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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

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Natural recombinant tobacco rattle tobravirus (TRV) isolates contain sequences from a different tobravirus, pea early browning virus (PEBV). To characterize the sequence requirements for viable recombinant formation hybrid cDNA clones of RNA2 of PEBV and TRV were assembled. Inclusion of 320 nt from the 5′ terminus of PEBV or 335 nt from the 5′ terminus of TRV in the hybrid RNAs was sufficient to permit their replication by, respectively, PEBV RNA1 or TRV RNA1 regardless of the origin of the 3′ terminal region. However, PEBV RNA1 but not TRV RNA1 was sometimes able to support low level replication of RNA2 containing the heterologous 5′ terminal region. *In vitro* translation of PEBV transcripts containing 5′ non-coding region deletions supported the hypothesis that *in vivo* the PEBV coat protein (CP) is expressed from a subgenomic RNA and that, therefore, in the recombinants the CP subgenomic promoter probably is recognized by the replicase of the heterologous virus.

The tobraviruses, which include tobacco rattle virus (TRV) and pea early browning virus (PEBV), have a genome comprising two single-stranded, positive-sense RNAs. The larger genomic RNA (RNA1, ca. 7000 nt) encodes all the functions necessary for replication and intraplant movement and can cause systemic infection in the absence of the smaller RNA (RNA2, ca. 2000–4000 nt) which encodes the coat protein and in some instances one or more additional proteins. The tobraviruses are differentiated from one another by host range, serological properties and by their ability to form viable pseudorecombinant isolates only when both RNAs are derived from the same virus. Thus, for example, RNA2 of different isolates of TRV can be successfully combined with RNA1 from any isolate of TRV but RNA2 of PEBV cannot be combined with TRV RNA1 (Harrison & Robinson, 1986; Robinson & Harrison, 1985).

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This situation is complicated by the existence of so-called anomalous isolates of TRV which cause infections typical of TRV but react with antisera specific for PEBV. Two such isolates have been studied in some detail; I6 is serologically related to the British serotype of PEBV (PEBV-B), while TCM is serologically related to Dutch PEBV (PEBV-D) (Angenent *et al.*, 1986; Robinson *et al.*, 1987). DNA sequencing of the termini of I6 RNA2 showed that about 275 nt at the 5′ end are from TRV but that the 3′ end was derived from PEBV (Robinson, 1994). TRV isolate TCM has an even more unusual construction; about 150 nt at the 5′ end are from TRV RNA2, the central 2100 nt are from PEBV-D RNA2, while 1100 nt at the 3′ terminus are from TRV RNA1 (Angenent *et al.*, 1986). These isolates are thought to have arisen by recombination during replication in plants infected with both viruses. Recombination between TRV RNAs 1 and 2 has been detected during passage of a TRV isolate derived from an infectious cDNA clone (Hernandez *et al.*, 1996) but experimental demonstration of recombination between TRV and PEBV has not been achieved.

Although natural recombinant isolates of TRV have been found, no isolates of PEBV that contain TRV sequences are known. This may be due to the relative paucity of PEBV isolates described so far or it may be due to a more fundamental property of the viruses. In this paper, we report the construction of an infectious cDNA clone of RNA2 of TRV isolate PPK20 (Ploeg *et al.*, 1992; Hernandez *et al.*, 1995) and the generation of hybrids between this clone and an infectious clone of PEBV RNA2 (MacFarlane *et al.*, 1996). Experiments were carried out to compare the ability of TRV RNA1 and PEBV RNA1 to replicate hybrid RNA2 molecules containing heterologous 5′-terminal or 3′-terminal regions. Apart from the 5′-terminal six nucleotides (AUAAAA), the 5′ non-coding regions (NCRs) of TRV PPK20 and PEBV TPA56 RNA2 have almost no sequence homology (there are only four blocks of four or more identical bases between the 556 nt TRV sequence and the 509 nt PEBV sequence). The 3′ NCR of TRV PPK20 RNA2 is 392 nt and that of PEBV TPA56 is 482 nt. They are identical over the 3′-terminal 25 bases, 70% similar over the

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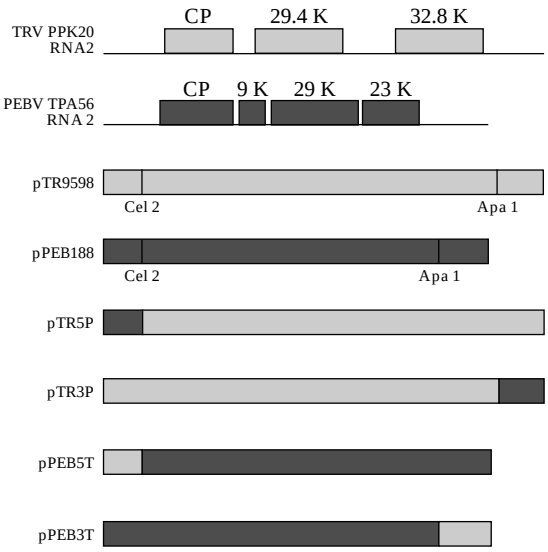

Fig. 1. Genome maps of TRV and PEBV RNA2, and structure of hybrid clones. PEBV sequences are black and TRV sequences are grey. The locations of the coat protein gene (CP) and additional non-structural genes are indicated.

next 140 bases upstream and only 44% similar over the remainder of the TRV NCR (the same figure obtained when comparing the RNA2 coding sequences).

A full-length cDNA clone of TRV PPK20 RNA2 was constructed by ligating a 3′-terminal 1·8 kb clone isolated from a viral cDNA library together with two 5′-terminal and central region clones derived by RT–PCR. The 5′-terminal PCR primer included a T7 RNA polymerase promoter sequence and allowed the synthesis of run-off transcripts of RNA2 after linearizing the full-length clone, pT72K20, at the 3′ terminus of the viral sequence with *Sma*I. The RNA2 transcripts were infectious to plants when co-inoculated with TRV PPK20 RNA1, obtained as a preparation of total RNA extracted from plants infected with TRV RNA1 only (data not shown).

New restriction sites were introduced in pT72K20 or pT72A56, an infectious, full-length cDNA clone of RNA2 of PEBV isolate TPA56 (MacFarlane & Brown, 1995; MacFarlane *et al*., 1996), using either the PCR ligase (Michael, 1994) or PCR-megaprimer (Steinberg & Gorman, 1994) methods. A *Cel*II and *Apa*I site were created at, respectively, nt 335 in the 5′ NCR and nt 3468 in the 3′ NCR of TRV RNA2. Clone pT72A56, which has a pre-existing *Cel*II site at position 320 in the 5′ NCR, was mutagenized to introduce an *Apa*I site at position 2910 in the 3′ NCR of PEBV RNA2. Restriction fragments containing the mutations were sequenced to ensure there were no second site changes, and the fragments were recloned into the full-length constructs. The resulting clones, pPEB188 (PEBV RNA2 with a novel 3′ *Apa*I) and pTR9598 (TRV RNA2 with novel 5′ *Cel*II and 3′ *Apa*I) were used to create further, hybrid molecules.

Four different hybrid clones with exchanges at the 5′ or 3′ termini of the viral cDNAs were constructed (Fig. 1). The 5′ NCR of PEBV RNA2 was replaced with that of TRV to produce clone pPEB5T. Replacement of the 5′ NCR of TRV with that of PEBV produced clone pTR5P. The 3′ NCR of PEBV RNA2 was replaced with that of TRV to produce clone pPEB3T, and the 3′ NCR of TRV was replaced with that of PEBV to produce clone pTR3P. Capped transcripts were synthesized from the wild-type and hybrid PEBV and TRV full-length cDNA clones using a commercial kit (MEGAscript T7, Ambion). Transcript RNA2 (30–50 μg) was combined with 100 μg of total RNA extracted from plants infected with either PEBV or TRV RNA1 and inoculated onto five *Nicotiana benthamiana* plants as described previously (MacFarlane *et al*., 1991).

Table 1. Co-replication results of combinations of wild-type and hybrid TRV and PEBV RNAs

RNA1	RNA2*	Replication of RNA2†	TRV ISEM‡	PEBV ISEM§
TRV	TRV[a]	5/5	+	−
PEBV	PEBV[b]	5/5	−	+
TRV	PEB5T	5/5	−	+
TRV	PEB3T	0/5	NT	−
TRV	TR5P	0/5	−	−
TRV	TR3P	5/5	+	NT
TRV	PEBV[b]	0/5	−	−
PEBV	PEB5T	0/5	−	−
PEBV	PEB3T	5/5	NT	+
PEBV	TR5P	5/5	+	−
PEBV	TR3P	1/5[a]	−	−
PEBV	TRV[a]	1/5[b]	−	−

* *a*, From clone pTR9598; *b*, from clone pPEB188.
† Detected by RT–PCR; results given as no. of positive plants over no. of plants tested. *a*, One positive, confirmed by digestion of PCR fragment by *Apa*I; *b*, one positive, identified by PCR amplification only.
‡ Particles trapped by anti-TRV antiserum.
§ Particles trapped by anti-PEBV antiserum.
NT, Not tested.

Replication of the hybrid RNAs was assessed by RT–PCR. Total RNA was isolated (Verwoerd *et al*., 1989) from leaf discs taken from upper, uninoculated leaves of each transcript-inoculated plant at 10–14 days post-inoculation. The RNA was annealed to a primer complementary to the 3′ terminus of all PEBV and TRV RNAs and transcribed using MuMLV reverse transcriptase (Life Technologies). Aliquots of the cDNA were amplified by PCR using different primer pairs specific to regions of TRV and PEBV RNAs 1 and 2. In addition, the presence of virus particles in inoculated plants was assayed by immunosorbent electron microscopy of sap extracted from leaf discs (Roberts, 1986).

The results of the inoculation experiments are summarized in Table 1. As would be expected, 'wild-type' TRV transcript

RNA2, synthesized from clone pTR9598 (and containing the novel restriction sites) was replicated by TRV RNA1 and produced particles that could be trapped by antibodies raised against TRV. Similarly 'wild-type' PEBV transcript RNA2, synthesized from clone pPEB188, was replicated by PEBV RNA1 and produced particles that were recognized by antibodies specific for PEBV.

Transcripts from clones pPEB5T and pTR3P were replicated by TRV RNA1 and produced particles that were trapped with, respectively, anti-PEBV and anti-TRV antibodies. By contrast, transcripts from clones pPEB3T and pTR5P were not replicated by TRV RNA1 and, consequently, did not produce virus particles. RT–PCR confirmed that in all these experiments TRV RNA1 replicated with or without any RNA2, producing a systemic infection in all the inoculated plants, and that there was no contamination with PEBV RNA1. In addition, the identity of the RNA2 molecules which were replicated in these experiments was confirmed by restriction enzyme digestion and sequencing of the amplified DNA fragments. These results show that, in agreement with sequence data from the natural TRV recombinant isolate I6, TRV RNA1 can replicate RNA2 containing the coding region and 3′ terminus of PEBV if it possesses 335 nt from the 5′ terminus of TRV RNA2. In addition, the presence of 3′-terminal sequences from PEBV RNA2 does not inhibit replication of TRV RNA2 by TRV RNA1. Conversely, any RNA2 which has 5′-terminal sequences derived from PEBV cannot be replicated by TRV RNA1.

Inoculation of plants with PEBV RNA1 and the various transcript RNAs produced similar results but included several differences. Transcripts from clones pPEB3T and pTR5P were replicated by PEBV RNA1 and produced virus particles that were trapped by antibodies to PEBV and TRV, respectively. PEBV RNA1 but not TRV RNA1 was detected by RT–PCR in the upper, uninoculated leaves of all the test plants. Whereas no RNA2 was detected in plants inoculated with transcripts from clone pPEB5T, traces of RNA2 transcribed from either clone pTR3P or pTR9598 were identified by RT–PCR in the upper, uninoculated leaves of one of five inoculated plants (data not shown). In the plant inoculated with transcript from pTR3P, sufficient amplified fragment was produced, that was of a size unique to this particular clone and that also contained the novel *Apa*I restriction site, to confirm the identity of this hybrid. In neither of these two cases were any virus particles detected. These results show that PEBV RNA1 can replicate RNA2 containing the coding region and 3′ terminus of TRV if it possesses 320 nt from the 5′ terminus of PEBV RNA2, and that the presence of 3′-terminal sequences from TRV RNA2 does not inhibit replication. Thus there appears to be no fundamental barrier to the existence of such recombinants in nature.

The occasional detection by RT–PCR of RNA2 carrying 5′-terminal sequences from TRV in systemic leaves of test plants inoculated with PEBV RNA1 and transcripts from pTR3P or pTR9598 suggests that these RNAs can be replicated to a limited extent by PEBV RNA1, and might suggest that there is a difference in the specificity of the replicase enzymes of these two viruses. This finding agrees with previous experiments in which a pseudorecombinant virus could be produced containing RNA1 from the SHE isolate of PEBV and RNA2 from the I6 isolate of TRV (Robinson *et al.*, 1987). Virus particles were found in plants infected with the pseudorecombinant but the isolate could not be maintained long enough for a more detailed investigation.

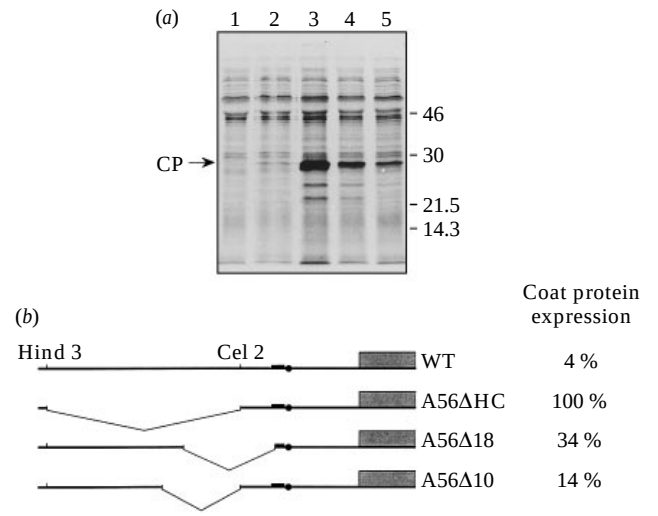

Fig. 2. Expression of the PEBV coat protein. (*a*) *In vitro* translation of coat protein with full-length or deleted upstream sequences. Lane 1, no RNA; lane 2, full-length (wild-type) transcript RNA2; lane 3, transcript of pA56ΔHC; lane 4, transcript of pA56Δ18; lane 5, transcript of pA56Δ10. The position of the coat protein (CP) is indicated at the left. Positions of protein size markers (in kDa) are indicated at the right. (*b*) Structure of clones used for translation. The horizontal black bar is the subgenomic promoter stem–loop and the black circle shows the start of the sgRNA. The CP is shown as a shaded rectangle. The numbers to the right are the amounts of CP expressed by each construct relative to A56ΔHC.

Compared to many other plant viruses the 5′ NCR of tobraviruses is unusually long and contains numerous (six or more) AUG triplets upstream of the coat protein initiation codon. The inhibitory effect of this region on the expression of the virus coat protein (CP) was shown by translation in rabbit reticulocyte lysate of transcripts of PEBV RNA2 containing different deletions. Full-length transcript produced only 4% of the amount of CP than did a transcript, A56ΔHC, deleted between bases 14 and 320 (*Hin*dIII and *Cel*II restriction sites in pT72A56) which has lost all of the upstream AUG triplets (Fig. 2). Two other transcripts, A56Δ18 (deleted between bases 230 and 376) and A56Δ10 (deleted between bases 196 and 332), which both retain five of the six upstream AUGs, produced 34% and 14% respectively of the A56ΔHC CP amount. The tobraviruses are thought to overcome this translation inhibition by expressing the CP from a subgenomic RNA (sgRNA). An encapsidated sgRNA for CP synthesis was isolated from one strain of TRV, and the 5′ termini of putative

CP sgRNAs of other isolates of TRV and PEBV have been sequenced (Robinson *et al.*, 1983; Cornelissen *et al.*, 1986; Wallis, 1992). The tobravirus CP sgRNA promoter has not been defined, although a potential stem–loop structure was found to occur immediately upstream of the 5′ termini of TRV and PEBV CP sgRNAs (Goulden *et al.*, 1990). The region between the *Cel*II site (nt 320) and the CP initiation codon of PEBV, which contains the stem–loop sequence, can function as a promoter when moved to a different part of the genome (S. MacFarlane, unpublished). Thus, for the hybrid clone pPEB5T in which the TRV 5′ terminus is joined to the PEBV CP promoter, the TRV replicase protein probably activates transcription from the heterologous sgRNA promoter. In addition, for clone pTR5P, in which the PEBV promoter region is replaced by TRV sequences, the PEBV replicase also probably recognizes the heterologous TRV promoter.

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