RNA:protein interactions associated with satellites of panicum mosaic virus

Bénédicte Desvoyes, Karen-Beth G. Scholthof*

Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX 77845-2132, USA

Received 15 August 2000; revised 17 October 2000; accepted 19 October 2000

First published online 2 November 2000

Edited by Hans-Dieter Klenk

Abstract The interactions between satellite panicum mosaic virus (SPMV) capsid protein (CP) and its 824 nucleotide (nt) single stranded RNA were investigated by gel mobility shift assay and Northwestern blot assay. SPMV CP has specificity for its RNA at high affinity, but little affinity for non-viral RNA. The SPMV CP also bound a 350 nt satellite RNA (satRNA) that, like SPMV, is dependent on panicum mosaic virus for its replication. SPMV CP has the novel property of encapsidating SPMV RNA and satRNA. Competition gel mobility shift assays performed with a non-viral RNA and unlabeled SPMV RNA and satRNA revealed that these RNA:protein interactions were in part sequence specific.

Key words: Panicum mosaic virus; Satellite virus; Satellite RNA; Encapsidation

1. Introduction

Satellite panicum mosaic virus (SPMV) is a part of an unusual complex of RNAs that are infectious on a few species within the Gramineae [1]. SPMV is dependent on panicum mosaic virus (PMV; genus *Panicovirus*, family Tombusviridae) for replication. On millet plants (*Setaria italica* and *Panicum miliaceum*), PMV causes mild symptoms, but as a mixed infection with SPMV the plants are significantly stunted, develop a severe chlorotic mottle and the systemic infection progresses ca. 1 day more rapidly than for PMV alone [2]. In addition, SPMV induces a synergism by increasing the titer of PMV in the infected plant 2–4-fold [2]. PMV, the helper virus, also supports the replication and spread of satellite RNAs (satRNA) [3,4]. SatRNAs are often observed in plant virus infections and to date all known satRNAs are encapsidated by the helper virus capsid protein (CP) [5].

Thus far, four satellite viruses have been described in plants, SPMV, satellite tobacco mosaic virus (STMV), satellite tobacco necrosis virus and satellite maize white line mosaic virus (reviewed by [1,6]). There is no inter-relationship between these satellite viruses, although they have the same superficial features and a requirement for a helper virus to support their replication in planta. The 824 nucleotide (nt) single stranded positive sense SPMV RNA encodes a 17 kDa CP [7]. A 16 nm icosahedral T = 1 particle [8,9] is formed from 60 copies of the 157 amino acid CP [1]. Based on analyses of the crystalline structure of SPMV particles, strong nucleic acid–protein interactions were not observed, which is in contrast to the RNA:protein interactions that were detected from the STMV crystallographic analysis [8,9].

In the southern United States, St. Augustinegrass (*Stenotaphrum secundatum*), is naturally infected with PMV alone, or in combination with SPMV and/or satRNA [10]. Preliminary evidence suggested that the ca. 350 nt PMV satRNAs were encapsidated in SPMV virions (A.O. Jackson, personal communication). In this study, we developed an assay to determine the specificity of the interactions between SPMV CP and SPMV RNA as well as satRNA, to define the encapsidation parameters of this viral complex. This physico-chemical study was undertaken because of our interest in elucidating the biological roles of each of these molecules (PMV, SPMV and satRNA) as well as inter- and intra-molecular interactions that affect replication, movement, disease development and encapsidation.

2. Materials and methods

2.1. Preparation of soluble SPMV and PMV CPs

Millet plants were mechanically inoculated with plant sap containing a mixture of PMV and SPMV [2]. At 10–15 days post-inoculation, the symptomatic leaves were harvested and homogenized in 4 volumes of 0.2 M sodium acetate (pH 5.2) and 0.2 mM dithiothreitol (DTT). PMV and SPMV virions were precipitated with 8% polyethylene glycol (PEG) MW 8000 and 0.2 M NaCl followed by resuspension in 0.05 M sodium acetate (pH 5.5) and 1% Triton X-100. The clarified extract was then loaded on a 10–40% sucrose density gradient in 0.05 M sodium acetate, pH 5.5 and centrifuged for 3 h at 35 000 rpm (SW41 rotor, Beckman). A good separation of the two virions, PMV and SPMV, was obtained in the gradient. PMV and SPMV were recovered individually using a density gradient fractionator (ISCO, Lincoln, NE, USA and pelleted by centrifugation for 1.5 h at 50 000 rpm (Ti 60 rotor, Beckman). SPMV CP and PMV CP were prepared by dissociation of the respective purified virions. PMV was dissociated as described by Wei and Morris [11]. Briefly, the pellet was resuspended in 500 μl of dissociation buffer (0.1 M Tris–HCl (pH 8.5), 0.5 M NaCl, 5 mM EDTA) and incubated on ice for 1 h. The viral RNA was then precipitated by addition of 1 volume of 4 M LiCl. SPMV virions were dissociated by incubation for 3 h in 0.1 M Tris–HCl (pH 8), 80 mM KCl, 10 mM magnesium acetate, 1 mM DTT and 8 M urea. The SPMV RNA was precipitated by overnight incubation at −20°C in 3.5 M LiCl. The SPMV CP was separated from the SPMV RNA by centrifugation at 10 000 rpm for 10 min and the supernatant was dialyzed against several changes of TMK buffer (0.1 M Tris–HCl (pH 8), 80 mM KCl, 10 mM magnesium acetate, 1 mM DTT). The purity of PMV CP and SPMV CP was confirmed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and the concentration was determined by a BCA assay (Pierce Chemical, Rockford, IL, USA).

*Corresponding author. Fax: (1)-979-845 6483.
E-mail: kbgs@acs.tamu.edu

PII: S0014-5793(00)02177-3

2.2. In vitro transcripts of SPMV and satRNA

Transcripts were generated in vitro from infectious cDNA clones of both SPMV and satRNA-S1 [4,12] using T7-RNA polymerase according to the manufacturer's instructions (Life Technologies, Rockville, MD, USA) with the addition of ^{32}P-UTP to generate a probe for the gel mobility shift assay and the Northwestern blot assay. A 1475 nt 3′-terminal portion of the full length PMV cDNA was amplified by polymerase chain reaction and used as a template to generate ^{32}P-labeled transcripts representing the PMV subgenomic (sg) RNA, as described previously [12]. Following each transcription reaction, the DNA template was degraded with 1 U RQ1-DNase (Promega, Madison, WI, USA) for 15 min at 37°C. The transcripts were extracted with phenol/chloroform (v/v), precipitated with 2.5 volumes of ethanol at −80°C, centrifuged and resuspended in 20 μl of TE buffer (10 mM Tris–HCl (pH 7.5), 1 mM EDTA).

2.3. Northwestern blot assay

Purified PMV and SPMV CP (1 μg) were boiled in 2% SDS-sample buffer, electrophoresed through 12.5% polyacrylamide gels and transferred to nitrocellulose membrane [13]. The membrane was then blocked for 2 h at room temperature (RT) in binding buffer (10 mM Tris–HCl (pH 7), 1 mM EDTA, 1×Denhardt's reagent) [14] containing 200 or 400 mM NaCl and 25 μg/ml yeast tRNA. The ^{32}P-labeled RNA probe was added and incubated with the blot for 2 h at RT. The membrane was washed three times with binding buffer plus NaCl and exposed to X-ray film for 12 h at −80°C.

2.4. Electrophoretic gel mobility shift assay

The gel mobility shift assays were performed as described by Wei and Morris [11]. Approximately 50 ng of ^{32}P-labeled RNAs were incubated with different concentrations of the PMV CP or SPMV CP in TMK buffer (0.1 M Tris–HCl (pH 8), 80 mM KCl, 10 mM magnesium acetate, 1 mM DTT) containing 150 mM NaCl. After 15 min incubation at RT, the reaction mixtures were supplemented with 2 μl of 50% glycerol containing 0.2% bromophenol blue and then electrophoresed through 4% polyacrylamide gels containing 45 mM Tris–borate (pH 8.3), 1 mM EDTA and 4.5% glycerol. The gels were dried and exposed to X-ray film for 12 h at −80°C.

3. Results and discussion

3.1. Purification of PMV and SPMV coat proteins

PMV and SPMV were easily purified on a sucrose density gradient from mixedly infected plants due to their different sedimentation coefficients, 109 S and 42 S, respectively. PMV CP was obtained by dissociation of the virus in 0.5 M NaCl under mild alkaline conditions [11]. The same procedure applied to SPMV showed that the CP preparation still contained SPMV RNA (data not shown), suggesting that stronger RNA:CP and/or CP:CP interactions govern the SPMV architecture. Stringent conditions, such as 8 M urea and 3.5 M LiCl, were necessary to complete the virus dissociation. Soluble SPMV CP was obtained after dialysis and was used for the protein:RNA binding assays. Fresh preparations of SPMV CP were suitable for the gel mobility shift assays, but its RNA binding properties decreased when the CP was stored for more than 1 week at 4°C or −20°C. This suggested the occurrence of spontaneous CP:CP aggregation which abolished the capacity of the SPMV RNA to bind to the CP in a gel mobility shift assay.

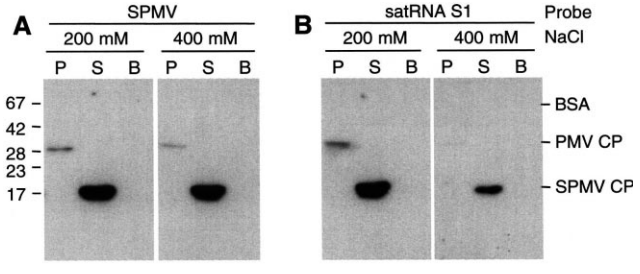

Fig. 1. Analyses of the SPMV RNA and satRNA-S1 binding activities of PMV CP and SPMV CP by Northwestern hybridization. PMV CP (P), SPMV CP (S) and BSA (B), 1 μg of each, were separated by SDS–12.5% PAGE. After transfer to a nitrocellulose membrane, the blot was hybridized with ^{32}P-labeled transcripts of SPMV (A) or satRNA S1 (B), in the presence of 200 or 400 mM NaCl, and autoradiographed. The positions of the protein molecular weight standards (in kDa) are indicated on the leftmost panel.

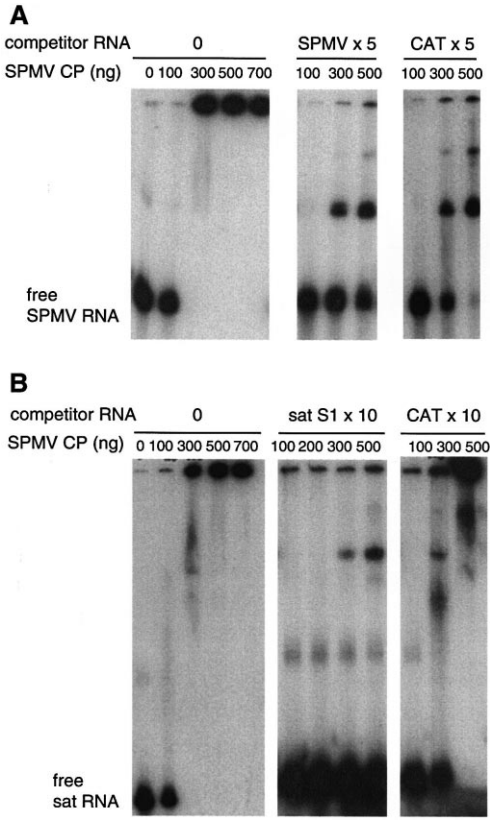

Fig. 2. Gel mobility shift assays of SPMV CP complexes interacting with SPMV RNA and satRNA S1. The indicated amount of purified SPMV CP or buffer only (0) were mixed with 50 ng ^{32}P-labeled transcripts of SPMV (A) or satRNA-S1 (B) in the presence or absence of competitive RNA as indicated on the top of each panel. The RNA:protein complexes were then separated on a 4% polyacrylamide gel, which was dried and autoradiographed. The position of the free RNA is indicated.

3.2. Specific interactions between SPMV CP and either SPMV RNA or satRNA-S1

The propensity for specific interactions between SPMV CP and its RNA were suggested by Northwestern blots. Fig. 1 shows that the SPMV RNA and the satRNA-S1 bind with a greater affinity to the 17 kDa SPMV CP than to the 26 kDa PMV CP in the presence of 200 mM NaCl, but do not bind bovine serum albumin (BSA). In the presence of 400 mM NaCl only very low binding between the satRNA and PMV CP could be detected and the SPMV-RNA signal decreased. CPs can generally be classified as nucleic acid binding proteins, but these data indicate a particular affinity of SPMV CP for both SPMV RNA and satRNA.

The preliminary results of the Northwestern analyses were

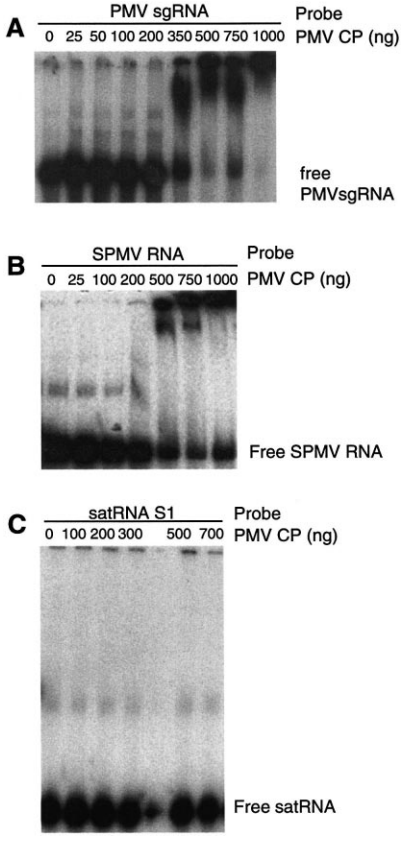

Fig. 3. Gel mobility shift assays of PMV CP interacting complexes with PMV RNA, SPMV RNA and satRNA S1. The indicated amount of purified PMV CP or buffer only (0) were mixed with 50 ng ^{32}P-labeled transcripts of PMV sgRNA (A), SPMV RNA (B) or satRNA S1 (C). See Fig. 2 legend for additional details.

extended with electrophoretic gel mobility shift assays in order to examine with more precision the affinity and the specificity of these interactions. With freshly prepared soluble SPMV CP we consistently observed that complete cooperative binding occurred with 300 ng of protein and 50 ng of ^{32}P-labeled SPMV RNA (Fig. 2A, left panel). Interestingly, a similar affinity was observed between the SPMV CP and the satRNA-S1 (Fig. 2B, left panel). To confirm this specificity, competition experiments were performed using cold SPMV RNA as a specific competitor and the ca. 800 nt bacterial chloramphenicol acetyl transferase (CAT) cDNA transcripts as an aspecific competitor. The presence of five times excess of cold SPMV RNA impaired binding to the extent that free ^{32}P-SPMV RNA could be detected even when 500 ng of protein was used in the binding reaction (Fig. 2A, middle panel). When CAT RNA was used as the competitor, a shifted RNA band was still obtained with 300 ng SPMV CP, but it considerably reduced the cooperative binding (Fig. 2A, right panel). This suggested that there are two different types of interactions. In the first case, a specific reaction occurs which is not affected by the presence of the CAT RNA and results in a shifted band. And in the second case, an aspecific reaction is observed that is inhibited by CAT RNA and subsequently resulted in the formation of large complexes that were unable to penetrate into the gel. Similar competition experiments performed with satRNA-S1 showed that the binding is inhibited by a 10 times excess of cold satRNA-S1 but not by the CAT RNA (Fig. 2B, middle and right panel). When BSA was used in place of the SPMV CP, a detectable complex was not observed for SPMV RNA or satRNA-S1 (data not shown), again indicating the specificity of the SPMV CP:RNA interaction in vitro. The gel shift data confirm and extend the Northwestern blot data in that a specific interaction occurs between SPMV CP and both SPMV RNA and satRNA-S1. As the satRNA and SPMV RNA have no sequence similarity except for ca. 5–7 nt at their respective extreme 5′- and 3′-termini, this suggests that there may be structural features on both RNAs that specifically capture the SPMV CP.

Binding properties of the PMV RNA, SPMV RNA and the satRNA S1 with the PMV CP were investigated as well. The 4326 nt full length PMV RNA could not be used in this assay because it is too big to be separated on a 4% polyacrylamide gel. In vitro transcripts corresponding to the 1475 nt PMV sgRNA were tested and found to be suitable as a riboprobe for the PMV CP binding assay. Previously, PMV RNA molecules of ~4000 and ~1300 nt in length, representing the PMV gRNA and sgRNA, were detected on denaturing agarose gels following RNA extraction of virions collected from sucrose density gradients [15]. This suggested that the PMV sgRNA would have an affinity for PMV CP. Fig. 3A shows that the PMV CP shifted the sgRNA probe at a concentration of 350 ng and cooperative binding was observed when the concentration of protein increased. Although PMV CP bound SPMV RNA at a lower affinity (500 ng), it did not show affinity for satRNA-S1 (Fig. 3B,C). These data confirmed the Northwestern assay in that SPMV RNA, but not satRNA, could bind the PMV CP when 400 mM NaCl was present in the binding buffer (Fig. 1).

The in vitro experiments demonstrate a specific affinity of SPMV RNA for its homologous CP, which may be important in the initiation of particle assembly. Thus far, these interactions have not been pinpointed from analysis of the crystalline structure [8,9]. In addition, SPMV CP, but not PMV CP, binds cooperatively and specifically to PMV satRNA which could explain why, in vivo, satRNAs are encapsidated in SPMV virions but not in the PMV virions. This fact is very peculiar because to our knowledge all other satRNAs are encapsidated by their respective helper virus [5]. However, in planta PMV satRNA does not require SPMV CP for viability and can systemically spread and replicate when co-infected with PMV alone [10]. Therefore, it is not clear what benefit SPMV CP affords the satRNA, other then protection of its ca. 350 nt RNA genome.

Acknowledgements: We thank WenPing Qiu and Herman Scholthof for critical reading of the manuscript. We appreciate the technical and greenhouse assistance provided by Beth Whitehead. This work was supported by the Texas Agricultural Experiment Station (H-8388) and USDA Competitive Grants 96-35303-3714 and 99-35303-7974.

References

[1] Scholthof, K.-B.G., Jones, R.W. and Jackson, A.O. (1999) Curr. Top. Microbiol. Immunol. 239, 123–143.
[2] Scholthof, K.-B.G. (1999) Mol. Plant-Microbe Interact. 12, 163–166.
[3] Cabrera, O., Roossinck, M. and Scholthof, K.-B.G. (2000) Phytopathology 90, 977–980.
[4] Monis, J., Sopher, D.S. and Jackson, A.O. (1992) Phytopathology 82, 1175.

[5] Roossinck, M.J., Sleat, D. and Palukaitis, P. (1992) Microbiol. Rev. 56, 265–279.
[6] Dodds, J.A. (1998) Ann. Rev. Phytopathol. 36, 295–310.
[7] Masuta, C., Zuidema, D., Hunter, B.G., Heaton, L.A., Sopher, D.S. and Jackson, A.O. (1987) Virology 159, 329–338.
[8] Ban, N. and McPherson, A. (1995) Nat. Struct. Biol. 2, 882–890.
[9] Ban, N., Larson, S.B. and McPherson, A. (1995) Virology 214, 571–583.
[10] Cabrera, O. and Scholthof, K.-B.G. (1999) Plant Dis. 83, 902–904.
[11] Wei, N. and Morris, T.J. (1991) J. Mol. Biol. 222, 437–443.
[12] Turina, M., Maruoka, M., Monis, J., Jackson, A.O. and Scholthof, K.-B.G. (1998) Virology 241, 141–155.
[13] Scholthof, K.-B.G., Hillman, B.I., Modrell, B., Heaton, L.A. and Jackson, A.O. (1994) Virology 204, 279–288.
[14] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[15] Masuta, C. (1986) M.Sc. Thesis, Purdue University, West Lafayette, IN.