Short Communication

Surface-exposed C-terminal amino acids of the small coat protein of *Cowpea mosaic virus* are required for suppression of silencing

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The small (S) coat protein of *Cowpea mosaic virus* (CPMV) has been identified previously as a virus-encoded suppressor of post-transcriptional gene silencing (PTGS). Deletions within the C-terminal 24 aa of this protein affect the yield and systemic spread of the virus, suggesting that the C-terminal amino acids of the S protein, which are exposed on the surface of assembled virus particles, may be responsible for the suppressor activity. To investigate this, versions of CPMV RNA-2 with deletions at the C terminus of the S protein were tested for their ability to counteract PTGS in leaf-patch tests. The results showed that the C-terminal 16 aa of the S protein are particularly important for suppressing PTGS and that these amino acids are virus-specific and cannot be substituted by the equivalent sequence from the related virus *Bean pod mottle virus*.

The genome of *Cowpea mosaic virus* (CPMV) consists of two molecules of separately encapsidated single-stranded, positive-sense RNA, termed RNA-1 and RNA-2. Both of these RNAs are translated to give precursor polyproteins, which are processed to give the mature viral proteins. RNA-1 encodes proteins involved in RNA replication and RNA-2, which is dependent on RNA-1 for its replication, encodes the viral movement protein and the two viral coat proteins, large (L) and small (S). Like many plant viruses, CPMV encodes a suppressor of post-transcriptional gene silencing (PTGS), though its activity appears to be relatively weak (Voinnet *et al.*, 1999). This suppressor function was subsequently mapped to the S coat protein (Liu *et al.*, 2004).

CPMV particles consist of 60 copies each of the L and S coat proteins arranged with pseudo $T=3$ ($P=3$) symmetry (Lin & Johnson, 2003). The only portion of the S protein not visible in the X-ray structure consists of the C-terminal 24 aa (aa 190–213). This sequence is exposed on the virus surface, is mobile and is frequently lost by proteolysis without affecting particle stability (Lin & Johnson, 2003). Analysis of mutants with deletions in the C-terminal 24 aa region showed that it played an important role in efficient growth and spread of the virus (Taylor *et al.*, 1999). Deletion of the entire 24 aa sequence (mutant DM4) reduced virus yield to less than 5 % of the wild-type level in inoculated leaves and substantially delayed systemic spread. Furthermore, lesions on the inoculated leaves were surrounded by rings of necrosis and virion preparations contained a dramatically increased proportion (70 %) of protein-only shells (empty capsids). Smaller deletions of 16 or 7 aa from the C terminus (mutants DM5 and DM6, respectively) again reduced virus yield and delayed systemic spread, but to a lesser extent than in DM4, the degree of debilitation being approximately proportional to the size of the deletion (Taylor *et al.*, 1999). Moreover, no necrosis was observed with DM5 and DM6 and virus preparations contained wild-type levels (5–10 %) of empty particles. Taken together, these results indicated that aa 190–197 of the S protein are involved in the insertion of RNA into virus particles and that the region downstream (aa 198–213), in some unspecified way, affected virus accumulation and spread (Taylor *et al.*, 1999). In view of the recent evidence that the CPMV S protein has suppressor activity, we have examined the ability of mutant forms of CPMV RNA-2 with deletions or a substitution at the C terminus of the S protein to suppress PTGS by the leaf-patch test method using the green fluorescent protein (GFP) (Voinnet *et al.*, 2000; Johansen & Carrington, 2001).

To create plasmids suitable for leaf-patch tests, the 2·0 kb *Bam*HI–*Eco*RI fragment encoding the wild-type CPMV coat proteins in the full-length RNA-2 *Agrobacterium* plasmid pBinPS2NT (Liu & Lomonossoff, 2002; Fig. 1a) was replaced with the equivalent fragments from RNA-2 mutants pCP-DM4, -DM5 or -DM6 (Taylor *et al.*, 1999). This resulted in the production of pBINPLUS (van Engelen *et al.*, 1995)-based plasmids pBinP-DM4, -DM5 and -DM6, which contained modified versions of RNA-2 with deletions of 24, 16 and 7 aa, respectively, at the C terminus of the S protein (Fig. 1c). To investigate whether properties of the C-terminal amino acids were sequence-specific, the entire C-terminal region was replaced with the equivalent region from the S protein of *Bean pod mottle virus* (BPMV). Oligonucleotide-directed mutagenesis of pCP2, a full-length

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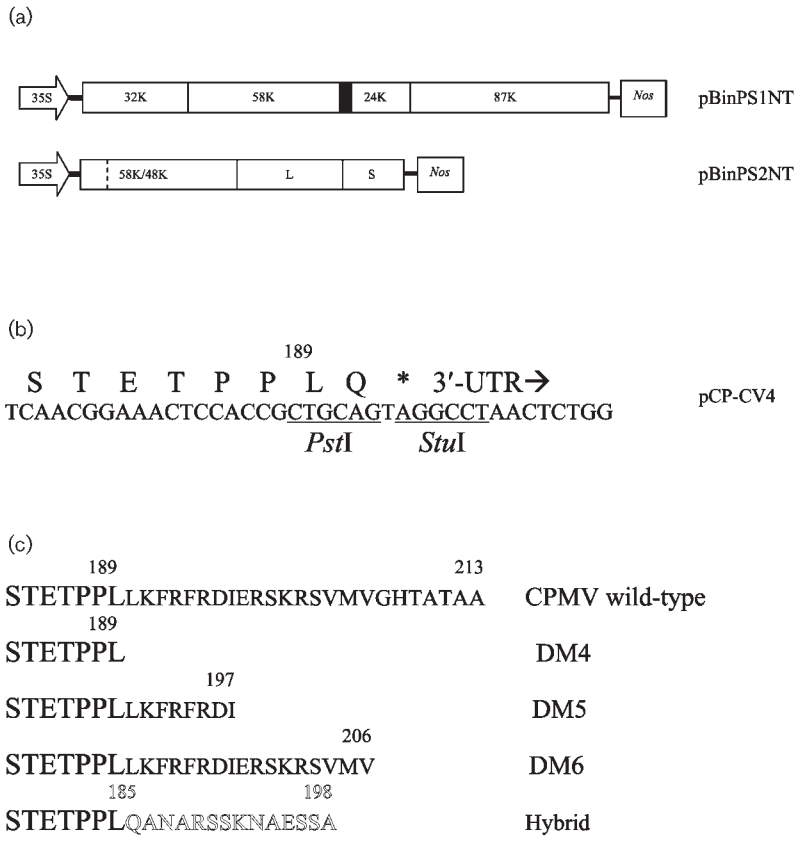

Fig. 1. Structure of CPMV RNA-1 and RNA-2 constructs. (a) Structure of the CPMV-specific portions of RNA-1- and RNA-2-based plasmids pBinPS1NT and pBinPS2NT, which contain full-length cDNA copies of CPMV RNA-1 and RNA-2, respectively, between the 35S promoter and *Nos* terminator in pBINPLUS. (b) Sequence around the C terminus of the S coat protein in the vector pCP-CV4, showing the unique *Pst*I and *Stu*I sites used for the construction of plasmid pCP-Hybrid. The amino acid sequence of the encoded protein is shown above the nucleotide sequence with the introduced termination codon shown as an asterisk (*). The start of the 3′-untranslated region (3′-UTR) is also indicated. (c) Amino acid sequences at the C terminus of the S protein in the wild-type and mutant forms of CPMV RNA-2 used in leaf-patch tests. The regions within the C-terminal 24 aa are shown as smaller characters. In the case of the wild-type S protein, the positions of the proteolytic cleavage site (aa 189) and the C terminus (aa 213) are shown. The position of the C-terminal amino acid in each of the deletion mutants, DM4, DM5 and DM6, is indicated. In the case of pBinP-Hybrid, the BPMV-specific portion is shown as open lettering, with numbering according the BPMV S protein sequence.

cDNA clone of RNA-2 (Dessens & Lomonossoff, 1993), was used to introduce unique *Pst*I and *Stu*I sites immediately downstream of the codon for Leu-189 of the S protein (Fig. 1b). Oligonucleotides encoding the C-terminal 13 aa of the BPMV S protein were ligated into *Pst*I/*Stu*I-digested pCP-CV4 to give plasmid pCP-Hybrid. This plasmid was infectious when inoculated on to cowpea (*Vigna unguiculata*) plants in the presence of RNA-1, the properties of the resulting infection being virtually identical to those seen with DM4, including necrotic rings around the sites of initial infection, delayed systemic movement, a low yield of virus particles and an increased proportion of empty capsids (V. Volpetti & G. P. Lomonossoff, unpublished). The 2·0 kb *Bam*HI–*Eco*RI fragment from pCP-Hybrid was subsequently substituted for the wild-type sequence in pBinPS2NT to give pBinP-Hybrid (Fig. 1c). All the CPMV RNA-2-based constructs were used to transform *Agrobacterium tumefaciens* strain C58C1.

To assess the suppressor properties of the mutant forms of the S protein, *Nicotiana benthamiana* leaves were agro-infiltrated with a mixture of 35S–GFP (pBIN61-GFP), 35S–RNA-1 (pBinPS1NT; Liu & Lomonossoff, 2002) and the desired RNA-2 plasmid as described previously (Liu *et al.*, 2004). CPMV RNA-1 is essential for the RNA-2-encoded suppressor activity to be manifested, since it is required for the processing of RNA-2 polyprotein (Liu *et al.*, 2004). Co-infiltration of 35S–GFP and 35S–RNA-1 with either the empty pBIN61 vector or a plasmid (35S–HcPro) expressing the well-characterized suppressor of silencing, HcPro, from *Potato virus Y* (Brigneti *et al.*, 1998) was used as a negative and positive control, respectively.

Leaves co-infiltrated with 35S–GFP, 35S–RNA-1 and pBIN61 showed no detectable fluorescence by 6 days post-inoculation, indicating that silencing of GFP expression had occurred by this time (Fig. 2a). By contrast, replacement of pBIN61 with either 35S–RNA-2 (pBinPS2NT) or 35S–HcPro resulted in clear fluorescence being maintained, the effect being stronger with 35S–HcPro (Fig. 2a). These results are consistent with the CPMV S protein being a weaker suppressor than HcPro (Voinnet *et al.*, 1999; Liu *et al.*, 2004). Infiltration with a version of RNA-2 lacking the C-terminal 7 aa of the S protein (pBinP-DM6) resulted in diminished but detectable fluorescence in all infiltrated leaves, while infiltration with pBinP-DM5 (lacking the C-terminal 16 aa from the S protein) resulted in only very faint fluorescence being visible in about 25 % of infiltrated leaves. The levels of fluorescence in leaves co-infiltrated with pBinP-DM4 or pBinP-Hybrid were similar to those seen with pBIN61. These results indicated that the suppressor activity of the S protein lies within the C-terminal 24 aa, that aa 198–213

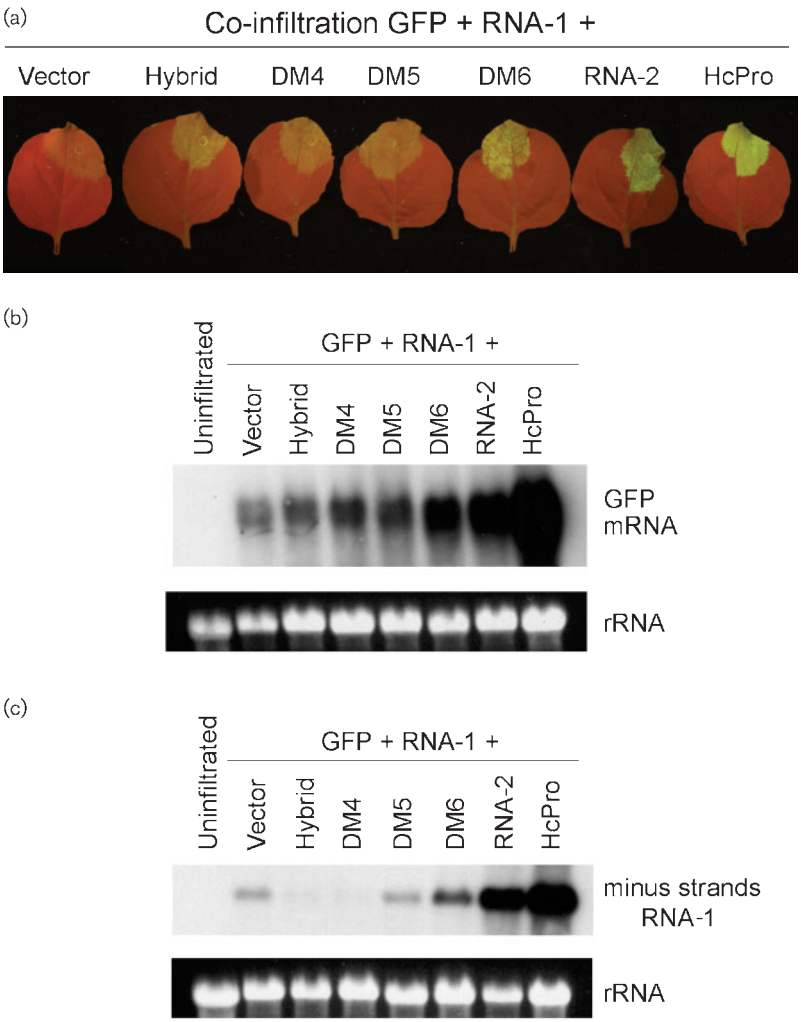

Fig. 2. Effect of potential suppressor activity on 35S–GFP expression after agro-infiltration of *N. benthamiana* leaves. (a) Photographs taken under UV light of *N. benthamiana* leaves 6 days after co-infiltration with *A. tumefaciens* harbouring 35S–GFP and 35S–RNA-1 in combination with either pBIN61 (vector), pBinP-Hybrid (Hybrid), pBinP-DM4 (DM4), pBinP-DM5 (DM5), pBinP-DM6 (DM6), 35S–RNA-2 (RNA-2) or 35S–HcPro (HcPro). (b) Northern blot of HMM RNA extracted from the infiltrated zones of the leaves in (a) hybridized with a digoxigenin-labelled GFP-specific probe. RNA extracted from an uninfiltrated leaf was used as a negative control. Bound probe was detected by chemiluminescence and the position of GFP mRNA is indicated. The lower panel shows ethidium bromide staining of ribosomal (r)RNA as a loading control. (c) Northern blot of HMM RNA extracted from the infiltrated zones of the leaves shown in (a) hybridized with a digoxigenin-labelled probe specific for CPMV RNA-1 minus strands. RNA extracted from an uninfiltrated leaf was used as a negative control. Bound probe was detected by chemiluminescence and the position of RNA-1 minus strands is indicated. The lower panel shows ethidium bromide staining of rRNA as a loading control.

are particularly important for this function and that the sequence cannot be substituted with that from the related virus BPMV.

To verify that the C-terminal 24 aa of the S protein affected RNA levels, high- and low-molecular-mass (HMM and LMM) RNA was isolated from the infiltrated leaf patches as described by Johansen & Carrington (2001). HMM RNA was fractionated on formaldehyde-containing agarose gels, transferred to nylon membranes and probed with digoxigenin-labelled strand-specific probes for GFP mRNA or RNA-1 minus strands as described previously (Liu *et al.*, 2004). Hybridization with the GFP-specific probe showed that levels of GFP mRNA correlated with levels of fluorescence in the leaf patch, being highest in the leaves co-infiltrated with 35S–HcPro or 35S–RNA-2 and lowest in those co-infiltrated with pBIN61, pBinP-Hybrid or pBinP-DM4 (Fig. 2b). Because all the agro-infiltrations involved co-infiltration with 35S–CPMV RNA-1, it was possible to assess the effect of the S protein-mutants on RNA-1 replication. To avoid any effects that the mutations in the S protein might have on RNA accumulation resulting from differences in the efficiency of RNA encapsidation, we examined the levels of minus-strand RNA-1, as these molecules are not encapsidated. Co-infiltration with pBIN61, pBinP-Hybrid, -DM4 or -DM5 resulted in low levels of RNA-1 minus-strand accumulation (Fig. 2c). Although there was some variation between the levels found with these samples, these were not consistently observed and were not thought to be significant. However, co-infiltration with those constructs that led to obviously increased levels of GFP expression (pBinP-DM6, 35S–RNA-2 and 35S–HcPro) also gave increased levels of RNA-1 minus-strands (Fig. 2c). These results demonstrated that, as with GFP expression, the C-terminal 24 aa, and particularly aa 198–213, of the S protein are important for efficient replication of RNA-1.

To investigate the mechanism whereby the C terminus of the S protein suppresses silencing, LMM RNA extracted from infiltrated patches was fractionated on polyacrylamide gels. After transfer to nylon membranes, the blots were analysed for small interfering (si)RNAs using probes for either GFP mRNA or RNA-1 plus strands as described previously (Liu *et al.*, 2004), except that the probes were hydrolysed with sodium carbonate before use. The Northern blot probed for GFP-specific sequences revealed two size

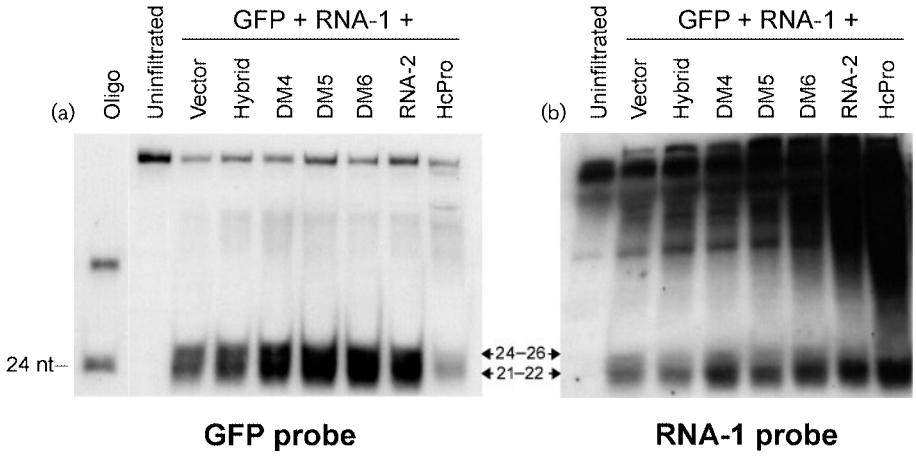

Fig. 3. Northern blot analysis of LMM RNAs extracted from patches of *N. benthamiana* leaves. RNA was extracted either from uninfiltrated leaves or from leaves infiltrated with 35S–GFP and 35S–RNA-1 in combination with either pBIN61 (vector), pBinP-Hybrid (Hybrid), pBinP-DM4 (DM4), pBinP-DM5 (DM5), pBinP-DM6 (DM6), 35S–RNA-2 (RNA-2) or 35S–HcPro (HcPro). Each lane contained 4 μg LMM RNA extracted from leaves and electrophoresis was on a 15 % (w/v) polyacrylamide gel containing 7 M urea. (a) Northern blot hybridized with a digoxigenin-labelled GFP-specific probe. Bound probe was detected by chemiluminescence. Ten picomoles of 24-mer DNA GFP oligonucleotide in the sense orientation was used as a size marker and the positions of siRNA are indicated as either 21–22 nt (short class) or 24–26 nt (long class). (b) Northern blot hybridized with a digoxigenin-labelled probe specific for CPMV RNA-1 plus strands. Bound probe was detected by chemiluminescence and the positions of siRNAs are indicated as either 21–22 nt (short class) or 24–26 nt (long class).

classes of siRNAs (21–22 and 24–26 nt) in approximately equal abundance (Fig. 3a). In plants, the shorter class (21–22 nt) has been implicated in mRNA degradation and the longer class (24–26 nt) in directing DNA methylation and in the systemic spread of silencing (Hamilton *et al.*, 2002; Himber *et al.*, 2003; Zilberman *et al.*, 2003). The two size classes of siRNAs are believed to arise via processing by two distinct dicer-like (DCL) proteins (Tang *et al.*, 2003) of double-stranded (ds)RNA molecules derived from the transgene-specific mRNA by the action of a cellular RNA-dependent RNA polymerase (RdRp) (Dalmay *et al.*, 2000; Sijen *et al.*, 2001; Vaistij *et al.*, 2002). Co-infiltration with the various forms of CPMV RNA-2 did not reduce the level of either size class of GFP-specific siRNAs below that found with co-infiltration with pBIN61. The only significant reduction was found in the samples of LMM RNA extracted from leaves co-infiltrated with 35S–HcPro, a result in line with the known properties of this suppressor in inhibiting siRNA production. These findings suggest either that the CPMV suppressor acts downstream of the production of siRNAs or that its activity on siRNA production is too weak to be seen in this assay. When Northern blots of LMM RNA were probed for CPMV RNA-1 plus strands, the smaller class of siRNA (21–22 nt) was predominant (Fig. 3b). This is consistent with the RNA-1-specific siRNAs being derived by DCL protein processing of dsRNA produced by the action of the virus-encoded rather than the cellular RdRp (Voinnet, 2001; Silhavy & Burgyan, 2004). None of the potential suppressors co-infiltrated into the leaves, including 35S–RNA-2 and 35S–HcPro, reduced the levels of the RNA-1-specific siRNAs, indicating that the replicating RNA-1 is a more potent inducer of silencing than 35S–GFP. However, in the samples infiltrated with pBinP-DM6, 35S–RNA-2 and 35S–HcPro, increased levels of HMM RNA were detected (Fig. 3b), consistent with the previous observation that the presence of these molecules allowed an increased rate of RNA-1 replication (Fig. 2c).

Overall, the results presented here provide compelling evidence that the suppressor activity of the CPMV S protein requires the C-terminal region, and aa 198–213 in particular. The data explain the debilitation of mutants harbouring deletions in this region (Taylor *et al.*, 1999) and the low level of replication often found when RNA-1 replicates on its own in protoplasts (de Varennes & Maule, 1985). The fact that the native CPMV sequence cannot be functionally substituted by the equivalent region of BPMV also explains the phenotype of the infection caused by pCP-Hybrid and why CPMV chimaeras with the natural C-terminal amino acids replaced by foreign epitopes grow extremely poorly (Taylor, 1997). Our analysis of the siRNAs does not provide clear evidence as to the site of action of the S protein in the silencing pathway. However, given the involvement of the C-terminal residues in interacting with RNA (Taylor *et al.*, 1999), it is tempting to speculate that they may be involved in binding to siRNAs in a manner analogous to p19 of tombusviruses (Silhavy *et al.*, 2002). Though a region of the coat protein of *Turnip crinkle virus* (TCV p38) has also been implicated in the suppression of PTGS (Qu *et al.*, 2003; Thomas *et al.*, 2003), the mechanisms of action appear to be different in the two cases, with p38 preventing the formation of siRNAs

(Qu *et al.*, 2003). Also, the region of TCV p38 involved in suppression lies on the inner surface of the virions and is unlikely to be functional once particle assembly has occurred (Thomas *et al.*, 2003). In CPMV, the sequence required for suppressor activity is exposed on the virus surface and will be available immediately when virus particles enter the cell, thus preventing silencing from being established.

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