Mapping of the RNA-binding domain of the cucumber mosaic virus movement protein

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A series of in-frame deletion mutants was used to identify a domain within the 3a protein of cucumber mosaic virus (CMV) that is required for RNA-binding activity. Deletions in the 3a gene were generated by PCR and restriction digestion, and the resulting mutated 3a sequences were cloned either in pT7-7 or in pGEX-5X3 expression vectors. The mutated 3a proteins or fusions with glutathione S-transferase (GST) were expressed in E. coli, purified, and their nucleic acid-binding activities analysed by photochemical UV cross-linking assays using digoxigenin–UTP-labelled RNA probes. Comparative analyses of seven mutated 3a proteins obtained from inclusion bodies and eight GST fusion proteins revealed that there is an RNA-binding domain located between amino acids 174 and 233. This RNA-binding domain is able to bind single-stranded RNA out of the context of the complete 3a movement protein and is highly conserved within both subgroups of CMV.

The cucumber mosaic virus (CMV) 3a protein has been generally considered to be the movement protein of the virus. Analysis of 3a gene deletion mutants revealed that the 3a protein is not required for replication, but is essential for the spread of infection in planta (Suzuki et al., 1991; Boccard & Baulcombe, 1993). Transgenic tobacco plants expressing the 3a gene have been shown to complement movement-deficient mutants (Kaplan et al., 1995). Furthermore, the CMV 3a protein shares several properties with other well-characterized viral movement proteins, such as cell-wall localization in transgenic and infected tobacco plants and the ability to modify the size-exclusion limit of plasmodesmata (Vaquero et al., 1994, 1996) and to traffic RNA from cell-to-cell (Ding et al., 1995).

The ability to bind nucleic acids in vitro is a common feature of plant virus movement proteins (Citovsky et al., 1990, 1991; Osman et al., 1992; Schoumacher et al., 1992a, b; Tacke et al., 1991; Rouleau et al., 1994; Ivanov et al., 1994; Bleykasten et al., 1996). Distinct domains essential for this binding activity have been identified in several movement proteins, such as the 30 kDa (‘30K’) protein of tobacco mosaic virus (TMV) (Citovsky et al., 1992), the 35 kDa protein of red clover necrotic mosaic virus (RCNMV) (Osman et al., 1993; Giesman-Cookmayer & Lommel, 1993), the P3 protein of alfalfa mosaic virus (AlMV) (Schoumacher et al., 1994) and the P1 protein of cauliflower mosaic virus (CaMV) (Thomas & Maule, 1995). The 30K protein of TMV comprises two domains: domain ‘A’, which interacts with nucleic acids only in the context of the complete movement protein, and has a low surface probability, and the independent domain ‘B’, which has a high surface probability (Citovsky et al., 1992). The CMV 3a protein also binds single-stranded (ss) nucleic acids in vitro (Li & Palukaitis, 1996). To define the domain of the 3a protein required to bind ssRNA, we employed deletion mutagenesis using mutated 3a proteins from inclusion bodies as well as soluble glutathione S-transferase (GST) fusion proteins.

The recombinant plasmid pT7-7C3a containing the cDNA sequence of the RNA 3 corresponding to nucleotides (nt) 99–1022 of a Spanish isolate of CMV (CMV-24) has been described previously (Vaquero et al., 1996). Deletions within the 3a gene (nt 99–938) were created by PCR amplification using plasmid pT7-7C3a as template and specific oligonucleotides containing appropriate restriction endonuclease sites as primers, or by exploiting the existing StyI or KpnI restriction sites in the 3a gene. Mutated 3a sequences were cloned either into NdeI- and Sall-digested pT7-7 expression vector (Tabor, 1990) to create the DNA constructs M1–M7, or into pGEX-5X3 (Pharmacia) between the BamHI and XhoI restriction sites for constructs M8–M15 (Fig. 1). The full-length 3a gene was cloned in pGEX-5X3 to create plasmid pGEX-5X3/3a. All recombinant plasmids were confirmed by sequence analysis.

The 3a protein and deletion proteins M1–M7 were expressed in E. coli BL21(DE3) (Novagen). The recovery of inclusion bodies was carried out as described by Vaquero et al. (1996), with the exception that bacteria were disrupted by sonication. The proteins were solubilized by resuspending inclusion bodies with 8 M urea in binding buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA, 50 mM NaCl, 1 mM PEFA-1095
block and 10% glycerol). Refolding was performed by dialysis against binding buffer containing decreasing amounts of urea until its complete removal. The yield of recovered soluble proteins varied with the expression construct used, ranging from 0.4 to 1 mg/litre. For preparation of the 3a protein and deletions M8–M15 as fusions to GST, the proteins were expressed in E. coli BL21(DE3), purified by affinity chromatography (Smith & Johnson, 1988) and dialysed against binding buffer. The yields of affinity-purified soluble fusion proteins ranged from 0.1 to 2 mg/litre of culture depending on the construct. SDS–PAGE and Western blot analyses revealed that each expressed protein constituted the major final product, although faint degradation products and minor bacterial contaminants were sometimes detectable (data not shown).

Initially, the RNA-binding capacity of purified 3a and mutant proteins was assayed with both radioactively and with digoxigenin (DIG)-labelled CMV ssRNA probes in UV crosslinking experiments. Comparable binding results and sensitivity (data not shown) indicated that RNA binding was not affected by DIG and therefore nonradioactively labelled ssRNA probes were used for subsequent experiments. DIG–UTP-labelled ssRNA probes using the DIG–UTP:UTP ratio (0.65:0.35) recommended by the manufacturer (Boehringer Mannheim) were obtained by in vitro transcription with T7 RNA polymerase (New England Biolabs) from XhoI-linearized pBluescript KS containing a 270 bp fragment of RNA 3 (nt 354–623). The purified proteins were incubated with 5 ng DIG-labelled RNA in binding buffer at 4°C for 30 min. The reaction mixtures were irradiated with 1±8 J of UV light (GS GEN LINKER; Bio-Rad), and treated with 0.5 µg RNase A at 37°C for 15 min. Samples were electrophoresed in duplicate SDS–polyacrylamide gels (0±1% SDS, 15% polyacrylamide) (Laemmli, 1970). One gel was stained with Coomassie blue to visualize the protein bands, the other was electro-blotted onto nitrocellulose filters for detection of bound DIG-labelled RNA using alkaline phosphatase-labelled anti-DIG Fab fragments (Boehringer). The chemiluminescent reaction was developed using CDP-Star (Boehringer), and the membranes were exposed to X-ray film overnight.

The ability of both 3a and GST–3a proteins to bind ssRNA was demonstrated by UV cross-linking using the DIG-labelled RNA probe. Increasing amounts of both proteins resulted in a concurrent increase of signals at the expected Mr positions. No signals at similar positions were detected when a total protein extract from BL21(DE3)/pT7-7 or GST protein alone was used as a control (data not shown). A band of Mr ~ 70000 was detected in the bacterial protein extract and also in protein samples purified from inclusion bodies (Fig. 2b) or GST fusions

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**Fig. 1.** (a) Deletion and GST fusion mutants of the CMV 3a protein and their ability to bind ssRNA. (b) Amino acid sequence of the RNA-binding domain of the CMV 3a protein, and amino acid residues that have the potential to interact with nucleic acids: (+) basic (H, K, R); (−) acid (D, E); (●) amides (N, Q); (Δ) polar (S, T); (□) aromatic (F, Y) amino acids.
Fig. 2. RNA-binding analysis of the CMV 3a protein (WT) and deletion mutant proteins (M1–M7) expressed in E. coli BL21 and purified from inclusion bodies by UV cross-linking. Five ng of DIG-labelled ssRNA probe was incubated with approximately 100 ng of WT and M5, 200 ng of M2, M3 and M4, and 500 ng of M1, M6 and M7. Reaction mixtures were subjected to UV treatment and digested with RNase A as described in the text. Samples were analysed in duplicate by (a) SDS–PAGE followed by Coomassie blue staining, and (b) Western blot hybridization using anti-DIG Fab labelled with alkaline phosphatase.

* Signal at $M_r \sim 70\,000$ corresponding to contaminating E. coli protein binding the ssRNA probe.

Fig. 3. RNA-binding analysis of the GST–3a and GST–deletion mutant proteins. Approximately 200 ng of GST, M8 and M9, 500 ng of WT and M15, and 1000 ng of M10, M11, M12, M13 and M14 were used for a UV cross-linking assay. (a) SDS–PAGE analysis; (b) Western blot hybridization analysis.

* Signal at $M_r \sim 70\,000$ corresponding to contaminating E. coli protein binding the ssRNA probe.

(Fig. 3b), although it was not clearly visible on Coomassie-stained gels. These bands were shown to be of bacterial origin by Western blotting using a polyclonal antiserum raised against BL21(DE3) bacterial proteins (data not shown). The presence of this co-purified bacterial protein in nucleic acid-binding assays has been reported for other virus movement proteins (Rouleau et al., 1994; Thomas & Maule, 1995).

To identify the region of the 3a protein involved in interaction with RNA, we tested the ability of deletion proteins M1–M7, isolated from inclusion bodies, to bind ssRNA (Figs 1 and 2). As shown in Fig. 2, proteins M1 and M2, carrying deletions at the N terminus ($\Delta 4–39$ and $\Delta 1–85$, respectively), and M3, with a deletion in the central region ($\Delta 86–170$), were fully competent for ssRNA binding. In contrast, all deletions at the C terminus located between amino acids (aa) 176 and 279 (M4–M7) completely abolished RNA binding. These results indicated that the region between aa 176 and 279 is involved in the protein–RNA interaction.

Previous studies on the nucleic acid-binding properties of virus movement proteins have shown that some deletion proteins were unable to bind RNA due to incorrect refolding (Citovsky et al., 1992; Osman et al., 1993). To exclude this possibility and to confirm the presence of an RNA-binding domain within the C-terminal region of the 3a protein, we tested 3a deletion proteins fused to GST (M8–M15) (Figs 1 and 3). Analyses of mutants M10 and M8, retaining two-thirds of the N terminus (aa 1–175) or C terminus (aa 86–279) of the protein, respectively, showed that only M8 retained the ability to interact with RNA, indicating that a domain located between aa 175–279 is responsible for RNA interaction. To find out whether any cryptic binding sites were hidden in M8, three deletions fused to GST, namely M11, M12 and M13, which retained the N-terminal (aa 1–107), central (aa 86–175) and C-terminal (aa 174–279) regions of the 3a protein, respectively, were assayed for their RNA-binding activity. Neither M11 nor M12, which both lack the C-terminal region, was able to interact with the ssRNA probe, while M13 strongly bound RNA. This result confirmed that the C-terminal RNA-binding site is active out of the context of the 3a protein. To further map the RNA-binding domain of the 3a protein, we tested the GST fusion proteins M9, M14 and M15, which contain aa 1–233, 1–6 and 174–256, and 1–6 and 174–233, respectively. As shown in Fig. 3, the removal of 23 (M14) or up to 46 aa (M9 and M15) from the most C-terminal region of the
protein did not affect the RNA-binding activity of the fusion proteins. Therefore, we concluded that there is an RNA-binding site located between aa 174 and 233 of the CMV 3a protein which is able to interact with ssRNA both in the context of the 3a protein and when fused to GST (M15).

Proteins M4 and M5 were purified in several independent experiments using different buffers for solubilization and refolding from inclusion bodies. In contrast to the results obtained with soluble M9, M14 and M15 GST fusion proteins, none of the purified M4 or M5 proteins bound RNA, suggesting that proteins carrying deletions within the C terminus were unable to adopt the correct conformation after denaturation and refolding.

The amino acid sequence of the 3a protein deduced from the nucleotide sequence of RNA 3 from CMV-24 (Vaquero et al., 1996) is identical to that of the 3a protein of CMV-Fny (Owen et al., 1990). Complementation studies using the Fny strain showed that deletion of 43 aa from the C terminus of the 3a protein did not affect the ability of the virus to infect non-transformed tobacco plants (Kaplan et al., 1995). Here, we show that proteins M9 and M15, which lack the C-terminal 46 aa, were fully competent for RNA binding, suggesting that this region is also not involved in the interaction with RNA. The function of this C-terminal portion of the 3a protein during virus infection remains unclear. Kaplan et al. (1995) also reported that a deletion of up to 70 aa (A209–279) at the C terminus resulted in the virus losing its ability to infect wild-type tobacco plants, indicating the importance of aa 209–236 of the CMV 3a protein for establishment of infection. Our results demonstrate that the region between aa 174 and 233 is an essential domain of the CMV 3a protein for protein–RNA interaction in vitro. The inability of the A209–279 CMV mutant to infect host plants was probably due to the inability of the mutated 3a protein to interact with the viral RNA, as has been proposed for other virus movement proteins (Citovsky et al., 1990, 1991).

The biological significance of the identified RNA-binding domain (aa 174–233) is also supported by its high degree of sequence conservation among the 3a proteins of both subgroups of CMV (Kaplan et al., 1995). The sequence between aa 174–233 of the CMV-24 3a protein shows 100% sequence identity with four strains of subgroup I (CMV-Fny, CMV-O, CMV-Y and CMV-M), and differs at only nine positions from three strains belonging to subgroup II (CMV-Q, CMV-Kin and CMV-Trk7). Furthermore, this stretch of 60 aa includes a large number of positively charged residues which could form ionic bonds with negatively charged phosphate groups, as well as many residues with the potential to interact with nucleic acids (Fig. 1b). Similar to RNA-binding domain ‘B’ of TMV (Citovsky et al., 1992), the RNA-binding domain of CMV 3a protein has a high surface probability.

In summary, the CMV-24 3a protein has an ssRNA-binding domain located between aa 174 and 233 which is able to interact with ssRNA of CMV as well as TMV and PRLV (data not shown) out of the context of the remainder of the movement protein. As this RNA-binding domain is highly conserved in both subgroups of CMV, ongoing experiments will further elucidate the role of this domain in virus movement.

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