Virology 279, 478–487 (2001)
doi:10.1006/viro.2000.0677, available online at http://www.idealibrary.com on IDEAL®

Tobravirus 2b Protein Acts *in Trans* to Facilitate Transmission by Nematodes

N. Vassilakos, E. K. Vellios, E. C. Brown, D. J. F. Brown, and S. A. MacFarlane[1]

Department of Pathology, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, United Kingdom

Received December 6, 1999; returned to author for revision January 4, 2000; accepted September 29, 2000

Analysis of RNA2 of TRV PaY4 showed it to be recombinant, carrying 3′-terminal sequences derived from RNA1. Virus produced using an infectious cDNA clone of PaY4 RNA2 was nematode transmissible, demonstrating that natural TRV recombinant isolates are not necessarily defective. Mutations introduced into PaY4 RNA2 showed that the 2b gene, but not the 2c gene, is required for transmission by both *Paratrichodorus pachydermus* and *P. anemones* nematodes. Experiments examined whether infection of plants with two different virus clones would impact upon nematode transmission of either virus. Simultaneous inoculation with TRV clones expressing green or red fluorescent proteins revealed that mixing of the two virus populations did not occur, although, in roots, adjacent cells were found containing green- or red-tagged viruses. Subsequently, in similar experiments it was found that a TRV PaY4 2b mutant was transmitted when combined with wild-type TRV PaY4. Also, transmission of a 2b mutant of an *in vitro* TRV/PEBV recombinant virus (TRV-C1) occurred after coinfection with wild-type virus. Thus, the tobravirus 2b transmission protein is *trans*-acting. Although TRV PaY4 and TRV PpK20 are both transmitted by *P. pachydermus,* a 2b mutant of TRV PaY4 was not transmitted when coinoculated to plants with TRV PpK20.

INTRODUCTION

The tobraviruses (*Tobacco rattle virus* [TRV], *Pea early-browning virus* [PEBV], *Pepper ringspot virus*) belong to one of only two genera of viruses that are transmitted between plants by nematodes. These viruses move rapidly through the root system of infected plants, where they are ingested by vector nematodes which feed primarily on epidermal cells located just behind the zone of root elongation near the root tip (Taylor and Brown, 1997). The transmission is a noncirculative process. Thus, virus particles initially are retained in the nematode's mouthparts and, during subsequent feeding episodes, they are released from the site of retention in the esophagus and introduced into new plants. Tobraviruses are transmitted only by nematodes belonging to the genera *Trichodorus* and *Paratrichodorus,* and some previous studies suggested that there is a high degree of specificity in the association of particular nematode species with particular viruses or virus isolates (Ploeg *et al.,* 1992a).

Efforts to understand the details of the interaction between vector nematode and transmitted virus are complicated by the large variation that occurs among tobravirus isolates. Although the tobraviruses have two single-stranded, genomic RNAs (RNA1 and RNA2), the larger RNA1 is able to multiply on its own and move systemically in plants. There is little or no selection pressure for the maintenance of RNA2 in an infected plant, and in crops that are vegetatively propagated, such as potato, it is quite common to find RNA1-only (also known as NM-) infections (Harrison *et al.,* 1983). Also, in isolates that retain RNA2, this RNA species often undergoes extensive deletion and rearrangement, a situation that is thought to be accelerated by repeated passaging of virus using mechanical inoculation (Hernandez *et al.,* 1996).

When the 12 published tobravirus RNA2 sequences are examined, only two (PEBV TpA56 and PEBV SP5) have an identical gene organization (MacFarlane, 1999). Each of the 12 sequenced isolates encodes a coat protein (CP), although deleted forms lacking the CP gene were previously cloned from infected plants (Hernandez *et al.,* 1996). There is, however, a wide variation in CP sequence, with TRV PpK20 and TRV PLB having 94% amino acid sequence similarity, whereas TRV TCM and TRV PLB are only 52% similar. This variability is reflected in the extreme serological diversity that is found among tobravirus strains. RNA2 of tobraviruses also appears to be highly susceptible to recombination (MacFarlane, 1997). The 3′ proximal region of RNA2 of eight of the 12 sequenced isolates is derived by recombination with RNA1. This can result in the inclusion of a complete, second copy of the RNA1-encoded 1b gene and, in some cases, part of the 1a (cell-to-cell movement) gene in RNA2. It is not clear whether such recombinants are fully

The PaY4 RNA2 sequence has been deposited in the EMBL database, accession number AJ250488.

[1] To whom correspondence and reprint requests should be addressed. Fax: +44 1382 562426. E-mail: s.macfarlane@scri.sari.ac.uk.

0042-6822/01 $35.00

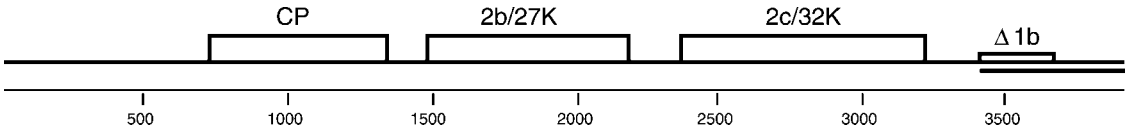

FIG. 1. Genome organization of TRV PaY4 RNA2. Virus genes are denoted by open boxes. The gene name and molecular weight appear above the gene. The 3′ part of the genome that is underlined corresponds to sequences derived from RNA1. The partial, frameshifted copy of the RNA1-encoded 16K gene is denoted as Δ1b.

functional or whether they represent a subset of self-limiting, defective viruses.

Depending on the location in RNA2 where recombination occurs, one or more RNA2-specific genes may be deleted by the recombination process. Mutagenesis studies have shown that the 2b and 2c genes, located on RNA2 downstream of the CP gene, are involved in transmission of tobraviruses by nematodes. Specifically, for PEBV isolate TpA56 it was found that the 2b gene was essential for transmission and that the 2c gene was not essential but greatly increased the efficiency of transmission (MacFarlane *et al.,* 1996). For TRV PpK20, however, although the 2b gene was essential, the 2c gene was completely dispensable for nematode transmission (Hernandez *et al.,* 1997). These studies also showed that PEBV TpA56 could be transmitted by *T. primitivus* but not by *P. pachydermus,* whereas TRV PpK20 was transmitted by *P. pachydermus* and not by *T. primitivus.*

Recently, using the yeast two-hybrid assay system, the 2b protein of TRV PpK20 was shown to interact with the C-terminal part of the virus coat protein (Visser and Bol, 1999). This finding has been interpreted as supporting the hypothesis that the tobravirus 2b protein acts as a bridge to link the virus particle and surface structures within the vector nematode mouthparts. A similar model was developed to explain the function of helper transmission protein in potyvirus transmission by aphids (Pirone and Blanc, 1996). Insect transmission studies, particularly of potyviruses and *Cauliflower mosaic virus,* are greatly aided by the use of membrane feeding techniques, whereby (partially) purified transmission helper protein can be mixed with virus *in vitro* before being taken up by the vector (Govier and Kasanis, 1974; Espinoza *et al.,* 1988). Such techniques are not available for the study of nematode transmission, making it necessary to develop new experimental approaches.

The detailed characterization of different nematode-transmissible tobravirus isolates is an important prerequisite for understanding the transmission mechanism. In a previous study, Ploeg *et al.* (1992b) isolated a number of serologically distinct strains of TRV from individual trichodorid nematodes collected at different sites in the United Kingdom, Sweden, and the Netherlands. Here we describe a study of the genome organization and transmission determinants of one of these TRV isolates, PaY4. This isolate initially was selected because, in different studies, *P. anemones,* its vector nematode, was reported to transmit both PEBV (Harrison, 1967) and TRV (Van Hoof, 1968). In recent tests TRV isolate PaY4 was shown to be transmitted both by *P. anemones* and by *P. pachydermus,* whereas PpK20, the only other nematode-transmissible TRV isolate that has been studied in detail, could not be transmitted by *P. anemones* (Vassilakos, MacFarlane, and Brown, unpublished). In addition, we describe transmission-complementation tests carried out using TRV PaY4, TRV PpK20, and a third, recombinant isolate, TRV-C1. The results of these tests provide insights into both the mechanics of and the specificity of the interaction between the virus and its vector nematode.

RESULTS

Sequence analysis of PaY4 RNA2

Sequencing of the full-length clone revealed that PaY4 RNA2 contains 3926 nucleotides (nt) and is thus the largest tobravirus RNA2 examined to date. PaY4 RNA2 encodes three large open reading frames (ORFs) (Fig. 1). The first ORF starts with the sixth AUG from the 5′-terminus at nucleotides 713–715 and extends to a UGA codon at nucleotides 1343–1345. This ORF encodes the CP, which has 209 amino acid (aa) residues and a molecular weight of 22.5 kDa. Phylogenetic analysis revealed that the tobravirus CP amino acid sequences can be clustered into several distinct groups (MacFarlane, 1999). The PaY4 CP is most similar (94.7% amino acid sequence identity) to that of the German TRV isolate SP, collected from spinach (Schmidt and Koenig, 1999). Also, in the same cluster is the German ON isolate (91.9% identity to PaY4). A second, less closely related cluster comprised TRV isolate TCM (83.7% identical to PaY4) and PEBV-D isolate E-116 (84.7% identical to PaY4).

The second ORF (2b) is separated from the coat protein ORF by 135 nt. It starts with an AUG codon at nucleotides 1475–1478 and extends to a UAA codon at nucleotides 2190–2192, encoding a protein of 237 aa residues with a molecular weight of 27 kDa. This protein has extensive amino acid similarity with the 2b nematode-transmission proteins that are encoded by some other tobravirus isolates. As with the above-mentioned CP, sequence analysis of the Pay4 2b protein shows that it most closely resembles the 2b proteins of TRV isolates ON (95.3% identity) and SP (92%

TABLE 1

Frequency of Transmission of TRV PaY4 by *Paratrichodorus sp.* Nematodes

	P. anemones				*P. pachydermus*[a]				Total	
Virus	SP[b]	%	BP[c]	%	SP	%	BP	%	BP total	%
WT	23/23	100	16/23	69	16/20	80	12/16	75	28/39	72
2bfs	23/25	92	0/23	0	18/20	90	0/18	0	0/41	0
2cfs	13/15	86	4/13	30	16/20	80	7/16	43	11/29	38
2cΔ	15/15	100	8/15	53	31/35	88	22/31	70	30/46	65

[a] Mixed population of *P. pachydermus* and *T. primitivus*.

[b] Source plants (number of plants with virus in roots over number of inoculated plants), totals from at least two experiments per virus/vector combination.

[c] Bait plants (number of plants with virus in roots over number of inoculated plants).

identity). The next most closely related group comprises TRV TCM (64.5% identical) and PEBV-D E116 (61.3%). The 2b proteins from three other virus isolates show significantly lower sequence identities (TRV PpK20, 42.8%; PEBV-B TpA56, 41.9%; TRV TpO1, 39.5%).

The third ORF (2c) is located 180 nt downstream of ORF2 and starts with an AUG codon at nucleotides 2368–2369, extending to a UAA codon at nucleotides 3225–3227. This ORF encodes a protein of 286 aa residues with a molecular weight of 32 kDa. The PaY4 2c protein very closely resembles the 2c (287 aa) protein of TRV SP (87.4% identical), and part of it is also almost identical to a small (9-kDa) ORF in TRV ON.

The 3′ part of TRV PaY4 RNA2, 170 nt downstream of the 2c gene, is derived from TRV RNA1 and includes part of the 1b (16-kDa) gene. However, this sequence is only 93% identical to TRV SYM RNA1, and the duplicated, partial 1b gene contains four premature translation termination codons.

Infectivity and transmission tests using cloned TRV PaY4

Western blotting and electron microscopy showed that RNA transcripts synthesized from the full-length clone of TRV isolate PaY4 RNA2 were infectious when inoculated to plants together with RNA1 of TRV PpK20 (data not presented). The cloned TRV PaY4 virus was transmitted by *P. anemones* nematodes (Table 1), showing that it retained the transmission properties of the noncloned, PaY4 field isolate, and further demonstrating that only RNA2 and not RNA1 determines vector specificity. Also, the cloned PaY4 virus was transmitted by *P. pachydermus* (Table 1) but not by *T. primitivus* (Vassilakos, MacFarlane, and Brown, unpublished).

Three PaY4 RNA2 mutants [2b frameshift (2bfs), 2c deletion (2cΔ), and 2c frameshift (2cfs)] generated as part of this study, multiplied and spread in leaves and roots of *Nicotiana benthamiana* plants with efficiencies similar to that of the wild-type virus. RT-PCR analyses demonstrated that the mutations were stably maintained, and electron microscopy revealed that the infected plants contained virus particles typical of TRV (data not shown). However, a 2b-deletion mutant was not stable in plants, undergoing further, multiple deletion during propagation. For this reason, the 2b-deletion mutant was not included in nematode transmission tests.

The three stable RNA2 mutants (2bfs, 2cΔ, and 2cfs) were each tested for transmission by both *P. anemones* and *P. pachydermus* in at least two separate experiments. Virus containing the wild-type, cloned PaY4 RNA2 was used as a positive control. The wild-type virus was found in the roots of all source plants (23 of 23) included in the *P. anemones* tests and in the roots of most (16 of 20) source plants used in the *P. pachydermus* tests. All of the mutants were found to infect source plant roots with similar efficiencies (Table 1).

The wild-type virus was transmitted to 28 of 39 plants (72%), with similar frequencies for *P. anemones* (69%) and *P. pachydermus* (75%). The 2bfs mutant was not transmitted to any of 41 bait plants, showing that this protein is essential for nematode transmission both by *P. anemones* and *P. pachydermus*. The 2c mutants were each transmitted by both of the nematode species. The 2cΔ mutant was transmitted at near wild-type frequency by both *P. anemones* (53%) and *P. pachydermus* (70%). The 2cfs mutant, however, was transmitted with lower frequency (*P. anemones*, 30%; *P. pachydermus*, 43%).

Mutagenesis and transmission testing of TRV-C1

TRV-C1 is an *in vitro*-constructed, recombinant virus containing TRV PpK20 RNA1 and RNA2 derived from clone pPEB5T (Mueller *et al.,* 1997). This RNA2 consists of the terminal part of the 5′ noncoding region (NCR) of TRV PpK20 RNA2, joined to the CP subgenomic promoter, structural and nonstructural genes, and the 3′NCR of PEBV TpA56 RNA2. Previously this virus was shown to replicate and spread efficiently in plants but was not tested for transmission. It was expected that TRV-C1 would be transmitted by *T. primitivus,* the known vector of

TABLE 2

Transmission of TRV 2b Mutants Following Coinoculation with Transmissible Virus

Virus	Wild type or mutant	Plant species	Source plants containing both viruses	Bait plants containing both viruses[a,b]
TRV PaY4 (Expt. 1)	2cΔ[c] + 2bfs	*N. clevelandii*	5/7	4/5
TRV PaY4 (Expt. 2)	2cΔ + 2bfs	*N. clevelandii*	ND	2/11
TRV PpK20 (wt) + TRV PaY4 2bfs		*N. benthamiana*	8/15	0/8[d]
TRV-C1	wt	*N. benthamiana*	3/3[e]	2/3[e]
TRV-C1	2bΔ	*N. benthamiana*	3/3[e]	0/3[e]
TRV-C1	Wt + 2bΔ	*N. benthamiana*	5/12	1/5

[a] Identity of transmitted virus checked by RT-PCR and sequencing.
[b] Nematodes from a mixed population of *Paratrichodorus pachydermus* and *Trichodorus primitivus*.
[c] Mutant 2cΔ is fully transmissible and, thus, is equivalent to wild type.
[d] No bait plants contained TRV PaY4 2bfs.
[e] Single virus only present in these plants.
Note. ND, not determined.

PEBV TpA56. In addition, a 2b-deletion mutation (29Δ), previously shown to prevent transmission of PEBV TpA56 (MacFarlane *et al.,* 1996), was also introduced into TRV-C1. As expected, although the wild-type TRV-C1 was transmissible, the TRV-C1 2b deletion (2bΔ) mutant was not transmissible (Table 2).

Coinfection of plants with TRV tagged with the green or red fluorescent protein

One approach to studying the mechanism of action of the tobravirus transmission protein would be to test whether the 2b protein, when expressed from one virus, could rescue the transmission of a second virus carrying a mutation in its own 2b gene. A successful result would indicate that, like the helper proteins of other, insect-transmitted viruses, the tobravirus 2b protein functions *in trans.* This would also provide a means to investigate transmission specificity, by examining combinations of viruses with different vector nematodes. Difficulties might occur, however, if the close sequence similarity between the viruses leads to interference by one virus with the replication or movement of the second virus, as happens during cross-protection (Ponz and Bruening, 1986). Indeed previous work showed that, in some circumstances, coinoculation of plants with tobacco mosaic virus expressing either green fluorescent protein (GFP) or red fluorescent protein (RFP) did not lead to mixed infection of single cells (S. Santa Cruz and K. Oparka, personal communication).

Previously, a clone of TRV PpK20 was constructed in which the 2b and 2c genes were replaced with the green fluorescent protein (GFP) gene (MacFarlane and Popovich, 2000). Infection of plants by this virus (TRV-GFP) can be followed by fluorescence microscopy. We inoculated plants with a mixture of TRV-GFP and a second virus (TRV-RFP) in which the GFP gene was replaced by the gene for a red fluorescent protein (Matz *et al.,* 1999). On inoculated leaves, both viruses accumulated in discrete infection foci, which came into contact with one another but did not appear to merge (Fig. 2). Examination of the roots of these plants showed that the viruses were able simultaneously to infect the same lateral root but that individual cells did not appear to contain both viruses. Although some regions of root had yellow areas, suggesting mixing of the green- and red-tagged viruses, optical sectioning showed that these were formed by the overlaying of separate green and red fluorescent cells.

Complementation of transmission of 2b-mutant viruses

In two experiments, transmissible TRV PaY4 2cΔ was mixed with nontransmissible TRV PaY4 2bfs and inoculated to *N. clevelandii* plants (Table 2). Use of the 2cΔ mutant instead of wild-type TRV PaY4 made it possible to test for the presence of both this virus and the 2bfs mutant in a single PCR assay (Fig. 3). In one experiment five source plants contained both the 2cΔ and 2bfs virus in the roots. The 2bfs mutant was transmitted from four of these plants to bait plants (Table 2). In the second experiment, two of 11 bait plants were found to contain the 2bfs mutant, although it was not determined how many of the 11 source plants contained both viruses in the roots. In a single experiment, the TRV-C1 2bΔ mutant was transmitted to one bait plant following infection of five source plants with both the mutant and wt TRV-C1 (Table 2).

A test was carried out to determine whether the 2b protein of TRV PpK20, which is transmitted by *P. pachydermus,* could complement transmission of the TRV PaY4 2bfs mutant, the parental clone of which is also transmitted by *P. pachydermus.* Although eight source plants were infected with both viruses, none of the cor-

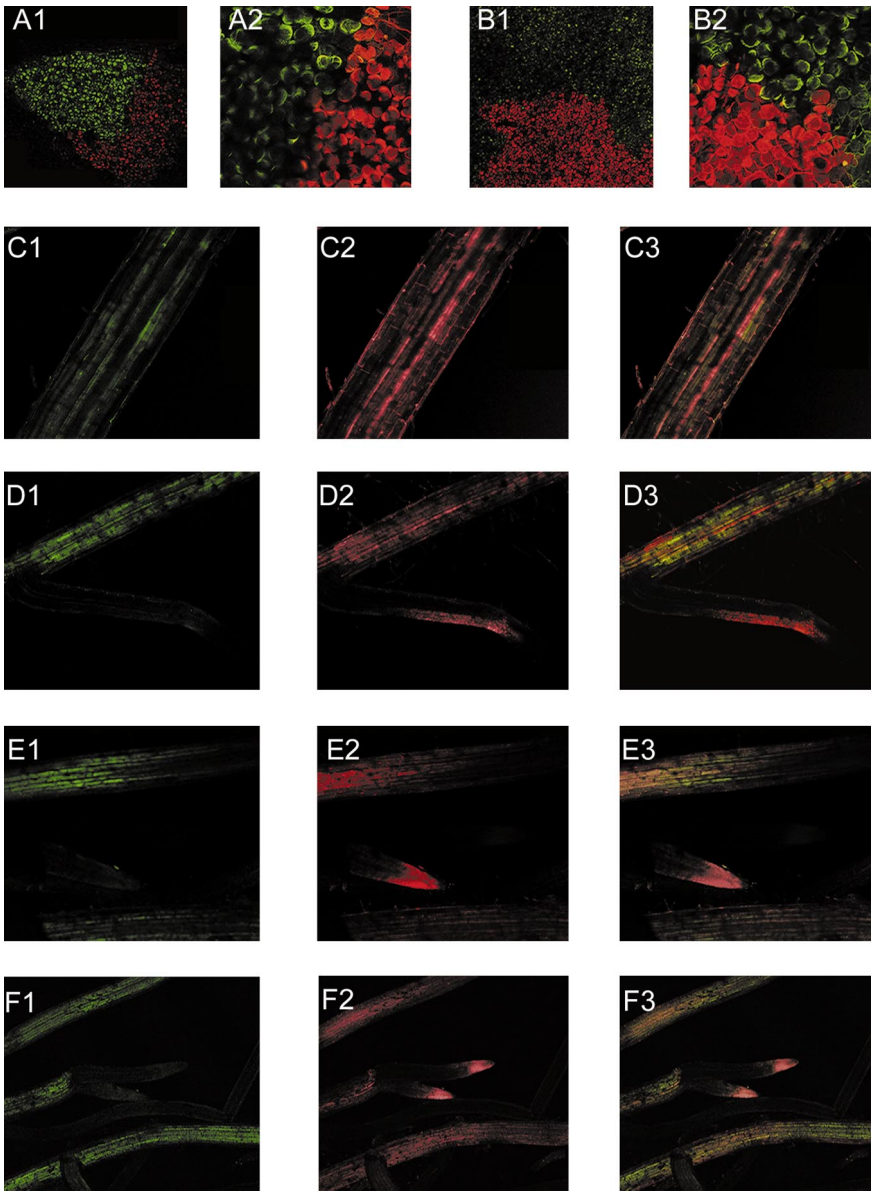

FIG. 2. Confocal fluorescence microscopy of plants coinfected with TRV-GFP and TRV-RFP. (A1) adjacent green- and red-fluorescent infection foci on an inoculated leaf (×1.6 magnification); (A2) same area (×10); (B1) inoculated leaf (×1.6); (B2) same area (×10); (C–F) roots infected with TRV-GFP and TRV-RFP. Image 1, GFP fluorescence; image 2, RFP fluorescence; image 3, red and green images overlayed.

responding bait plants became infected with the TRV PaY4 2bfs mutant (Table 2).

DISCUSSION

This study shows that, as has been found for the majority of tobravirus isolates examined to date, the RNA2 of TRV PaY4 is a recombinant molecule that carries RNA1-derived sequences at its 3′-terminus. Transcripts of a cDNA clone of PaY4 RNA2 were combined with RNA1 from TRV isolate PpK20 to produce virus for nematode transmission testing. Although TRV PpK20 itself is transmitted only by *P. pachydermus,* the pseudorecombinant PpK20/PaY4 virus was transmitted both by *P. pachydermus* and *P. anemones* (the natural vector of the field isolate of TRV PaY4). This is further evidence that RNA1 does not have a role in determining vector specificity, and is in agreement with earlier work which suggested that RNA2 contains the determinants of nematode transmission (Ploeg *et al.,* 1993).

TRV PaY4 is the first recombinant tobravirus experimentally demonstrated to be nematode transmitted. It is not absolutely certain but is very likely that the recombination did not occur during the limited mechanical passaging of the virus prior to cloning and,

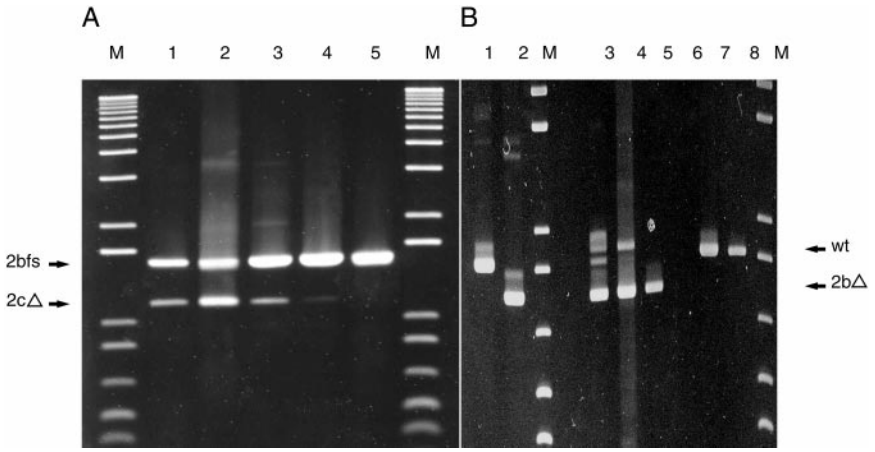

FIG. 3. Confirmation by RT-PCR of complementation of nematode transmission of TRV 2b-mutants. (A) TRV PaY4. Lanes 1 and 2, source plants after inoculation with both 2cΔ and 2bfs mutant. Lanes 3 and 4, corresponding bait plants with transmitted virus. Lane 5, wild-type plasmid. (B) TRV-C1. Lane 1, wild-type plasmid; lane 2, 2bΔ plasmid; lane 3 (source plant) and lane 4 (bait plant) after inoculation with both wild-type and 2bΔ mutant; lane 5 (source plant) and lane 6 (bait plant) after inoculation with 2bΔ mutant only; lane 7 (source) and lane 8 (bait plant) after inoculation with wild-type virus only.

thus, it is probable that the field isolate of PaY4 is a recombinant. In many of the other similar tobravirus recombinants that were previously sequenced the duplicated 1b gene is identical to the RNA1-encoded homolog. For PaY4, the 1b-duplicate contains several frameshift mutations. This could be taken as evidence that the recombination event did not occur recently, and that the recombinant virus has been propagated in the field for a significant period.

In addition, TRV PaY4 is the first sequenced tobravirus isolate that is known to be transmitted by more than one species of vector nematode. Construction of an infectious, nematode-transmissible cDNA clone of TRV PaY4, and comparison with TRV PpK20, makes it possible now to investigate the molecular details of vector selection by these viruses.

The virus CP is essential and fundamental for nematode transmission because unencapsidated viral RNA would not remain viable when ingested by a vector nematode. Deletion of different parts of the C-terminal domain of the PEBV CP markedly reduced or even abolished transmission of the virus, even though virus particle formation was unaffected (MacFarlane *et al.,* 1996). Similarly, the DI7 mutant of TRV PpK20, which has the 15 C-terminal residues of the CP replaced with three nonviral amino acids, was found to be encapsidated but not to be nematode-transmissible (Hernandez *et al.,* 1996). This supports earlier suggestions that the C-terminal CP domain, which is unstructured and is thus "flexible," could be involved in binding interactions with specific surface molecules inside the vector nematode (Mayo *et al.,* 1994). In the yeast two-hybrid assay, the TRV PpK20 CP was shown to interact with the 2b transmission protein (Visser and Bol, 1999). However, replacement of the 19 C-terminal residues with 15 nonviral amino acids prevented interaction of the CP with the 2b protein.

Although this domain is involved in the transmission process, it is not known whether it plays any part in vector specificity. Comparison of the C-terminal CP domain from different tobravirus isolates shows that there is significant amino acid sequence conservation in this region (Fig. 4). The C-terminus of the flexible region is

```
                    . .  ..        .... **...*
TRV PaY4        WVAGAAGTPAGVNAVAGGGTPSGGGRGPNS   P. anemones/P. pachydermus
TRV ON          WATGAAGAPASGNAVSGGGTPPGGGRGPNP
TRV SP          WVAGTAGAPAGGNAVASGGTPPGGGRGPGP
PEBV-D E116     WSAT---APAAA-----GGTPAGGGRGPGA
TRV TCM         W---TAAAPVAAA----GGTPPGGGRGPGT
TRV TpO1        W-AGS--APA---------TSSGGGKGPVV   T. primitivus
PEBV-B TpA56    WKETPQQQQNVTGPTVPA-TSSGGGKGPGVA  T. primitivus
TRV PpK20       F--GGAAASSSAPPPA-----SGGPIRPNP   P. pachydermus
```

FIG. 4. Comparison of the C-terminal "flexible" region of the coat proteins of different tobraviruses. Dots above the sequences identify residues that are identical in at least five of the eight isolates. Asterisks above the sequences identify residues that are identical in all of the isolates. The nematode vector(s) of each isolate (where known) appears at the right of the alignment.

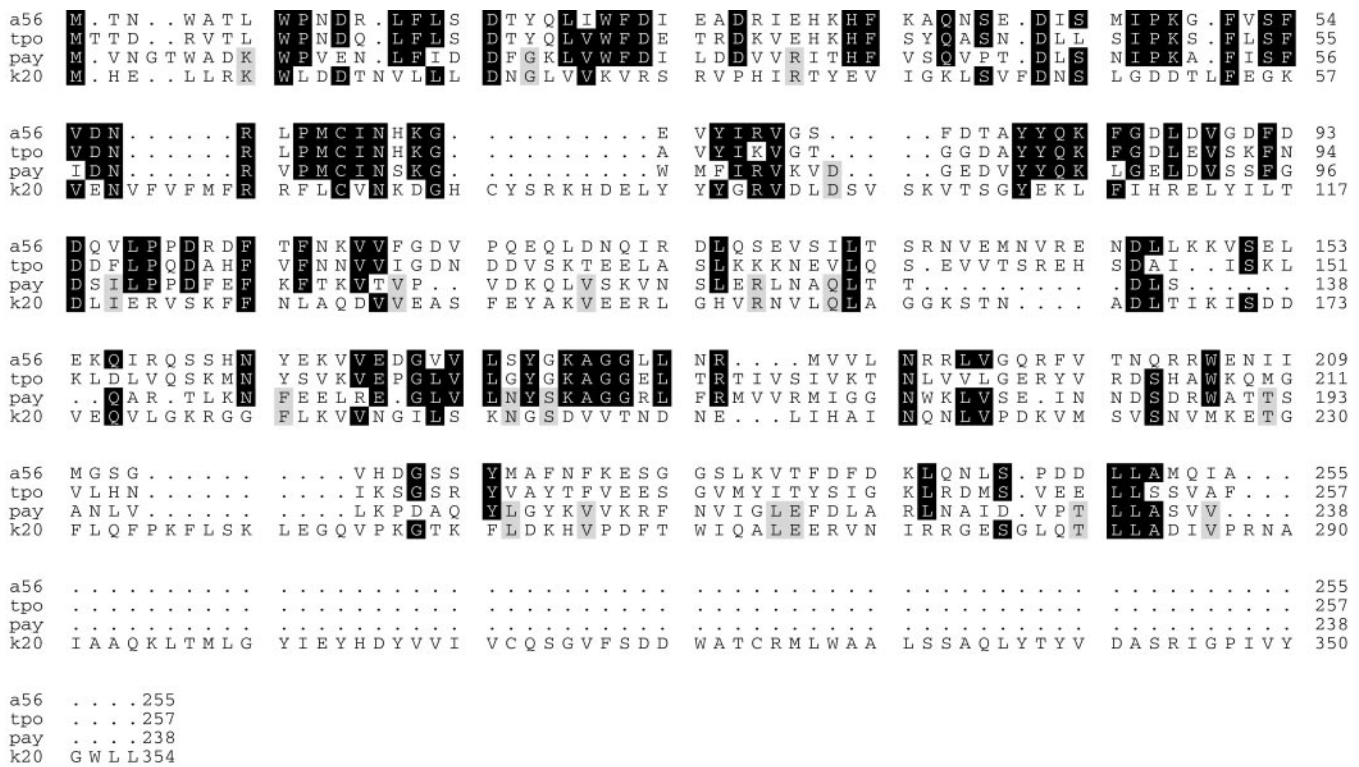

FIG. 5. Amino acid sequence alignment of the 2b transmission helper proteins from four tobraviruses with known vector nematodes. Residues which are identical in three or more of the viruses are boxed in black. Residues which are identical only between TRV PaY4 and TRV PpK20 are boxed in gray. Virus names are a56 (PEBV TpA56), tpo (TRV TpO1), pay (TRV PaY4), and k20 (TRV PpK20).

most highly conserved between TRV PaY4, TRV ON, TRV SP, TRV TCM, and PEBV-D. It is not known what species of nematode transmitted TRV ON, TRV SP, TRV TCM, or PEBV-D. However, in one study, a TRV isolate belonging to the PEBV-D serotype was transmitted by *P. teres,* but was not transmitted by *P. pachydermus* (Ploeg *et al.,* 1996), which is a vector of TRV PaY4. Furthermore, it is clear that the C-terminal domains of the TRV PaY4 and PpK20 CP are very different, even though these isolates are both transmitted by *P. pachydermus.* Thus, it is possible that the CP C-terminal domain is not involved in the specific recognition of vector nematodes.

The PaY4 2b protein has a central role in the transmission process and, as was found with the TRV PpK20 and PEBV TpA56 2b proteins, is absolutely required for transmission to occur. In addition, the PaY4 2b protein is essential for transmission by both *P. anemones* and *P. pachydermus.* The 2b proteins of TRV PaY4 and TRV PpK20 are very dissimilar, even though both of these viruses are transmitted by *P. pachydermus* (Fig. 5). The TRV PpK20 2b protein is significantly larger than all of the other tobravirus 2b proteins, and amino acid sequence alignment suggests that the PpK20 2b protein includes a C-terminal domain that is not present in the other 2b proteins. The 2b proteins of PEBV TpA56 and TRV TpO1 (both transmitted by *T. primitivus*) have extensive regions of amino acid sequence in common with the 2b protein of TRV PaY4. Few of these shared-sequence motifs are present in the TRV PpK20 2b protein. Consequently, it is not clear which, if any, regions of the 2b protein might be involved in vector selection. It is possible that regions of both the CP and the 2b protein act together to determine which vector species will transmit the virus.

Transmission testing using plants infected with mixtures of viruses showed, for the first time, that the 2b protein functions *in trans* and can complement nematode transmission of mutant viruses that are usually nontransmissible. Thus, the tobravirus 2b protein is a true "helper" protein and, as has been proposed for the potyvirus helper component, might act as a bridge linking the virion and vector mouthparts. In the case of the TRV PaY4 transmission complementation experiments, PCR analysis appeared to show that the bait plants contained much more of the 2bfs mutant than of the fully transmissible 2cΔ clone (Fig. 3A, lanes 3 and 4). This suggests that infection of the bait plants by the 2b mutant is very efficient. Thus, it is likely that helper-complementation acts at the stage of virus acquisition from the source plant, and that the 2b protein is not involved in release of the virus from the nematode into the bait plant. The inability of wild-type TRV PpK20 to complement transmission of the TRV PaY4 2bfs mutant suggests that each 2b protein interacts only with its cognate virus CP. Use of this system may enable us to determine which se-

quences in the CP and 2b protein are responsible for nematode vector selection.

Coinoculation experiments with green- and red-tagged TRV PpK20 appeared to show that, although these two viruses are almost identical in sequence, they did not simultaneously infect the same root cell. Although the reasons for this interference are not clear, the phenomenon has implications for the nematode transmission process. If the TRV PaY4 or C1 "wild-type" and 2b-mutant viruses are segregated in a similar manner, then complementation can take place only when the contents of two or more cells are combined. Before feeding, trichodorid nematodes inject pharyngeal gland secretions into the root cell to aggregate the cytoplasm at the feeding site and liquefy the cell contents. The feeding process usually kills the cell but can cause nonlethal, physiological changes in other cells up to two layers below the epidermis (Taylor and Brown, 1997). These changes may cause leakage of cell contents, including virus, which may be taken up by the feeding nematode. Thus, viruses from more than one infected cell might be acquired during a single feeding episode. Alternatively, the complementation might have occurred inside the nematode esophagus, with active 2b protein being retained during a first feed, and the virus particle interacting with the retained 2b protein during a second feeding episode. Whatever the mechanism, however, these results demonstrate that the process of nematode transmission of tobraviruses has similarities with membrane feeding during aphid transmission tests, where helper component functions following physical mixing with virus particles.

MATERIALS AND METHODS

Virus propagation

Ploeg *et al.* (1992b) described the recovery of TRV from nine individual *P. anemones* nematodes extracted from soil collected in York, UK. In $F(ab')_2$-ELISA tests none of these isolates (PaY1 to PaY9) reacted strongly with any of eight different TRV serotype-specific antisera, although PaY8 did react very weakly with antisera to TRV SYM and TRV RQ. Also it was found that isolate PaY8 could not be transmitted by a mixed population (*P. pachydermus, T. primitivus,* and *T. cylindricus*) of nonviruliferous nematodes from Woodhill (Scotland) (Ploeg *et al.,* 1992a). For the work described here, isolate PaY4 was selected at random, and mechanically inoculated to *N. benthamiana* plants for propagation and further investigation.

Cloning and sequencing

Total RNA was extracted from virus-infected plants using the method of Verwoerd *et al.* (1989). First-strand cDNA synthesis was carried out using a primer complementary to the 3′-terminus of all TRV RNAs. A full-length clone of PaY4 RNA2 was obtained by long template PCR using a 5′ primer incorporating a T7 RNA polymerase promoter and a 3′ primer carrying a *Sma*I restriction site downstream of the virus sequence (MacFarlane, 1996). The PCR fragment (~3.8 kb) was ligated into pT7Blue (Novagen, Madison, WI), and a clone containing the correct viral 5′- and 3′-terminal sequences, as determined by sequencing using vector-specific primers, was selected for further study. The full-length clone was completely sequenced on both strands by creating a series of overlapping internal deletions, and by using virus-specific, internal primers that were designed as the sequence became available. Sequence analysis was carried out using Wisconsin Package programs (Genetics Computer Group, Madison, WI).

Construction of mutants

Mutations were introduced by Klenow treatment of restricted DNA or deletion between preexisting restriction sites using standard methods. Mutant 2bΔ contains a deletion between an *Msc*I site (position 1503) and a *Pfl*MI site (position 1675). Mutant 2bfs was created by Klenow treatment following restriction at an *Xba*I site (position 1608). Mutant 2cΔ contains a deletion between an *Aat*II site (position 2580) and an *Nsi*I site (position 2956). Mutant 2cfs was created by Klenow treatment following restriction at the *Aat*II site.

The construction of a cDNA clone (pPEB5T) of TRV-C1 RNA2 was described previously (Mueller *et al.,* 1997). A TRV-C1 2b mutant was constructed by deletion between two *Bgl*II sites (positions 1723 and1837 in PEBV TpA56 RNA2) within the 2b gene.

TRV-RFP was constructed by PCR amplification of the RFP gene from plasmid pDsRed (Clontech, Palo Alto, CA) using modified primers to insert an *Nco*I site at the initiation codon and a *Kpn*I site immediately downstream of the termination codon of the gene. The *Nco*I–RFP–*Kpn*I fragment was used to precisely replace the *Nco*I–GFP–*Kpn*I fragment from pTRV-GFPc (MacFarlane and Popovich, 2000)

Infection of and detection of virus in plants

The full-length and mutant clones were linearized by digestion with *Sma*I (which cuts at the 3′-terminus of the virus sequence) and *Pst*I, which cuts between the T7 promoter immediately upstream of the virus sequence and a second T7 promoter in the cloning vector. Capped transcripts were prepared using a commercial kit (Ambion, Austin, TX), mixed with total plant RNA containing RNA1 of TRV isolate PpK20 and inoculated to *N. benthamiana* as described before (MacFarlane *et al.,* 1996). Following inoculation with transcripts, infection of plants was confirmed by electron microscopy and by RT-PCR. Tissue samples were taken from the leaves and

roots of infected plants and prepared for Northern blotting as described before (MacFarlane *et al.,* 1996). Northern blots were probed with virus-specific, minus-sense transcripts labeled with alkaline phosphatase according to the manufacturer (AlkPhos Direct; Amersham Pharmacia Biotech, Piscataway, NJ).

For detection of virus by RT-PCR, first-strand cDNA synthesis was carried out as described above. Amplification of RNA2 to confirm the identity of the TRV PaY4 2b mutants was carried out using primer 238, which corresponds to nucleotides 1084–1103 of PaY4 RNA2, and primer 245, which is complementary to nucleotides 1983–2005. Amplification of the TRV PaY4 2c mutants was carried out using primer 267, which corresponds to nucleotides 2453–2473, and primer 244, which is complementary to nucleotides 3514–3534. Amplification of TRV-C1 was carried out using the PEBV TpA56-specific primers 122, corresponding to nucleotides1197–1214, and 154, which is complementary to nucleotides 2043–2059.

Nematode transmission testing

Nematode transmission tests were carried out essentially as described before (MacFarlane *et al.,* 1996), except that two populations of nematodes were used in this study. Virus-free *P. anemones* nematodes were obtained as a homogeneous population from York (England), whereas virus-free *P. pachydermus* nematodes were obtained from Woodhill (Scotland) mixed together with *T. primitivus*. Successful transmission of virus to *N. clevelandii* bait plants was revealed by inoculating a homogenate of the bait plant roots to local lesion indicator plants (*Chenopodium amaranticolor*). The identity of the transmitted virus (wild-type or mutant) was confirmed by RT-PCR and sequencing.

Detection of green and red fluorescence

Virus-infected leaf and root tissues were examined with a Bio-Rad MRC 1000 confocal laser scanning microscope (Bio-Rad, Richmond, CA), using methods described previously (Oparka *et al.,* 1995). GFP-fluorescence was detected following excitation at 488 nm using a krypton-argon laser with a 522-nm emission filter. Excitation of RFP was at 488 nm with detection between 570 and 620 nm.

ACKNOWLEDGMENTS

We thank Sheena Lamond for help with the nematode transmission tests. N.V. is supported by an EU TMR Grant (FAIR-BM-96-2556); E.V. is supported by an EU TMR Grant (FAIR-BM-97-3730); and E.B. is funded by Large Scale Biology Inc. (Vacaville, CA). The Scottish Crop Research Institute (SCRI) is grant-aided by the Scottish Executive Rural Affairs Department.

REFERENCES

Espinoza, A. M., Markham, P. G., Maule, A., and Hull, R. (1988). *In vitro* biological activity associated with the aphid transmission factor of cauliflower mosaic virus. *J. Gen. Virol.* **69,** 1819–1830.

Govier, D. A., and Kassanis, B. (1974). Evidence that a component other than the virus particle is needed for aphid transmission of potato virus Y. *Virology* **57,** 285–286.

Harrison, B. D. (1967). Pea early-browning virus (PEBV). *In* "Annual Report for 1966 of Rothamstead Experimental Station," p. 115.

Harrison, B. D., Robinson, D. J., Mowat, W. P., and Duncan, G. H. (1983). Comparison of nucleic acid hybridisation and other tests for detecting tobacco rattle virus in narcissus plants and potato tubers. *Ann. Appl. Biol.* **102,** 331–338.

Hernández, C., Carette, J. E., Brown, D. J. F., and Bol, J. F. (1996). Serial passage of tobacco rattle virus under different selection conditions results in deletion of structural and non-structural genes in RNA2. *J. Virol.* **70,** 4933–4940.

Hernández, C., Visser, P. B., Brown, D. J. F., and Bol, J. F. (1997). Transmission of tobacco rattle virus isolate PpK20 by its nematode vector requires one of the two non-structural genes in the viral RNA2. *J. Gen. Virol.* **78,** 465–467.

MacFarlane, S. A. (1996). Rapid cloning of uncharacterised tobacco rattle virus isolates using long template (LT) PCR. *J. Virol. Methods* **56,** 91–98.

MacFarlane, S. A. (1997). Natural recombination among plant virus genomes: Evidence from tobraviruses. *Semin. Virol.* **8,** 25–31.

MacFarlane, S. A. (1999). The molecular biology of the tobraviruses. *J. Gen. Virol.* **80,** 2799–2807.

MacFarlane, S. A., and Popovich, A. H. (2000). Efficient expression of foreign proteins in roots from tobravirus vectors. *Virology* **267,** 29–35.

MacFarlane, S. A., Wallis, C. V., and Brown, D. J. F. (1996). Multiple genes involved in the nematode transmission of pea early browning virus. *Virology* **219,** 417–422.

Matz, M. V., Fradkov, A. F., Labas, Y. A., Savitsky, A. P., Zaraisky, A. G., Markelov, M. L., and Lukyanov, S. A. (1999). Fluorescent proteins from nonbioluminescent Anthozoa species. *Nat. Biotech.* **17,** 969–973.

Mayo, M. A., Robertson, W. M., Legorburu, F. J., and Brierley, K. M. (1994). Molecular approaches to an understanding of the transmission of plant viruses by nematodes. *In* "Advances in Molecular Nematology" (F. Lamberti, C. De Giorgi, and D. McK. Bird, Eds.), pp. 277–293. Plenum Press, New York.

Mueller, A.-M., Mooney, A. L., and MacFarlane, S. A. (1997). Replication of *in vitro* tobravirus recombinants shows that the specificity of template recognition is determined by 5′ non-coding but not 3′ non-coding sequences. *J. Gen. Virol.* **78,** 2085–2088.

Oparka, K. J., Roberts, A. G., Prior, D. A. M., Chapman, S., Baulcombe, D. C., and Santa Cruz, S. (1995). Imaging the green fluorescent protein in plants: Viruses carry the torch. *Protoplasma* **189,** 131–141.

Pirone, T. P., and Blanc, S. (1996). Helper-dependent vector transmission of plant viruses. *Ann. Rev. Phytopathol.* **34,** 227–247.

Ploeg, A. T., Brown, D. J. F., and Robinson, D. J. (1992a). Acquisition and subsequent transmission of tobacco rattle virus isolates by *Paratrichodorus* and *Trichodorus* nematode species. *Neth. J. Plant Path.* **98,** 291–300.

Ploeg, A. T., Brown, D. J. F., and Robinson, D. J. (1992b). The association between species of *Trichodorus* and *Paratrichodorus* vector nematodes and serotypes of tobacco rattle tobravirus. *Ann. Appl. Biol.* **121,** 619–630.

Ploeg, A. T., Robinson, D. J., and Brown, D. J. F. (1993). RNA2 of tobacco rattle virus encodes the determinants of transmissibility by trichodorid vector nematodes. *J. Gen. Virol.* **74,** 1463–1466.

Ploeg, A. T., Zoon, F. C., de Bree, J., and Asjes, C. J. (1996). Analysis of

the occurrence and distribution of tobacco rattle virus in field soil and disease in a subsequent tulip crop. *Ann. Appl. Biol.* **129,** 461–469.

Ponz, F., and Bruening, G. (1986). Mechanisms of resistance to plant viruses. *Ann. Rev. Phytopathol.* **24,** 355–381.

Schmidt, K., and Koenig, R. (1999). Genetic analysis of large-sized RNA2 species of a TCM-like tobacco rattle virus source from spinach. *Arch. Virol.* **144,** 503–511.

Taylor, C. E., and Brown, D. J. F. (1997). "Nematode Vectors of Plant Viruses." CAB International, New York.

Van Hoof, H. A. (1968). Transmission of tobacco rattle virus by Trichodorus species. *Nematologica* **14,** 20–24.

Verwoerd, T. C., Dekker, B. M. M., and Hoekema, A. (1989). A small-scale procedure for the rapid isolation of plant RNAs. *Nucleic Acids Res.* **17,** 2362.

Visser, P. B., and Bol, J. F. (1999). Nonstructural proteins of *Tobacco rattle virus* which have a role in nematode-transmission: Expression pattern and interaction with viral coat protein. *J. Gen. Virol.*, **80,** 3272–3280.