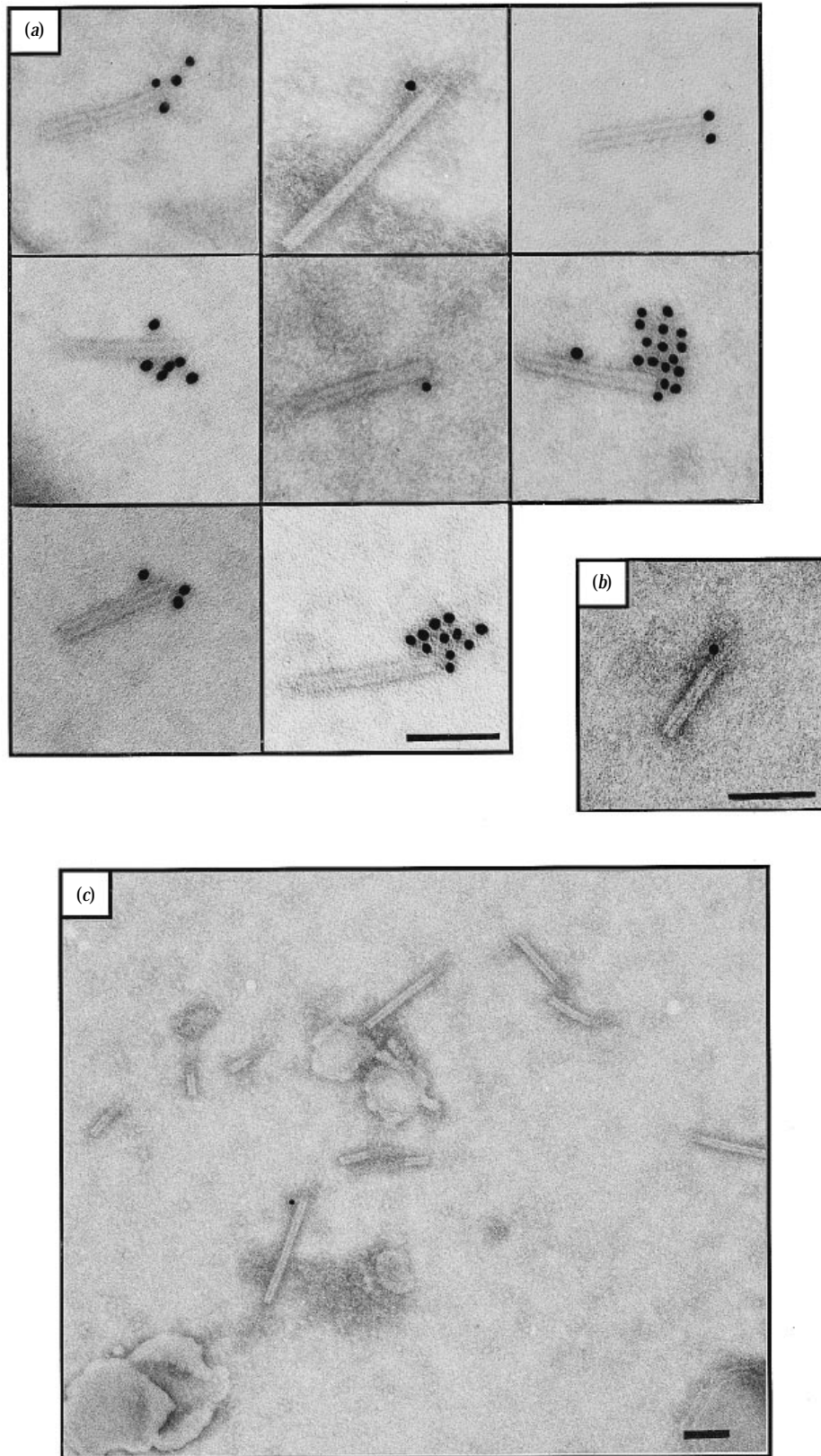


Fig. 2. (a). Electron micrographs of PMTV particles labelled with anti-RT-immunogold conjugate. (b) Labelled PMTV particle showing uncoiling. (c) Low magnification image; note absence of background staining with gold particles. Bars represent 100 nm.

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