

# Immunological detection and mutational analysis of the RNA2-encoded nematode transmission proteins of pea early browning virus

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Pea early browning virus (PEBV) is transmitted between plants by root-feeding trichodorid nematodes. Mutagenesis studies have implicated two non-structural viral proteins in the transmission process. These two proteins [the 29 kDa ('29K') protein and the 23K protein] were expressed in bacteria and used to raise antibodies. In Western blotting experiments, the antibodies detected both of these virus proteins in leaves and roots of infected *Nicotiana bethamiana* and *N. clevelandii* plants. Periodate treatment of proteins transferred to nitrocellulose membranes suggested that the PEBV 23K protein may be glycosylated. A PEBV mutant was constructed lacking the complete 23K coding sequence. The mutant was able systemically to infect

*Nicotiana* spp. but caused striking chlorotic ringspot leaf symptoms and stunting of both leaves and roots. These symptoms were absent in plants doubly-infected with the mutant and wild-type PEBV. The 23K gene deletion mutant was transmitted by nematodes at a much reduced frequency compared to wild-type virus, indicating that the 23K protein is involved in but not essential for vector transmission. Western immuno-blot and ELISA experiments revealed that the reduction in the nematode-transmissibility of PEBV carrying mutations in the 23K gene did not result from interference in the expression of the 29K transmission protein or from gross changes in the titre of virus in the roots of infected plants.

## Introduction

The transmission of plant viruses by their vector organisms, whether insect, mite, fungus or nematode, can be divided into three phases – acquisition by the vector, retention by the vector and release from the vector into a recipient plant. An integral component of this complex process involves the physical interaction of the virus particle and structures within (or on the surface of) the vector. Moreover, the recognition mechanism between virus and vector is highly specific; frequently a particular virus, and sometimes a particular isolate of a virus, is transmitted by few or only one vector species (Brown & Weischer, 1997). While relatively little is known about the particular vector components that are necessary for

virus transmission, molecular studies are providing information about the viral proteins that take part in the process.

Studies of insect-transmission have shown that, for some viruses, the only viral protein involved in the transmission process is the coat protein (Chen & Francki, 1990). Alternatively, some other viruses encode additional proteins, often referred to as helper proteins, which act together with the coat protein to facilitate transmission (Pirone & Blanc, 1996). Much less is known about the processes involved in the transmission of viruses by nematodes although the phenomenon itself has been recognized for nearly 40 years (Taylor & Brown, 1997). Only two types of viruses are transmitted by nematodes; the tobnaviruses and the nepoviruses. The construction of infectious cDNA clones of two nematode-transmissible isolates of the tobnaviruses tobacco rattle virus (TRV) and pea early browning virus (PEBV) has permitted reverse-genetics experiments to be carried out which have identified several viral determinants of vector transmission.

The tobnaviruses have two single-stranded, positive-sense genomic RNAs each separately encapsidated in rod-shaped particles. The larger RNA (RNA1, about 7000 nt) encodes

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proteins involved in virus replication and cell-to-cell spread, and on its own causes a systemic, so-called NM infection of plants (Harrison & Robinson, 1986). The smaller RNA (RNA2) varies in length from about 1800–3800 nt depending on the particular isolate, and encodes the coat protein (CP) often together with one to three other proteins. The RNA2 of two nematode-transmissible isolates of PEBV (TpA56) and TRV (PpK20) that have been sequenced are 3374 nt and 3856 nt respectively (MacFarlane & Brown, 1995; Hernandez *et al.*, 1995). PEBV TpA56 RNA2 encodes the CP and potentially three other proteins with molecular masses of 9, 29 and 23 kDa (9K, 29K and 23K proteins). TRV PpK20 RNA2 encodes the CP and potentially two other proteins with molecular masses of 37 and 32.8 kDa (37K and 32.8K proteins). Mutagenesis experiments showed that these RNA2-encoded non-structural proteins may be involved in the nematode transmission process. For PEBV possibly all of the non-structural proteins have roles in transmission. The involvement of the 9K protein is unclear, although a frameshift mutation of the 9K gene reduced the frequency of transmission by nearly 90%. A deletion within the 29K gene and a frameshift of the 23K gene abolished transmission, although a small deletion within the 23K gene permitted transmission at a frequency of 4% (MacFarlane *et al.*, 1996). To investigate the transmission process in more detail, antibodies against the PEBV 29K and 23K proteins have been prepared, and their activity tested in virus-infected plants. Also, we have extended our analysis of 23K gene mutants with regard to infection of the plant and to transmission by nematodes.

## Methods

■ **Preparation of antibodies against the PEBV 29K and 23K proteins.** The 29K and 23K genes were amplified from a full-length cDNA clone of RNA2 of PEBV isolate TPA56 carried on the plasmid pT72A56 (MacFarlane *et al.*, 1996) by PCR using *Taq* polymerase. The oligonucleotide primers used for the amplifications included an *Nco*I restriction site (5' primer) and a *Bam*HI site (3' primer). The amplified DNAs were cloned into pT7Blue (Novagen), sequenced to ensure that no unwanted mutations had been introduced by the *Taq* polymerase and recloned as *Nco*I–*Bam*HI fragments into the *Escherichia coli* expression vector pET-32a (Novagen). This vector allows the expression of heterologous proteins fused at the N terminus to a poly-histidine tag and also part of the *E. coli* thioredoxin protein, which in some instances has been shown to increase the solubility of recombinant fusion proteins. These clones were transformed into *E. coli* strain BL21 LysE and protein expression was induced by growth at 25–28 °C in the presence of 0.3 mM IPTG for 2 h. Samples were analysed by electrophoresis through 12.5% SDS–polyacrylamide gels. Cell fractionation experiments showed that, despite the presence of the thioredoxin tag, both the 29K and 23K proteins accumulated as insoluble inclusions (data not shown). Thus, recombinant protein was purified by sonication of pelleted cells, solubilization in 6 M urea, followed by binding to an immobilized nickel affinity matrix according to the manufacturer's instructions (Novagen). The recombinant proteins were eluted from the column in the presence of 1 M imidazole and 6 M urea, dialysed at 4 °C in 15 mM Tris–HCl, pH 8, 150 mM NaCl, 0.05% Tween 20 with firstly 2 M urea, then 1 M

urea and finally without urea, before storage at –70 °C. Rabbits were injected subcutaneously with approximately 50 µg of either the purified 29K or 23K proteins, mixed with an equal volume of Freund's incomplete adjuvant, followed by one (29K) or two (23K) additional injections. Serum was tested for the presence of antibodies against the recombinant proteins by Western blotting of *E. coli* lysates containing each of the proteins.

■ **Construction and testing of a 23K gene deletion mutant.**

Unique restriction sites were introduced at the 5' and 3' ends of the 23K gene carried on plasmid pT72A56 using the PCR 'megaprimer' method (Steinberg & Gorman, 1994). Primer 194 (5' CTGTTATGCATGCTCTTAAATTTACC 3') was used to insert an *Nsi*I site at the 23K gene initiation codon and primer 188 (5' CTAAGAAGGGCCCAAGTACAGTC 3') was used to insert an *Apa*I site 15 nt downstream of the 23K gene termination codon. The region surrounding these novel sites was sequenced and recloned into pT72A56 to create plasmid pTP188.94. This clone was digested with *Nsi*I and *Apa*I, blunted by treatment with Klenow polymerase and religated to produce plasmid pA56Δ23. This clone was linearized at the 3' terminus of the viral sequence using *Sma*I, and run-off transcripts were synthesized using a commercial kit (Ambion). Transcripts were mixed with PEBV RNA1 (extracted as total RNA from NM-infected plants) and inoculated to *Nicotiana benthamiana*. The mutant virus produced by this process was designated as PEBV 23Δ1. Infection of the plants was tested by immunosorbent electron microscopy, and maintenance of the mutation was confirmed by sequencing of viral cDNA amplified by RT–PCR, as described previously (MacFarlane *et al.*, 1996). Infected leaf tissue was homogenized in tap water, inoculated to *Nicotiana clevelandii* seedlings and tested for transmission by *Trichodorus primitivus* nematodes (MacFarlane & Brown, 1995).

■ **Antibody detection of viral proteins.**

Leaf and root samples of *N. benthamiana* and *N. clevelandii* plants infected with PEBV were harvested at different times post-inoculation. The tissue was homogenized in 1 ml/g (leaves) or 3 ml/g (roots) 0.1 M (1 ×) PBS, pH 7.4 and analysed by electrophoresis through denaturing 12.5% polyacrylamide gels. The separated proteins were transferred to nitrocellulose, probed with antisera raised against the viral proteins and detected by reaction with alkaline phosphatase-conjugated goat anti-rabbit serum, as described by the supplier (Sigma). Roots of infected plants (16 days post-inoculation) were washed free of soil and extracted with 3 ml/g of buffer (1 × PBS, 2%, w/v, polyvinylpyrrolidone, 0.05% Tween 20) using a Pollahne press. A comparison of virus titre in the root extracts was carried out using double antibody sandwich (DAS) ELISA (Clark & Adams, 1977) with the trapping anti-PEBV antibody used at a dilution of 1/10 000 and the anti-PEBV–alkaline phosphatase conjugate used at 0.01 mg/ml. After the addition of alkaline phosphatase substrate, the plates were incubated at 4 °C overnight before determining the absorbance at 405 nm

## Results

### Antibody reaction with *E. coli*-expressed protein

Antisera raised against the PEBV 29K and 23K proteins expressed in *E. coli* were able to detect the recombinant proteins when present in bacterial lysates. In Western blots the recombinant 29K and 23K proteins could be detected when each of the antisera was diluted by as much as 1/40 000 (data not shown); subsequently, both antisera were routinely used at a dilution of 1/10 000 (29K) or 1/5000 (23K). The recombinant proteins were purified as fusions to thioredoxin and both antisera contain anti-thioredoxin antibodies which caused

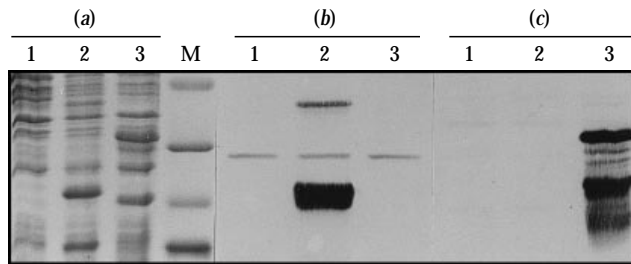


Fig. 1. Western blot of PEBV 23K and 29K proteins expressed in *E. coli*. *E. coli* proteins separated by SDS-PAGE and detected by (a) Coomassie staining, (b) anti-23K serum and (c) anti-29K serum. Lane 1 is an extract of cells containing expression plasmid pET-16b, lane 2 is an extract of cells containing pET-16b/23K and lane 3 is an extract of cells containing pET-16b/29K. Lane M contains pre-stained marker proteins (Rainbow Marker, Amersham) with molecular masses (descending) 46 kDa, 30 kDa, 21.5 kDa and 14.3 kDa.

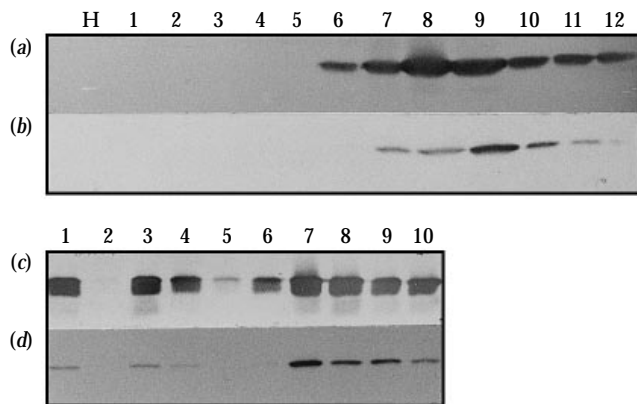


Fig. 2. Western blot of PEBV coat protein and 29K protein expressed in plants. (a, b) Time-course (1–12 days p.i.) of viral protein expression in systemically infected *N. benthamiana* leaves. Lane H is a healthy plant control. (c, d) Viral proteins detected in root samples of ten *N. clevelandii* plants following exposure to viruliferous nematodes. (a, c) Probed with anti-PEBV CP serum; (b, d) probed with anti-29K serum.

extensive cross-reaction when bacterial lysates containing the pET-32a constructs were examined. To demonstrate the specificity of the antisera, further constructs were made by cloning the 29K and 23K genes into plasmid pET-16b (Novagen), which allowed the expression of the viral proteins fused only to a small poly-histidine peptide. In Western blots of bacterial lysates containing the pET-16b/23K fusion protein the anti-23K serum detected a major protein of molecular mass about 23 kDa and a minor protein of molecular mass about 40 kDa (possibly a 23K dimer) (Fig. 1). In addition, the antiserum weakly recognized a bacterial protein of molecular mass about 29 kDa which was also present in extracts from cells containing the parental pET-16b plasmid or the recombinant pET-16b/29K plasmid. In lysates containing the pET-16b/29K fusion protein the anti-29K serum recognized a protein of molecular mass about 33 kDa and a number of smaller proteins, especially a protein of molecular mass about 24 kDa. These additional bands possibly represent either

truncated or processed forms of the 29K protein. The anti-29K serum also reacted very weakly with several bacterial proteins in both the pET-16b and pET-16b/23K lysates.

### Identification of the 29K protein in plants

Expression of the 29K protein in systemically infected leaves was examined in a time-course study. Samples were removed from the leaf immediately above the inoculated leaf in a series of plants at daily intervals [1–12 days post-inoculation (p.i.)] and analysed by Western blotting. Virus coat protein was detected at 6 days p.i. using an anti-PEBV CP serum, rising to a maximum concentration at 9 days p.i. and was still readily detectable at 12 days p.i. (Fig. 2a). The 29K protein was faintly detected at 6 days p.i. using the anti-29K serum; its expression rose to a maximum at 9 days p.i. and it was just detectable at 12 days p.i. (Fig. 2b). Thus apparently the expression of the 29K protein follows that of the coat protein with perhaps a slight delay in the start of 29K protein synthesis and also a slight advance in the cessation of expression or protein turnover.

For the 29K protein to facilitate the transmission of PEBV by nematodes it would be expected to be present in the roots of infected plants. To mimic the infection cycle that occurs in the field, virus was transmitted to plants using nematodes. Thus, a group of non-viruliferous *T. primitivus* nematodes was allowed to acquire PEBV by feeding on the roots of *N. clevelandii* plants that had previously been mechanically inoculated on the leaves with the virus. After 3 weeks the nematodes were extracted from the soil and transferred to pots containing healthy plants. After a weeks the roots of these plants were washed to remove the nematodes and homogenized in buffer for Western blotting. Virus coat protein was detected in eight plants, faintly detected in one plant and not detected in one plant (Fig. 2c). The 29K protein was detected in the eight plants which also contained coat protein, but was not detected in the two other plants (Fig. 2d).

### Identification of the 23K protein in plants

The anti-23K serum reacted to virus-specific proteins in leaf or root extracts of PEBV-infected plants but gave a more heterogeneous signal than that of the anti-29K or anti-virus coat protein sera. In one experiment, leaves of plants were manually inoculated with either wild-type PEBV or the mutant 23Δ1 (complete 23K gene deletion) and a series of total root samples was taken at daily intervals. Virus coat protein was detected at 4 days p.i. increasing to a maximum concentration at 7 days p.i. for the wild-type virus infection, and was also detected in samples taken at 5 and 7 days p.i. from plants infected with mutant 23Δ1 (Fig. 3a). The coat protein migrated as a sharply defined band indicating that the gel was not overloaded with root extract. By contrast, in equivalent samples of the same root extracts, proteins which reacted to the anti-23K serum were not clearly defined, and produced a

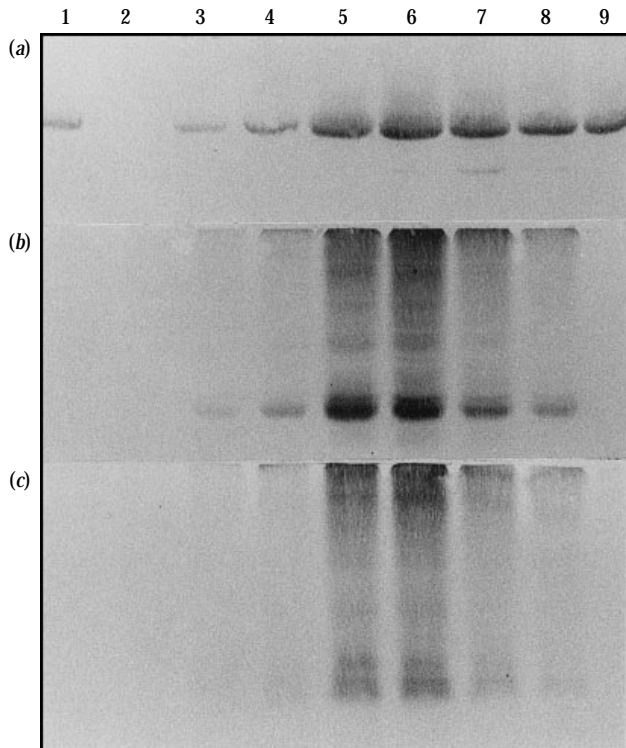


Fig. 3. Western blot of root extracts of *N. benthamiana*. Lanes 1 and 9, plants infected with PEBV mutant 23 $\Delta$ I at 5 days p.i. and 7 days p.i., respectively. Lanes 2–8, plants infected with wild-type PEBV at 3–9 days p.i. (a) Probed with anti-PEBV CP serum; (b) blotted proteins treated with sodium periodate and probed with anti-23K serum; (c) probed with anti-23K serum only.

smear signal throughout the gel (Fig. 3c). To examine the possibility that in plants the 23K protein is modified by glycosylation, which would affect its migration pattern in denaturing PAGE gels, the root extracts were treated with sodium periodate and sodium borohydride after transfer to the nitrocellulose membrane (Woodward *et al.*, 1985). The anti-23K serum detected several proteins with molecular masses between 21.5 and 30 kDa in samples of wild-type virus-infected roots at 4–9 days p.i. but did not react with any proteins in the mutant 23 $\Delta$ I-infected samples (Fig. 3b). Although this treatment did not remove the background smear, the major reacting band of molecular mass about 21.5 kDa was much stronger and more clearly defined. This suggests that the anti-23K serum, which was raised against (unglycosylated) *E. coli*-expressed protein, initially reacted only weakly with glycosylated 23K protein expressed in the plant.

#### Accumulation and nematode transmission of a 23K gene deletion mutant

To unequivocally assess the involvement of the 23K gene in nematode transmission, a mutant was constructed lacking the entire coding sequence. This mutant, 23 $\Delta$ I, was able to



Fig. 4. Detached, uninoculated leaves of *N. clelandii* infected with (clockwise from top right) wild-type PEBV, mutant 23 $\Delta$ I, mutant 23 $\Delta$ , mutant  $\Delta$ BE and mutant 23FS.

infect systemically both *N. benthamiana* and *N. clelandii*, and was encapsidated (data not shown). Also, this mutant produced striking chlorotic ring spot symptoms on both inoculated and systemically infected leaves of these plants. This is in contrast to infection by wild-type PEBV and by three other mutants,  $\Delta$ BE (a large deletion of RNA2, removing part of the 9K gene and all of the 29K and 23K genes), 23FS (a frameshift mutation in the 23K gene) and 23 $\Delta$  (a small, in-frame deletion in the 23K gene) (MacFarlane *et al.*, 1996), all of which produced no visible symptoms on infected plants (Fig. 4). In addition, plants infected with mutant 23 $\Delta$ I were stunted when compared to plants infected with the other viruses (results not shown).

Mutant 23 $\Delta$ I was tested for vector transmission as described before (MacFarlane *et al.*, 1996). Two separate tests were carried out, the first involving 10 *N. clelandii* source plants each inoculated with virus and then exposed to 60–80 non-viruliferous nematodes, and in a second test the virus was inoculated to 29 source plants, each being exposed to at least 60 nematodes. Combining the results of these tests, mutant 23 $\Delta$ I was transmitted from 6 of 39 source plants, while in the same series of experiments wild-type PEBV was transmitted from 18 of 20 source plants (Table 1). The identity of the transmitted mutant in the first experiment was confirmed by RT-PCR and sequencing; the transmitted mutants from the second experiment were confirmed by RT-PCR (data not shown). Thus, the 23K gene is not essential for nematode transmission but does have an unidentified role in maintaining the frequency of transmission.

Northern blot analyses of co-infected plants revealed that mutant 23 $\Delta$ I and wild-type PEBV could replicate together in

**Table 1.** Transmission of wild-type and mutant PEBV by nematodes\*

Virus	Source plants†	Bait plants‡
<b>Experiment 1</b>		
WT PEBV‡	10/10	8/10
23ΔI§	10/10	1/10
WT + 23ΔI¶	10/10	7/10
<b>Experiment 2</b>		
WT PEBV	10/10	10/10
23ΔI	29/30	5/29

\* Mixed population of *Trichodorus primitivus* and *Paratrichodorus pachydermus* nematodes in a ratio of 2:1 from Woodhill, Barry (Scotland), UK.

† Proportion of plants tested which were found to have virus in the roots.

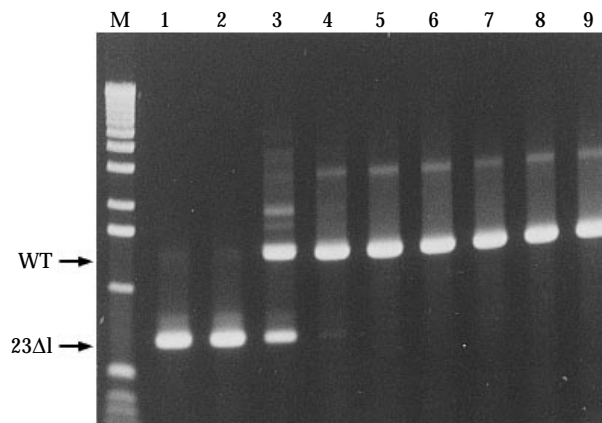
‡ Derived from infectious cDNA clones of PEBV SP5 RNA1 and PEBV TpA56 RNA2.

§ Derived from infectious cDNA clones of PEBV SP5 RNA1 and PEBV TpA56 mutant 23ΔI RNA2.

¶ Derived by inoculating plants with a mixture of leaf extracts from plants infected with either virus.

the same plant, and that in dual-infected plants the chlorotic phenotype of mutant 23ΔI was suppressed (data not shown). Subsequently, experiments were carried out to examine whether in dual-infected plants the wild-type virus would affect the efficiency of nematode transmission of mutant 23ΔI, or vice versa. From ten source plants manually infected with both viruses, seven transmissions to bait plants were achieved while in the same experiment wild-type PEBV alone was transmitted from eight of ten infected source plants (Table 1). One of the plants from the dual virus transmission showed strong chlorotic ring symptoms, and one plant had a mild mottle while the remaining five plants showed no symptoms. Each plant was examined by RT-PCR which showed that the strongly symptomatic plant contained only the deletion mutant (Fig. 5, lane 2), the mildly mottled plant contained both viruses (Fig. 5, lane 3) and that four of the remaining five asymptomatic plants contained only the wild-type virus (Fig. 5, lanes 5–9), with the fifth plant containing wild-type virus and only a trace of the deletion mutant (Fig. 5, lane 4).

An explanation for the reduced transmission frequency of mutant 23ΔI, compared to that of wild-type virus, might include an alteration in the level of virus in the roots of infected plants, or even an indirect effect on the expression of the viral 29K transmission protein. To address these two possibilities, plants were inoculated with wild-type PEBV and four mutants (ΔBE, 23FS, 23Δ and 23ΔI, see above) and the roots of each plant were harvested 16 days p.i. The root systems of plants infected with mutant 23ΔI were obviously more stunted than those from plants inoculated with the other mutant or wild-



**Fig. 5.** RT-PCR analysis of virus transmitted by nematodes to *N. clelandii*. Lane 1, source plant infected only with mutant 23ΔI. Lanes 2–9, source plants infected with both wild-type PEBV and mutant 23ΔI. Arrows indicate the sizes of amplified DNA fragments expected for each virus.

**Table 2.** Accumulation of wild-type and mutant PEBV in *N. benthamiana* roots

Virus	ELISA value*		Mean weight of roots (g)
	1/5 dilution†	1/50 dilution†	
Healthy‡	0.163	0.153	
WT PEBV§	1.475	0.846	0.379
ΔBE¶	1.394	0.743	0.528
23FS¶	1.555	0.335	0.336
23Δ¶	1.580	0.811	0.368
23ΔI¶	1.130	0.454	0.113

\* Mean absorbance value ( $A_{405}$ ) after incubation with substrate overnight at 4 °C.

† Initial root extracts further diluted in extraction buffer before ELISA testing.

‡ Single plant tested.

§ Six plants tested.

¶ Eight plants tested.

type viruses (Table 2), as was noted for the leaves. When the virus content of each root sample was compared by ELISA, mutant 23ΔI appeared to accumulate to slightly lower levels than did the wild-type virus. However, this apparent reduction in virus titre was not due primarily to the lack of functional 23K protein, as the other mutants, which lacked or had alterations in the 23K gene, did not differ greatly in virus titre from the wild-type virus (Table 2). Most likely, the stunting of the root system of plants infected with mutant 23ΔI made it more difficult to extract a fully representative sample of root tissue from the soil, especially the fine roots. Examination by Western blotting of root extracts containing these viruses revealed that

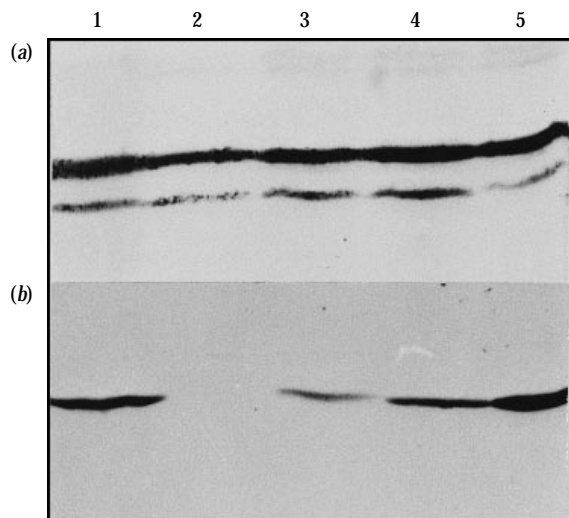


Fig. 6. Western blot of root extracts of *N. benthamiana* infected with wild-type and mutant PEBV. Lane 1, infected with wild-type PEBV; lane 2, infected with mutant  $\Delta$ BE; lane 3, infected with 23FS; lane 4, infected with 23 $\Delta$ ; lane 5, infected with 23 $\Delta$ I. (a) Probed with anti-PEBV CP serum, (b) probed with anti-29K serum.

PEBV coat protein was present at similar levels for each of the viruses (in this particular experiment the CP migrated as a doublet) (Fig. 6a). Probing the same root extracts with anti-29K serum showed that mutant  $\Delta$ BE (which lacks both the 29K and 23K genes) did not express 29K protein. Conversely, deletion or frameshifting of the 23K gene did not prevent expression of the 29K protein (Fig. 6b).

## Discussion

Current ideas about the mechanism of nematode transmission of tobnaviruses are based mainly on models proposed for the transmission of viruses by insects, for which extensive data are available (Pirone & Blanc, 1996). The principal hypothesis is that the virus-encoded helper proteins act as a bridge between the virus particle and specific regions of the insect mouthparts or gut. This possibly occurs by the interaction of different domains of the helper protein with the virus coat protein and a surface molecule at the site of virus retention within the vector. Alternatively, the helper protein might promote or stabilize a direct contact between the virus particle and the virus retention site (Pirone & Blanc, 1996; Blanc *et al.*, 1997).

Previous studies on virus transmission by nematodes revealed a variation in the degree of specificity between virus isolates and their vector nematode species. For example, in glasshouse tests PEBV isolate TpA56 is transmitted by two nematodes, *Trichodorus primitivus* and *Paratrichodorus anemones*, whereas TRV isolate PpK20 is transmitted by *P. pachydermus* but not by *T. primitivus* or *P. anemones* (N. Vassilakos, S. MacFarlane & D. Brown, unpublished). Also, *P. pachydermus*

was shown to be a vector of four different isolates of TRV each of which belonged to the PRN serogroup (which includes PpK20) but did not transmit five other TRV isolates belonging to different serogroups (Ploeg *et al.*, 1992a, b). In addition, *P. pachydermus* did not transmit PEBV from Britain but did transmit PEBV from Holland, although neither of these isolates is serologically related to TRV PRN (van Hoof, 1962; N. Vassilakos, S. MacFarlane & D. Brown, unpublished). Electron microscope studies examining the location of TRV in trichodorid nematodes revealed particles of TRV associated with the oesophageal cuticle of *P. pachydermus* (Taylor & Robertson, 1970) and *T. similis* (Brown *et al.*, 1996). In the former study virus particles were found concentrated in regions of the nematodes' feeding apparatus that are covered by a mucous layer.

The studies described in this paper provide data necessary for developing an understanding of the molecular basis of nematode transmission of tobnaviruses. The 29K protein of PEBV TPA56 and the 37K protein of TRV PPK20 have about 48% amino acid sequence similarity, and have both been shown by mutagenesis of infectious viral cDNA clones to be required for nematode transmission (MacFarlane *et al.*, 1996; Hernandez *et al.*, 1997). The demonstration here that expression of the PEBV 29K protein is closely associated with that of the virus CP, and particularly that the 29K protein is abundant in the roots of infected plants, is consistent with its role as a vector transmission factor. The leaf expression data compare well with a previous study which identified the 29K protein in infected peas at 4 days p.i. (Johnsen *et al.*, 1991). The antiserum prepared against the PEBV protein will permit immunogold localization studies in plants and nematodes, and may also allow the development of *in vitro* assays to examine the possible interaction of the 29K protein with virus particles.

Data from the 23K gene studies are more difficult to interpret. Deletion of the 23K gene produced a substantial difference in the phenotype of PEBV infection. Previously, all other *in vitro*-generated mutants of PEBV have no obvious alterations in infection symptomatology, which is generally confined to limited leaf curling with occasional faint mottling on *Nicotiana* spp. (Harrison, 1973). Mutant 23 $\Delta$ I, lacking the entire 23K gene, caused chlorotic ringspot symptoms on inoculated and systemically infected leaves. As a frameshift or a small deletion mutation in the 23K gene did not induce symptoms, this effect presumably is not associated with a lack of the 23K protein but arises because of some novel feature of the RNA sequence or secondary structure created at the deletion junction or in the flanking regions. Such RNA effects have been demonstrated for various plant virus satellite RNAs (Taliensky & Robinson, 1997). Co-inoculation experiments revealed that the large deletion mutant could replicate in the presence of wild-type RNA2, and that in this combination chlorotic symptoms were absent.

Western blotting experiments revealed that the 23K protein is expressed in the leaves and roots of PEBV-infected plants. As

mentioned above for the 29K protein, expression in roots is considered essential for any protein involved in nematode transmission. However, the status of the PEBV 23K protein as a transmission factor is not certain. Sequencing studies have identified a third open reading frame, analogous in position to the 23K gene, in TRV isolate PpK20 (Hernandez *et al.*, 1995) and in two other nematode transmissible isolates of TRV (S. MacFarlane, unpublished), although these putative proteins do not share obvious amino acid sequence homology. In previous transmission studies, whereas wild-type virus was transmitted to 26 of 30 bait plants, with two 23K gene mutants of PEBV, a small, internal deletion mutation reduced vector transmission frequency (1/27 plants) and a frameshift mutation abolished nematode transmission (0/27 plants) (MacFarlane *et al.*, 1996). These results suggested that the 23K protein is probably essential for transmission and that the small deletion mutation (which was in-frame) possibly reduced the activity of the protein without leading to the complete loss of its function. In contrast, deletion of the third, 32.8K gene from TRV PpK20 had no effect on the frequency of nematode transmission (Hernandez *et al.*, 1997). The studies reported here confirm that while the 23K gene (and its translation product) is not essential for nematode transmission it does have some role in the process. The 23K protein may be involved in the release of virus particles from the nematode mouthparts during feeding. For example, the virus coat protein and/or 29K protein may facilitate the retention (binding) of virions in the nematode oesophagus. Release of particles probably occurs by the washing action of secretions from the oesophageal gland passed along the nematode mouthparts during feeding (Taylor & Brown, 1997). The 23K protein may have an enzymatic function which is integral to this process. Evidence mitigating against this hypothesis could be the apparent inability, in dual infection experiments, of the 23K protein encoded by the wild-type virus to increase the frequency of transmission of mutant 23Δ1 to wild-type levels. However, these studies were complicated by the feeding of more than one nematode on the dually-infected plants. A better estimate of the degree of complementation between the two viruses probably requires the demonstration that a single nematode can simultaneously transmit more than one virus (or combination of mutants/isolates).

Alternatively the 23K protein may affect vector transmission by altering the amount and/or distribution of virus in the plant roots, thus facilitating its availability to a feeding nematode. An *in vitro* nematode feeding system, similar to the feeding of aphids through artificial membranes, is not available; therefore, the concentration of virus (either purified or in roots) which is necessary to permit efficient vector transmission cannot be determined. Although a large difference in the amount (as detected by ELISA) of wild-type and 23K mutant virus in roots was not observed, it is possible that minor differences in virus concentration may have a significant effect on transmission frequency. There is relatively little information

on the feeding behaviour of trichodorid nematodes, especially in relation to virus acquisition and transmission. It is possible that virus might only be acquired when it is present in a particular cell type/position in the root and the 23K protein might function by ensuring appropriate localization of the virus.

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