

The C-terminal 33 amino acids of the cucumber mosaic virus 3a protein affect virus movement, RNA binding and inhibition of infection and translation

Sang Hyon Kim,¹ Natalia O. Kalinina,^{1,2} Igor Andreev,³ Eugene V. Ryabov,⁴ Alexander G. Fitzgerald,³ Michael E. Taliansky¹ and Peter Palukaitis¹

Correspondence

Peter Palukaitis
ppaluk@scri.sari.ac.uk

¹Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK

²A.N. Belozersky Institute of Physico-chemical Biology, Moscow State University, Moscow 119899, Russia

³Dept of Electronic Engineering and Physics, University of Dundee, Dundee DD1 4NH, UK

⁴Horticulture Research International-East Malling, ME19 6BJ, UK

The capsid protein (CP) of *Cucumber mosaic virus* (CMV) is required for cell-to-cell movement, mediated by the 3a movement protein (MP). Deletion of the C-terminal 33 amino acids of the CMV 3a MP (in the mutant designated 3aΔC33 MP) resulted in CP-independent cell-to-cell movement, but not long-distance movement. RNA-binding studies done *in vitro* using isolated bacterially expressed MP showed that the 3aΔC33 MP bound RNA more strongly, with fewer regions sensitive to RNase and formed cooperatively bound complexes at lower ratios of protein : RNA than the wild-type (wt) 3a MP. Analysis of the architecture of the complexes by atomic force microscopy showed that the wt 3a MP formed a single type of complex with RNA, resembling beads on a string. By contrast, the 3aΔC33 MP formed several types of complexes, including complexes with virtually no MP bound or thicker layers of MP bound to the RNA. Assays showed that protein–RNA complexes containing high levels of either MP inhibited the infectivity and *in vitro* translatability of viral RNAs. The 3aΔC33 MP inhibited these processes at lower ratios of protein : RNA than the wt 3a MP, consistent with its stronger binding properties. The apparent contradiction between these inhibition data and the CP-independent cell-to-cell movement of CMV expressing the 3aΔC33 MP is discussed.

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INTRODUCTION

The cell-to-cell and long-distance movement of *Cucumber mosaic virus* (CMV) involve the participation of each of the five encoded proteins of CMV, expressed from the three genomic and two subgenomic RNAs (Ding *et al.*, 1995a, b; Gal-On *et al.*, 1994; Hellwald & Palukaitis, 1995; Kaplan *et al.*, 1995; Suzuki *et al.*, 1991). Nevertheless, the 3a protein, expressed from RNA 3 of CMV, is designated the virus movement protein (MP), since this protein is involved in a number of functions associated with movement (Canto & Palukaitis, 1999a, b; Ding *et al.*, 1995a; Gal-On *et al.*, 1995; Kaplan *et al.*, 1995; Li & Palukaitis, 1996; Li *et al.*, 2001). The 3a MP binds ssRNA cooperatively (Li & Palukaitis, 1996), forming tightly packed nucleoprotein complexes (Nurkiyanova *et al.*, 2001). This virus-encoded protein also interacts with plasmodesmata and facilitates the movement of viral RNA from one cell to another, as well as into and out of the vasculature (Blackman *et al.*, 1998; Canto *et al.*, 1997; Ding *et al.*, 1995a). Different cell types can form

barriers to the movement of RNA by various mutants of the 3a MP (Canto & Palukaitis, 1999a, b; Kaplan *et al.*, 1997). The 3a MP expressed in transgenic plants can complement the movement of various, but not all, 3a protein mutants (Canto *et al.*, 1997; Kaplan *et al.*, 1995; Li *et al.*, 2001).

The capsid protein (CP), but not virus particles, was also shown to be essential for the cell-to-cell movement of CMV (Kaplan *et al.*, 1998), and mutants in the CP also have host-specific effects on CMV movement (Kaplan *et al.*, 1998; Ryu *et al.*, 1998; Takahashi *et al.*, 2001; Takeshita *et al.*, 2001; Wong *et al.*, 1999). It was speculated that the CP might have a role in altering the conformation of the 3a MP, facilitating its interaction with plasmodesmata (Ryabov *et al.*, 1999). It is conceivable that the C-terminal region of the 3a MP is involved in altering the RNA-binding affinity, because this region was shown to be adjacent to the region involved directly in RNA binding (Vaquero *et al.*, 1997) and was found to be dispensable for infection (Kaplan *et al.*, 1995; Nagano *et al.*, 1997, 2001). If this were the case, then the CP

might be required to alter the stability of MP–RNA complexes, while the C-terminal region of the 3a MP would be expected to have some effect on the binding of MP to RNA. No evidence could be obtained for a direct interaction between the CP and MP *in vitro* (Nagano *et al.*, 2001; D. Szilassy, T. Canto & P. Palukaitis, unpublished data). Therefore, in this study we tested the hypothesis that deletion of the C-terminal 33 aa of the MP might alter its binding to viral RNA, and hence CP would not be required to effect the stability of the mutant 3a protein–RNA complexes. Specifically, we examined the role of the C-terminal 33 aa in RNA binding and the biological properties of the protein–RNA complexes.

METHODS

Generation of cDNA clones and *in vitro* transcripts. The generation of infectious transcripts *in vitro* with T7 RNA polymerase using cDNA clones of RNAs 1, 2 and 3 of the Fny-strain of CMV has been described previously (Rizzo & Palukaitis, 1990). A chimaeric cDNA clone of CMV RNA 3 (pFL. ORF4.GFP), in which the 3a gene was replaced with ORF4 of *Groundnut rosette virus* (GRV) and the CP gene was replaced with the gene encoding the green fluorescent protein (GFP) (Ryabov *et al.*, 1999), was used to construct a cDNA clone in which the GRV ORF4 was replaced by a truncated CMV 3a gene, designated pFL:3aΔC33/GFP (Fig. 1). The sequences encoding the C-terminal 33 aa of the 3a protein were absent from pFL:3aΔC33/GFP. The truncated 3a gene was generated by PCR using plasmid pFny309 (Fig. 1), a primer corresponding to sequences upstream of the T7 promoter containing the *Bam*HI site, and a primer complementary to nucleotides 832 to 857 of the 3a gene and containing an *Spe*I site, using conditions previously described (Ryabov *et al.*, 1999). The resulting fragment, digested with *Bam*HI and *Spe*I, was used to replace the corresponding *Bam*HI–*Spe*I fragment of pFL. ORF4.GFP to generate the cDNA clone pFL:3aΔC33/GFP. The generation of cDNA clones, which contained either the wild-type (wt) 3a gene and the CP gene replaced by the gene encoding the GFP (pL:3a/GFP), or just the 3a gene replaced by the gene encoding the GFP (pF:GFP/CP), was described previously (Canto *et al.*, 1997). RNA transcripts from any of the above plasmids were generated using T7 RNA polymerase, as described previously (Zhang *et al.*, 1994).

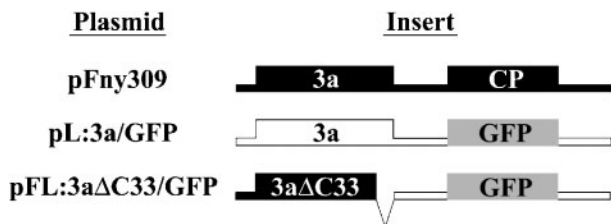


Fig. 1. Gene organization of RNA 3 expressed as T7 RNA transcripts from the specified plasmids. The wt RNA 3 is transcribed from the plasmid pFny309, and contains ORFs for the 3a MP and the CP. RNA 3 containing an ORF for the GFP in place of the CP was transcribed from plasmid pL:3a/GFP. RNA 3 in which the ORF for the CP was replaced with the ORF for the GFP and the ORF for the 3a MP was truncated to delete the sequences encoding the C-terminal 33 aa was transcribed from plasmid pFL:3aΔC33/GFP.

Expression, isolation and renaturation of the MP. Cloning and overexpression of the wt 3a gene and the truncated 3a gene in *Escherichia coli* were done as follows. The ORFs of the wt and mutant MP genes were amplified by PCR from plasmids pFny309 and pFL:3aΔC33/GFP, respectively, and were inserted into plasmid pQE60 (Qiagen) digested with *Nco*I and *Hind*III. The resultant expressed proteins contained six C-terminal histidine residues. The ligated plasmids were transformed into cells of *E. coli* strain TG1, and 500 ml overnight cultures containing plasmid DNA were induced by 50 μM IPTG at room temperature for 2 h before harvest. The cells were collected by centrifugation and were disrupted by resuspending in a solution containing 6 M guanidinium.HCl in 20 mM Tris/HCl, pH 7.9, and 10 mM 2-mercaptoethanol, and incubating for 1 h on ice. The material was centrifuged at 20 000 g for 1 h and the resulting supernatant was passed through a column of Ni–NTA agarose (Qiagen). Unbound proteins were washed with 10 vols of buffer containing 20 mM Tris/HCl, pH 7.0, 10 mM 2-mercaptoethanol, 6 M urea and 25 mM imidazole, before the bound protein was eluted with 5 column vols of the same buffer containing 400 mM imidazole. Approximately 25 mg of protein was obtained from a 500 ml culture of cells. Protein at a concentration of no less than 0.25 mg ml⁻¹ was renatured overnight at 4 °C in a solution containing 50 mM Tris/HCl, pH 8.0, 50 mM NaCl, 2.5 mM DTT and 1 M NDSB201 (Novagen), and dialysed extensively against water to remove the NDSB201. Proteins soluble in water were concentrated to 10 mg ml⁻¹ using 10 a kDa-cut Centrplus Centrifugal Filter Device (Amicon), according to the manufacturer's instructions.

A plasmid containing the gene encoding the tobacco mosaic virus (TMV) 30K MP fused to sequences encoded six histidine residues at the N terminus of the 30K protein was obtained from Sean Chapman (SCRI). The TMV 30K MP was overexpressed, purified and renatured as described above for the CMV MP.

***In vitro* RNA binding assays.** ³²P-labelled RNA transcripts of CMV RNA 3 were prepared from linearized pFny309 using 5 μCi of α-[³²P]UTP (Amersham) and the mMESSAGE mMACHINE T7 kit (Ambion). The labelled transcripts (4 ng) were mixed with different amounts of purified protein, either wt 3a MP or the truncated 3a MP, in 15 μl of binding buffer (50 mM Tris/HCl, pH 7.0, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mg BSA ml⁻¹ and 10% glycerol). After incubation on ice for 30 min, the mixtures were subjected to electrophoresis in non-denaturing gels of 1% agarose. The gels were dried and subjected to autoradiography. Treatment of MP–RNA complexes with RNase A (200 ng) and electrophoresis was done as described previously (Li & Palukaitis, 1996). The formation of complexes in different concentrations of NaCl and the detection of labelled RNA in complexes by filtration of the solutions and liquid scintillation counting were done as described previously (Li & Palukaitis, 1996).

Atomic force microscopy (AFM). TMV RNA, total CMV RNA or purified RNA transcripts from plasmid pF:GFP/CP were used for the formation of MP–RNA complexes visualized by AFM. RNA (0.1 μg) was denatured by heating at 95 °C for 30 s and quenching on ice. The denatured RNA was mixed on ice with 20 μl of MP in RNase-free water, at different weight ratios of MP:RNA, for 30 min. The complexes were diluted 10-fold in RNase-free water before image analysis. MP–RNA complexes (5 μl) were placed onto freshly cleaved mica strips for 5 min. The mica strips were then rinsed with deionized water and vacuum dried at room temperature. Sometimes AP-mica strips were used instead of untreated mica (which contains a negative surface charge), prepared as described by Lyubchenko *et al.* (1993a, b). That is, the mica strips were incubated in an atmosphere of 3-aminopropyltriethoxylane (APTES) to cover

the surface with amino groups. Imaging of the complexes and measuring of heights were done as described previously (Nurkiyanova *et al.*, 2001).

Infectivity assays. RNA transcripts of CMV RNAs 1 and 2 were mixed with RNA 3 transcribed from either pFL:3aΔC33/GFP or pL:3a/GFP and inoculated directly onto Carborundum-dusted leaves of *Nicotiana benthamiana* or tobacco (*N. tabacum* cv. Samsun NN). Plants were maintained in a shaded greenhouse for the times indicated. Infection was detected by fluorescence due to GFP expression, using either a confocal microscope or a hand-held UV lamp, as described previously (Ryabov *et al.*, 1999).

Fny-CMV RNAs (50 ng) or TMV RNA (100 ng) were mixed with *E. coli*-expressed MP at different MP:RNA ratios from 1:25:1 to 100:1 for CMV RNA and 5:1 to 120:1 for TMV RNA, on ice for 1 h, in 20 to 40 μl of deionized water. CMV RNAs either pre-incubated with water alone or with various amounts of MP were inoculated to opposite half-leaves of *Chenopodium amaranticolor* plants. TMV RNA pre-incubated with water or with various amounts of MP was inoculated to opposite half-leaves of either non-transgenic tobacco (*N. tabacum* cv. Xanthi XHFD8) or transgenic tobacco expressing the R-CMV CP gene (line CP-R.9A). The numbers of local lesions were scored at 4 days post-inoculation (p.i) for both sets of experiments. The above inoculations (CMV and TMV) were each done in four separate experiments. The mean and standard error values for the percentage reduction in infectivity at each MP:RNA ratio were calculated.

In vitro translation and analysis of proteins. CMV RNA 3 transcripts (0.5 μg), derived from plasmid pF:GFP/CP, were denatured at 95 °C for 30 s, quenched on ice and incubated at 0 °C for 30 min with different amounts of either the wt 3a MP or the truncated 3a MP. Free or MP-complexed RNA was translated *in vitro* in rabbit reticulocyte cell-free lysate, using the Transcend tRNA non-radioactive detection system (Promega), following the manufacturer's instructions. The translation products were fractionated by 10% SDS-PAGE. The proteins were transferred onto a PVDF membrane (Bio-Rad) and the membrane was processed to detect the biotinylated GFP using alkaline-phosphatase-conjugated streptavidin followed by incubation with BCIP/NBT. Denaturing gels containing the complexes were stained with Coomassie blue to visualize the MP (Sambrook & Russell, 2001).

RESULTS

Movement of CMV with and without CP

We sought first to confirm the results of Nagano *et al.* (2001), using Fny-CMV lacking the C-terminal 33 aa of the 3a MP (referred to here as 3aΔC33), since the results concerning the movement of the 3a MP lacking the C-terminal 43 aa differed between Fny-CMV and Y-CMV (Kaplan *et al.*, 1995; Nagano *et al.*, 2001). Specifically, the gene encoding the 3a MP of Fny-CMV was modified in a biologically active cDNA clone of CMV RNA 3 to express a truncated MP, in which the C-terminal 33 aa were absent. In addition, the gene expressing the CP was replaced with the gene expressing the GFP, yielding plasmid pFL:3aΔC33/GFP (Fig. 1). Transcription of RNA 3 from pFL:3aΔC33/GFP, and inoculation to *N. benthamiana* (Fig. 2A and C) or tobacco (Fig. 2D) along with transcripts of cDNA clones of Fny-CMV RNAs 1 and 2, led to a spreading infection in the inoculated leaves (Fig. 2A, C and D). By contrast, CMV in

which the CP gene was replaced with the gene encoding the GFP and the 3a MP was wt replicated, but did not move cell to cell (Fig. 2B), as previously reported (Canto *et al.*, 1997). Inoculation of the latter virus to transgenic tobacco line CP-R.9A, expressing the CMV CP, but which was not resistant to CMV infection (Jacquemond *et al.*, 2001), resulted in complementation of cell-to-cell movement (data not shown).

The above results confirmed and extended the observations of Nagano *et al.* (2001), using a different strain of CMV in two other plant species. Moreover, infection in either *N. benthamiana* (Fig. 2C) or tobacco (Fig. 2D) did not progress to leaves above the inoculated leaves. To assess whether CP expressed from the transgenic plants could complement long-distance movement of the virus expressing the mutant 3aΔC33 MP, the viral RNAs were inoculated to the transgenic tobacco line CP-R.9A. In none of the plants tested was there movement of the GFP-expressing virus to upper, non-inoculated leaves (data not shown). Thus, the transgenically expressed CP was not able to complement movement of the viral RNA into and/or out of the vascular system.

To test whether the Fny-CMV wt 3a MP interfered with the movement of CMV expressing the mutant 3aΔC33 MP, CMV RNAs expressing both the mutant 3aΔC33 MP and the GFP were inoculated to transgenic tobacco expressing high levels of the wt Fny-CMV 3a protein (Kaplan *et al.*, 1995). The transgenically expressed wt 3a MP did not inhibit cell-to-cell movement of the CMV mediated by the 3aΔC33 MP (Fig. 2E).

Binding kinetics, nuclease sensitivity and stability of the protein–RNA complexes

E. coli-expressed and solubilized MPs were used to determine whether the mutant 3aΔC33 MP showed differences in RNA binding from the wt 3a MP. The wt 3a MP bound to CMV RNA 3 in a cooperative fashion (Fig. 3A), as described previously for wt 3a MP solubilized by a different procedure (Li & Palukaitis, 1996). About half of the labelled RNA was bound to protein and formed complexes with 200 ng of wt 3a MP, while in the presence of 400 ng of wt 3a MP, all of the available RNA was associated with 3a MP (Fig. 3A). The mutant 3aΔC33 MP also bound RNA cooperatively, but much less protein was required to convert all of the free RNA to protein–RNA complexes than for wt 3a MP (Fig. 3B vs Fig. 3A). About half of the viral RNA was associated with protein–RNA complexes when between 20 and 50 ng of the 3aΔC33 MP was available, and in the presence of 100 ng of 3aΔC33 MP, all of the RNA was bound to protein (Fig. 3B). This suggested that the mutant MP did not bind with the same degree of cooperativity (i.e. saturation) and/or it had a greater affinity for RNA. In the former situation, more protein would be available to bind to other RNA molecules. In the latter case, the MP–RNA complexes formed between 3aΔC33 MP and RNA might be expected to be more stable than those formed

between wt 3a MP and RNA. The same results were obtained using different viral RNAs (data not shown).

To determine whether there were differences in the nature of the cooperative protein–RNA complexes formed by the wt 3a MP vs the mutant 3a Δ C33 MP, the complexes were

treated with RNase A and were analysed by gel electrophoresis. The RNA associated with the complexes formed using wt 3a MP showed a migration in the gel faster than that of either the untreated 3a MP–RNA complex or free RNA (Fig. 4A, lanes 1 and 2), as also reported previously for the wt 3a MP solubilized by a different procedure (Li & Palukaitis, 1996). This indicates that segments of the viral RNAs were protected from RNase, but that the cooperatively bound proteins did not protect the entire RNA. By contrast, the RNA associated with the mutant 3a Δ C33 MP showed both highly protected RNA, although as only a minor fraction of the total RNA, and RNA of a similar size distribution to that protected by the wt 3a MP (Fig. 4A, lanes 3 and 4, vs lanes 1 and 2). This was the case at both high and low ratios of 3a Δ C33 MP : RNA (Fig. 4A, lanes 3 and 4). Thus, the mutant 3a Δ C33 MP appeared to form complexes with most of the viral RNA similar to the wt 3a MP but, in addition, was able to form complexes in which the viral RNA did not appear to have as many gaps of unprotected RNA.

The stability of the protein–RNA complexes formed *in vitro* using wt 3a MP vs the mutant 3a Δ C33 MP was compared by examining the effects of salt on the ability of the respective proteins to form complexes with RNA. The complexes formed by the wt 3a MP showed an almost 2-fold lower stability to salt than those formed by the 3a Δ C33 mutant MP (Fig. 4B). This indicates that the mutant 3a Δ C33 MP formed complexes of greater stability than the wt 3a MP, and suggests that the mutant 3a Δ C33 MP may have a greater affinity for RNA than the wt 3a MP. There also was no effect on the relative stability of the protein–RNA complexes by using different viral RNAs (data not shown).

Architecture of the protein–RNA complexes

The architecture of MP–RNA complexes has been examined before by AFM, both for the wt CMV 3a MP and the 30K MP encoded by TMV (Kiselyova *et al.*, 2001; Nurkiyanova *et al.*, 2001). In the case of the wt 3a MP, large aggregates of protein–RNA complexes were formed. Using a slightly different sample treatment for the AFM (see Methods) allowed visualization of non-aggregated protein–RNA complexes (Fig. 5A). Most of the RNA within these complexes appeared coated (Fig. 5A, panels 1 and 2), but at higher resolution the complexes formed between wt 3a

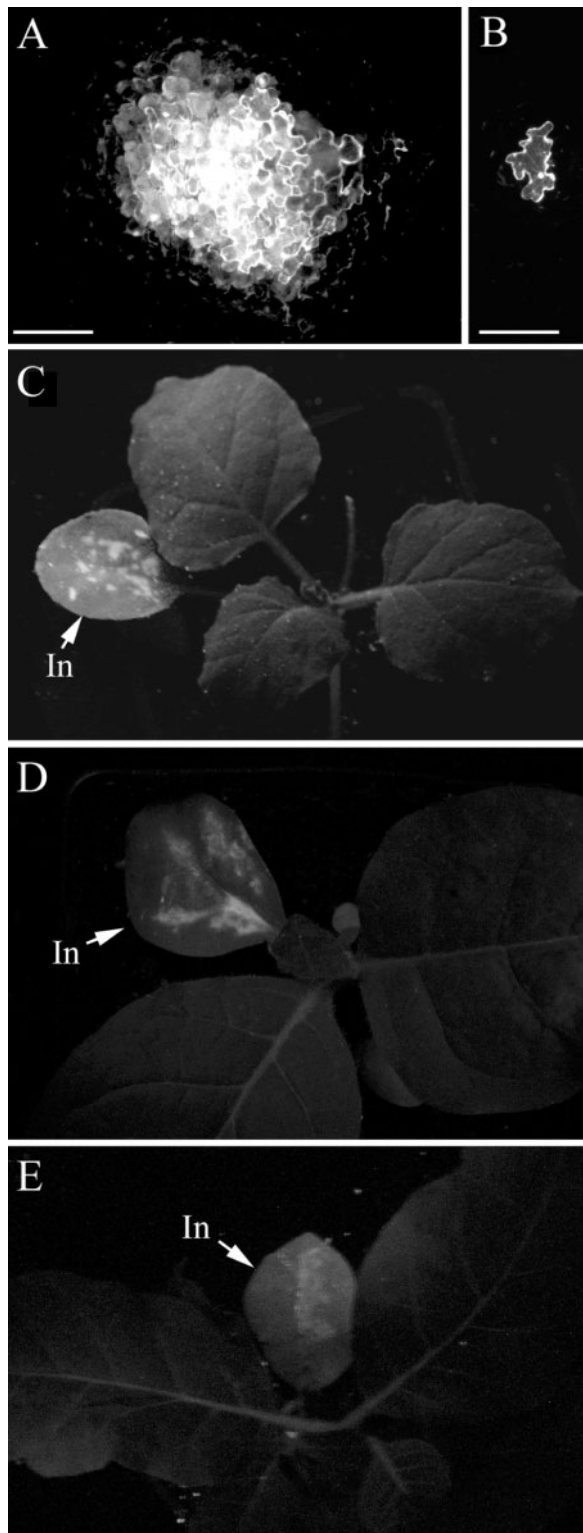


Fig. 2. Infectivity of GFP-expressing CMV RNAs. *N. benthamiana* plants (A–C) or tobacco plants (D–E) were inoculated with transcripts of CMV RNAs 1, 2 and 3 in which the CP gene in RNA 3 had been replaced by the gene encoding the GFP and expressing either the mutant 3a Δ C33 MP (A and C–E) or the wt 3a MP (B). Tobacco plants were either non-transgenic (D) or expressing the CMV MP (E). Fluorescence due to the expression of GFP was detected by either fluorescence microscopy at 3 days p.i. (A and B), or under a long-wavelength UV lamp at 10 days p.i. (C–E). Bars in A and B represent 250 μ m.

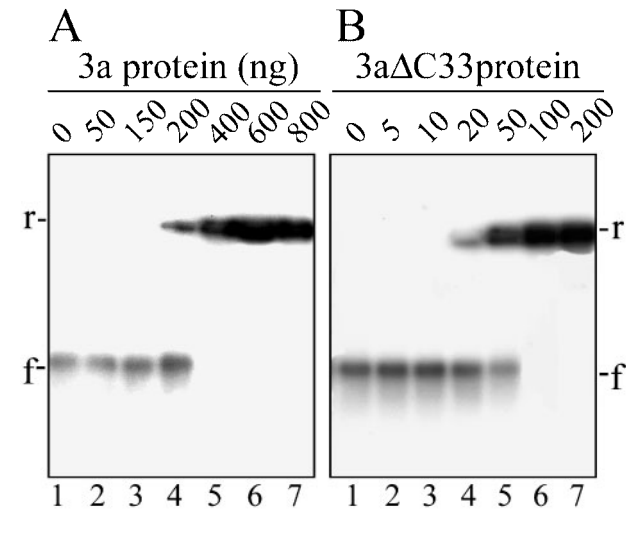


Fig. 3. Gel retardation assay for RNA binding by MP. Increasing amounts of wt CMV 3a MP (A) and the mutant 3aΔC33 MP (B) were incubated with 4 ng of ^{32}P -labelled CMV RNA 3 transcript and the mixture was fractionated by non-denaturing gel electrophoresis in 1% agarose. The amounts of MP added are indicated. The electrophoretic positions of free (f) and retarded (r) RNAs are marked.

MP and RNA showed alternating thicker and thinner regions (Fig. 5A, panel 3), with an apparent periodicity of approximately 15 nm (Fig. 5A, panel 4). These complexes had some similarities to the ‘beads-on-a-string’ complexes described for the TMV MP (Kiselyova *et al.*, 2001), although the ‘string’ was not naked RNA but, based on its thickness, was also coated with MP. All of the various complexes formed between the wt 3a MP and RNA showed the same pattern and periodicity. By contrast, the complexes observed between the mutant 3aΔC33 MP and RNA were not uniform (Fig. 5B, panels 1–4) and showed three structural elements, which may be present on the same RNA molecule (Fig. 5B, panel 1): (a) protein forming thick beads on a coated string (Fig. 5B, panel 5), similar to what was observed for the wt 3a MP (Fig. 5A, panel 3), with an apparent periodicity of 20 nm (Fig. 5B, panel 7); (b) a more densely coated string (Fig. 5B, panel 6), with an apparent periodicity of 20 or 30 nm, but with a low amplitude between the bead units (Fig. 5B, panel 8); and (c) structures that may be described as white nodules (asterisks in Figs 5B, panels 1, 2 and 4). The same results were obtained using various viral RNAs to form the protein–RNA complexes.

RNase treatment of 3aΔC33 MP–RNA complexes yielded two types of linear fragments with modal lengths of approximately 30 to 50 nm and 120 nm (data not presented). The former was also seen after RNase digestion of wt 3a MP–RNA complexes. The white nodules completely disappeared after RNase treatment (data not shown), indicating that they were loose globular structures of

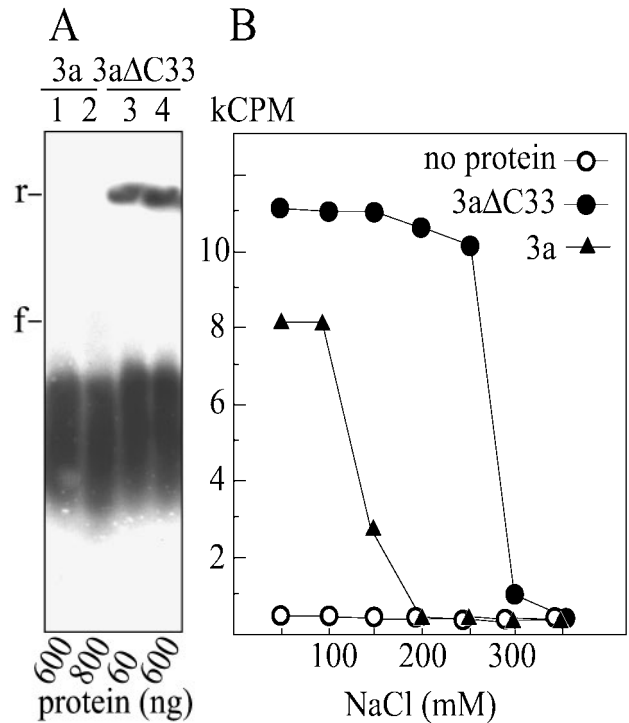


Fig. 4. Characterization of CMV MP–RNA complexes. (A) The accessibility of MP–RNA complexes to RNase A was evaluated after incubation of MP–RNA complexes formed with either the wt 3a MP (lanes 1 and 2) or the mutant 3aΔC33 MP (lanes 3 and 4). The RNase-treated complexes were analysed by electrophoresis on a non-denaturing 1% agarose gel. Two levels of MP were used for the formation of the complexes analysed, as indicated. The electrophoretic positions of unbound and non-digested free (f) and MP-complexed and retarded (r) RNAs are marked. (B) The ability of the MP–RNA complexes to form at different salt concentrations was determined by a filter-binding assay. ^{32}P -labelled RNA (4 ng) was incubated with no protein or with 600 ng of either wt 3a MP or mutant 3aΔC33 MP in binding buffer containing different concentrations of NaCl and the mixtures were filtered through a nitrocellulose membrane. RNA binding was quantified by liquid scintillation counting of the radioactivity remaining on the membrane.

either RNA coated with only a few molecules, or naked RNA, as seen previously when free RNA was examined by AFM (Nurkiyanova *et al.*, 2001).

Infectivity of protein–RNA complexes

If the mutant 3aΔC33 MP binds more efficiently to viral RNA than the wt 3a MP, then complexes formed *in vitro* between CMV RNA and the two MPs would be expected to differ in their infectivities. Therefore, CMV RNAs were incubated with different amounts of the two MPs and were inoculated to *C. amaranticolor*, to assess the effect of the MPs on the infectivity of the viral RNAs. The infectivity of CMV RNAs pre-incubated without protein was assessed on

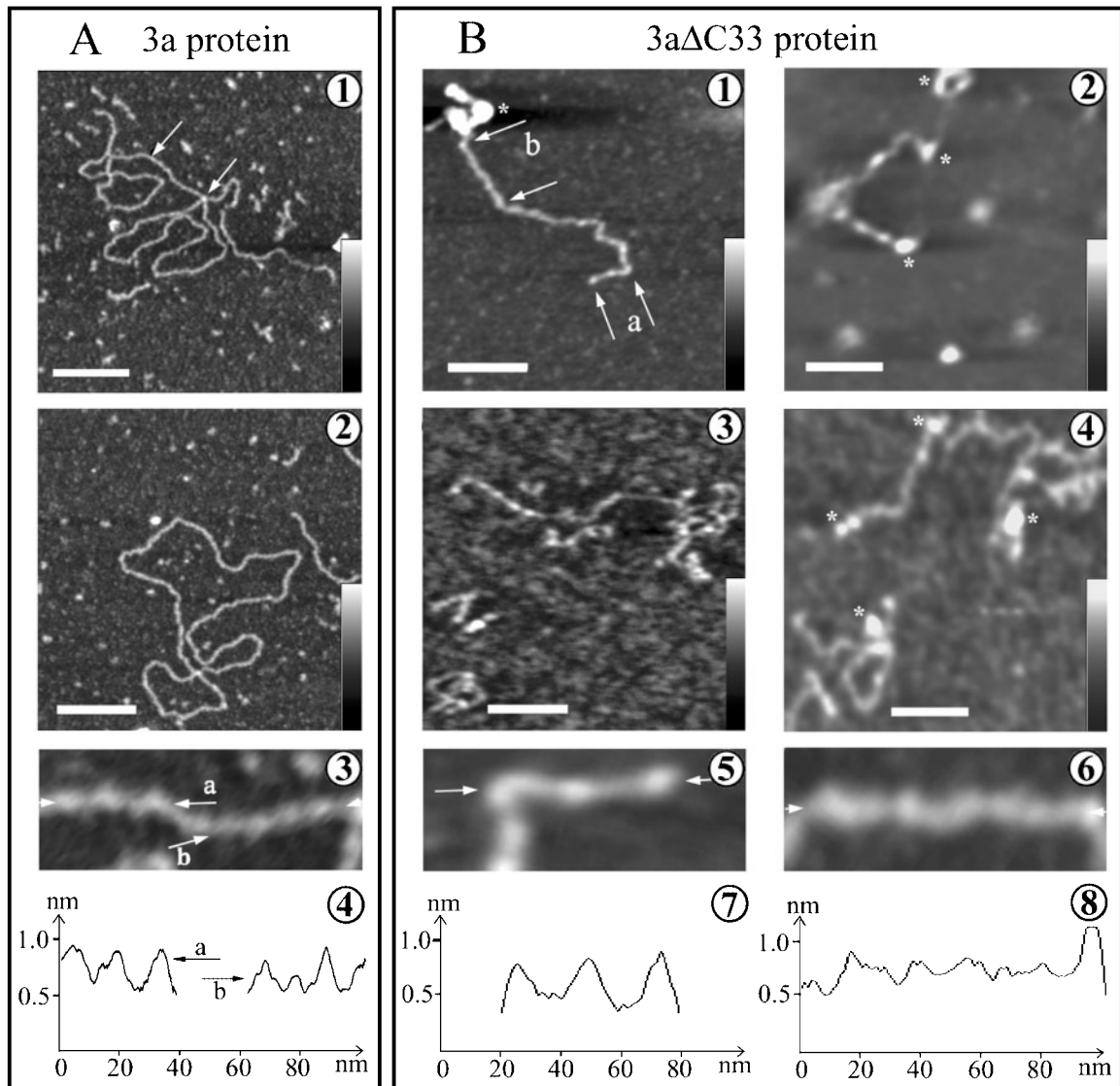


Fig. 5. AFM analysis of MP–RNA complexes. Either wt 3a MP (A) or mutant 3aΔC33 MP (B) was used to form complexes with total CMV RNA in solution. Samples containing complexes were applied to mica strips and were processed for imaging by AFM, shown in panels A1, A2 and B1–B4. All samples contained 4 ng of RNA, while the mutant 3aΔC33 MP was used at two levels: 60 ng in panels B1 and B3, and 600 ng in panels B2 and B4. Panel A3 shows a view of the area between arrows in panel A1 at a higher magnification, and panel A4 shows the heights of the complexes and the periodicity of the peaks in this area. Panels B5 and B6 show an enlarged view of the areas marked ‘a’ and ‘b’ in panel B1, and panels B7 and B8 show the heights of the complexes and periodicity for regions ‘a’ and ‘b’, respectively. Asterisks in B1, B2 and B4 indicate globules of RNA nodules. For A1, A2 and B1–4, the horizontal bars represent 100 nm and the vertical bars represent 2.5 nm.

opposite half leaves in four separate experiments. There was considerable variation in the number of local lesions obtained for the same concentration of inocula in different experiments and on different half leaves (Fig. 6A), but this is typical of CMV (Habibi & Francki, 1974; Lakshman & Gonsalves, 1985; Nagano *et al.*, 2001; Rao & Francki, 1981). Nevertheless, the data show that at high ratios of MP : RNA, the infectivity of both types of MP–RNA complex was inhibited. However, the ratio of MP : RNA required to inhibit the infectivity of the viral RNA was less for the

mutant 3aΔC33 MP than for the wt 3a MP (Fig. 6A). That is, the wt 3a MP showed a drop in relative infectivity to 50% at a MP : RNA ratio of about 25 : 1. By contrast, the relative infectivity of complexes formed by the mutant 3aΔC33 MP and CMV RNA dropped to 50% at a MP : RNA ratio of about 15 : 1 (Fig. 6A).

To ensure that the loss of infectivity was not due to the presence of increasing amounts of protein per se interfering with uptake of the viral RNAs, complexes were also formed

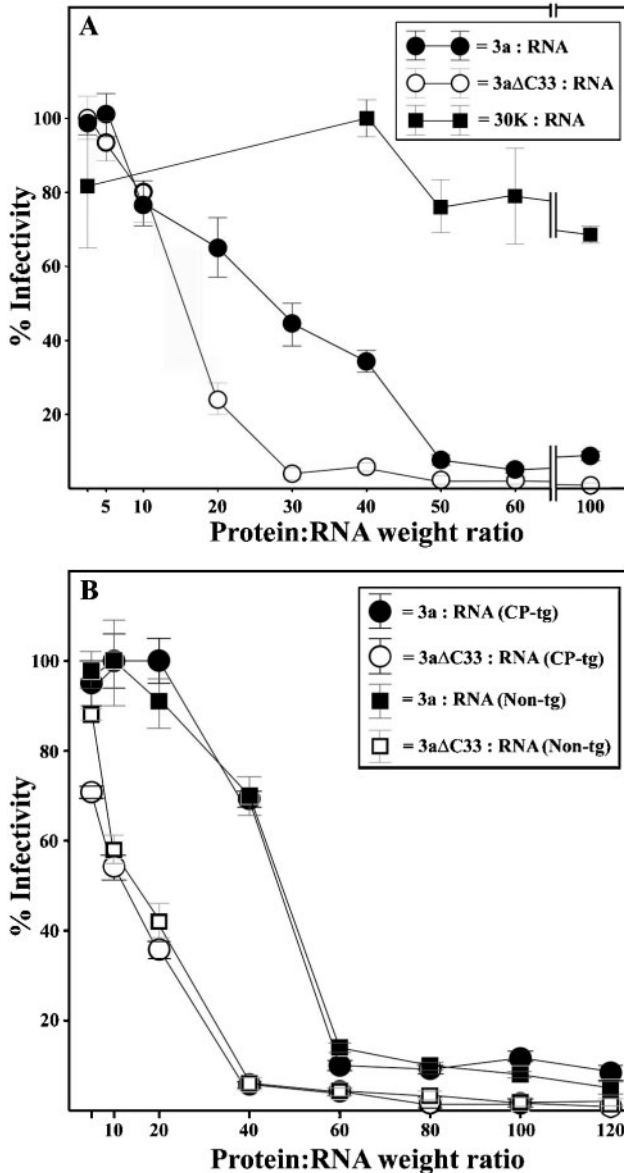


Fig. 6. Inhibition of infectivity by prior formation of MP-RNA complexes. Different amounts of wt CMV 3a MP, the mutant 3aΔC33 MP or TMV 30K MP were incubated with CMV RNAs 1–3 (A) or TMV RNA (B) and were inoculated to either *C. amaranticolor* (A) or tobacco (B). TMV RNA complexed with wt CMV 3a MP and the mutant 3aΔC33 MP were tested on non-transgenic tobacco as well as on transgenic tobacco expressing the CMV CP (B). Lesions formed on opposite half-leaves by the MP-RNA complex vs free RNA, with the former taken as a percentage of the latter, were averaged for four experiments to determine the extent of inhibition of infection. The mean and standard error for the percentage inhibition of infection at each protein:RNA ratio tested are shown.

between the TMV MP and the CMV RNAs and were tested for infectivity. It has been shown that the TMV MP did not affect infectivity of TMV RNA in inoculated plants (Karpova

et al., 1997). CMV RNA complexed with the TMV MP did not show the same loss of infectivity as CMV RNA complexed with either the wt 3a MP or the mutant 3aΔC33 MP (Fig. 6A). Thus, the negative effects on infectivity are specific to the CMV MPs. Moreover, deproteinization with phenol of the MP-RNA complex formed using the mutant 3aΔC33 MP, followed by ethanol precipitation, yielded RNA that gave similar numbers of local lesions as did CMV RNA treated similarly, but without prior incubation with MP (data not shown). Thus, inhibition of infectivity by the wt MP and the mutant 3aΔC33 MP was not due to the presence of some contaminating RNase.

It is conceivable that the CP is able to interact with the wt 3a MP associated with the viral RNA, disrupting the MP-RNA complex. However, CMV CP could not be added to such complexes *in vitro*, since CP capsomers isolated from virions are insoluble. Therefore, transgenic tobacco plants expressing high levels of CP, but not showing resistance to infection by CMV (Jacquemond *et al.*, 2001), were used as a source of CP to determine whether the presence of CP had any effects on the inhibition of viral infectivity by MP. Unfortunately, as CMV RNA coated with wt CMV MP retained some basal level of infection even at MP:RNA ratios of 50 to 100:1 (Fig. 6A), yielding three to ten local lesions on *C. amaranticolor* (data not presented), tobacco, a systemic host for CMV, could not be used to assess quantitative effects on infection. However, these transgenic tobacco plants also contained the *N* gene, which gives a hypersensitive response after infection by TMV. Thus, to quantify the effects of CP, as well as different levels of MP on the infection of the viral RNAs, TMV RNA was combined with different amounts of wt 3a MP or mutant 3aΔC33 MP and was inoculated to transgenic tobacco plants expressing the CMV CP, as well as to non-transgenic plants of the same tobacco cultivar. Inoculation of non-transgenic tobacco plants with TMV complexed with either the wt 3a MP or the mutant 3aΔC33 MP resulted in the formation of local lesions and a reduction in the specific infectivity of the TMV RNA with increasing amounts of MP (Fig. 6B). Moreover, the mutant 3aΔC33 MP again showed greater inhibition of infection at lower protein:RNA ratios than did the wt 3a MP, with infectivity reduced to about 50% by a protein:RNA ratio of 15:1 for the mutant 3aΔC33 MP vs 50:1 for the wt 3a MP (Fig. 6B). When tobacco plants transgenic for the CMV CP were inoculated with the same protein-RNA complexes, TMV RNA complexed with either the mutant 3aΔC33 MP or the wt 3a MP showed the same effects on inhibition of infection as shown in the non-transgenic tobacco plants (Fig. 6B). These data indicate that the transgenically expressed CMV CP was not able to destabilize the protein-RNA complexes formed *in vitro* using either the wt 3a MP or the mutant 3aΔC33 MPs.

Inhibition of translation of CMV RNA 3 in MP-RNA complexes

To determine whether complexes formed between CMV RNAs and MPs were able to interfere with translation of the

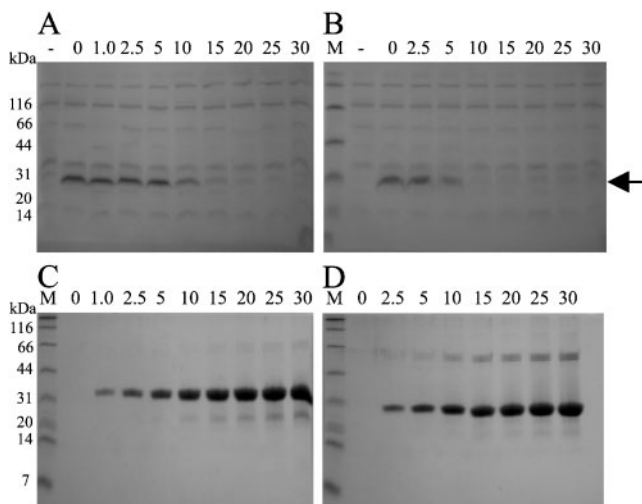


Fig. 7. Inhibition of translation *in vitro* by prior formation of MP–RNA complexes. *In vitro* transcripts of CMV RNA 3 (0.5 µg) with the ORF for the GFP replacing the 3a MP ORF were combined on ice with different amounts (1–30 µg) of either wt CMV 3a MP (A and C) or the mutant 3aΔC33 MP (B and D), as indicated. The complexes were assayed for translatability *in vitro* in a rabbit reticulocyte cell-free lysate by SDS-PAGE and the translation products (A and B) were detected using a colorimetric detection system. The GFP translation product is indicated by an arrow. The added CMV MPs were detected by staining the gels with Coomassie blue (C and D). ‘-’ indicates that no RNA was added to the translation system. Lane M contains protein markers, for which the molecular mass is given in kDa on the left.

RNA, CMV RNA 3 was mixed with various amounts of either wt 3a MP or mutant 3aΔC33 MP and assayed for translatability. The CMV RNA used for these assays contained the gene encoding the GFP in place of the native 3a gene. Analysis of the translation products showed that at low ratios of MP:RNA there was no inhibition of translation (Fig. 7A and B), while as the amount of MP increased (shown in Fig 7C and D), translation of the GFP gene was inhibited (Fig. 7A and B). Inhibition of translation by the wt 3a MP occurred over a broad range of MP:RNA ratios, starting between 20:1 and 30:1, with complete inhibition occurring at 60:1 (Fig. 7A). By contrast, inhibition of translation by the mutant 3aΔC33 MP occurred sharply between MP:RNA ratios of 10:1 and 20:1 (Fig. 7B). Therefore, MP–RNA complexes formed *in vitro* inhibited gene expression of the coated viral RNAs, and somewhat less MP was required for the mutant 3aΔC33 MP than for the wt 3a MP to confer this inhibition.

DISCUSSION

The C terminus of the CMV 3a MP has several effects on the function of the CMV MP. Deletion of the C-terminal 33 aa resulted in CP-independent cell-to-cell movement,

although CP was still required for long-distance movement (Fig. 2). This is consistent with earlier observations on the role of the CP in long-distance movement separate from its role in cell-to-cell movement (Kaplan *et al.*, 1998; Wong *et al.*, 1999). Deletion of the C-terminal 33 aa of the CMV MP also resulted in a stronger binding of the MP to the viral RNA (Fig. 4B). This resulted in less mutant 3aΔC33 MP than wt 3a MP being required for binding and gel retardation of the viral RNA (Fig. 3), as well as for inhibition of infectivity (Fig. 6) and translatability (Fig. 7) of the viral RNAs. These observations might suggest that the truncated MP had a greater affinity for the viral RNA than did the wt 3a MP. The biological differences could also have been a consequence of differences in the architecture of binding of the two MPs.

The wt 3a MP showed a distinctive, but repetitive pattern in the coating of the viral RNA, in which complexes appeared as ‘beads on a thick string’ (Fig. 5A). There were also gaps present on the ‘string’, which allowed access to RNase giving only smaller protected fragments (Fig. 4A). By contrast, the mutant 3aΔC33 MP was able to interact with the viral RNA to produce several types of complexes, at both high and low ratios of protein:RNA. These included complexes that were thicker than those produced by the wt 3a MP, in which the MP appeared to have more layers, as well as complexes in which more of the RNA was exposed (Fig. 5B). The former may also have led to more stable complexes (Fig. 4B), as well as complexes that offered better protection against RNase (Fig. 4A) and did not require complete coating of the viral RNA to affect inhibition of several processes. This is consistent with the observations that lower concentrations of the mutant MP were required to inhibit infection than for the wt MP (Fig. 6).

TMV RNA complexed with TMV MP was found to be infectious on plants, but not translatable (Karpova *et al.*, 1997). By contrast, the CMV MP was able to inhibit infection of either CMV RNAs or TMV RNA in plants (Fig. 6), as well as translation (Fig. 7), whereas the TMV MP also did not inhibit the infection of CMV RNAs in plants (Fig. 6A). For TMV, this has been explained by the observation that at high TMV MP:RNA ratios, translation of TMV RNA was inhibited, but transfer of the associated RNA from one cell to another was not (Karpova *et al.*, 1997). At high TMV MP:RNA ratios, the complexes were found to adopt a thickened string architecture (Kiselyova *et al.*, 2001). In the case of the CMV MP, the thickened string structure only was observed for complexes involving the truncated 3a MP, and not the wt 3a MP (Figs 5B vs 5A).

In contrast to the formation of the TMV beads-on-a-string complexes, in which binding of the TMV MP to RNA was done under non-cooperative-binding conditions (Kiselyova *et al.*, 2001), CMV 3a MP–RNA complexes were formed under cooperative interaction conditions (Fig. 3A). Moreover, in contrast to the TMV complexes, in which the RNA was fully accessible to RNase (Kiselyova *et al.*, 2001), the CMV 3a MP–RNA complexes were partially resistant to

RNase, producing linear fragments 30 to 50 nm in length. The 3a Δ C33 MP–RNA complexes produced similar RNase resistant fragments, as well as fragments with a length of approximately 120 nm. These two different sized fragments could have originated from structural elements described as resembling beads on a string and more densely packed beads, respectively, and may correspond to the two types of RNase-protected complexes observed in Fig. 4(A) (lanes 3 and 4). Interestingly, the length of the former (30 to 50 nm) corresponds to two or three units of the uniform structure of the wt 3a MP–RNA complexes of 15 nm periodicity (Fig. 5A, panel 3). Such segments are separated presumably by short gaps of free RNA, accessible to RNase, but not resolvable by AFM.

Contrary to our initial expectations, the 3a Δ C33 MP bound RNA more strongly than the wt 3a MP. During infection, the MP has to compete with the virus replicase, the CP and ribosomes for binding to the viral RNA. Thus, to promote virus movement, the CMV MP may need to bind RNA either very strongly, or with a higher affinity early during infection. It is conceivable that the CMV CP could alter the MP conformation to increase its binding efficiency. Thus, deleting the C terminus of the MP might have the same effect on the overall conformation as adding CP. This effect of the CP could not be mimicked *in vivo* after inoculation of pre-formed MP–RNA complexes to tobacco plants expressing CP (Fig. 6B). This might be because MP had already formed a complex, and the CP did not affect the stability of such complexes. Thus, we propose that the role of the CMV CP would not be to destabilize the MP–RNA complexes already formed, as originally envisaged, but rather would be one of modifying the type of complexes initially formed, by altering the conformation of the MP available for complex formation. CP may also not interact directly with the MP, but via some host protein, which may explain the failure to detect direct interactions between MP and CP.

The studies of Nagano *et al.* (2001) showed that CMV containing the 3a Δ C33 MP was able to infect *N. benthamiana* systemically, in the presence but not in the absence of CP. Therefore, if the C terminus of the MP is not required for cell-to-cell or long-distance movement, why is it maintained? These sequences may have other host-dependent functions that are not obvious from the limited plant species so far inoculated. In addition, differences in the binding affinities between the wt 3a MP and 3a Δ C33 MP to RNA may have different consequences for infection in different plant backgrounds. Future studies should allow us to distinguish between such possibilities.

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REFERENCES

- Blackman, M. L., Boevink, P., Santa Cruz, S., Palukaitis, P. & Oparka, K. J. (1998). The movement protein of cucumber mosaic virus traffics into sieve elements in minor veins of *Nicotiana glauca*. *Plant Cell* **10**, 525–537.
- Canto, T. & Palukaitis, P. (1999a). The hypersensitive response to cucumber mosaic virus in *Chenopodium amaranticolor* requires virus movement outside the initially infected cell. *Virology* **265**, 74–82.
- Canto, T. & Palukaitis, P. (1999b). Are tubules generated by the 3a protein necessary for cucumber mosaic virus movement? *Mol Plant Microbe Interact* **12**, 985–993.
- Canto, T., Prior, D. A. M., Hellwald, K.-H., Oparka, K. J. & Palukaitis, P. (1997). Characterization of cucumber mosaic virus. IV. Movement protein and coat protein are both essential for cell-to-cell movement of cucumber mosaic virus. *Virology* **237**, 237–248.
- Ding, S.-W., Li, W.-X. & Symons, R. H. (1995a). A novel naturally occurring hybrid gene encoded by a plant RNA virus facilitates long distance virus movement. *EMBO J* **14**, 5762–5772.
- Ding, B., Li, Q., Nguyen, L., Palukaitis, P. & Lucas, W. J. (1995b). Cucumber mosaic virus 3a protein potentiates cell-to-cell trafficking of CMV RNA in tobacco plants. *Virology* **207**, 345–353.
- Gal-On, A., Kaplan, I., Roossinck, M. J. & Palukaitis, P. (1994). The kinetics of infection of zucchini squash by cucumber mosaic virus indicate a function for RNA 1 in virus movement. *Virology* **205**, 280–289.
- Gal-On, A., Kaplan, I. & Palukaitis, P. (1995). Differential effects of satellite RNA on the accumulation of cucumber mosaic virus RNAs and their encoded proteins in tobacco vs zucchini squash with two strains of CMV helper virus. *Virology* **208**, 58–66.
- Habili, N. & Francki, R. I. B. (1974). Comparative studies of tomato aspermy and cucumber mosaic viruses. III. Further studies on relationship and construction of a virus from parts of the two viral genomes. *Virology* **61**, 443–449.
- Hellwald, K.-H. & Palukaitis, P. (1995). Viral RNA as a potential target for two independent mechanisms of replicase-mediated resistance against cucumber mosaic virus. *Cell* **83**, 937–946.
- Jacquemond, M., Teycheney, P.-Y., Carrère, I., Navas-Castillo, J. & Tepfer, M. (2001). Resistance phenotypes of transgenic tobacco plants expressing different cucumber mosaic virus (CMV) coat protein genes. *Mol Breed* **8**, 85–94.
- Kaplan, I. B., Shintaku, M. H., Li, Q., Zhang, L., Marsh, L. E. & Palukaitis, P. (1995). Complementation of virus movement in transgenic tobacco expressing the cucumber mosaic virus 3a gene. *Virology* **209**, 188–199.
- Kaplan, I. B., Gal-On, A. & Palukaitis, P. (1997). Characterization of cucumber mosaic virus. III. Localization of sequences in the movement protein controlling systemic infection in cucurbits. *Virology* **230**, 343–349.
- Kaplan, I. B., Zhang, L. & Palukaitis, P. (1998). Characterization of cucumber mosaic virus. V. Cell-to-cell movement requires capsid protein but not virions. *Virology* **246**, 221–231.
- Karpova, O. V., Ivanov, K. I., Rodionova, N. P., Dorokhov, Y. L. & Atabekov, J. G. (1997). Nontranslatability and dissimilar behavior in plants and protoplasts of viral RNA and movement protein complexes formed *in vitro*. *Virology* **230**, 11–21.
- Kiselyova, O. I., Yaminsky, I. V., Karger, E. M., Frolova, O. Y., Dorokhov, Y. L. & Atabekov, J. G. (2001). Visualization by atomic

force microscopy of tobacco mosaic virus movement protein–RNA complexes formed in vitro. *J Gen Virol* **82**, 1503–1508.

Lakshman, D. K. & Gonsalves, D. (1985). Genetic analysis of two large-lesion isolates of cucumber mosaic virus. *Phytopathology* **75**, 758–762.

Li, Q. & Palukaitis, P. (1996). Comparison of the nucleic acid- and NTP-binding properties of the movement protein of cucumber mosaic cucumovirus and tobacco mosaic tobamovirus. *Virology* **216**, 71–79.

Li, Q., Ryu, K. H. & Palukaitis, P. (2001). Cucumber mosaic virus–plant interactions: identification of 3a protein sequences affecting infectivity, cell-to-cell movement, and long-distance movement. *Mol Plant Microbe Interact* **14**, 378–385.

Lyubchenko, Y. L., Oden, P. I., Lampner, D., Lindsay, S. M. & Dunker, K. A. (1993a). Atomic force microscopy of DNA and bacteriophage in air, water and propanol: the role of adhesion forces. *Nucleic Acids Res* **21**, 1117–1123.

Lyubchenko, Y. L., Shiyakhtenko, L., Harrington, R., Oden, P. & Lindsay, S. (1993b). Atomic force microscopy of long DNA: imaging in air and under water. *Proc Natl Acad Sci U S A* **90**, 2137–2140.

Nagano, H., Okuno, T., Mise, K. & Furusawa, I. (1997). Deletion of the C-terminal 33 amino acids of cucumber mosaic virus movement protein enables a chimeric brome mosaic virus to move from cell to cell. *J Virol* **71**, 2270–2276.

Nagano, H., Mise, K., Furusawa, I. & Okuno, T. (2001). Conversion in the requirement of coat protein in cell-to-cell movement mediated by the cucumber mosaic virus movement protein. *J Virol* **75**, 8045–8053.

Nurkianova, K. M., Ryabov, E. V., Kalinina, N. O., Fan, Y., Andreev, I., Fitzgerald, A. G., Palukaitis, P. & Taliansky, M. (2001). Umbravirus-encoded movement protein induces tubule formation on the surface of protoplasts and binds RNA incompletely and non-cooperatively. *J Gen Virol* **82**, 2579–2588.

Rao, A. L. N. & Francki, R. I. B. (1981). Comparative studies on tomato aspermy and cucumber mosaic viruses. VI. Partial compatibility of genome segments for the two viruses. *Virology* **114**, 573–575.

Rizzo, T. M. & Palukaitis, P. (1990). Construction of full-length cDNA clones of cucumber mosaic virus RNAs 1, 2 and 3: generation of infectious RNA transcripts. *Mol Gen Genet* **222**, 249–256.

Ryabov, E. V., Roberts, I. M., Palukaitis, P. & Taliansky, M. (1999). Host-specific cell-to-cell and long-distance movements of cucumber mosaic virus are facilitated by the movement protein of groundnut rosette virus. *Virology* **260**, 98–108.

Ryu, K. H., Kim, C.-H. & Palukaitis, P. (1998). The coat protein of cucumber mosaic virus is a host range determinant for infection of maize. *Mol Plant Microbe Interact* **11**, 351–357.

Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Suzuki, M., Kuwata, S., Kataoka, J., Masuta, C., Nitta, N. & Takanami, Y. (1991). Functional analysis of deletion mutants of cucumber mosaic virus RNA3 using an in vitro transcription system. *Virology* **183**, 106–113.

Takahashi, H., Suzuki, M., Natsuaki, K., Shigyo, T., Hino, K., Teraoka, T., Hosokawa, D. & Ehara, Y. (2001). Mapping the virus and host genes involved in the resistance response in cucumber mosaic virus-infected *Arabidopsis thaliana*. *Plant Cell Physiol* **42**, 340–347.

Takeshita, M., Suzuki, M. & Takanami, Y. (2001). Combination of amino acids in the 3a protein and the coat protein of cucumber mosaic virus determines symptom expression and viral spread in bottle gourd. *Arch Virol* **146**, 697–711.

Vaquero, C., Liao, Y.-C., Nähring, J. & Fischer, R. (1997). Mapping of the RNA-binding domain of the cucumber mosaic virus movement protein. *J Gen Virol* **78**, 2095–2099.

Wong, S., Thio, S. S., Shintaku, M. H. & Palukaitis, P. (1999). The rate of cell-to-cell movement in squash of cucumber mosaic virus is affected by sequences of the capsid protein. *Mol Plant Microbe Interact* **12**, 628–632.

Zhang, L., Hanada, K. & Palukaitis, P. (1994). Mapping local and systemic symptom determinants of cucumber mosaic cucumovirus in tobacco. *J Gen Virol* **75**, 3185–3191.