RNA-binding properties and mapping of the RNA-binding domain from the movement protein of *Prunus necrotic ringspot virus*

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INTRODUCTION

*Prunus necrotic ringspot virus* (PNRSV) is the causal agent of several diseases affecting most cultivated stone fruits, including cherry, sour cherry, almond, peach, apricot and plum. PNRSV is a seed- and pollen-transmitted plant virus (Mink, 1992; Aparicio et al., 1999) with a tripartite RNA genome and belongs to the *Bromoviridae* family (type species: *Tobacco streak virus*, TSV). Its genome organization and translation products are similar to those of *Bromovirus*, *Cucumovirus*, *Oleavirus* and *Alfalfa mosaic virus* (AMV), which belong to the family *Bromoviridae* (van Regenmortel et al., 2000). RNAs 1 and 2 encode the polymerase proteins P1 and P2; RNA 3 is translated into the movement protein (MP), whereas the encoded coat protein (CP) is translated from a subgenomic RNA 4.

MPs of plant viruses are involved in viral nucleic acid transport through plasmodesmata. At least five different types of MP have been described: the products of the triple gene block of potexviruses and related viruses, the tymovirus MPs, a series of small polypeptides (less than 10 kDa) encoded by carmo-like viruses and some geminivirus, the hsp70-like proteins of closterovirus and the well characterized ‘30K superfamily’, related to the 30 kDa *Tobacco mosaic virus* (TMV) MP. Comparative sequence analyses have assigned PNRSV MP to the ‘30K superfamily’ (Mushegian & Koonin, 1993), a meaningful grouping of proteins related in sequence and structure (Melcher, 2000) whose members are characterized by presenting a motif of 30 highly conserved amino acids, which may comprise a hydrophobic interaction domain (Mushegian & Koonin, 1993). Previous sequence comparison analysis (Sánchez-Navarro & Pallás, 1997) has revealed that AMV and ilarvirus MPs have a basic region preceding the 30K motif, with a high surface probability, which makes them good candidates for RNA-binding interactions, and a transmembrane domain within the 30K motif, which could be involved in targeting of the MP to the cell wall (Berna, 1995; van der Wel et al., 1998).

In this study, we have demonstrated the RNA-binding properties of the PNRSV MP, which have not been previously described for any member of the *Ilarvirus* genus. In addition, the RNA-binding domain of this protein was mapped to aa 56–88, located at the N terminus of the MP. Interestingly, the RNA-binding domains of *Alfamovirus* and *Ilarvirus* are located at the N terminus, whereas those of *Bromovirus* and *Cucumovirus* are at the C terminus of the MP. Although no significant sequence homology was found among these
RNA-binding domains, they share several important features that could reflect a common origin.

METHODS

Production in bacteria and purification of PNRSV MP. PNRSV MP gene was amplified by PCR from a plasmid containing the RNA 3 of PNRSV PV32 (Sánchez-Navarro & Pallás, 1997) using VP38 sense primer (5'-AGTGAATCCATGCCCGGGTCTGCTAAAC-3', containing a BamHI site, underlined) and VP104 antisense primer (5'-ACATAAGGCTTCACCTCGCCAGAC-3', with a HindIII site). Plasmid pQE-PNRSV MP (pQE-MP) was constructed through insertion of the BamHI/HindIII-digested product from PCR into pQE-30 (Qiagen), fusing the MP sequence after a sequence encoding a sixHistidine tag (MRGS). E. coli M15 (pREP4) cells (Qiagen) transformed with either pQE-MP or pQE-30 (control) were selected on LB medium containing 100 mg ampicillin L\(^{-1}\) and 25 mg kanamycin L\(^{-1}\). Transformants were grown at 37°C. Induction of gene expression with 1 mM IPTG for 5 h and protein purification using Ni-NTA agarose columns (Qiagen) under denaturing conditions were carried out essentially as described by the manufacturer. Purified PNRSV MP was finally eluted from the column in a buffer containing 100 mM sodium phosphate, 10 mM Tris/HCl, pH 6-3, 8 M urea and 400 mM imidazole. For gel electrophoretic mobility shift assays (EMSA; see below), purified MP preparations were dialysed at 4°C for 24 h against sterile water followed by gel filtration chromatography through Sephadex G-25 NAP-5 columns (Pharmacia Biotech) using sterile water as the eluent. The purified sample was concentrated in a Speed Vac and the protein concentration was estimated by the Bradford method.

Nucleic acid binding assay. Protein-RNA binding studies were performed by EMSA. Plus-strand PNRSV RNA 4 was generated by in vitro transcription of a full-length cDNA copy (Sánchez-Navarro & Pallás, 1994). The plasmid was linearized with SacI and transcribed with T3 RNA polymerase as previously described (van der Kuyl et al., 1991). For the EMSA, 5 ng of plus-strand PNRSV RNA 4 transcript (mixed with either equal or with tenfold mass excess of competitors, as specified) were heated for 5 min at 85°C and cooled at room temperature for 15 min. Various amounts of purified PNRSV MP (600 ng of MP for salt and competition experiments) were added and incubated for 30 min at room temperature in a 10 µl final volume of binding buffer (BB; 10 mM Tris/HCl, pH 8-0, 100 mM NaCl, 50% glycerol, 2 units HPR1 RNase inhibitor). When indicated, the samples were incubated with 6 M urea at 60°C for 15 min after the binding reaction.

Following incubations, 2 µl of tracking dye was added and the samples were separated by electrophoresis through a 1% agarose gel at 60 V in TAE (40 mM Tris/acetate, 1 mM EDTA, pH 8-0). RNAs were transferred to positively charged nylon membranes (Roche Diagnostics) by electrotransference at 350 mA in TAE for 1 h at 4°C. RNAs were fixed to the membranes with a UV cross-linker (700 x 100 µm/cm\(^2\)). Hybridization and detection of DIG-RNA probes was conducted as previously described (Pallás et al., 1998).

Synthesis of DIG-labelled riboprobe. Synthesis of the PNRSV RNA 4 DIG-labelled riboprobe was carried out by in vitro transcription of a PNRSV RNA 4 full-length cDNA copy (Sánchez-Navarro & Pallás, 1994). The plasmid was linearized with KpnI and transcribed with T7 RNA polymerase as previously described (van der Kuyl et al., 1991) in the presence of DIG-UTP.

Cloning of the PNRSV MP and mutated forms in an expression vector. PNRSV MP was amplified from PNRSV PV32 isolate (Sánchez-Navarro & Pallás, 1997) by PCR using VP200 sense primer (5'-P-ATGCCGGGTCTCAGTAAAAAC-3') and VP104 antisense primer (5'-ACATAAGGCTTCACCTCGCCAGAC-3', containing a HindIII site, underlined) The MP start and stop codons are included in the sense and antisense primers, respectively. After HindIII digestion, the PCR-amplified fragment was ligated into the bacterial expression vector pMal-c2x digested with Asp700 and HindIII, to generate the recombinant plasmid pMal-200/104. The mutants were amplified from PNRSV PV32 RNA 3 plasmid by PCR using appropriate primers and cloned into the vector pMal-c2x digested with Asp700 and HindIII (see Fig. 4A). pMal-246/104, in which the first 52 amino acids of the MP were deleted, was obtained using VP246 sense primer (5'-AACCTCCGAATCTCAATTGA-CTAAGA-3') and VP104 antisense primer. In pMal-238/104, the first 86 amino acids of the protein were deleted using VP239 sense primer (5'-GGCCGGTGATTTCTGTTATGTA-3') and VP104 antisense primer. To delete the first 111 amino acids of the MP, the PCR amplification was carried out using VP234 (5'-AAGTTGCAGAATCCGTCAACAG-3') sense primer and VP104 antisense primer to obtain pMal-234/104. pMal-200/201 was obtained by cloning the first 125 amino acids after amplifying the fragment of the protein resulting from using VP200 sense primer and VP201 antisense primer (5'-GGGAGGTATACCAAAATG-3') and the C-terminal region, which follows the domain, by using VP277 sense primer (5'-TTGGTTATACCTCGCCAGAC-3', containing a Smal site) and VP104 antisense primer. The PCR products obtained were then mixed and used as templates in a third amplification using VP200 sense primer and VP104 antisense primers.

Expression, purification and analysis of PNRSV MP and the mutant forms. All the constructions were used to transform E. coli DH5\(\alpha\) cells; the cultures were grown overnight and then diluted 1:100 into 100 ml growth medium (LB medium containing 100 mg ampicillin ml\(^{-1}\) and 0.2% glucose). Cultures were incubated at 37°C until the OD\(_{600}\) reached 0.5. To induce protein expression, 0.3 mM IPTG was added and the cells were grown for 3 h at 37°C. All subsequent steps were carried out at 4°C. The cells were centrifuged at 4000 r.p.m. for 20 min and resuspended in 5 ml column buffer (20 mM Tris/HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol). The cells were ruptured with 1 mg lysozyme ml\(^{-1}\) followed by sonication. To eliminate cellular debris, the cells were centrifuged at 9000 r.p.m. for 30 min and the supernatants were diluted 1:5 with column buffer and loaded on to 2 ml amylose columns (New England Biolabs) equilibrated with the same buffer. The column was washed with the column buffer to eliminate unbound protein and the retained proteins were eluted in column buffer containing 10 mM maltose. The purified proteins were analysed by 12% SDS-PAGE and stained with Coomassie brilliant blue.

Northwestern assays. Purified proteins were electrophoresed through 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). Northwestern blot assays were performed as described previously (Pallás et al., 1999). Membranes were incubated four times in RN buffer (10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 0.05% Triton X-100, 1 x Denhardt’s reagent) followed by a 3 h incubation in RN buffer in the presence of the DIG-labelled riboprobe and three 15 min washes in washing buffer (10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl). Finally, the DIG-labelled riboprobe was detected as describe above except that the Tween-20 was omitted from the washing solutions and a colorimetric method was used.
RESULTS

RNA-binding properties of PNRSV MP

To study the RNA-binding capabilities of the movement protein of PNRSV, its ORF was fused to a sequence encoding a six-histidine tag and expressed in *E. coli*. Induction of expression resulted in the synthesis of a protein with the expected size of the MP (32 kDa) (data not shown). The binding of the purified His–MP to PNRSV ssRNA was assayed by EMSA (Carey, 1991). A constant amount of RNA transcript (PNRSV RNA 4) was incubated with increasing amounts of His–MP (0–15–6 μM) and the electrophoretic retardation of RNA was detected using complementary DIG-labelled riboprobes. The ribonucleoprotein complex became detectable at 0·3 μM, and at 2·4 μM of protein the free RNA band disappeared (Fig. 1A, lanes 3 and 6, respectively). Interestingly, the ribonucleoprotein complex was able to enter the electrophoresis gel when the protein amount was over 0·8 μg (Fig. 1A, lanes 6–8), strongly suggesting the formation of two different complexes, one fully retarded complex operating at low protein : RNA ratios and an intermediate complex that is formed at high protein : RNA ratios and migrates into the gel. We reasoned that the first type of complex could adopt a rod-like structure that is not able to enter the agarose gel, whereas at higher amounts of protein, an increase in protein–protein interactions could result in a globular form able to enter the gel. In the latter case, the RNA–protein interactions would be protected from adverse treatments. To check this
hypothesis, both types of ribonucleoprotein complex were subjected to treatment with 6 M urea at 60°C for 15 min. As shown in Fig. 1(B), complexes formed at a 120:1 protein:RNA ratio were disturbed after this treatment, whereas complexes formed at a 400:1 ratio were not affected, revealing that in this type of complex the RNA–protein interactions are more protected.

Disappearance of the free RNA band is evidence of complex formation (Carey, 1991) and was therefore quantified by film densitometry to calculate the apparent $K_d$ of the RNA–MP interaction. Data from three independent experiments, similar to that shown in Fig. 1(A), were used to generate a linear regression curve (Fig. 1C) from which the MP concentration at which half of the RNA bound was estimated to be $1 \times 10^{-4} \text{M}$. To quantify cooperativity, a Hill transformation was used to analyse the data, as previously described (Daròs & Carrington, 1997; Marcos et al., 1999). The Hill plot revealed a Hill coefficient ($c$) of $1.5$; this value above $1$ ($c = 1$ is indicative of no cooperativity) can be taken as a weak indication of positive cooperativity.

The PNRSV MP binding specificity was examined by competition experiments (Fig. 2). BSA did not produce electrophoretic retardation of the RNA probe (Fig. 2, lane 3). The ssRNA from a heterologous origin (Lettuce big vein virus; LBVV) only competed at 10-fold excess (Fig. 2, lanes 4 and 5), whereas tRNA did not compete (lanes 6 and 7), as expected for a highly structured RNA (Li & Palukaitis, 1996; Marcos et al., 1999). The dsRNA, either containing probe sequences (Fig. 2, lanes 8 and 9) or not (lanes 10 and 11), partially displaced the binding only at a 10-fold ratio (lanes 9 and 11), whereas ss- and dsDNAs did not produce any effect on the electrophoretic retardation (lanes 12 and 15). These results indicate that the PNRSV MP has a preference for ssRNA binding, although it is able to bind with a lower affinity to dsRNAs.

The dependence of RNA binding on electrostatic interactions was evaluated by increasing the NaCl concentration of the incubation mixtures, and the dissociation of the complex was analysed by quantifying the appearance of free RNA by film densitometry. The NaCl concentration at which binding was reduced to 50% of the maximal level was estimated to be 750 mM (Fig. 3), a value significantly higher than that obtained for some other viral RNA-binding proteins (Osman et al., 1992; Brantley & Hunt, 1993; Li & Palukaitis, 1996; Richmond et al., 1998; Wobbe et al., 1998; Marcos et al., 1999). The results suggested that, in addition for ssRNA binding, although it is able to bind with a lower affinity to dsRNAs.
Characterization of the RNA-binding domain of PNRSV MP

It has been previously 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; Vaquero et al., 1997), complicating the appropriate interpretation in binding experiments. To circumvent this problem and to characterize the RNA-binding domain of the PNRSV MP, the protein was fused to the maltose-binding protein (MBP) and deletion mutants were generated (Fig. 4A).

Equivalent amounts of different deletion mutants were used to delimit the RNA-binding domain of the MBP–MP fusion protein. The proteins were separated by SDS-PAGE (Fig. 4B), electroblotted on to a nitrocellulose membrane, renatured and incubated with the DIG-labelled riboprobe as described previously (Aparicio et al., 2003). Analysis of mutants 200/201 and 234/104, retaining the C-terminal half or the N-terminal half of the protein, respectively, showed that only mutant 200/201 retained the RNA-binding activity (Fig. 4C, compare lanes 1 and 2). Analysis of two N-terminal deletion mutants (239/104 and 246/104) mapped the RNA-binding domain between aa 52 and 86 (Fig. 4C, lanes 3 and 4), which fits well with the region (aa 56–88) previously predicted (Sánchez-Navarro & Pallás, 1997). To define this region further, a mutant was generated in which the region between aa 56 and 88 was removed (mutant 200Δ104). As shown in Fig. 4(C, lane 5), this mutant lacked RNA-binding activity. Therefore, the analysis defined the nucleic acid binding domain between aa 56 and 88, since only constructs containing this region were able to bind efficiently to RNA (Fig. 4C, lanes 1, 4 and 6). Interestingly, the RNA-binding domain described here and that described for AMV are located at the N terminus of the MP, whereas similar domains previously characterized in viruses of the genera Bromovirus and Cucumovirus are present at the C terminus, reflecting their phylogenetic relationships.

DISCUSSION

I larvirus MPs belong to the ‘30K superfamily’. A variety of activities have been demonstrated for this group of proteins (Leisner, 1999), including nucleic acid binding, accumulation in the plasmodesmata where MPs are involved in increasing the plasmodesmata size exclusion limit, movement of RNA to neighbouring cells, formation of tubular structures in some cases and interaction with the cytoskeletal elements. However, no previous evidence has been presented demonstrating the RNA-binding capability of the MP of any of the ilarviruses characterized so far.

In this work, in vitro RNA-binding properties of PNRSV MP were demonstrated and the RNA-binding domain mapped. In gel retardation experiments, PNRSV MP bound preferentially to ssRNA in a non-sequence-specific manner. A cooperative binding of PNRSV MP to RNA was demonstrated with a quantitative approach, which was supported by the absence of detectable intermediate binding complexes.

Fig. 4. (A) Schematic representation of the wild-type and mutated forms of the MBP–MP fusion proteins. Nomenclature of the mutated forms refers to the primers used to make the different constructions. Numbers in the construct refer to the amino acid residue position in the wild-type protein. (B) SDS-PAGE analysis of the purified MBP–MP fusion protein and its mutated forms in a 12 % gel stained with Coomassie blue. The positions of the molecular mass markers (lane 7) are indicated on the right. Lane 0, pMal; lane 1, pMal-200/201; lane 2, pMal-234/104; lane 3, pMal-239/104; lane 4, pMal-246/104; lane 5, pMal-200Δ104; lane 6, pMal-200/104. (C) Northwestern blot analysis of MBP–MP and its mutated forms. The fusion proteins (3 μg) were separated by 12 % SDS-PAGE, transferred to a nitrocellulose membrane and was incubated with the PNRSV RNA 4 riboprobe. The probe was visualized using a colorimetric method.
Thus, the first MP molecule binds and boosts subsequent MP binding, most likely as a result of protein–protein interactions. Interestingly, in vitro binding assays revealed the existence of two different complexes between the PNRSV MP and RNA. One of the complexes was able to enter the electrophoresis gel and was formed at a 400 : 1 protein : RNA ratio, whereas at 120 : 1 the complex formed was fully retarded without entering the gel. The use of a protein-denaturing agent strongly suggests that these two complexes adopt very different forms. That formed at the lower protein : RNA ratio could adopt a rod-like structure, whereas that formed at the higher protein : RNA ratio could adopt a globular structure. The observation that the urea treatment mainly affected the MP–RNA complexes that were unable to enter the gel, and the other complexes to a much lesser extent, can be explained by the fact that that urea interacts directly with polar residues and the peptide backbone, thereby stabilizing non-native conformations (Bennion & Daggett, 2003). It is reasonable to assume that in the complexes formed at low protein : RNA ratio, where all the protein molecules must be in direct contact with the RNA, modification of protein conformation by urea would result in the lost of the capability to bind RNA, since it has been shown that binding of MPs to RNA is structure-dependent (Li & Palukaitis, 1996). In the faster-migrating complexes, formed at a higher protein : RNA ratio, only the more external protein molecules would be affected, whereas those directly involved in the RNA binding would not. Interestingly, two different complexes have recently been reported for the TMV-encoded MP with TMV RNA (Kiselyova et al., 2001). The MP distribution along the chain of RNA depended on the molar MP : RNA ratios at which the complexes were formed. The structure of TMV-specific complexes was visualized by atomic force microscopy. A rise in the amount of protein relative to the RNA led to a structural change of the complexes from RNase-sensitive ‘beads-on-a-string’ into a ‘thick string’ form that was partly resistant to RNase treatment. These complexes may be considered equivalent to the rod-like and globular complexes, respectively, suggested here for the PNRSV MP–RNA interaction.

The dissociation constant \( (K_d) \) value for the PNRSV MP–RNA complex was estimated to be 1·4 \( \mu M \), which is within the range of values reported for other sequence-non-specific RNA binding, such as the major hnRNP proteins \( (K_d=0·1–1·3 \mu M) \) (Burd & Dreyfuss, 1994), the poliovirus 3D polymerase \( (K_d=3 \mu M) \) (Pata et al., 1995), the Nla from TEV \( (K_d=1·1–1·3 \mu M) \) (Daró & Carrington, 1997) and the p7 movement protein of Carnation mottle carmovirus \( (K_d=0·7 \mu M) \) (Marcos et al., 1999; Vilar et al., 2001). This lack of specificity supports the idea that many MPs have the ability to bind different nucleic acids. In this case, heterologous ssRNA and dsRNA partially competed for PNRSV MP homologous ssRNA binding.

As previously mentioned, a sequence comparison analysis revealed that AMV and ilarviruses MPs have a basic motif preceding the 30K motif, with a high surface probability, which makes them good candidates for RNA–protein interactions (Sánchez-Navarro & Pallás, 1997). The results presented here confirmed this prediction and located this basic domain to aa 56–88 at the N terminus of the PNRSV MP. Further evidence demonstrating the functionality of this characterized domain was obtained using a synthetic peptide, which behaved like the PNRSV MP in in vitro RNA-binding experiments (M. C. Herranz, A. Sauri, I. Mingarro & V. Pallás, unpublished data). The biological significance of the identified RNA-binding domain is also supported by a high degree of sequence conservation in this region among the MPs of the PNRSV isolates characterized so far (Aparicio & Pallás, 2002).

The MP RNA-binding domain of PNRSV was compared with the other three previously characterized domains of members of the family Bromoviridae. Interestingly, a first visual inspection revealed that in AMV and PNRSV MPs, the RNA-binding domains are located at the N terminus, whereas those of Cucumber mosaic virus (CMV) and Brome mosaic virus (BMV) are at the C terminus of the MP (Fig. 5). Although the alignment of sequences did not reveal any obvious similarities, several common features can be drawn from this analysis: (i) a high proportion of basic amino acids (Arg or Lys) is present at the C terminus of the four domains; within or preceding this motif there is an unusually high number of aliphatic amino acids (Ile, Leu); (ii) secondary structure predictions revealed the presence of two \( \alpha \)-helices; the presence of \( \alpha \)-helices in a basic environment has been described for other proteins that bind RNA (Tau et al., 1993; Tau & Frankel, 1995; Marcos et al., 1999; Vilar et al., 2001; Gómez & Pallás, 2001); and (iii) similar to the RNA-binding domain ‘B’ of TMV (Citovsky et al., 1992), the four RNA-binding domains compared here have a high surface probability.

**Fig. 5.** Schematic representation of the MPs of members of the family Bromoviridae indicating the location of the RNA-binding domain showing sequence alignment of the four RNA-binding domains characterized so far for members of the family Bromoviridae. Predicted \( \alpha \)-helices are shadowed. Basic residues are indicated in bold. Sequences were aligned on the consensus sequence L/M-V/R-L/M (boxed).
The similar location of the RNA-binding domains of the MPs of AMV and PNRSV strongly reflects their close phylogenetic relationship and is consistent with previous observations demonstrating that an AMV RNA 3 bearing the PNRSV MP is competent for limited cell-to-cell movement (Sánchez-Navarro et al., 1997). Similarly, MP genes of CMV and BMV can be exchanged for the cell-to-cell movement function provided that their cognate CP is present (Nagano et al., 1999).

Emerging evidence suggests that many plant proteins are made up of spatially defined domains that are associated with assigned functions (Lam & Blumward, 2002). In many cases, the homology within a protein domain family could be low and might only be conserved in some crucial functional residues. On the other hand, it has been reported that some viral MPs show structural and functional similarities to some phloem proteins (Xoconostle-Cázares et al., 1999; Vilar et al., 2001; Gómez & Pallás, 2001). This has led to the hypothesis that genes encoding these viral MPs have been acquired from the plant genome, providing a connection between the evolution of the virus and its plant host (Lucas & Wolf, 1999). It is tempting to speculate that the four RNA-binding domains compared here have been acquired by a common ancestor from the plant host or that this ‘capture’ could have happened in two separate events, one for AMV and the genus Ilarvirus and the other for the genera Cucumovirus and Bromovirus. The differences among them could be explained as an adaptation of these viruses to the special requirements of their different hosts, without modifying the critical amino acid residues required for protein function.

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