Mapping the RNA-binding domain on the Apple chlorotic leaf spot virus movement protein

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The RNA-binding properties of the cell-to-cell movement protein (MP) of Apple chlorotic leaf spot virus were analysed. MP was expressed in Escherichia coli and was used in UV-crosslinking analysis, using a digoxigenin–UTP-labelled RNA probe and gel-retardation analysis. The analyses demonstrated that MP bound cooperatively to single-stranded RNA (ssRNA). When analysed for NaCl dependence of the RNA-binding activity, the majority of the MP could bind ssRNA even in binding buffer with 1 M NaCl. Furthermore, competition binding experiments showed that the MP bound preferentially to ssRNA and single-stranded DNA without sequence specificity. MP deletion mutants were used to identify the RNA-binding domain by UV-crosslinking analysis. Amino acid residues 82–126 and 127–287 potentially contain two independently active, single-stranded nucleic acid-binding domains.

Additionally, when MP–GFP was expressed transiently in leaf cells, the fusion protein spread from the cells that produced the protein into neighbouring cells (Satoh et al., 2000). For this reason, it was speculated that the ACLSV genome is transported as an MP–RNA complex that uses the ability of the MP to move from cell to cell.

In this study, we have demonstrated in vitro RNA-binding properties of the MP, which have not been described for any other member of the genus Trichovirus, and its RNA-binding domains have been mapped.

Wild-type and mutated ACLSV MP genes (Fig. 1a) were amplified from plasmids for transient expression (Isogai et al., 2003) by PCR and were inserted into bacterial expression vectors pET-3a and pET-32a (Novagen). To express the full-length MP and its deletion mutants (Fig. 1a), each plasmid was transfected into Escherichia coli BL21(DE3)pLysS competent cells and expressed as indicated in the manufacturer’s instructions (Novagen). Cells were harvested and lysed by sonication for 10 min on ice in suspension buffer [50 mM Tris/HCl (pH 8.0), 2 mM EDTA]. Proteins expressed from pET-32a constructs contained protein tags (Trx; thioredoxin tag, histidine tag and S-protein tag) at their N-terminal ends, whereas proteins expressed from pET-3a constructs were non-fused proteins. Those expressed proteins that were detected in the soluble fraction (Trx–ΔGC2 and Trx–ΔGCR2; Fig. 1a) were purified by His-Bind resin, as indicated in the manufacturer’s instructions (Novagen). On the other hand, the expressed proteins that were detected in the insoluble fraction (all proteins except for Trx–ΔGC2 and Trx–ΔGCR2; Fig. 1a) were washed three times in suspension buffer. Before use, each protein was denatured.

Two types of plant virus cell-to-cell movement have been characterized (Carrington et al., 1996). In the first type, the movement protein (MP) interacts with RNA to form an MP–RNA complex that uses the ability of the MP to move from cell to cell. In the second, mature virions are transported through viral MP-containing tubules that are assembled inside the plasmodesmal pore. However, some studies have raised questions on whether these two forms of movement are necessary, as movement of Cauliflower mosaic virus and Cowpea mosaic virus occurs through tubules formed by MP (Citovsky et al., 1991; Carvalho et al., 2003), but both MPs also can bind RNA. Additionally, earlier studies have shown that viruses that form an MP–RNA RNA complex to move from cell to cell can also form tubules on the surface of infected protoplasts (Canto & Palukaitis, 1999).

Apple chlorotic leaf spot virus (ACLSV) has a flexuous, filamentous particle (740–760 nm in length and 12 nm in width) and is the type species of the genus Trichovirus (van Regenmortel et al., 2000). The ACLSV genome consists of single-stranded RNA (ssRNA) (7552 nt) with a poly(A) tract at its 3′ end and contains three ORFs that encode a replication-associated protein, MP and a coat protein (Sato et al., 1993). The MP has been assigned to the ‘30K superfamily’ by comparative sequence analyses (Mushegian & Koonin, 1993). Immuno-electron microscopy with antiserum against the MP showed that the protein is localized to plasmodesmata within virus-infected cells without the formation of tubules; virions were not observed in cell-wall plasmodesmata (Sato et al., 1995; Yoshikawa et al., 1999), although tubular structures protruded from the cell surface when MP fused to green fluorescent protein (MP–GFP) was expressed transiently in protoplasts (Satoh et al., 2000).
in 6 M urea and renatured overnight at 4 °C in renaturation solution [50 mM Tris/Cl (pH 8.0), 50 mM NaCl, 2.5 mM dithiothreitol and 1 M NDSB-201 (Calbiochem)] and dialysed against water to remove the NDSB-201 (Kim et al., 2004).

The RNA-binding ability of the full-length MP was assayed by UV-crosslinking analysis using a digoxigenin (DIG)-UTP-labelled RNA probe, as described by Vaquero et al. (1997). The MP with the DIG-labelled RNA showed a positive reaction with anti-DIG Fab fragments coupled to alkaline phosphatase (anti-DIG–AP; Roche Applied Science), although MP alone did not cross-react (Fig. 2a, lanes 1 and 9). These results suggested that the MP was competent for ssRNA binding. We also investigated the RNA-binding

Fig. 1. (a) Summary of MP deletion mutants and their ability to bind ssRNA. The remaining portions of wild-type MP are represented by grey bars. Trx indicates proteins that contain thioredoxin, histidine and S-protein tags. Domains A and B of the MP have the potential to interact with ssRNA. (b) Amino acid sequence analysis of domains A and B. Amino acid sequences of domains A and B and distribution of basic (H, K, R; denoted by +), acidic (D, E; denoted by –), amide (N, Q; denoted by □), polar (S, T; denoted by △) and aromatic (F, Y; denoted by ●) amino acids are indicated. Underlined amino acid residues in domains A and B show the principal conserved motif of the ‘30K superfamily’ and a region of positively charged residues, respectively.

Fig. 2. RNA-binding analysis of the MP wild-type and deletion mutant proteins by UV crosslinking. Five nanograms of a DIG-labelled actin RNA probe (588 nt; Novagen) was incubated with the proteins in 10 μl binding buffer [10 mM Tris/Cl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 10% glycerol]. Reaction mixtures were subjected to UV crosslinking and digested with RNase A. The top panels show SDS-PAGE of the purified MP and its mutated proteins in 12·5% polyacrylamide gels that were stained with Coomassie blue. The bottom panels show the ability of the proteins to bind ssRNA as detected by UV crosslinking. Trx in front of the names of MP deletion mutants indicates that the proteins are fused to protein tags (thioredoxin, histidine and S-protein tags) at the N-terminal end. The MP does not cross-react with anti-DIG–AP that was used to detect protein binding to the DIG-labelled actin RNA probe [(a), lane 9]. Asterisks indicate detected bands that are objects of the analyses in (a–d).
properties of the MP by gel-retardation analysis, as described by Herranz & Pallàs (2004). A constant amount of ssRNA (5 ng), 1000 nt from the 5′ end of the ACLSV genome, was incubated with an increasing amount of MP (0–640 ng) in 10 μl binding buffer and the electrophoretic retardation of the ssRNA was detected by using complementary DIG-labelled riboprobes (Fig. 3a). We chose ssRNA that was 1000 nt long to minimize aggregation of MP complexed to ssRNA (Herranz & Pallàs, 2004) and confirmed that 1 mg BSA did not show a mobility shift of the ssRNA in this analysis, as a negative control (data not shown). At low concentrations of MP (up to 40 ng), ssRNA migrated to the same distance as protein-free RNA (Fig. 3a, lanes 1–3). In contrast, when 80 ng or more MP was added, the free RNA signals disappeared (Fig. 3a, lanes 4–7), indicating that a ribonucleoprotein complex had formed and the complex barely entered the gel matrix from the well of the agarose gel, despite using ssRNA of only 1000 nt in length. The absence of intermediate bands, representing a few protein molecules bound to ssRNA, is characteristic of cooperative protein binding (Lohman et al., 1986; Citovsky et al., 1990). The complex was subjected to treatment with 0·1 % SDS at room temperature for 1 min. The complex was affected by the 0·1 % SDS treatment (data not shown), suggesting that the RNA-binding properties are dependent on the structure of the protein.

The binding specificity of MP was determined by competition experiments using gel-retardation analysis (Fig. 3b). Competition binding assays were performed by incubation of ACLSV ssRNA and competitor nucleic acids with MP. Fragments (approx. 800 nt long) of ssRNA and single-stranded DNA (ssDNA) from a bacterial vector (pBluescriptII KS+) were able to compete efficiently with the ACLSV ssRNA (Fig. 3b, lanes 3–4 and 7–8). The results show that MP binds ssRNA and ssDNA molecules of various, unrelated nucleotide sequence. On the other hand, the double-stranded RNA (dsRNA) genome of Rice black streaked dwarf virus (Isogai et al., 1995) and linearized double-stranded DNA (dsDNA) from the plasmid vector did not considerably displace the binding of the ACLSV ssRNA (Fig. 3b, lanes 5–6 and 9–10). These results indicate that the MP has a preference for binding single-stranded nucleic acids, and does not bind double-stranded nucleic acids significantly.

To identify the MP domain that is involved in interaction with ssRNA, we expressed and purified a series of deletion mutants, ΔAA–ΔAG, to test for ssRNA binding by UV-crosslinking analysis (Figs 1a and 2a). ΔAA–AG showed positive reactions (Fig. 2a, lanes 2–8). These results suggested that the mutants were competent for ssRNA binding and may be explained if the MP contains at least two independently active ssRNA-binding domains, so that most single deletions would leave at least one of the domains intact. To test this hypothesis, we constructed another set of MP deletion mutants (ΔAΔC, ΔAΔD, ΔAΔC1–ΔAΔC3, ΔAΔ, FΔA, FΔE, FΔF)

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**Fig. 3.** Electrophoretic gel-retardation assay of RNA binding by the MP extracted from *E. coli*. (a) Cooperative binding of the MP to ssRNA. The indicated amount of MP was incubated in 10 μl binding buffer with 5 ng ssRNA containing the 5′-terminal 1000 nt of the ACLSV genome. (b) Specificity of the RNA-binding properties of the MP analysed by electrophoretic gel retardation. The MP (160 ng) was incubated in 10 μl binding buffer with 5 ng ssRNA in the absence (lanes 1 and 2) or presence (lanes 3–10) of the indicated amount of competitors: ssRNA (from plasmid vector (pBluescriptII KS+), approx. 800 nt long; lanes 3 and 4), dsRNA (Rice black streaked dwarf virus; lanes 5 and 6), ssDNA (heat-denatured plasmid DNA (pBluescriptII KS+), approx. 800 nt long; lanes 7 and 8), dsDNA (plasmid DNA, 800 bp; lanes 9 and 10). (c) Dependence of ACLSV MP RNA-binding activity on NaCl concentration. The MP (160 ng) was incubated with 5 ng ssRNA in 10 μl binding buffer with the indicated concentrations of NaCl (lanes 1–7). Lane 8 (cont) shows ssRNA incubated without MP as a control. The positions designated ‘free’ and ‘well’ indicate the positions of the free RNA and the wells of 1 % agarose gel, respectively. Asterisks indicate free RNA dissociated from the MP–RNA complex.
FAF, ΔGC and FAC; Fig. 1a) and analysed them for RNA-binding ability by UV crosslinking (Fig. 2b). It has previously been reported that certain deletion proteins are unable to adopt the correct conformation after denaturation and refolding if they are not fused to other proteins (Citovsky et al., 1992). To characterize the RNA-binding domain of FAF and ΔGC, the proteins were fused to the Trx tag at their N-terminal ends (Trx–FAF and Trx–ΔGC, respectively). The Trx tag alone does not bind ssRNA (data not shown), but Trx–ΔC1 binds to ssRNA in a similar manner to the non-fused ΔC1 (Fig. 2b, lanes 4 and 11). Mutants ΔC1–ΔC3, FΔD, FΔE, Trx–FΔF and Trx–ΔGC each retained RNA-binding ability, but the RΔC and RΔD mutants did not bind RNA (Fig. 2b). These results indicate that amino acid residues 1–126 (FAF) and 127–287 (ΔGC) function independently as RNA-binding domains.

To elucidate the RNA-binding domains of FAF and ΔGC, we expressed Trx–FAFF, Trx–FΔFR, Trx–ΔGC2, Trx–ΔGC2R and Trx–ΔFC (Fig. 1a) and analysed them by UV crosslinking (Fig. 2c and d). Within the RNA-binding domain of FAF, Trx–FΔFR was able to bind ssRNA, whereas Trx–FAFF was not (Fig. 2c). These results indicated that the RNA-binding domain of FAF was delineated by amino acid residues 82–125 (Fig. 1b; designated domain A). In addition, the deletions in mutants Trx–ΔGC2, Trx–ΔGC2R and Trx–ΔFC each affected RNA-binding activity. The RNA-binding domain of ΔGC was longer than and directly downstream of binding domain A, between amino acid residues 126 and 287 (Fig. 1b; designated domain B).

Our previous work shows that ΔC1 fused to cyan fluorescent protein (AC1–CFP) expressed transiently in leaf epidermis can spread from the cells that produced ΔC1–CFP into neighbouring cells without other viral proteins. However, all infectious cDNA clones of ACLSV tested to date that contain deletions in the MP have lost their infectivity (Satoh et al., 2000; Isogai et al., 2003). The movement-competent ΔC1–CFP protein contains both domains A and B, but ΔC2–CFP, which has only lost the RNA-binding ability of domain B, cannot spread. To test whether MP mutants with domains A and B can move from cell to cell, we constructed Δ82C1 (aa 82–287) fused to CFP and expressed it transiently in the leaf epidermis by using particle bombardment. Observation by fluorescence microscopy 24 h after bombardment showed that Δ82C1–CFP was restricted to single cells (data not shown). This result indicates that the RNA-binding ability of both domains A and B is necessary, but not sufficient, for the movement function of the MP. Domain B contains a hydrophilic region in the C-terminal half of the domain (Fig. 1b, domain B) (Hopp & Woods, 1983). This region has a large number of positively charged residues between aa 239 and 283 (Fig. 1b, underlined amino acid residues in domain B), which could form ionic bonds with negatively charged phosphate groups, similar to RNA-binding domains of other viral MPs (Vaquero et al., 1997). Conversely, domain A does not consist of highly hydrophilic residues and the probability is low that domain A is on the surface of the MP structure (Fig. 1b, domain A). We analysed RNA-binding ability of the MP (160 ng) in 10 μl binding buffer with different NaCl concentrations (Fig. 3c). At NaCl concentrations of 600 mM or more, free RNA increased as the concentration of salt increased (Fig. 3c, lane 5), suggesting that the RNA binding is mediated by electrostatic interactions (Gómez & Pallás, 2001; Carvalho et al., 2003). However, free RNA observed at 1 M NaCl was < 5 ng (Fig. 3c, lanes 7 and 8). The binding between the ACLSV MP and RNA is significantly stronger than that of MPs of other viruses, suggesting that, in addition to electrostatic interactions, other interactions are involved in this binding (Gómez & Pallás, 2001; Carvalho et al., 2003).

The MPs of ACLSV and Tobacco mosaic virus (TMV) are the only examples that contain two adjacent RNA-binding domains (Citovsky et al., 1992). Similarly to the ACLSV MP, domain A of the TMV MP does not consist of highly hydrophilic residues, whereas the TMV MP domain B has a large number of positively charged residues. The ACLSV MP domain A, however, includes the principal conserved motif in a vast superfamily of plant MPs, although the TMV MP domain A does not include it (Fig. 1b, underlined amino acid residues in domain A) (Mushegian & Koonin, 1993). Furthermore, the ACLSV MP domain A has two aspartic acids (at aa 95 and 112). D112 is referred to as the D motif and is almost completely conserved within MPs belonging to the ‘30K superfamily’ (Koonin et al., 1991). Chen & Bruening (1992) suggested that the D motif is part of the GTP-binding site; when the Cowpea mosaic virus MP D motif was replaced with alanine, the protein failed to bind GTP–agarose beads (Carvalho et al., 2003). Future experiments will address whether the D motif is involved in the RNA-binding ability of the ACLSV MP.

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References


RNA-binding domain of ACLSV movement protein


