The 2b protein of *Pea early-browning virus* (PEBV) is required for transmission of the virus by nematodes. Comparison of the 2b proteins of highly transmissible (TpA56) and poorly transmissible (SP5) isolates of PEBV identified two amino acid substitutions (G90S and G177R) that might be responsible for the poor transmission of isolate SP5. Hybrid viruses were created in which the TpA56 2b protein carried SP5-specific substitutions at residue 90 or 177, and in which the SP5 2b protein carried TpA56-specific substitutions at these positions. Transmission tests showed that the G177R substitution is sufficient to prevent nematode transmission of the virus. Examination of the 2b proteins from PEBV and other tobraviruses predicted the presence of a coiled-coil domain in the central region of the protein. This structural element is important for the association of interacting proteins and, thus, might mediate interaction of the 2b protein with the virus coat protein or with the vector nematode.

Tobraviruses, which include *Pea early-browning virus* (PEBV), *Tobacco rattle virus* (TRV) and *Pepper ringspot virus* (PepRSV) are transmitted between plants by soil-inhabiting nematodes (Taylor & Brown, 1997). Glasshouse tests have demonstrated that particular tobravirus isolates can be transmitted only by certain nematode species. For example, TRV isolate PpK20 can be transmitted by *Paratrichodorus pachydermus* but not by *P. anemones* or *Trichodorus primitivus* (Hernández et al., 1997). In contrast, TRV isolate PaY4 can be transmitted by both *P. pachydermus* and *P. anemones* (Vassilakos, 2000; Vassilakos et al., 2001), whereas PEBV isolate TpA56 is transmitted by *T. primitivus* but not by *P. pachydermus* (MacFarlane & Brown, 1995).

Tobraviruses have two positive-stranded genomic RNAs, each of which is encapsidated separately in a rod-shaped particle. The larger RNA (RNA1) encodes proteins involved in the replication and spread of the virus in plants, and can infect plants systemically in the complete absence of the second, smaller genomic RNA (RNA2) (Harrison & Robinson, 1986). RNA2 was shown to carry the determinants for nematode transmission by the analysis of pseudorecombinant isolates of TRV in which the genomic RNAs from the nematode-transmissible PpK20 isolate and the non-transmissible PLB isolate were re-assorted (Ploeg et al., 1993). These experiments showed that inclusion of RNA2 from TRV PpK20 led to the transmission of TRV PLB RNA1, and the replacement of PpK20 RNA2 with PLB RNA2 resulted in failure to transmit PpK20 RNA1. Thus, RNA2 of TRV PpK20 encoded a transmission determinant(s) that was lacking in RNA2 of TRV PLB.

RNA2 has been sequenced from many tobravirus isolates, revealing a great variation in the size and gene content (MacFarlane, 1999). All isolates encode a coat protein (CP), although deleted forms of RNA2 lacking the CP gene can be generated in infected plants (Hernández et al., 1996). Many isolates include at the 3′ end of RNA2 sequences derived by recombination from RNA1. In some instances RNA2 carries partial and even complete 1a and 1b genes from RNA1. Downstream of the CP, a few isolates carry a gene encoding a putative 9K protein, and many isolates carry genes encoding the 2b and 2c proteins. Some of this sequence variation is thought to result from prolonged maintenance of virus by mechanical transmission in the glasshouse or by long-term infection of vegetatively propagated crop plants such as potato. In these situations virus spread does not require transmission by nematodes, thus removing any selection pressure to retain functional RNA2-encoded genes involved in the vector transmission process.

Full-length cDNA clones of RNA1 and RNA2 of the SP5 isolate of PEBV were constructed and found to be infectious (MacFarlane et al., 1991); however, virus derived from these clones was not transmitted by nematodes. This isolate originally was transmissible by *T. primitivus* but subsequently was maintained by frequent mechanical passage before being cloned. An infectious clone of RNA2 of PEBV isolate TpA56, a recently obtained, nematode-transmissible isolate, was then constructed. Virus consisting of SP5 RNA1 and TpA56 RNA2 was transmissible by *T. primitivus* nematodes, suggesting that a mutation in SP5 RNA2 prevented transmission of this isolate.
Sequence analysis of PEBV SP5 and PEBV TpA56 showed that RNA2 of both isolates was identical in size and that each encoded CP, 9K, 2b and 2c proteins. Furthermore, the two RNAs differed at only 11 of 3374 nucleotides, and only three of the base changes affected the amino acid sequences of the virus gene products. One base change produced a conservative substitution in the CP of valine to isoleucine, and was considered unlikely to be the cause of the difference in nematode transmissibility of the two isolates. Two of the base changes caused amino acid substitutions in the 2b protein. An adenine (SP5) to guanine (TpA56) change at nucleotide 1736 resulted in a serine (SP5) to glycine (TpA56) substitution at amino acid 90, and an adenine (SP5) to guanine (TpA56) change at nucleotide 1997 resulted in an arginine (SP5) to glycine (TpA56) substitution at amino acid 177. These results provided the first evidence that the tobavirus 2b protein is involved in nematode transmission. Subsequently, mutagenesis studies have demonstrated directly that the 2b protein is required for nematode transmission of PEBV TpA56 (MacFarlane et al., 1996), TRV PpK20 (Hernández et al., 1997) and TRV PaY4 (Vassilakos et al., 2001).

We constructed hybrids of RNA2 of PEBV SP5 and TpA56 in which nucleotides 1736 or 1997 were exchanged individually. Mutagenesis was carried out using a PCR strategy in which overlapping fragments were generated with complementary primer pairs carrying single-base changes. A 770 bp Bcl–PfJMI subfragment incorporating each of the mutations was re-cloned into the parental full-length RNA2 plasmids pT72 (isolate SP5: MacFarlane et al., 1991) or pT72A56 (isolate TpA56: MacFarlane et al., 1996). Clone TpA56/S1736 carries RNA2 of isolate TpA56 in which nucleotide 1736 is derived from SP5 resulting in a G90S substitution (Fig. 1A). Three other clones, TpA56/S1997, SP5/T1736 and SP5/T1997, resulting in G177R, S90G and R177G substitutions, respectively, were also constructed. Transcripts from these clones were mixed with RNA1 of PEBV SP5 and inoculated to N. benthamiana plants, as described previously (MacFarlane et al., 1991). The hybrid viruses caused systemic infections indistinguishable from those of the parental SP5 and TpA56 viruses, and Western blot analysis showed that expression of CP and 2b protein of each of the hybrid viruses was identical to that of the parental viruses (Fig. 1B).

Infected N. benthamiana plants were used as source plants in transmission tests with a mixture of predominantly T. primitivus and P. pachydermus nematodes isolated from soil collected at Woodhill near Dundee (MacFarlane & Brown, 1995). The nematodes were allowed to feed on the roots of virus-infected source plants for 3 weeks, after which time they were transferred to pots containing healthy N. benthamiana bait plants for a further 3 weeks. Transmission of virus was demonstrated by homogenization in tap-water of one-half of the root system of each source and bait plant, and inoculation of the root extract to Chenopodium amaranticolor plants. Development of pin-prick necrotic lesions on the inoculated leaves of these plants indicated that virus was present in the roots of the source and bait plants. To confirm the identity of the virus in these plants, the remaining part of each root system was processed to isolate total RNA using the method of Verwoerd et al. (1989). Viral RNA was amplified by RT–PCR using primer 501 (5′ GGACCCTTAATTAGGGTGCC), which corresponds to nucleotides 1384–1404, upstream of the PEBV 2b gene, and primers 502 (5′ CATTAAACAGTATGAAACACC) or 456 (5′ CTGCGCTGAGATCGAGCC) or 2675 (5′ CGTCGCGTGGATCGCAGGCTAAATAGGAGGTGCCC), which correspond to nucleotides 2245–2270 or 2694–2675, respectively, downstream of the 2b gene. The entire 2b gene from one example of each mutant collected from bait plants was completely sequenced to confirm maintenance of the mutation and lack of additional mutations. Thereafter, amplified PCR fragments were analysed by restriction digestion to confirm the identity of the virus in each plant. Specifically, isolate SP5 contains an Asel site (CTTATAT) at position 1655 which is lacking in isolate TpA56 (ATTAAC). Isolate TpA56 contains an AvaI site (CTCGGG) at position 2381 which is lacking in isolate SP5 (CTCAGG). One of the base changes under study in the 2b gene, G to A at position 1736, produces a diagnostic HphI site in TpA56 (GGTGAGA) which is absent in SP5 (AGTGAA). The G to A base change at
position 1997 could not be detected by restriction digestion; thus this region of the PCR products from every bait plant was sequenced to confirm the identity of the two hybrids (TpA56/S1997 and SP5/T1997) carrying base changes at this position.

The results of the transmission tests are shown in Table 1. As expected, PEBV isolate TpA56 was fully transmissible (51/51 plants); however, surprisingly, isolate SP5 was also transmitted in these tests although at a very low frequency (2/27 plants). RT–PCR and sequencing confirmed the identity of SP5 in the two, positive bait plants. This contrasts with previous tests in which SP5 was not transmissible, although a greater number of plants and vector nematodes were included in the tests reported here. Soil from Woodhill does contain a small number of trichodorid nematodes other than *P. pachydermus* and *T. primitivus*; however, it is not known whether these additional species are able to transmit PEBV. Nevertheless, replacement of nucleotide 1736 from TpA56 with that of SP5 did not markedly reduce transmission of the virus (27/30 plants) and, conversely, the SP5 hybrid in which nucleotide 1736 was replaced with that of TpA56 was not transmitted (0/23 plants). Thus, the base difference at nucleotide 1736 that substitutes glycine in TpA56 for serine in SP5 is not responsible for the very poor nematode transmission of PEBV SP5.

Exchange of nucleotide 1997 between TpA56 and SP5 RNA2 had a very pronounced effect on the transmission of these viruses. Alteration of SP5 to include nucleotide 1997 from TpA56 significantly increased the transmission frequency from 7% (2/27 plants) for the parental SP5 isolate to 38% (8/21 plants) for SP5/T1997. The reciprocal exchange reduced transmission of the virus from 100% (TpA56) to only 3% (1/30 plants; TpA56/S1997). Thus, the base difference at nucleotide 1997 that substitutes glycine in TpA56 for arginine in SP5 at amino acid 177 of the 2b protein has a substantive effect on the transmission of PEBV.

Phylogenetic analysis of the 2b proteins from different tobaviruses isolates showed them to partition into at least four different groups (MacFarlane, 1999). The 2b proteins from TRV ORY and TRV PpK20 each have little sequence homology to any other 2b protein. The TRV TpO1 and PEBV SP5/TpA56 2b proteins group together, and the PEBV E116, TRV TCM, TRV PaY4, TRV SP and TRV ON 2b proteins form a fourth group. An alignment of a selection of 2b sequences from these groups is shown in Fig. 2(A). The N-terminal part of the 2b proteins is the most conserved region, and the PpK20 and ORY proteins are larger than the other 2b proteins, with additional C-terminal sequences.

Structural analysis of the 2b proteins was carried out using the PIX package of programs (Williams & Faller, 1999) to search for functional motifs. These analyses predicted the existence of a coiled-coil domain in the central part of all of the different 2b proteins (Fig. 2A). For example, the PEBV TpA56 2b protein was predicted to form a coiled-coil between residues 115 to 171 (P > 0.9) that possibly has two α-helical segments. In structural terms a coiled-coil is a bundle of α-helices that are wound into a superhelix. The sequence of each helix taking part in the formation of the coiled-coil contains a heptad repeat of chemically conserved residues, with hydrophobic amino acids spaced regularly at the first and fourth position, and hydrophilic amino acids occupying the other positions (Lupas, 1996). However, most coiled-coils contain discontinuities in which the heptad pattern is disrupted at some point by missing or duplicated residues. Coiled-coils were first identified as a structural element in which peptide domains are stabilized via association between the hydrophobic residues from two or more α-helices. Latterly, coiled-coils have been found to be responsible for the oligomerization of a wide variety of proteins. The coiled-coil domains predicted for the tobavirus 2b proteins are shown in Fig. 2(B). Although these domains are located in the same, central region of the 2b protein, the domains differ in size and have only limited sequence homology to one another. It is possible to speculate that the 2b coiled-coil domain is responsible for the interaction of the 2b protein with the virus CP or with a protein(s) at the virus retention site in the pharyngeal tract of the vector nematode (Karanastasi, 2000; Karanastasi et al., 2000). Possibly, the sequence variation apparent in the 2b coiled-coil domains

<table>
<thead>
<tr>
<th>Virus isolate/hybrid</th>
<th>No. of bait plants infected/no. of source plants infected*</th>
<th>Transmission rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TpA56</td>
<td>51/51</td>
<td>100</td>
</tr>
<tr>
<td>SP5</td>
<td>2/27</td>
<td>7</td>
</tr>
<tr>
<td>TpA56/S1736</td>
<td>27/30</td>
<td>90</td>
</tr>
<tr>
<td>TpA56/S1997</td>
<td>1/30</td>
<td>3</td>
</tr>
<tr>
<td>SP5/T1736</td>
<td>0/23</td>
<td>0</td>
</tr>
<tr>
<td>SP5/T1997</td>
<td>8/21</td>
<td>38</td>
</tr>
</tbody>
</table>

* At least 70 *T. primitivus* nematodes were added to each source plant for transmission testing.

**Table 1. Transmission efficiencies of parental and hybrid viruses**
Fig. 2. (A) Alignment of amino acid sequences of 2b proteins from PEBV (TpA56, E116) and TRV (TpO1, PaY4, TCM, PpK20, ORY) isolates produced using ClustalW default parameters (Thompson et al., 1994). Dark shading indicates conserved residues in all sequences at a given position, medium shading is conservation in 80% of sequences, lightest shading is conservation in 60% of sequences. Predicted coiled-coil domains are indicated by underlining. The locations of glycine-90 and glycine-177 in the PEBV TpA56 2b protein are indicated by asterisks above the sequence. (B) Expanded view of predicted coiled-coil domains. Hydrophobic residues (A, I, L, M, V) at positions 1 and 4 of the heptad repeat are in bold.

is responsible for the specificity in the association between different virus isolates and nematode species. Interestingly, a coiled-coil domain promotes the interaction between the PIII and PII proteins of Cauliflower mosaic virus during transmission by vector aphids (Leclerc et al., 1998; Leh et al., 1999). Two mutations of the PEBV TpA56 2b protein are known to
severely affect nematode transmission of the virus. Mutant 29A carries a 38 amino acid in-frame deletion of residues 87 to 124 that prevents accumulation of the protein in plants and abolishes transmission (MacFarlane et al., 1996). This deletion extends into the beginning of the predicted coiled-coil domain and may, thus, prevent association of the 2b protein with other proteins. The TpA56/S1997 mutation, created as part of this work, results in a glycine to arginine substitution at residue 177, just downstream of the predicted coiled-coil. Possibly, introduction of the charged arginine residue at this position might affect oligomerization of the 2b protein with the CP or a nematode protein. Further studies are necessary to confirm the existence of this domain and to examine its putative role in the transmission process.

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