Mapping of viral RNA sequences required for assembly of peanut clump virus particles

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RNA sequences required for assembly into rod-shaped virions of RNA-1 and RNA-2 of *Peanut clump virus* (PCV) were mapped by testing the ability of different RNA-1 and -2 deletion mutants to be encapsidated *in vivo* in an RNase-resistant form. Encapsidation of RNA-1 was found to require a sequence domain in the 5′-proximal part of the P15 gene, the 3′-proximal gene of RNA-1. On the other hand, the subgenomic RNA which encodes P15 was not encapsidated, suggesting that other features of RNA-1 are important as well. Two sequences which could drive encapsidation of RNA-2 deletion mutants were located. One was in the 5′-proximal coat protein gene and the other in the P14 gene near the RNA 3′ terminus. There were no obvious sequence homologies between the different assembly initiation sequences.

INTRODUCTION

Since viral particles are generally the extracellular infectious entity, their assembly is an important step in the virus infection cycle. The best studied virus assembly system is that of *Tobacco mosaic virus* [TMV; see Culver (2002) for a recent review]. TMV has rod-shaped virions consisting of one copy of the single-stranded genome RNA and multiple copies of the capsid protein (CP) arranged as a rigid nucleoprotein helix. There are three nucleotide residues per CP subunit. Initiation of TMV assembly involves a concerted interaction between a preformed two-layer disk of CP subunits and several RNA hairpin structures located about 900 residues from the 3′-end of the viral RNA. The 'origin-of-assembly sequence' (OAS) has a strong order 3 repeat of G residues and selective binding of the multiple subunits of the disk to the OAS during assembly initiation is thought to reflect a slight preference of one of the three RNA-binding sites of the TMV CP for G residues (Turner *et al.*, 1988). The hairpin secondary structure is believed to be important in 'presenting' the repeating sequence to the initiating disk(s).

In contrast to the wealth of information concerning TMV, very little is known about the assembly of other rod-shaped plant viruses. There is evidence that the OAS of the potyvirus *Tobacco vein mottling virus* (TVMV) is situated near the 5′-extremity of the viral RNA (Wu & Shaw, 1998), and the 5′-extremity of the viral RNA is also the site of the OAS on RNA of *Papaya mosaic virus* (PMV) (Sit *et al.*, 1994). This does not, however, appear to be a general feature for *Potexviridae* since both the genomic and the 3′-co-terminal subgenomic RNAs of *Bamboo mosaic virus*-V isolate (BaMV-V) are packaged, suggesting that the OAS resides somewhere in the 3′-terminal 1000 nucleotides (nt) of these viral RNAs (Lee *et al.*, 1998).

In this paper we have investigated the location of RNA sequences important for the assembly of virions of *Peanut clump virus* (PCV; genus *Pecluvirus*). PCV has rigid rod-shaped virions which superficially resemble those of TMV, except that the virus particles display a bimodal length distribution (190 and 245 nm; Fritsch & Dollet, 2000). The genome of PCV is composed of two plus-sense RNAs which display little sequence homology except for the 3′-terminal 273 nt, which are 95 % identical between the two RNAs (Manohar *et al.*, 1993). RNA-1 is 5·9 kb in length and encodes two proteins involved in viral RNA replication (P131 and P191, a C-terminally extended form of P131 produced by translational readthrough of the P131 stop codon) (Herzog *et al.*, 1994), plus P15, a suppressor of post-transcriptional gene silencing (PTGS) (Dunoyer *et al.*, 2002a) (Fig. 1). RNA-2 (4·5 kb) encodes five proteins: the 23 kDa viral CP, P39, a putative vector transmission factor, and the triple gene block (TGB) movement proteins P51, P14 and P17 (Manohar *et al.*, 1993; Herzog *et al.*, 1994) (Fig. 1). P15 on RNA-1 and P51 on RNA-2 are translated from relatively abundant subgenomic RNAs. Subgenomic RNAs responsible for synthesis of P14 and P17 have not been detected. Analogy with other TGB-containing viruses, however, suggests that both proteins are probably synthesized from a low abundance subgenomic RNA with a 5′ terminus upstream of the P14 gene (Verchot *et al.*, 1998)

In this paper we have analysed the encapsidation *in vivo* of the major subgenomic RNAs and of a large collection of replicable PCV RNA-1 and -2 transcripts carrying different internal deletions. The results indicate that the OAS on PCV RNA-1 is situated within the P15 gene. On the other hand, we were unable to unambiguously localize a unique OAS in RNA-2.
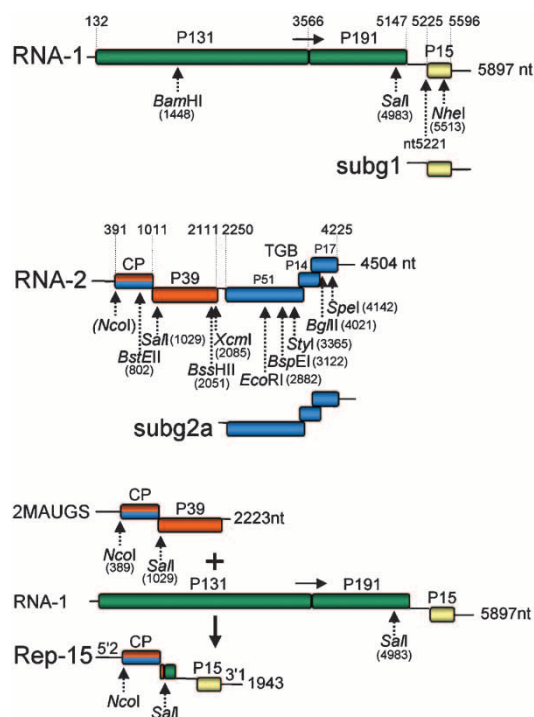
Fig. 1. Structure of PCV RNA-1, RNA-2 and Rep-15. Genes encoding the different viral proteins are indicated by a colour code: P131/P191 in green (the arrow indicates translation readthrough to produce P191), P15 in yellow, CP in red and blue, P39 in red and the TGB proteins (P51, P14 and P17) in blue. The coordinates of restriction sites used to produce the different deletion mutants and the boundaries of important genes are shown on the maps. The structure of the two abundant subgenomic RNAs subg1 and subg2a are shown below the RNA-1 and RNA-2 maps, respectively. The strategy used to create the RNA-1–RNA-2 chimera Rep-15 (Dunoyer *et al.*, 2001) is shown below. The number to the right of each structure indicates the predicted length of the transcript in nt.

METHODS

Construction of mutant plasmids. Unless otherwise stated, plasmids pPC1 and pPC2, corresponding to full-length cDNA clones of PCV RNA-1 and RNA-2 (Herzog *et al.*, 1998), or Rep-15, a chimera containing 5′-terminal cDNA sequence from RNA-2 and 3′-terminal sequence from RNA-1 (Dunoyer *et al.*, 2001), were used as starting material for mutant constructs. For some mutants, plasmid 2MAUGS (Herzog *et al.*, 1995), a pPC2 derivative consisting of the first 2223 nt of RNA-2 and containing a novel *Nco*I site, introduced at the position of the CP initiation codon by PCR-mediated overlap extension mutagenesis (Ho *et al.*, 1989), was employed in the construction. Preliminary experiments established that the introduction of the novel *Nco*I site did not alter the replication or encapsidation of full-length RNA-2 and Rep-15 transcripts (data not shown). Most of the deletion mutants were obtained by cleavage of the plasmid with a pair of restriction enzymes having unique sites in the cDNA, fill-in of the restriction sites by treatment with the Klenow fragment of DNA polymerase I if they were not compatible, and recircularization of the plasmid DNA with bacteriophage T4 DNA ligase. The sites (see Fig. 1) indicated in parentheses below were used to generate the following deletion mutants: 1ΔBS (*Bam*HI and *Sal*I), 1ΔBN

(*Bam*HI and *Nhe*I), RepΔCP1 (*Nco*I and *Sal*I), RepΔNN (*Nco*I and *Nhe*I), 2CPΔ2 (*Bst*EII and *Sal*I), 2Δ39 (*Sal*I and *Bss*HII), 2ΔCP39 (*Nco*I and *Bss*HII), 2ΔNcoEco (*Nco*I and *Eco*RI), 2ΔNcoSty (*Nco*I and *Sty*I), 2ΔNEΔStSp (*Nco*I and *Eco*RI, then *Sty*I and *Spe*I), 2Δ2 (*Bss*HII and *Eco*RI), 2Δ3 (*Eco*RI and *Bsp*EI), 2Δ4 (*Bsp*EI and *Sty*I), 2Δ5 (*Sty*I and *Bgl*II), 2Δ5b (*Sty*I and *Spe*I), 2ΔTGB (*Xcm*I and *Spe*I), 2Δ39TGB (*Sal*I and *Spe*I), 2CPΔ2Δ39TGB (*Bst*EII and *Spe*I) and 2CPΔ3Δ39TGB (*Nco*I and *Bst*EII, then *Sal*I and *Spe*I).

Clone 2CPΔ1, which contains a 172 nt deletion in the CP gene (nt 808–982), was produced by replacing the *Bam*HI–*Nhe*I fragment from pPC2 (the *Bam*HI site is upstream of the insert in pPC2; *Nhe*I is at nt 1620 of the cDNA insert) by the corresponding fragment from the clone 2MΔ172 (Herzog *et al.*, 1995). Clone 2ΔCP1Δ39TGB was produced by digestion of 2ΔCP1 with *Sal*I and *Spe*I, fill-in with Klenow fragment and recircularization. To produce RepΔCP2, a *Sal*I site was created by PCR just upstream of the position corresponding to nt 5221 of RNA-1 (the novel *Sal*I site was built in to the primer used in the PCR reaction). The resulting PCR fragment extended to the 3′ terminus of the cDNA and was cleaved by *Sal*I and *Mlu*I (the *Mlu*I site is at the 3′ end of the cDNA sequence). The *Sal*I–*Mlu*I fragment was used to replace the *Nco*I–*Mlu*I fragment of Rep-15 to produce RepΔCP2. The same approach was used to create 1ΔB5220 by using the *Sal*I/*Mlu*I-cleaved PCR product to replace the *Bam*HI–*Mlu*I fragment of pPC1. The *Sal*I site was also used to produce a *Sal*I–*Nhe*I fragment (nt 5221–5513) of the P15 gene to replace the *Sal*I–*Spe*I fragment of 2CPΔ1 to produce 2CPΔ1Δ39TGB-15Nh. The validity of all constructs was confirmed by digestion with appropriate restriction enzymes and sequence analysis.

Synthesis of transcripts and purification of viral RNA. Capped *in vitro* transcripts were obtained with a Ribomax transcription kit (Promega) following the manufacturer's instructions. pPC1- and Rep-15-derived plasmids were linearized with *Mlu*I and pPC2-derived plasmids with *Hin*dIII before transcription. Viral RNA was extracted from purified PCV (isolate PCV2) (Manohar *et al.*, 1993).

Detection of viral proteins *in vitro* and *in vivo*. *In vitro* translation in wheat germ (WG) extract and rabbit reticulocyte lysate (RRL) were as described (Hemmer *et al.*, 1993; Herzog *et al.*, 1995) using [^{35}S]methionine to radiolabel newly synthesized proteins. The ^{35}S-labelled translation products were detected by autoradiography following electrophoresis through a 10 % polyacrylamide gel in denaturing conditions (SDS-PAGE; Hemmer *et al.*, 1993).

Total protein extracts from protoplasts were separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore). Western blotting procedures and the antiserum directed against the PCV P51 protein have been described (Erhardt *et al.*, 1999; Dunoyer *et al.*, 2001, 2002b).

Inoculation of plants and protoplasts. Inoculation of *Chenopodium quinoa* with virion RNA (1·25 μg in 50 μl for each of two leaves) was as described (Herzog *et al.*, 1998). Protoplasts (10^6 protoplasts in 0·5 ml) from BY-2 tobacco cells (Nagata *et al.*, 1992) were prepared and inoculated with transcripts (Dunoyer *et al.*, 2001). Five μg of each transcript was employed per inoculation unless a transcript smaller than ∼2000 nt was to be included in the inoculum mix. In this case, the shorter transcript strongly interfered with productive replication of the longer transcripts, resulting in accumulation of only very low amounts of progeny RNA-1 and -2. Therefore, preliminary tests were carried out using 5 μg each of pPC1 and pPC2 transcript plus a range of concentrations of each smaller transcript to determine the maximum amount of the latter which could be added to the inoculum mix without greatly inhibiting replication of the larger RNAs. Amounts of the smaller transcripts ranging from 0·05 μg (Rep-15 and its derivatives) to

0·2–0·5 μg (short pPC2-derived transcripts such as 2ΔNcoSty, 2Δ39TGB etc.) were found to meet this requirement and were used in subsequent experiments.

Northern blot analysis of viral RNA. Total RNA was purified from inoculated leaves (0·2 g) of infected *C. quinoa* plants 11 days post-inoculation (p.i.) (Herzog *et al.*, 1998) and from 10^6 inoculated BY-2 protoplasts 48 h p.i. by phenol/chloroform extraction (Hemmer *et al.*, 1993; Dunoyer *et al.*, 2001). For analysis of encapsidated RNA, the protoplasts (10^6) were collected by centrifugation for 2 min at 100 **g** in a swinging bucket rotor and the pellet was resuspended in 600 μl of 50 mM Tris/HCl, pH 7·5, 10 mM MgCl₂ in a 1·5 ml microtube. The protoplasts were disrupted by three cycles of 10 s ultrasonication followed by 30 s in ice. Ultrasonication was carried out with a Vibracell Ultrasonicator (Sonic & Materials, Danbury, Conn., USA) equipped with a microprobe and operated at position 5 (70 W theoretical power output). After the third cycle, the disrupted protoplast extract was incubated at 37 °C for 30 min to degrade nonencapsidated viral RNAs, followed by low-speed centrifugation to remove cell debris. Encapsidated RNA was then obtained by phenol/chloroform extraction as above. The encapsidated RNA corresponding to 250 000 protoplasts and the total RNA corresponding to 80 000 protoplasts was then subjected to electrophoresis through a 1 % agarose/formaldehyde gel, followed by capillary transfer to Hybond membrane (Amersham). Viral RNAs were detected by hybridization with specific *in vitro*-transcribed ^{32}P-labelled antisense riboprobes. Riboprobe 1 was complementary to nt 5193–5515 of RNA-1, riboprobe 2a to nt 390–1624 of RNA-2 and riboprobe 2b to nt 3593–4024 of RNA-2. Riboprobe 1+2 was complementary to the 3′-terminal 124 nt of RNA-1 and RNA-2, which are identical in sequence in this region (Matsuda *et al.*, 2000). Radioactive signals on the blot were detected by autoradiography.

RESULTS AND DISCUSSION

Encapsidation of PCV subgenomic RNAs

Since P15 and the TGB proteins are encoded by viral subgenomic RNAs derived from the 3′-terminal parts of RNA-1 and RNA-2, respectively (Fig. 1), their capacity to undergo encapsidation *in vivo* can provide information about the positions of the OAS on the genomic RNAs. In earlier experiments (Herzog *et al.*, 1995) we characterized viral proteins synthesized in RRL programmed with RNA extracted from purified PCV virions. The observed viral proteins included (i) P131 and P191, which are both presumably synthesized from full-length RNA-1, (ii) the viral CP, which is translated starting from the first AUG on full-length RNA-2, and (iii) P39, which is translated from full-length RNA-2 by a leaky scanning mechanism starting from the second AUG (Herzog *et al.*, 1995).

We have now carried out *in vitro* translation experiments with purified virion RNA in WG extract. ^{35}S-Labelled P39, P131 and P191 translation products corresponding to the species observed in RRL (Fig. 2, lane 1) were also obtained in the WG extract (Fig. 2, lane 3), although P39 accumulated less abundantly than in RRL. The relatively low expression of P39 in WG extract could indicate that the leaky scanning mechanism which provides access to the P39 initiation codon is less efficient in this system. CP (which contains only one methionine residue: at the N terminus) was not observed in translations using either the WG extract
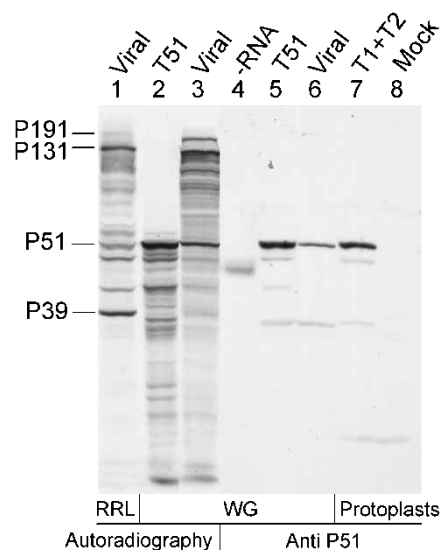

Fig. 2. Evidence that subg2a RNA is encapsidated. RNA extracted from purified PCV virions (obtained from PCV-infected leaves 11 days p.i.) was translated in RRL (lane 1) or WG extract (lanes 3 and 6). Other samples translated in WG extract correspond to T51, a transcript containing the TGB sequence (lanes 2 and 5), and a translation-negative control with no added template RNA (lane 4). Lanes 7 and 8 were loaded with proteins extracted from 50 000 protoplasts which had been infected with RNA-1 and RNA-2 full-length transcripts T1 and T2 (lane 7) or mock-inoculated (lane 8). All samples were separated by SDS-PAGE (Hemmer *et al.*, 1993) and important viral proteins are identified to the left. [^{35}S]Methionine-labelled translation products in lanes 1–3 were detected by autoradiography. P51 in lanes 4–8 was detected by Western blot using a P51-specific antiserum following transfer of proteins in the gel to Immobilon-P membrane (Millipore).

or RRL (Fig. 2, lanes 1 and 3). It was readily detected, however, in both translation systems when [^{3}H]leucine rather than [^{35}S]methionine was used as the radioactive tracer amino acid (Herzog *et al.*, 1995; and data not shown). In addition to the species expected to be translated from full-length viral RNA-1 and -2, the WG extract contained an abundant ∼51 kDa translation product (Fig. 2, lane 3) which was also detected in smaller amounts in the RRL (Fig. 2, lane 1). This species co-migrated with the major product of translation of T51 (Fig. 2, lane 2), an RNA transcript which contains the 3′-terminal half of RNA-2 starting just upstream of the gene for P51. The ∼51 kDa species was immuno-detected by a P51-specific antiserum from WG extract programmed with T51 or total viral RNA (Fig. 2, lanes 5 and 6), and from a total protein extract of tobacco BY-2 protoplasts transfected with the full-length PCV RNA-1 and -2 transcripts T1+T2 (Fig. 2, lane 7).

The foregoing observations indicate that subg2a, the subgenomic RNA which presumably directs translation of P51 in PCV-infected cells (see Fig. 1), is present in the

encapsidated RNA preparation. Additional evidence that subg2a is encapsidated was obtained by Northern blot analysis of RNase-resistant PCV RNAs in extracts from viral RNA-infected *C. quinoa* leaves. A crude cellular extract from the infected leaves was divided into two equal parts, one of which was immediately treated with phenol/chloroform to obtain a total (T) RNA fraction which will contain both unencapsidated and encapsidated viral RNAs. The rest of the extract was incubated at 37 °C for 30 min prior to phenol/chloroform extraction to obtain the encapsidated (E) RNA fraction. Preliminary experiments in which extracts from healthy plants were spiked with either naked viral RNA or purified virions before being carried through the aforesaid procedure established that unencapsidated viral RNA was totally degraded during the 37 °C incubation step whereas encapsidated RNA was protected from degradation (data not shown).

When analysed by Northern blot with riboprobe 2b, which is specific for the 3′ region of RNA-2 (Fig. 3A, top), a band of the size expected for subg2a was detected in both the T RNA and E RNA samples (Fig. 3A, lanes 3 and 4), consistent with the conclusion that the subgenomic RNA is encapsidated. No such band was visible in either the T or E samples when the blot was hybridized with riboprobe 2a, which is specific for the 5′-terminal half of RNA-2 (Fig. 3A, lanes 1 and 2), confirming that the band in question originates from the 3′-terminal half of RNA-2.

An effort was also made to detect encapsidated subg1, the previously observed subgenomic RNA which directs synthesis of the RNA-1-encoded species P15 (Dunoyer *et al.*, 2001). When hybridized with a riboprobe specific for the P15 gene (probe 1; Fig. 3B), a band of the size predicted for subg1 appeared clearly in the T RNA fraction at 6 days p.i. and reached maximum abundance at 8 days p.i. before declining slightly at 12 days p.i. (Fig. 3B, lanes 1–4). No corresponding band was detected in the E RNA fraction at any time p.i. (Fig. 3B, lanes 5–8), suggesting that subg1 is not significantly encapsidated.

Deletion mapping of sequences important for virion assembly on RNA-1

To obtain additional information about sequences important for assembly of virions containing PCV RNA-1, long deletions extending from a *Bam*HI site at nt 1448 to different downstream sites were produced in a full-length cDNA corresponding to RNA-1 (Fig. 4A). In designing the deletion mutants, the 5′- and 3′-noncoding regions
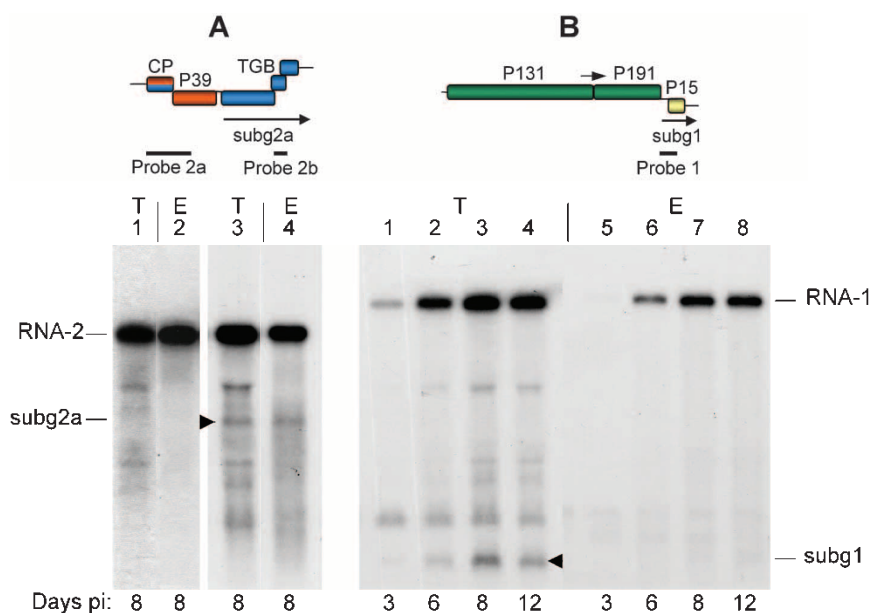

Fig. 3. Northern blot analysis of RNase-resistant PCV RNA in extracts of infected plants reveals that subg2a but not subg1 is encapsidated. Total RNA (T) and encapsidated viral RNA (E) fractions were extracted as described in Methods from inoculated leaves of PCV-infected *C. quinoa* at the day p.i. indicated below each lane. RNA was separated by electrophoresis through a formaldehyde-containing agarose gel and transferred to Hybond membrane (Amersham). (A) Viral RNA-2 sequences were detected by hybridization with ^{32}P-labelled antisense RNA Probes 2a (lanes 1 and 2) and 2b (lanes 3 and 4), which are complementary to the portions of the RNA-2 sequence indicated below the RNA-2 map. (B) Viral RNA-1 sequences were detected by hybridization with ^{32}P-labelled antisense RNA Probe 1, which is complementary to the portion of the RNA-1 sequence indicated below the RNA-1 map. The bands corresponding to subg2a and subg1 are indicated by triangles on the figure.
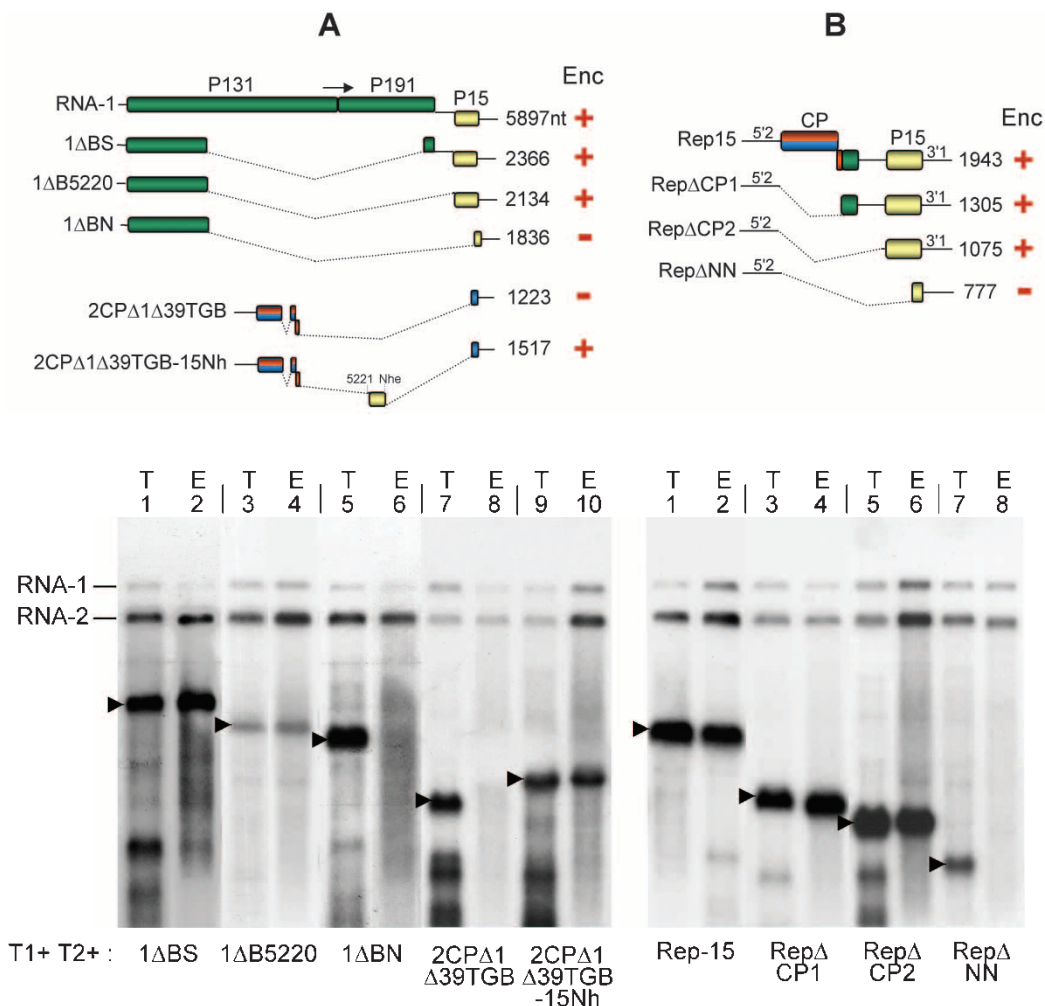
Fig. 4. Northern blot analysis of RNase-resistant PCV RNA-1 and Rep-15 deletion mutants from infected BY-2 protoplasts identifies a region in the P15 gene which is important for RNA-1 encapsidation. The protoplasts were inoculated with T1 and T2 plus a deleted transcript derived either from RNA-1 or RNA-2 (A) or Rep-15 (B). The structures of the mutant constructs are shown above and the length of each transcript and whether or not it is encapsidated (ENC) is indicated to the right. Total RNA (T; odd-numbered lanes) and encapsidated viral RNA (E; even-numbered lanes) fractions were extracted from the protoplasts 48 h p.i. as described in Methods. RNA was separated by electrophoresis through an agarose gel containing formaldehyde and transferred to Hybond membrane. Viral RNA sequences were detected by hybridization with ^{32}P-labelled antisense RNA Probe 1 + 2, which is complementary to the 3′-proximal 124 nt of both RNA-1 and RNA-2. The bands corresponding to the different deletion mutants are indicated by triangles.

were left intact since sequences required *in cis* for viral RNA replication are likely to reside there. Encapsidation of the mutants *in vivo* was assayed as follows. *In vitro* run-off transcripts obtained from the cloned deleted cDNAs were inoculated to tobacco BY-2 cell protoplasts along with full-length run-off transcripts of RNA-1 and -2. T and E RNA fractions were prepared from the protoplasts 48 h p.i. as described in Methods and the RNAs were analysed by Northern blot. The analysis revealed that the RNA-1 constructs 1ΔB5220 and 1ΔBS, in which the deletion stopped at the beginning of the P15 gene (1ΔB5220) or further upstream (1ΔBS), were protected, as the deleted species was present in both T and E fractions (Fig. 4A, lanes

1–4). In contrast, 1ΔBN, in which the sequence extending from nt 1453 to nt 5513 (84 nt upstream of the 3′ end of the P15 gene) had been deleted, was present in the T RNA fraction (Fig. 4A, lane 5) but not in the E fraction (Fig. 4A, lane 6). Consequently, we conclude that this deleted form of RNA-1 can replicate but does not undergo significant encapsidation. These observations suggest that the 5′-terminal region of the P15 gene contains an OAS sequence.

In a second series of experiments designed to further test the importance of P15 gene sequences in assembly, we performed deletion analysis on a chimeric 'replicon', Rep-15 (Dunoyer *et al.*, 2001). Rep-15 contains the 5′-terminal part

of RNA-2 (including the CP and the 19 first nt of the P39 gene) and the 3′ part of RNA-1 (including the P15 gene, the upstream non-translated region and the last 165 nt of the P191 gene) (Fig. 4B). Rep-15 transcript replicates (Dunoyer *et al.*, 2001) and is encapsidated (Fig. 4B, lane 1 and 2) when coinoculated with transcripts T1 and T2 to BY-2 protoplasts. Preparation of T and E RNA fractions from the infected protoplasts and Northern blot analysis as described above revealed that deletion of internal sequences up to the end of the CP gene (RepΔCP1) (Fig. 4B) or to the beginning of the P15 gene (RepΔCP2) (Fig. 4B) did not abolish RNase resistance (Fig. 4B, lanes 3–6). In contrast, RepΔNN, in which the deletion is extended to nt 5513 of the P15 gene (Fig. 4B), displayed no RNase-resistance (Fig. 4B, lanes 7 and 8), consistent with the conclusion based on the experiments with the deleted RNA-1 transcripts indicating that the OAS resides in the P15 gene, and more particularly within the 5′-terminal part of this gene.

Direct evidence that the 5′ portion of the P15 gene contains an OAS was provided by experiments using a deleted, encapsidation-defective form of PCV RNA-2. As will be discussed more fully below, deletion of most of the central portion of RNA-2 interferes with its assembly into virions. Thus, the RNA-2 mutant 2CPΔ1Δ39TGB, which contains a short internal deletion in the CP gene plus a deletion of almost all of the P39 gene and the TGB (Fig. 4A), replicates but is not protected from RNase degradation in extracts of protoplasts co-inoculated with the transcript plus transcripts T1 and T2 (Fig. 4A, lanes 7 and 8). However, addition of a sequence containing the first 293 nt of the P15 gene from RNA-1 into the aforesaid RNA-2 deletion mutant to produce 2CPΔ1Δ39TGB-15Nh (Fig. 4A) renders the mutant RNA resistant to RNase in the E fraction (Fig. 4A, lanes 9 and 10), indicating that the P15 gene sequence in question is sufficient to drive virion assembly in the absence of other RNA-1 sequences.

The foregoing observations map a *cis*-acting assembly sequence to the 5′-proximal part of the P15 gene of RNA-1. At present, we do not know why subg1, which would contain this putative OAS, is not encapsidated during a normal virus infection. Possibly, subg1 is very actively translated and the translating ribosomes shield the OAS from interaction with CP. Another possibility is that the position of the OAS in the RNA molecule is important. Thus, although the 5′ extremity of subg1 has not been mapped, the electrophoretic mobility of the subgenomic RNA in denaturing agarose gel indicates that subg1 contains approx. 750 nt, which would place its 5′ extremity about 80 nt upstream of the beginning of the P15 gene. In the short encapsidated deletion mutants such as RepΔCP2 and 2CPΔ1Δ39TGB-15Nh, the sequence upstream of the P15 gene is much longer, 402 nt and 866 nt, respectively. Perhaps a certain minimum length of sequence upstream of the OAS may be required for productive assembly initiation to occur.

Encapsidation of RNA-2 containing internal deletions

We have shown above that subg2a is encapsidated during a normal PCV infection, suggesting that an OAS resides somewhere within the 3′-terminal half of RNA-2. To test this idea directly, deletions were introduced in different parts of a cDNA corresponding to full-length PCV RNA-2, beginning with the 5′-proximal CP and P39 genes (see Fig. 5 for mutant structures). *In vitro* transcripts obtained from the deleted cDNAs were inoculated to BY-2 protoplasts along with full-length transcripts of RNA-1 and -2. In the case of constructs containing short deletions in the CP gene (2CPΔ1 and 2CPΔ2), transcript of Rep-15 rather than full-length RNA-2 was included in the inoculum as source of CP. T and E RNA fractions were then prepared from the protoplasts and analysed by Northern blot. The results illustrate that part (2CPΔ1 and 2CPΔ2) or all (2ΔCP39) of the CP gene can be eliminated without interfering with assembly of virions containing the rest of the RNA-2 sequence (Fig. 5, lanes 5–8 and 11–12). Similarly, the P39 gene can be deleted (mutants 2Δ39, 2ΔCP39 and 2ΔNcoEco) without inhibiting virion assembly (Fig. 5, lanes 9–14).

The above experiments are consistent with the presence of an OAS in the 3′-terminal half of RNA-2, as predicted by the encapsidation of subg2a in plants inoculated with wild-type PCV (see above). In order to map this putative OAS more precisely, a series of deletions was engineered into the 3′ half of RNA-2 to eliminate different parts of the TGB sequence. RNA-2 deletion mutants in which the deletion extended from the RNA-2 5′-noncoding region (nt 396) to nt 2882 (mutant 2ΔNcoEco; Fig. 5) or from nt 394 to nt 3369 (mutant 2ΔNcoSty; Fig. 5) in the P51 gene were replicated and encapsidated in protoplasts when coinoculated with RNA-1 and -2 transcripts (Fig. 5, lanes 13–16). On the other hand, mutant 2ΔNEΔStSp (Fig. 5), which is identical to mutant 2ΔNcoEco except for an additional deletion of nt 3370–4142 containing the P14 gene and most of the P17 gene, replicated but was not encapsidated (Fig. 5, lanes 17 and 18), indicating the 3′-proximal OAS is situated between nt 3370 and 4142.

Additional deletions in RNA-2 identify a second OAS

Replication/encapsidation assays were also carried out with a series of RNA-2 deletion mutants in which different internal sequences had been removed without eliminating the CP gene. Together, these mutations eliminated part or all the RNA-2 sequence between the beginning of the P39 gene and nt 4142 near the 3′ terminus of the gene encoding the 3′-proximal TGB protein P17 (Fig. 6). The various RNA-2 transcripts were inoculated to BY-2 protoplasts along with RNA-1 transcript and viral RNAs in the T and E fractions were analysed by Northern blot as above. All of the RNA-2 deletion mutants were encapsidated (Fig. 6). Interestingly, the encapsidated mutants included constructs
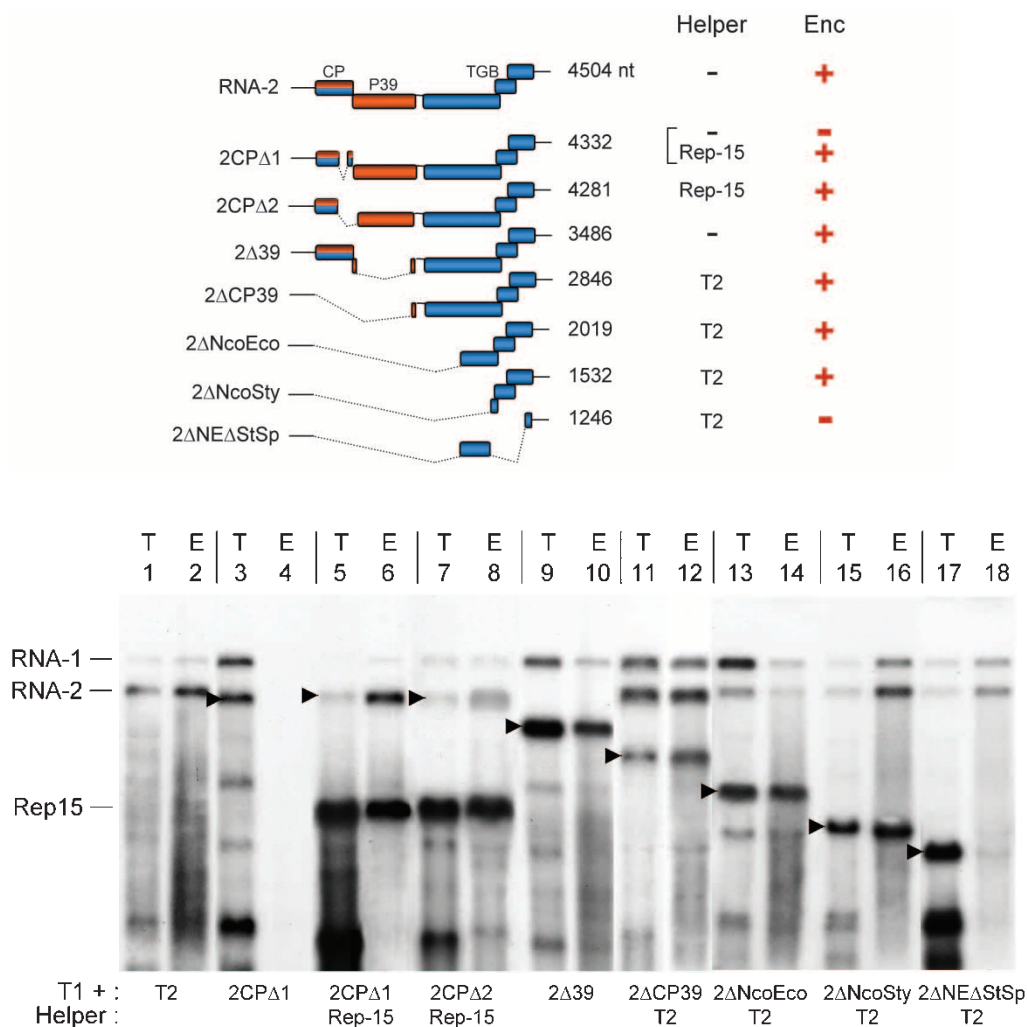
Fig. 5. Northern blot analysis of RNase-resistant PCV RNA-2 deletion mutants in extracts of infected BY-2 protoplasts identifies a region in the 3′-terminal half of RNA-2 which is required for encapsidation. The protoplasts were inoculated with T1 plus the deleted RNA-2 species shown below each pair of lanes (1 and 2, 3 and 4, etc.). In experiments in which the CP gene had been deleted from the RNA-2 construct, either Rep-15 (lanes 5–8) or full-length RNA-2 transcript (T2; lanes 11–18) was added to the inoculum as a 'helper' to provide a source of CP. Northern hybridization was carried out as described in the Fig. 4 legend. Total RNA samples (T) are in odd-numbered lanes and encapsidated RNA samples (E) are in even-numbered lanes. Bands corresponding to the different deletion mutants are identified by triangles. Other details as in Fig. 4.

such as 2Δ5, 2Δ5b, 2ΔTGB and 2Δ39TGB (Fig. 6) in which the region between nt 3370 and 4142 (identified above as the locus of a 3′-proximal OAS) was removed. These experiments thus indicate that there is a second OAS located within the CP gene.

Finally, an *in vivo* replication/encapsidation assay was carried out with an RNA-2 mutant in which the entire coding region (except for the 3′-terminal 83 nt of the TGB P17 gene) was deleted (mutant 2ΔCP39TGB; Fig. 7). This mutant replicated when co-inoculated to protoplasts with RNA-1 and -2 transcripts but was not detectably encapsidated (Fig. 7, lanes 1 and 2). Several deletion mutants

similar to 2ΔCP39TGB except for the presence of different segments of the viral CP gene (mutants 2CPΔ1Δ39TGB, 2CPΔ2Δ39TGB and 2CPΔ3Δ39TGB; Fig. 7) were also poorly encapsidated (Fig. 7, lanes 3–8). These observations suggest that the putative OAS located in the CP gene is unlikely to consist of a short sequence motif, although additional fine-scale deletion mapping of the CP gene will be required to define the exact domain implicated.

Concluding remarks

It is not known how many residues of PCV RNA interact with each CP monomer in a viral particle. Examination of
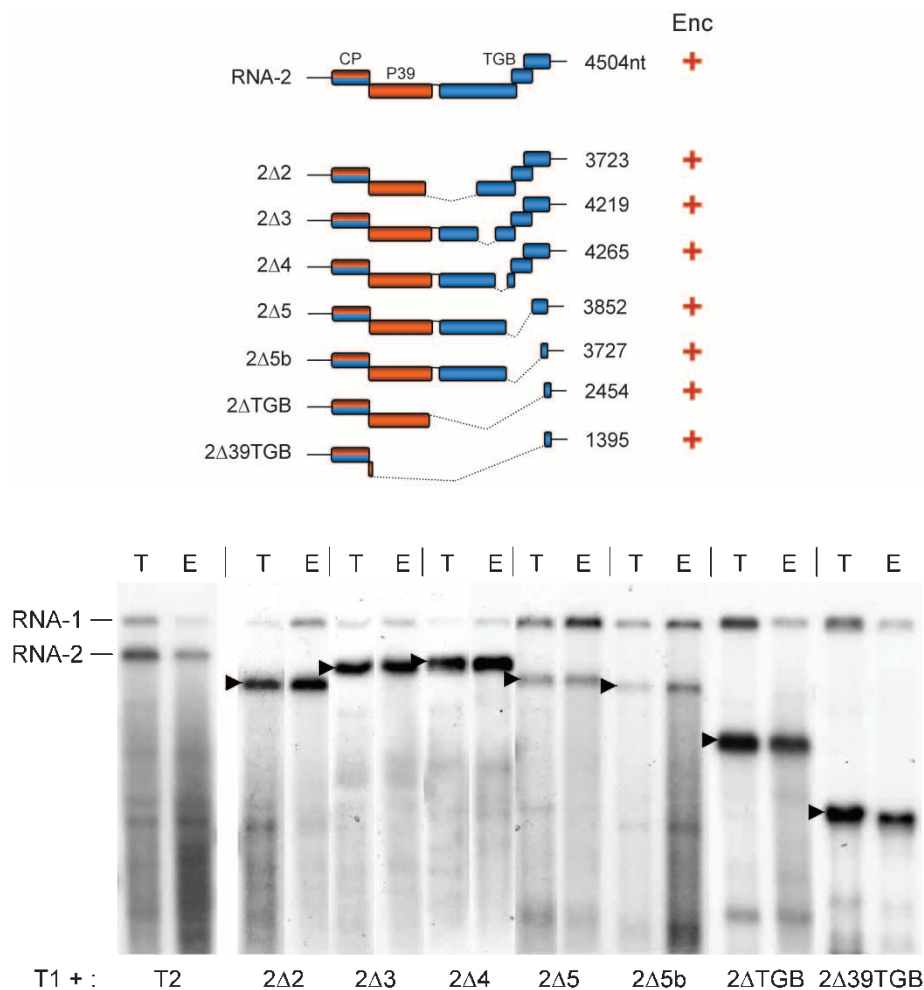
Fig. 6. The 5′-terminal part of RNA-2 contains a sequence which can promote RNA-2 encapsidation. BY-2 protoplasts were inoculated with T1 plus either T2 or one of the RNA-2 deletion mutants shown in the upper part of the figure. Total RNA (T) and encapsidated RNA (E) fractions were prepared 48 h p.i. and analysed by Northern hybridization as described in the Fig. 4 legend. The bands corresponding to the different RNA-2 deletion mutants are indicated by triangles. Other details as in Fig. 4.

the RNA-1 sequence in the region thought to contain the OAS, however, did not reveal any periodic repeats of A, G, C or U of order 3, 4 or 5 that were longer than similar repeats elsewhere in the sequence. For example, the longest threefold repeat of G residues in the RNA-1 OAS-containing domain (nt 5220–5513) is five triplets long, but this is equalled or exceeded in length at five other sites in RNA-1, sites which are apparently not active in assembly initiation. Thus initiation of PCV assembly, like that of TMV, probably involves interaction of CP with a relatively short sequence which is presented by RNA secondary structure in a special configuration. Fine-scale mutagenesis of the RNA-1 OAS region in the transcript 2CPΔ1Δ39TGB-15Nh should help to delineate the critical sequence/structure motif more precisely.

Taken at face value, our results suggest that PCV RNA-2 contains two OAS sequences: a 5′-proximal OAS in the CP gene and a 3′-proximal OAS in the central part of the TGB. It is however, extremely unlikely that assembly of viral nucleoprotein helices would initiate at two distant loci on the same viral RNA since this would present serious steric problems in properly encapsidating the RNA at the point where the two growing helices meet. We suggest rather that only one of the two observed OAS sequences is active on full-length RNA-2 and the second sequence is a pseudo-OAS which is disfavoured kinetically for assembly initiation compared to the authentic OAS. Significant assembly-initiation activity at the pseudo-OAS would be detected only in those mutants in which the authentic OAS has been eliminated by deletion. Thus, if the authentic OAS was present in the TGB coding region, for example, and a pseudo-OAS was in the CP gene (or vice versa), this would explain why mutants such as 2ΔCP39 (Fig. 5) and 2Δ39TGB (Fig. 6) are both encapsidated while the mutant 2ΔCP39TGB (Fig. 7), which would lack both the authentic
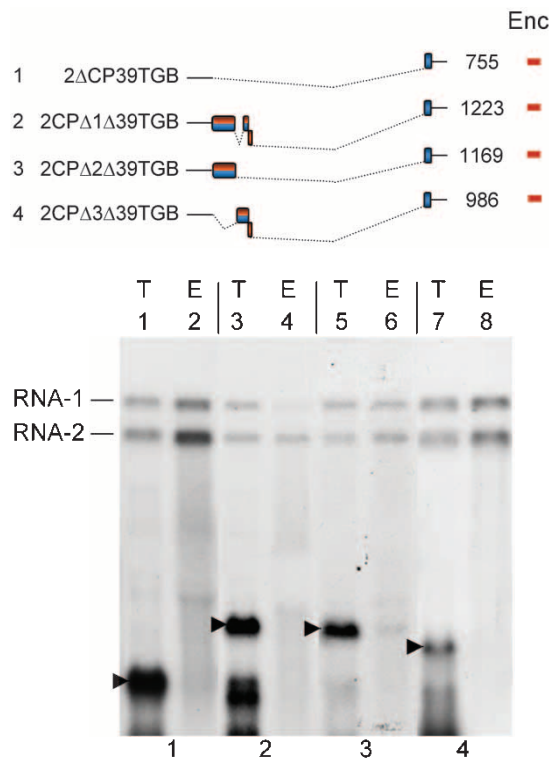
Fig. 7. Large deletions in RNA-2 abolish encapsidation. BY-2 protoplasts were inoculated with T1 and T2 plus one of the RNA-2 deletion mutants shown in the upper part of the figure. Total RNA (T) and encapsidated RNA (E) fractions were prepared 48 h p.i. and analysed by Northern hybridization as described in the Fig. 4 legend. The bands corresponding to the different RNA-2 deletion mutants are indicated by triangles. Other details as in Fig. 4.

and pseudo-OAS, is not. It is interesting to note that RNA of the TMV common strain also contains a pseudo-OAS, located in the CP cistron (Guilley *et al.*, 1975). It has been suggested that this pseudo-OAS, which is no longer functional on full-length TMV common strain RNA but displays structure and sequence homology with the authentic OAS, may be the ancestral assembly origin and that the common strain functional OAS derived from its duplication (Zimmern, 1977). The pseudo-OAS is in fact used to initiate assembly on viral RNA of some strains of TMV (Fukuda *et al.*, 1980), however, raising the possibility that different strains of PCV could similarly employ one or the other of the RNA-2 sequences mapped in this paper as functional OAS.

Wherever the authentic OAS of RNA-2 is located it is likely to share critical features of sequence and structure with the OAS of RNA-1. Thus, a fuller characterization of the RNA-1 OAS will provide a useful template for identifying similar motifs on RNA-2, which can then be more fully tested for their capacity to initiate assembly by substitution into assembly-defective transcripts of RNA-1.

REFERENCES

Culver, J. N. (2002). Tobacco mosaic virus assembly and disassembly: determinants in pathogenicity and resistance. *Annu Rev Phytopathol* **40**, 287–308.

Dunoyer, P., Herzog, E., Hemmer, O., Ritzenthaler, C. & Fritsch, C. (2001). Peanut clump virus RNA-1-encoded P15 regulates viral RNA accumulation but is not abundant at viral RNA replication sites. *J Virol* **75**, 1941–1948.

Dunoyer, P., Pfeffer, S., Fritsch, C., Hemmer, O., Voinnet, O. & Richards, K. E. (2002a). Identification, subcellular localization and some properties of a cysteine-rich suppressor of gene silencing encoded by peanut clump virus. *Plant J* **29**, 555–567.

Dunoyer, P., Ritzenthaler, C., Hemmer, O., Michler, P. & Fritsch, C. (2002b). Intracellular localization of the peanut clump virus replication complex in tobacco BY-2 protoplasts containing green fluorescent protein-labeled endoplasmic reticulum or Golgi apparatus. *J Virol* **76**, 865–874.

Erhardt, M., Stussi-Garaud, C., Guilley, H., Richards, K. E., Jonard, G. & Bouzoubaa, S. (1999). The first triple gene block protein of peanut clump virus localizes to the plasmodesmata during virus infection. *Virology* **264**, 220–229.

Fritsch, C. & Dollet, M. (2000). Genus *Pecluvirus*. In *Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses*, pp. 913–917. Edited by M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle & R. B. Wickner. San Diego: Academic Press.

Fukuda, M., Okada, Y., Otsuki, Y. & Takebe, I. (1980). The site of initiation of rod assembly on the RNA of a tomato and a cowpea strain of tobacco mosaic virus. *Virology* **101**, 493–502.

Guilley, H., Jonard, G., Richards, K. E. & Hirth, L. (1975). Sequence of a specifically encapsidated RNA fragment originating from the tobacco-mosaic-virus coat-protein cistron. *Eur J Biochem* **54**, 135–144.

Hemmer, O., Oncino, C. & Fritsch, C. (1993). Efficient replication of the *in vitro* transcripts from cloned cDNA of tomato black ring virus satellite RNA requires the 48 K satellite RNA-encoded protein. *Virology* **194**, 800–806.

Herzog, E., Guilley, H., Manohar, S. K., Dollet, M., Richards, K., Fritsch, C. & Jonard, G. (1994). Complete nucleotide sequence of peanut clump virus RNA 1 and relationships with other fungus-transmitted rod-shaped viruses. *J Gen Virol* **75**, 3147–3155.

Herzog, E., Guilley, H. & Fritsch, C. (1995). Translation of the second gene of peanut clump virus RNA-2 occurs by leaky scanning *in vitro*. *Virology* **208**, 215–225.

Herzog, E., Hemmer, O., Hauser, S., Meyer, G., Bouzoubaa, S. & Fritsch, C. (1998). Identification of genes involved in replication and movement of peanut clump virus. *Virology* **248**, 312–322.

Ho, S. N., Junt, H. D., Horton, R. M., Puller, J. K. & Pease, L. R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**, 51–59.

Lee, Y. S., Lin, B. Y., Hsu, Y. H., Chang, B. Y. & Lin, N. S. (1998). Subgenomic RNAs of bamboo mosaic potexvirus-V isolate are packaged into virions. *J Gen Virol* **79**, 1825–1832.

Manohar, S. K., Guilley, H., Dollet, M., Richards, K. & Jonard, G. (1993). Nucleotide sequence and genetic organization of peanut

clump virus RNA 2 and partial characterization of deleted forms. *Virology* **195**, 33–41.

Matsuda, D., Dunoyer, P., Hemmer, O., Fritsch, C. & Dreher, T. W. (2000). The valine anticodon and valylatability of peanut clump virus RNAs are not essential but provide a modest competitive advantage in plants. *J Virol* **74**, 8720–8725.

Nagata, T., Nemoto, Y. & Hasezawa, S. (1992). Tobacco BY-2 cell line as the ''HeLa'' cell in the cell biology of higher plants. *Int Rev Cytol* **132**, 1–30.

Sit, T. L., Leclerc, D. & AbouHaidar, M. G. (1994). The minimal 5′ sequence for in vitro initiation of papaya mosaic potexvirus assembly. *Virology* **199**, 238–242.

Turner, D. R., Joyce, L. E. & Butler, P. J. (1988). The tobacco mosaic virus assembly origin RNA. Functional characteristics defined by directed mutagenesis. *J Mol Biol* **203**, 531–547.

Verchot, J., Angell, S. M. & Baulcombe, D. C. (1998). In vivo translation of the triple gene block of potato virus X requires two subgenomic mRNAs. *J Virol* **72**, 8316–8320.

Wu, X. & Shaw, J. G. (1998). Evidence that assembly of a poty-virus begins near the 5′ terminus of the viral RNA. *J Gen Virol* **79**, 1525–1529.

Zimmern, D. (1977). The nucleotide sequence at the origin for assembly on tobacco mosaic virus RNA. *Cell* **11**, 463–482.