

Similarities in the genome organization of tobacco rattle virus and pea early-browning virus isolates that are transmitted by the same vector nematode

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Although sequence data have been obtained for several tobnavirus isolates, only two of these isolates are nematode-transmissible. Tobacco rattle virus (TRV) PpK20 is transmitted by *Paratrichodorus pachydermus*, whereas pea early-browning virus (PEBV) TpA56 is transmitted by *Trichodorus primitivus*. To clarify whether differences in the genome structure of these isolates are relevant to the specificity of interactions with particular vector nematodes, or merely reflect a taxonomic difference between TRV and PEBV, we have sequenced RNA2 of a new isolate of TRV (TpO1) that is transmitted by the same vector nematode as PEBV TpA56 but is not transmitted by the nematode vector of TRV PpK20. TRV TpO1 RNA2 encodes, in 5' to 3' order, a coat protein (CP), a 9K protein, a 2b (29K) protein and a 2c (18K) protein. Amino acid sequence comparison shows that both the CP and 2b proteins of TRV TpO1 resemble more closely the analogous proteins from PEBV TpA56 than those from TRV PpK20. Also, the TRV TpO1 9K protein has similarities with the PEBV 9K protein whereas this protein is lacking in TRV PpK20.

Tobnaviruses are one of only two types of plant virus that are transmitted from plant to plant by nematodes (Harrison & Robinson, 1986; Taylor & Brown, 1997). Tobnavirus transmission is carried out by nematodes belonging to the genera *Trichodorus* and *Paratrichodorus*, which acquire the virus while feeding on the roots of infected plants. Examination of sections of *P. pachydermus* and *T. similis* nematodes by electron microscopy has revealed tobacco rattle tobnavirus (TRV) particles associated with the cuticular lining of the oesophagus, tending to lie parallel with the long axis of the food canal

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(Taylor & Robertson, 1970; Brown *et al.*, 1996). The retention of virus inside the nematode probably requires a specific recognition and interaction between surface features of both the virus particle and the oesophageal cuticle of the vector nematode. This is supported by the finding that particular tobnavirus isolates are transmitted only by certain nematode species and, conversely, that particular nematode species may transmit only certain virus isolates. For example, the PpK20 isolate of TRV is transmitted by *P. pachydermus* but not by *T. primitivus*, and *P. pachydermus* transmits some TRV isolates (PpK20, PpB1, PpW1) but not others (TvC47, TpE1) (Brown *et al.*, 1989; Ploeg *et al.*, 1991, 1992a).

Although partial genome sequences have been determined for at least six different isolates of TRV, only one of these isolates is transmissible by nematodes. Indeed, the genome organization of each of the five non-nematode transmissible TRV isolates is very different from one another, and from the transmissible isolate. This reflects the common occurrence of deletion and recombination of sequences in TRV RNA2, particularly when isolates are maintained by repeated mechanical inoculation (Hernandez *et al.*, 1996; MacFarlane, 1997). Thus, in order to study the genetic determinants of TRV that are involved in the nematode transmission process, it is necessary to obtain the nucleotide sequence of isolates that are obtained directly from nematodes in field soil, or can be demonstrated to be transmissible by nematodes in glasshouse experiments.

Previous experiments showed that RNA2 of the nematode-transmissible PpK20 isolate of TRV encodes three genes: the coat protein (CP) gene, the 2b gene (molecular mass 40 kDa) and the 2c gene (molecular mass 32.8 kDa) (Hernandez *et al.*, 1995). This isolate is transmitted by *P. pachydermus* but not by *T. primitivus* (Ploeg *et al.*, 1992a). Mutagenesis of an infectious cDNA clone of this isolate showed that the 2b gene but not the 2c gene was required for transmission to occur (Hernandez *et al.*, 1997). A single, nematode-transmissible isolate of the related tobnavirus pea early-browning virus (PEBV) has also been cloned and sequenced (MacFarlane & Brown, 1995). In contrast to TRV PpK20, PEBV TpA56 is transmitted by *T. primitivus* but not by *P. pachydermus*. RNA2 of this isolate encodes four genes: the CP, 2b (molecular mass 29 kDa), 2c

Table 1. Transmission of TRV isolate TpO1 by trichodorid nematodes

Virus-free nematodes of both species were extracted from soil collected at Woodhill, Scotland, UK. Values in the table are no. of individual nematodes transmitting virus over total no. of nematodes examined.

Nematode species	Males	Females	Juveniles	Total
<i>Trichodorus primitivus</i>	27/56	25/54	3/16	55/126
<i>Paratrichodorus pachydermus</i>	0/19	0/37	0/21	0/77

(molecular mass 23 kDa) and a small ('9K') gene located between the CP and 2b genes. Mutagenesis experiments showed that all four genes play some role in nematode-transmission (MacFarlane *et al.*, 1996; Schmitt *et al.*, 1998), although transmission can occur infrequently in the absence of the 2c gene. In addition, the role of the 9K gene in transmission is unclear: a frameshift mutant was transmitted at very low frequency, whereas a mutant lacking an AUG translation initiation codon was transmitted at wild-type frequency (MacFarlane *et al.*, 1996).

Comparison of the transmission behaviour of these two tobnavirus isolates raises several questions. Why is the 2c gene of PEBV TpA56 involved in transmission whereas the TRV PpK20 2c gene apparently is not involved? Is the 2c gene necessary for transmission by *T. primitivus* but not *P. pachydermus*? Similarly, why does TRV PpK20 have no homologue to the PEBV 9K gene? Is vector specificity determined by particular amino acid sequences in the CP, or in the 2b protein or perhaps in both proteins?

In order to address these questions we have cloned and sequenced RNA2 of a new, nematode-transmissible isolate of TRV. This isolate, TpO1, was originally obtained from a single *T. primitivus* nematode extracted from an Oxford soil sample (Ploeg *et al.*, 1992*b*). This isolate was tested for transmission by single *T. primitivus* and *P. pachydermus* nematodes extracted from soil obtained from Woodhill, near Monifieth, Scotland, UK, using techniques described previously (MacFarlane & Brown, 1995). These tests (Table 1) showed that TRV TpO1 behaves like PEBV TpA56 rather than TRV PpK20 in that it is transmitted by *T. primitivus* but not by *P. pachydermus*. However, serological tests showed that TpO1 (RQ serotype) differed from both TRV PpK20 (PRN serotype) and PEBV TpA56 (SP5 serotype) (Ploeg *et al.*, 1992*a*).

Total RNA was isolated from *Nicotiana benthamiana* plants infected with TRV isolate TpO1, and virus-specific single-strand cDNA was synthesized as described previously (MacFarlane, 1996). The 5' primer used for long template (LT)-PCR of TpO1 RNA2 included a T7 RNA polymerase

promoter sequence. A 3 kb PCR product was cloned, without gel purification, into pT7Blue (Novagen) and a single clone (23.3) was chosen for further analysis. The 5' and 3' termini of the viral insert in clone 23.3 were sequenced using vector-specific primers confirming that it consisted of a complete viral cDNA (data not shown). The clone was linearized at the 3' terminus of the viral sequence by digestion with *Sma*I, and capped transcript RNA2 was tested for infectivity by inoculation to *N. benthamiana* plants in combination with total plant RNA containing RNA1 of TRV PpK20. Extracts of upper, uninoculated leaves, sampled at 7 days post-inoculation, were examined by electron microscopy following trapping of virus particles with homologous (RQ serotype) antiserum. These tests confirmed that clone 23.3 is fully infectious. The complete sequence of clone 23.3 was obtained from an overlapping series of 5' or 3' terminally deleted subclones prepared using the exonuclease III deletion method of Henikoff (1984). Nucleotide sequence analysis was carried out using Wisconsin Package programs (Genetics Computer Group) and amino acid sequence comparisons used the CLUSTALW program (Thompson *et al.*, 1994).

RNA2 of TRV isolate TpO1 was found to contain 3216 nucleotides (nt). The 5' and 3' non-coding regions of TRV TpO1 RNA2 are 474 nt and 415 nt, respectively. There are four significant open reading frames (ORFs) encoded by the viral RNA. The first ORF starts at nt 475 (the sixth AUG codon from the 5' terminus) and extends to nt 1062. This ORF encodes the CP, which has a molecular mass of 21 514 Da. Amino acid sequence comparisons show that the CPs of TRV TpO1 and PEBV TpA56, which are transmitted by the same vector nematode, are slightly more similar (60.2%) to one another than they are to TRV PpK20 (TpO1: PpK20, 57.1%; TpA56:PpK20, 54.6%), which is transmitted by a different nematode (Fig. 1). The C terminus of the tobnavirus CP is predicted to extend away from the surface of the rod-shaped virion, and has been suggested as a recognition domain which could influence the specificity of the interaction between the virus and vector nematode (Mayo *et al.*, 1994). This flexible domain is different in size in all three of the virus isolates [TpO1, 17 amino acids (aa); TpA56, 29 aa; PpK20, 22 aa]. It was previously shown that removal of the C-terminal 15 aa of the CP (but retaining the terminal alanine residue) abolished nematode transmission of PEBV (MacFarlane *et al.*, 1996). This region in TRV TpO1 (PATSSGGGKGPVV) is almost identical to PEBV TpA56 (PATSSGGGKGPVVA), whereas the same region in TRV PpK20 is very different (PPPASGGPIRPNP). However, it might be expected that residues at the extreme C terminus of the flexible CP domain, rather than the more internal residues, would be most important in specific recognition of vector surfaces or transmission helper protein, and in this respect all three virus sequences are different.

The second ORF extends from an AUG codon at nt 1111 to nt 1353 and encodes a protein with a molecular mass of 9273 Da. This protein ('9K') is similar in size to the 9K protein

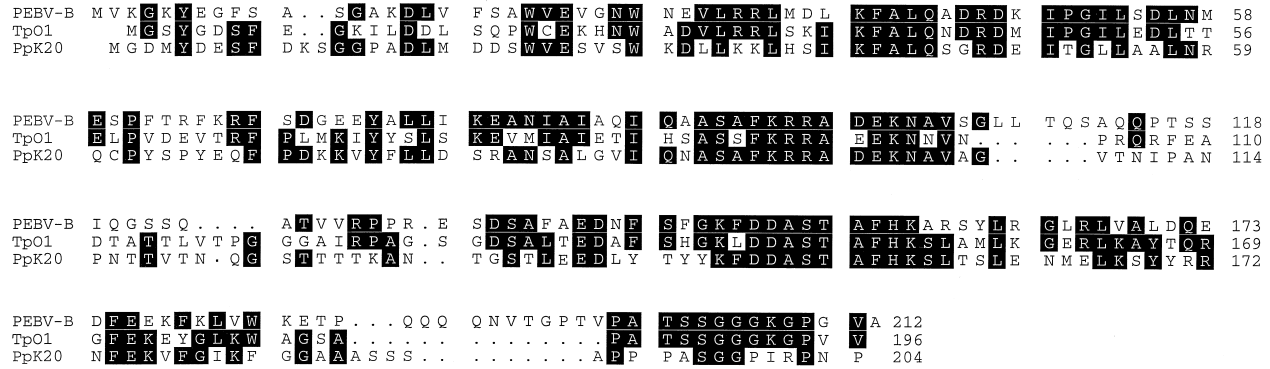


Fig. 1. Amino acid sequence comparison of the CPs of nematode-transmissible tobnavirus isolates PEBV-B (PEBV TpA56), TpO1 (TRV TpO1) and PpK20 (TRV PpK20). Alignments were carried out using CLUSTALW default parameters. Dots indicate gaps introduced to optimize the alignments.

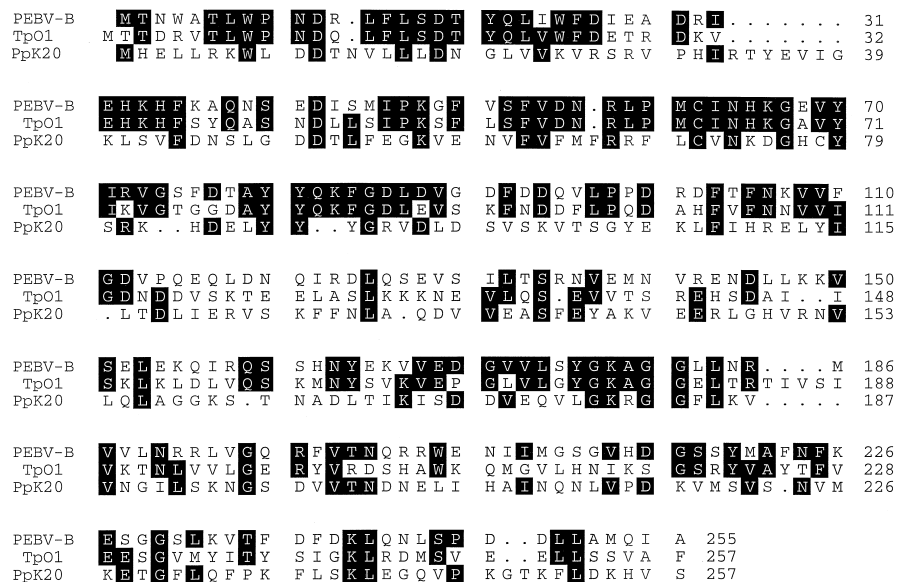


Fig. 2. Amino acid sequence comparison of tobnavirus 2b, nematode-transmission proteins. The PpK20 (TRV PpK20) sequence includes only 257 aa at the N terminus of the 2b protein (full size 354 aa).

encoded by PEBV TpA56 RNA2. In addition, the TRV TpO1 and PEBV TpA56 9K proteins have low (24%) but statistically significant sequence identities. As with PEBV, the TRV TpO1 9K ORF is located just downstream of, and in-frame with, the CP gene. Readthrough translation of the TRV TpO1 CP termination codon would result in the expression of a 31.9 kDa molecular mass CP–9K fusion protein. Both the PEBV TpA56 and TRV TpO1 CP genes terminate with a UGA codon; however, there is very little nucleotide sequence conservation either upstream or downstream of the CP termination codon, which suggests that these two viruses do not share a common readthrough mechanism. Also, thus far the PEBV TpA56 9K or CP–9K fusion proteins have not been detected in Western blots of extracts of virus-infected plants using either antibodies raised against the CP or against the 9K protein (S. A. MacFarlane & C. Schmitt, unpublished).

The third ORF extends from nt 1388 to nt 2158, encoding a protein with a molecular mass of 29099 Da which has 45% amino acid sequence identity with the 2b, nematode transmission proteins of PEBV TpA56 (Fig. 2). These proteins have much lower sequence identity with the 2b nematode transmission protein of TRV PpK20. However, the identities are increased (TRV TpO1:TRV PpK20, 17% identity; PEBV TpA56:TRV PpK20, 19% identity) if comparisons are carried out using only the N-terminal 29 kDa portion of the PpK20 (40 kDa) protein. This suggests that the TRV PpK20 2b protein may include an additional domain at its C terminus that is not present in the TRV TpO1 and PEBV TpA56 2b proteins.

The fourth ORF extends from nt 2337 to 2801 and encodes a protein of molecular mass 18093 kDa. We propose to call this protein the 2c protein, and, similarly, to refer to the proteins encoded by the 3'-proximal ORFs of PEBV TpA56

RNA2 and TRV PpK20 RNA2 also as the 2c protein. These proteins are of different sizes and share no amino acid sequence identity, making it not possible to infer a role in nematode transmission for the TRV TpOI 2c protein.

Comparison of RNA2 of the three nematode-transmissible tobamoviruses described here allows us to make some comments on the viral determinants involved in the specific recognition of vector nematodes. Both the CP and 2b proteins of the two viruses transmitted by *T. primitivus* (TRV TpOI and PEBV TpA56) are more similar to each other than they are to the same proteins of TRV PpK20, which is transmitted by *P. pachydermus*. In addition, both of the *T. primitivus*-vectored viruses encode a 9K protein downstream of the CP, whereas TRV PpK20 does not. However, the proteins of TRV PpK20 differ sufficiently from those of the two other viruses that it is difficult to identify particular domains which might be responsible for discriminating between the two vector nematodes. A better understanding of the process of nematode transmission of tobamoviruses will require fine-detail mutagenesis of the viral CP and 2b proteins, particularly targeting residues that are predicted to be surface located.

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