

Molecular biology of the tobnaviruses

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Introduction

The genus *Tobravirus* comprises of three different viruses, tobacco rattle virus (TRV), pea early-browning virus (PEBV) and pepper ringspot virus (PepRSV) (Harrison, 1973; Robinson & Harrison, 1989*a, b*). The tobnavirus genome is divided into two positive-sense, single-stranded RNAs, each of which is encapsidated separately into rod-shaped particles. TRV, in particular, has a widespread distribution, can infect a very large number of plant species and causes economically significant disease in potato, tobacco and ornamental bulbs. PEBV has been found in northern Europe and North Africa, where it infects primarily legumes, including pea and field bean. PepRSV, which has been described only in Brazil, causes disease in pepper, tomato and globe artichoke. Tobnaviruses are unusual in being one of only two types of virus that are transmitted from plant to plant by soil-inhabiting nematodes (Taylor & Brown, 1997). There is a highly specific relationship between virus and nematode, so that particular virus isolates are transmitted only by certain vector nematode species. An additional, distinguishing feature of tobnaviruses is their ability to cause two types of infection. Plants infected with a so-called NM-type virus isolate contain only the larger viral RNA (RNA1), which can multiply and spread in the complete absence of the second, smaller RNA (RNA2). As RNA2 encodes the coat protein (CP), infection with RNA1 alone does not produce virus particles. Isolates containing both viral RNAs, referred to as M-type isolates, are encapsidated. Several earlier reviews describe in detail the biology and epidemiology of the tobnaviruses (Harrison & Robinson, 1978, 1986; Boulton, 1996). This paper aims to compile more recent molecular data obtained from the sequencing of various tobnavirus isolates and from site-directed mutagenesis of infectious, cloned tobnavirus genomes.

Complete sequence data are now available for RNA1 from two isolates each of TRV and PEBV and one isolate of PepRSV (Table 1). For RNA2, nine isolates of TRV, three isolates of PEBV and one isolate of PepRSV have been sequenced (Table 1). A system was proposed that denoted the internal genes of RNA1 according to the decreasing size of the subgenomic (sg) RNAs from which they probably are expressed (Robinson,

1983). Thus, the two internal genes of RNA1 were referred to as the 1a and 1b genes and their translation products as the 1a and 1b proteins. Despite some anomalies, this system is particularly useful when considering the highly variable tobnavirus genomic RNA2, and will be used throughout this paper.

RNA1

The RNA1 molecules of each virus are similar in length (TRV isolate SYM, 6791 nt; PepRSV isolate CAM, 6828 nt; PEBV isolate SP5, 7073 nt) and has a conserved gene organization (Fig. 1). As was deduced initially from hybridization studies in solution (Robinson & Harrison, 1985*a*), there is only limited RNA1 nucleotide sequence similarity (58–65%) between the three viruses. However, the RNA1 sequences of the two TRV isolates that have been sequenced (SYM and ORY) are 99% identical and the RNA1 sequences of two PEBV isolates are 92% identical.

The 5' non-coding regions (NCR) of RNA1 of PEBV (126 nt) and PepRSV (201 nt) share 64% sequence identity, whereas the 5' NCR of TRV (202 nt) is less identical (44–46%), although a few shared sequence motifs are apparent. The TRV RNA1 5' NCR contains imperfect direct repeats of 22 nucleotides, which are also present in TRV RNA2 (Hamilton *et al.*, 1987). In both RNA1 and RNA2, the 3' NCRs of TRV and PEBV are 73% identical in sequence over the 3'-terminal 170 nt, whereas PepRSV is 45% identical to PEBV and 51% identical to TRV in this region. RNA2 of many TRV isolates and of PepRSV CAM is recombinant (see below), and up to 1 kb of the 3' region is either identical or nearly identical to RNA1. For PEBV SP5, which is not recombinant, the 3' NCRs of RNA1 and RNA2 are, nevertheless, 96% identical over the 3'-terminal 267 nt. Although this sequence conservation presumably reflects a common function of these regions in RNA replication, absolute identity between the 3' termini of RNA1 and RNA2 is not essential. Indeed, a TRV isolate composed of TCM RNA1 and PLB RNA2 retained the different, RNA-specific 3'-terminal sequences even after a series of 25 passages in tobacco (Angenent *et al.*, 1989*b*). The 3'-terminal 140 nt of RNA2 of TRV PSG (and by analogy all other tobnavirus RNAs) was found to adopt a tRNA-like structure consisting of two hairpin loops and a pseudoknot (van Belkum *et al.*, 1987). TRV RNA2 could be adenylated at

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Table 1. Sources of complete tobnavirus sequences

Virus	Isolate	Accession no.	Reference	Infectious clone?	Reference
RNA1					
TRV	SYM	D00155	Hamilton <i>et al.</i> (1987)	+	Hamilton & Baulcombe (1989)
	ORY	AF034622	Sudarshana & Berger (1998)	–	–
PEBV	SP5	X14006	MacFarlane <i>et al.</i> (1989)	+	MacFarlane <i>et al.</i> (1991, 1992)
PEBV-BBYBV	Algerian	NA	Ferguson (1999)	–	–
PepRSV	CAM	L23972	Unpublished	–	–
RNA2					
TRV	PSG	X03686	Cornelissen <i>et al.</i> (1986)	–	–
	PLB	J04347	Angenent <i>et al.</i> (1989 <i>b</i>)	+	Angenent <i>et al.</i> (1989 <i>c</i>)
	ORY	AF034621	Sudarshana & Berger (1998)	–	–
	TCM	X03955	Angenent <i>et al.</i> (1986)	–	–
	PpK20	Z36974	Hernández <i>et al.</i> (1995)	+	Hernández <i>et al.</i> (1995)
	Rostock	NA	Unpublished	–	–
	TpO1	AJ009833	MacFarlane <i>et al.</i> (1999)	–	–
	PaY4	NA	Unpublished	–	–
	ON	Z97357	Uhde <i>et al.</i> (1998)	–	–
PEBV-B	SP5	X51828	Goulden <i>et al.</i> (1990)	+	MacFarlane <i>et al.</i> (1991, 1992)
	TpA56	X78455	MacFarlane & Brown (1995)	+	MacFarlane <i>et al.</i> (1996)
PEBV-D	E116	AJ006500	Swanson & MacFarlane (1999)	–	–
PepRSV	CAM	X03241	Bergh <i>et al.</i> (1985)	–	–

NA, Not available.

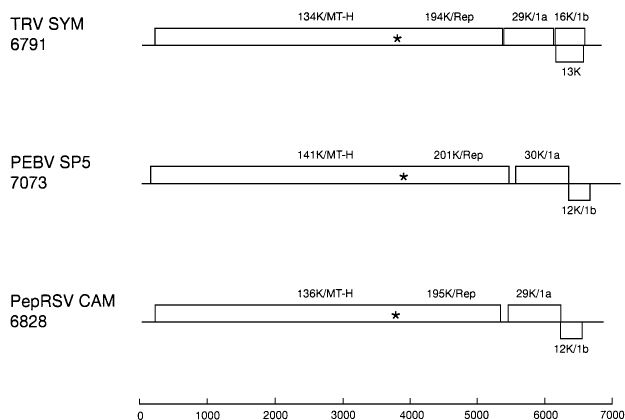


Fig. 1. Genome organization of tobnavirus RNA1. ORFs are boxed. Protein sizes (in kDa; indicated by K) and names appear above each gene. MT, Methyltransferase domain; H, helicase domain; Rep, RNA-dependent RNA polymerase. Asterisks denote a leaky translation termination codon. The PEBV and PepRSV 1a and 1b genes and the TRV 1b and 13K genes overlap. The RNA lengths (nt) appear under the isolate names.

the 3' terminus but, unlike most other plant viruses with tRNA-like terminal sequences, it could not be aminoacylated.

Replicase gene

The 5'-proximal gene on RNA1 encodes a protein of molecular mass 134 kDa (TRV), 141 kDa (PEBV) or 136 kDa

(PepRSV) that contains amino acid motifs associated with methyl transferase and nucleotide-binding/helicase proteins derived from viruses within the Sindbis-like supergroup (Rozanov *et al.*, 1992; Koonin, 1991). Immediately downstream of the helicase gene and in the same reading frame is a gene that encodes a protein with amino acid motifs typical of RNA-dependent RNA polymerases. *In vitro* translation experiments with both TRV and PEBV suggested that this gene is expressed from full-length genomic RNA1 by readthrough translation of the termination codon of the helicase gene (Pelham, 1979; Hughes *et al.*, 1986). Subsequently, it was shown that translation termination at the TRV helicase UGA (opal) codon could be suppressed by some chloroplast and cytoplasmic tRNAs, leading to the incorporation of either tryptophan or cysteine residues at this position (Zerfass & Beier, 1992; Urban *et al.*, 1996). The complete helicase/RNA polymerase (replicase) gene occupies nearly 75% of RNA1. Sequence comparisons of the replicase proteins show that PepRSV (195 kDa) and PEBV (201 kDa) are more closely related to each other than to TRV (194 kDa). As a group, the three tobnaviruses are most similar to plant viruses from the genera *Hordeivirus* and *Tobamovirus*. The C-terminal, '54K' region of the PEBV replicase gene was transformed into *Nicotiana benthamiana* plants and conferred resistance to infection of the plants by different isolates of PEBV (MacFarlane & Davies, 1992). As with other examples of 'replicase-mediated resistance' (Lomonosoff, 1995), PEBV 54K-mediated resistance was highly

sequence-specific and did not protect against infection by TRV or PepRSV.

1a (movement protein) gene

The 1a gene, located downstream of the polymerase gene, encodes a 29 kDa (TRV, PepRSV) or 30 kDa (PEBV) protein that has sequence similarity to the cell-to-cell movement protein (MP) of tobacco mosaic virus (TMV). The TRV 29 kDa protein was shown to be a virus MP by mutagenesis of an infectious cDNA clone of TRV SYM RNA1 (Hamilton & Baulcombe, 1989). Deletion of the TRV 1a gene prevented accumulation of the virus in inoculated leaves of tobacco (Ziegler-Graff *et al.*, 1991). However, inoculation of the TRV 1a mutant together with TMV, or to transgenic tobacco expressing the TMV MP, did result in the accumulation of TRV in inoculated leaves. Unexpectedly, it was found that inoculation of wild-type TRV RNA1 to TMV MP-transgenic plants resulted in a 5- to 10-fold increase in the level of the TRV RNA (Ziegler-Graff *et al.*, 1991). The TRV 1a gene probably is translated from an sgRNA that Northern blot and *in vitro* translation data suggest is encapsidated (Pelham, 1979; Robinson *et al.*, 1983; Boccara *et al.*, 1986). However, the 1a sgRNA of PEBV and PepRSV probably are not encapsidated (Hughes *et al.*, 1986; Bergh & Siegel, 1989).

1b (cysteine-rich protein) gene

The 3'-proximal gene of RNA1 encodes a 16 kDa (TRV) or 12 kDa (PEBV, PepRSV) protein. The N-terminal part of the 1b protein is rich in cysteine residues, whereas the C-terminal region includes several basic residues. Small, cysteine-rich proteins (CRPs) are encoded by a number of other plant viruses and have been implicated in virus gene expression (Donald & Jackson, 1994; Hehn *et al.*, 1995) and seed transmission (Edwards, 1995). Sequence alignments have revealed amino acid motifs that are shared between the CRPs of some viruses (Diao *et al.*, 1999) and have also suggested a similarity between the 1b protein and high-mobility-group chromatin proteins (Koonin *et al.*, 1991).

As with the CRP of barley stripe mosaic virus, the PEBV 1b gene has a role in seed transmission. Deletion of the 1b gene prevented seed transmission in pea plants without affecting virus accumulation (Wang *et al.*, 1997). However, a PEBV 1b frame-shift mutant did not accumulate in pea, suggesting that this gene may have more than one function. Indeed, when inoculated to *N. benthamiana*, both the deletion and frame-shift mutants accumulated to much lower levels than did wild-type PEBV (S. MacFarlane, unpublished). The initiation codons of the PEBV and PepRSV 1b genes, but not the TRV 1b gene, overlap the termination codon of the 1a gene. The overlap is preceded by a run of six adenine residues and is reminiscent of a so-called 'slippery sequence' that is responsible for frame-

shifting, for example, during translation of the potato leafroll virus replicase gene (Prüfer *et al.*, 1992). This raises the possibility that the PEBV and PepRSV 1b proteins may in some circumstances be expressed as fusions with the C terminus of the 1a protein.

The TRV 1b protein has been detected by Western blotting in protoplasts, where it was incorporated in a high molecular-mass complex, possibly together with host proteins (Angenent *et al.*, 1989a), and was shown by immunogold (IGL) electron microscopy to be localized predominantly in the nucleus (Liu *et al.*, 1991). Thus far it has not been examined for any possible role in seed transmission. In earlier studies, deletion of the 1b gene from TRV isolate SYM had no effect on virus accumulation in tobacco (Guilford *et al.*, 1991). However, more recently, deletion of the 1b gene from TRV isolate PpK20 was shown to reduce virus accumulation greatly in both protoplasts (*N. benthamiana*) and whole plants (*Nicotiana tabacum* and *N. benthamiana*) (S. MacFarlane, unpublished). Currently, the differences in these observations remain unexplained. A smaller open reading frame (ORF) encoding a putative polypeptide of 13 kDa (13K) was located within the TRV 1b gene but in a different reading frame (Angenent *et al.*, 1989b). It is not known whether this ORF is expressed, but transformation of plants with the 13K ORF or the 1b gene had no obvious effect on subsequent infection by TRV (Angenent *et al.*, 1990).

RNA2

In contrast to the situation with RNA1, hybridization studies have shown that different isolates of TRV have very little nucleotide sequence identity in RNA2 (Robinson & Harrison, 1985a). Fewer isolates of PEBV have been described, and so strain variation is not well understood, and only one isolate of PepRSV has been studied in any detail. However, PEBV has been grouped into three different serotypes corresponding to British PEBV (PEBV-B), Dutch PEBV (PEBV-D) and broad bean yellow band virus (PEBV-BBYBV) (Russo *et al.*, 1984). RNA2 molecules of isolates of the three serotypes did not appear to be closely related, although sequences from two isolates of PEBV-B (SHE and SP5) were similar to one another (Robinson & Harrison, 1985b).

The extreme variation in the RNA2 sequences of different tobavirus isolates makes it very difficult to describe a 'wild-type' or 'complete' genome organization (Fig. 2). RNA2 almost always encodes a gene for the virus CP, although deleted forms lacking the CP gene can be found in infected plants (Hernández *et al.*, 1996). Tobaviruses can only persist in the field if they retain the ability to be transmitted by nematodes. Recent studies have shown that one or more non-structural genes, located on RNA2 downstream of the CP gene, are involved in the transmission process (Ploeg *et al.*, 1993a; MacFarlane *et al.*, 1995, 1996; Hernández *et al.*, 1997). However, isolates that lack transmission genes can arise either

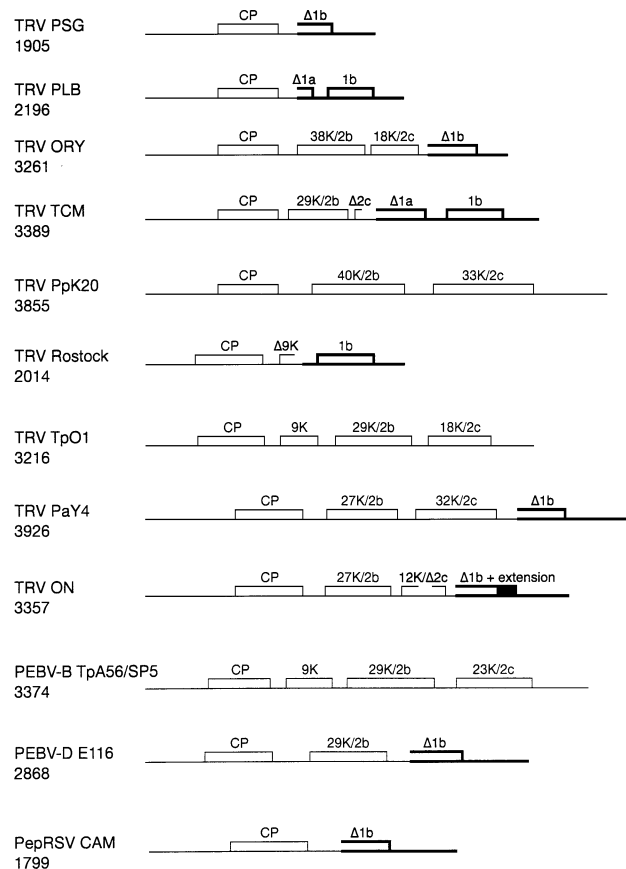


Fig. 2. Genome organization of tobnavirus RNA2. CP, Virus coat protein. Open-ended boxes denote partial/truncated ORFs. Sections in bold are derived from RNA1 by recombination.

in the glasshouse by repeated mechanical inoculation or in the field by vegetative propagation of infected crop plants such as potato or bulbous ornamentals.

RNA2 recombination

An additional source of variation in RNA2 results from recombination (Robinson *et al.*, 1987; MacFarlane, 1997). RNA1 and RNA2 of all isolates of each virus have nearly identical sequences in the non-coding part of the extreme 3' terminus. However, nine of the 13 tobnavirus RNA2 species sequenced are recombinant and have both coding and non-coding sequences at the 3' terminus that are derived from RNA1. The recombined region can be more than 1 kb in length and can encode partial or complete copies of the RNA1-encoded 1a and 1b genes. Recombination may also occur between two different tobnaviruses. Thus, RNA2 of TRV isolate TCM has a 5' terminus derived from TRV RNA2, a central region probably derived from PEBV RNA2 and the 3' region derived from TRV RNA1 (Angenent *et al.*, 1986; Goulden *et al.*, 1991). Such recombinant isolates should not necessarily be thought of as defective as, for example, during

a survey of the coastal bulb-growing areas of the Netherlands, 30% of the tobnavirus isolates recovered from fields were found to be TRV/PEBV recombinants (Ploeg *et al.*, 1991). Also, a cDNA clone of RNA2 of TRV isolate PaY4 was found to include an RNA1-derived sequence at the 3' terminus. Transcript RNA synthesized from this clone can give rise to infectious virus that retains its nematode-transmissibility (N. Vassilakos, D. Brown & S. MacFarlane, unpublished).

Two striking examples of RNA2 sequence conservation do exist. TRV isolates PSG and PLB, both infecting the same stock of potato plants from the Netherlands, were found to be identical for 1376 nt at the 5' terminus of RNA2 and, thus, encoded identical CPs. The 3' region of each of these isolates was derived from RNA1 but differed in exact sequence and length (Cornelissen *et al.*, 1986; Angenent *et al.*, 1989b). Perhaps more surprisingly, the 5' 1312 nt of RNA2 of the Rostock isolate of TRV, obtained from potato in Germany, was found to be 98% identical to TRV TpO1, which was isolated from a potato field in Oxford, UK (P. Willingmann, personal communication). However, TRV Rostock RNA2 is recombinant and has a duplicate 1b gene at the 3' terminus, whereas TRV TpO1 RNA2 is not recombinant but in the 3' region encodes non-structural genes involved in nematode-transmission.

RNA2 cis-acting sequences

The 5' NCR of RNA2 of different tobnavirus isolates varies considerably in length (470–710 nt). Protoplast studies with infectious cDNAs showed that TRV PLB RNA2 that retained 35 nt at the 5' terminus and 450 nt at the 3' terminus was still capable of replication (Angenent *et al.*, 1989c; Angenent, 1989). However, inclusion of only 109 nt at the 3' terminus was insufficient to permit replication. For PEBV, clones retaining 80 nt or more at the 5' terminus were able to replicate (Mooney, 1998). As described above, the 3' NCRs of TRV and PEBV are more than 70% identical over the 3'-terminal 170 nt and probably adopt a similar tRNA-like structure. As a result, recombinants in which the 3' NCR of TRV RNA2 is replaced with that of PEBV, or vice versa, are able to replicate efficiently. However, inclusion of the PEBV 5' NCR in TRV, or vice versa, prevented replication of RNA2 (Mueller *et al.*, 1997). Although the tobnavirus CP gene is the 5'-proximal gene on RNA2, it is not translated directly from the genomic RNA but rather from an sgRNA. This unusual strategy is necessary because the 5' NCR includes as many as seven translation initiation (AUG) codons upstream of the functional AUG codon. Deletion mutagenesis experiments confirmed that, whereas the full-length RNA2 of TRV PLB and PEBV SP5 was not translated *in vitro*, removal of sequences including the upstream AUG codons permitted the expression of virus CP. Earlier studies, in which the CP was translated from genomic RNA2, probably used preparations contaminated with CP sgRNA. A recent paper reported the translation of CP and concomitant ex-

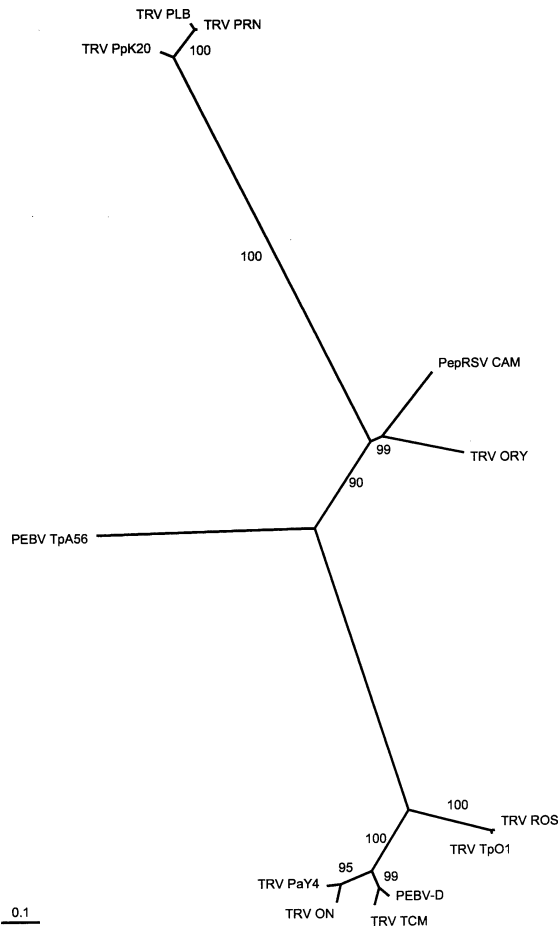


Fig. 3. Phylogenetic relationships estimated by approximate maximum-likelihood analysis of CP amino acid sequences of tobroviruses derived by using the quartet-puzzling method (Strimmer & von Haeseler, 1996). The JTT amino acid substitution matrix was used. Rate heterogeneity among positions in the sequence was modelled by using a gamma distribution with an alpha parameter of 1.02. Branch lengths are proportional to genetic distance. Bootstrap percentages less than 70% are not shown.

pression of two other, downstream genes from cloned full-length RNA2 of TRV ORY, but did not present data (Sudarshana & Berger, 1998).

The 5' ends of the TRV PSG and PEBV SP5 CP sgRNAs have been cloned and sequenced (Cornelissen *et al.*, 1986; Wallis, 1992) and the TRV SYM CP sgRNA was purified from virus particles and translated *in vitro* (Robinson *et al.*, 1983). Sequence analysis suggested that (part of) the tobrovirus CP sgRNA promoter might include a putative stem-loop structure that is conserved in size but not sequence, and is located immediately upstream of a GCAUA motif that includes the 5' end of the sgRNA (AUA) (Goulden *et al.*, 1992). Site-directed mutagenesis experiments confirmed that base-pairing of residues to maintain the stem-loop structure is essential for CP expression (Mooney, 1998). Similar sequences, but often with imperfect stem-loops and with GCAUU motifs, can be found upstream of the 1a and 1b genes as well as some of the other RNA2-encoded genes. For this reason, it is expected that these

genes also are expressed from sgRNAs, although direct evidence to support this hypothesis has not been obtained.

Virus coat protein

As was observed with the replicase proteins (above), the tobrovirus CP most closely resembles those of hordeiviruses and tobamoviruses, which have the same rod-shaped particle structure (Dolja *et al.*, 1991). Alignment of the amino acid sequences of tobrovirus CPs with that of TMV, whose particle structure has been studied extensively (Namba & Stubbs, 1986), revealed that the CP subunits of these viruses folded similarly. Thus, the CP subunits of tobroviruses form a tight helical array with their N and C termini located on the external surface of the virus particle (Goulden *et al.*, 1992).

In particular, the C-terminal domain of the tobrovirus CP is much larger than the equivalent part of the TMV CP. This part of the TRV CP is highly immunogenic and therefore located on the surface (Legorburu *et al.*, 1996). NMR studies of PepRSV revealed that the CP C-terminal region is unstructured and presumably extends away from the surface of the virus particle (Brierley *et al.*, 1993). This 'protruding', flexible domain was suggested to have a potential role in the recognition of tobrovirus particles by vector nematodes during transmission (Mayo *et al.*, 1994). Subsequent experiments showed that deletion of the CP C-terminal domain of PEBV prevented nematode transmission of the virus (MacFarlane *et al.*, 1996).

Phylogenetic analysis of the different tobrovirus CPs reveals a complex relationship (Fig. 3). PepRSV most closely resembles TRV ORY (both isolates originate in the Americas). Two Dutch (PSG and PLB) and one Scottish TRV isolate (PpK20) form a distinct cluster. PEBV-D (E116) most closely resembles the recombinant TRV TCM and is more similar to the German TRV ON and TRV Rostock and British TRV PaY4 and TRV TpO1 isolates than it is to PEBV-B.

Transgenic plants that expressed the TRV CP were shown to be resistant to subsequent infection by the virus (van Dun & Bol, 1988). Interestingly, the resistance was homology-dependent, as plants containing the TRV TCM CP resisted infection with TRV TCM but not TRV PLB and vice versa (the CPs of these isolates share only 39% amino acid sequence similarity; Angenent *et al.*, 1990). Also, the TRV TCM CP plants were largely protected against a Dutch isolate of PEBV, the RNA2 of which is highly homologous to TRV TCM RNA2. The CP-mediated resistance was effective against infection by virions but not viral RNA and operated in intact plants but not in protoplasts. It also appeared that CP-mediated resistance affected the replication of both RNA1 and RNA2, even though RNA1 has no sequence homology with the RNA2-derived CP gene and does not require RNA2 to infect untransformed plants. Although CP-transgenic plants were resistant to virions after mechanical inoculation, the same plants were susceptible to virus inoculation by nematodes (Ploeg *et al.*, 1993 *b*).

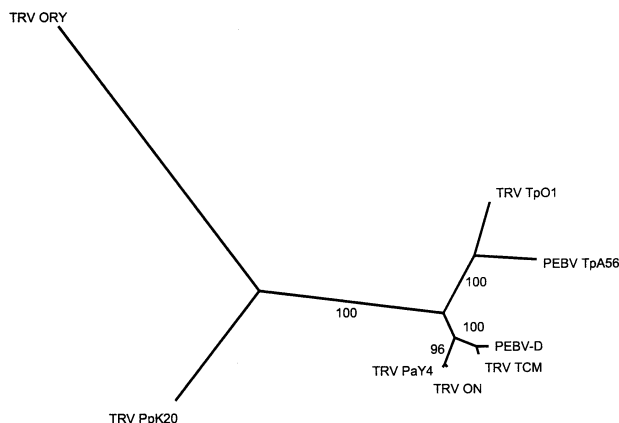


Fig. 4. Phylogenetic relationships of the 2b protein amino acid sequences of tobnaviruses. Rate heterogeneity was modelled by using a gamma distribution with an alpha parameter of 2.29.

RNA2-encoded non-structural proteins

TRV PpK20 RNA2 encodes two additional genes downstream of the CP gene. Adopting the system of Robinson (1983), and considering that the CP (or 2a protein) is expressed from an sgRNA, the two additional genes will be referred to as 2b and 2c.

The TRV PpK20 2b protein has limited sequence similarity to proteins encoded by several other TRV and PEBV isolates (Fig. 4). Much greater similarity exists between the 2b proteins of TRV PaY4 and TRV ON or between TRV TCM and PEBV-D E116. Mutagenesis studies have demonstrated that the 2b protein is absolutely required for virus transmission by nematodes (MacFarlane *et al.*, 1996; Hernández *et al.*, 1997). It is not known how the protein functions, although one mechanism might involve a direct interaction between the 2b protein and specific retention sites within the nematode oesophagus. Although the TRV and PEBV 2b proteins can be detected by Western blotting in the leaves and roots of infected plants (Johnsen *et al.*, 1991; Schmitt *et al.*, 1998; P. Visser, personal communication), it has not been possible by using IGL to localize these proteins either in plants or in nematodes (I. Karanastasi, personal communication). The 2b amino acid sequence alignment grouped together PEBV TpA56 and TRV TpO1 (Fig. 4), whereas these two viruses were not grouped together when the CPs were compared (Fig. 3). These two virus isolates are both transmitted by the same vector nematode, *Trichodorus primitivus*, which does not transmit any of the other isolates that have been sequenced. Thus, it is possible that transmissibility and vector specificity are both determined by the virus 2b protein. Transgenic plants were produced that expressed the 2b gene of TRV TCM (Angenent *et al.*, 1990), but these plants were not resistant to virus after mechanical inoculation and were not tested for their effect on nematode transmission.

The 2c proteins from different TRV and PEBV isolates generally share very little sequence similarity. However, small

ORFs can be found in TRV TCM and TRV ON that encode truncated proteins that are very similar to the 2c protein of TRV PaY4. Specifically, the protein encoded by the TRV TCM 2c ORF, which is disrupted after 51 nucleotides by a recombination with RNA1 sequences, has eight of 17 residues identical to the N terminus of the TRV PaY4 2c protein. The published sequence of TRV ON encodes a 2c-like protein of 77 amino acids that is 90% identical to the N terminus of PaY4 2c. However, removal of one base from the TRV ON sequence extends the protein by another 20 residues, all of which are identical to the C terminus of PaY4 2c. In this case, the TRV ON 2c protein would be 93% identical to the PaY4 2c protein but with a central deletion of 189 amino acids. The function of the 2c protein is not understood. Deletion or frame-shift mutation of the PEBV TpA56 2c gene did not affect virus multiplication, but resulted in a significant reduction in the frequency of nematode transmission (MacFarlane *et al.*, 1996; Schmitt *et al.*, 1998). This protein has been detected by Western blotting in infected plants, where it appears to be glycosylated. In contrast, mutation of the 2c genes of TRV PpK20 (Hernández *et al.*, 1997) and TRV PaY4 (N. Vassilakos, D. Brown & S. MacFarlane, unpublished) had no effect on nematode transmission. Whether this result reflects differences between TRV and PEBV or between the different vector nematode species remains to be discovered.

Three virus isolates (PEBV SP5, PEBV TpA56 and TRV TpO1) include an additional ORF, located between the CP and 2b genes (Fig. 2). Computer analysis suggested that this ORF (which encodes a putative 9 kDa protein designated 9K) was unlikely to be expressed and the 9K protein was not detected in plants by Western blotting (C. Schmitt & S. MacFarlane, unpublished). Nevertheless, nematode-transmission of a PEBV 9K mutant was reduced significantly, suggesting that the gene is functional and that the protein may be expressed as a C-terminal fusion to the CP (MacFarlane *et al.*, 1996). Further experiments are necessary to clarify these results.

Conclusions

The last few years have seen a significant effort to clone and sequence tobnavirus isolates from diverse sources. This has made very clear the high degree of sequence conservation in RNA1, contrasting with the extreme variability in RNA2, and has led to the availability of infectious clones of tobnavirus isolates with different biological properties. Using these clones, it will be possible to pursue tobnavirus research into a more detailed, functional analysis of viral proteins. Particular areas of study that could benefit from this approach include: (i) vector transmission, where highly specific interactions occur between virus and nematode, (ii) systemic invasion of plants by the tobnavirus RNA1 without the need for encapsidation, and perhaps also (iii) the understanding and deployment in crop species of host resistance, both natural and transgenic, against these viruses.

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