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M. Rossi, A. Genre & M. Turina

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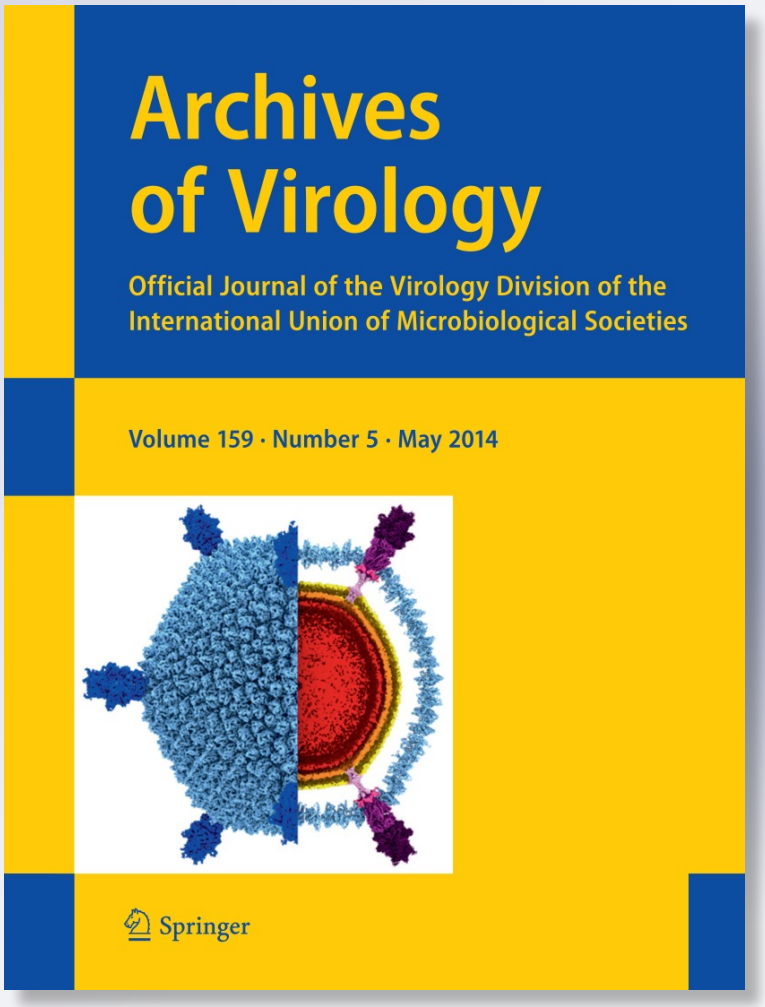

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BRIEF REPORT

Genetic dissection of a putative nucleolar localization signal in the coat protein of ourmia melon virus

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Abstract Ourmiaviruses became the object of recent attention for their unusual taxonomic placements among plant viruses. The ourmia melon virus (OuMV) RNA3 encodes a 22-kDa coat protein (CP). Besides its role in virion formation, the OuMV CP facilitates systemic virus spread. In *Nicotiana benthamiana*, an eGFP-CP fusion protein was localized in the nucleus and preferentially in the nucleolus. By bioinformatics analysis, we identified an arginine- and lysine-rich region at the N-terminus of the CP. Here, we demonstrate by deletion and alanine scanning mutagenesis that this region in the CP is responsible for its preferential accumulation in the nucleolus of host cells.

Keywords *Ourmiaviridae* · Ourmia melon virus · Nucleolus

Ourmia melon virus (OuMV) is the type member of the genus *Ourmiavirus* [21]. Three plus-strand RNA molecules form its genome, and each segment encodes only a single protein, making this one of the simplest non-defective virus genomes able to carry out a full infection cycle in nature. We have determined that RNA1 encodes an RNA-dependent RNA polymerase (RdRp), RNA2 encodes a movement protein (MP), and RNA3 encodes a 22-kDa coat protein (CP) [22]. Phylogenetic analysis showed that ourmiaviruses likely originated through a peculiar evolutionary process based on reassortment, possibly between a mycovirus and a plant virus. In particular, OuMV RdRp shares distant similarity with the RdRps of members of the *Narnaviridae*, a family of viruses that infect mainly fungi, yet the MP is distantly related to MPs of plant viruses of the family *Tombusviridae*. The CP has limited but significant similarity to CPs of plant and mammal viruses, but its origin remains uncertain [22]. The virion morphology, determined by the CP, is unique among plant RNA viruses: bacilliform particles of three different lengths with pointed ends [15]. In *Nicotiana benthamiana*, the mere expression of the CP and RNA3 segment is not sufficient for virion formation: virus particles are only formed when RNA1 is co-expressed and active replication occurs [3]. The CP is dispensable for cell-to-cell and long-distance movement in *N. benthamiana* but is crucial for efficient virus spread. In fact, without CP, RNA1 and 2 can move from inoculated leaves through the phloem and reach the upper leaves in *N. benthamiana*, but infection foci at the exit site remain limited [3].

In agroinfiltrated *N. benthamiana* leaves, eGFP-CP localizes in the nucleus and preferentially in the nucleolus of epidermal cells (Fig. 2A) [3]. Given the various roles played by the nucleolus in metabolic processes, such as ribosome synthesis and assembly, stress response, mitosis, cell growth, differentiation, and RNA silencing in plant cells [9, 14, 18], it would be important to establish the specific roles of the ourmiavirus CP in infection. It is well established that the majority of DNA viruses and retroviruses alter nucleolar functions or produce nucleolus-targeted molecules [10]. Nevertheless, the role of the nucleus and nucleolus in RNA virus replication and assembly is

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M. Rossi · M. Turina (✉)
Sez. di Torino, CNR, Istituto di Virologia Vegetale, Strada delle Cacce 73, 10135 Torino, Italy
e-mail: m.turina@ivv.cnr.it

A. Genre
Dipartimento di Scienze della Vita e Biologia dei Sistemi, Università di Torino, Viale P.A. Mattioli 25, 10125 Torino, Italy

Table 1 Oligonucleotides used for PCR and site-directed mutagenesis

Construct name[a]	Forward primer (5′-3′)	Reverse primer (5′-3′)
CP-eGFP(*)	TGGTAATCACGCACCGTATGATG	TGGCGCTCAGGGAAGTCCGTAAG
eGFP-N(1-17)	ACTTACTGGACTTCCCTGAGC	GTTCTTCTTCTTCTCATTACG
eGFP-N(1-11)	ACTTACTGGACTTCCCTGAGC	ACGTCGATTCTTTCTCTTTGGG
eGFP-N(1-9)	ACTTACTGGACTTCCCTGAGC	ATTCTTTCTCTTTGGGAGTCTG
eGFP-Δ(5-11)	AATGAGAAGAAGAAGAACGCACG	GAGTCTGGCCATGGGCTTGTACAGC
eGFP-Δ(14-16)	AACGCAAACGCATCGAGGGTCCAAAATG	CTCATTACGTCGATTCTTTCTCTTTGG
eGFP-10 R/A	CTCCCAAAGAGAAAGAATGCACGT AATGAGAAGAA	TTCTTCTCATTACGTGCATTCTTTCTC TTTGGGAG
eGFP-6,7-KR/AA	CCAGCGGCAAAGAATCGACGTAATG	GAGTCTGGCCATGGGCTTGTACAGC
eGFP-6,7,10-KRR/AAA (**)	CTCCCAAAGAGAAAGAATGCACGTA ATGAGAAGAA	TTCTTCTCATTACGTGCATTCTTTCTC TTTGGGAG
eGFP-Δ(194-201)	ACCGCGACTTACTGGACTTCCCTG	CACCGATTGCTTGTGTACTAGATC

(*) Template: pGC-RNA3

(**) Template: eGFP-6,7-KR/AA

[a] Template: pGC-GFP-CP

still under debate. Although a few studies have suggested that the nucleus and nucleolus are not involved in mammalian virus replication [5, 27], the role of these cellular compartments in RNA virus infection has become more evident in recent years [9]. For plant viruses, the best example of a functional interaction between an RNA virus protein and the nucleolus is the interaction of the ORF3-encoded protein of groundnut rosette virus (GRV) with fibrillarin. This interaction results in the formation of ring-like structures called ribonucleoprotein (RNP) particles [1], which are essential for long-distance movement of this umbravirus through the phloem [11, 12].

The aim of this study was to determine the region in the CP sequence responsible for the preferential accumulation of an eGFP-CP fusion protein in the nucleolus.

The targeting of a protein fusion is often expected to vary according to the relative position of the GFP and native protein in the engineered construct [17]. Since only a GFP fusion to the CP N-terminus had been tested so far, we constructed a clone encoding a C-terminal CP-eGFP fusion protein. For this purpose, an *Nco*I restriction site was inserted exactly at the 3′ end of ORF3 by PCR-directed mutagenesis using the primers listed in Table 1 and the pGC-RNA3 clone as a template, using conditions described previously [3]. The eGFP-encoding sequence was inserted in the *Nco*I restriction site (Fig. 2). The use of this CP-eGFP construct confirmed that CP targets eGFP to the nucleolus irrespective of the fusion arrangement (Fig. 2E).

All putative nucleolar localization signals (NoLSs) that have been characterized so far are rich in arginine and lysine residues [4, 6, 9]. Significantly, we identified one such region next to the N-terminus of the OuMV CP. Here, the basic amino acid stretch $_{6}$KRKNRRNEKKK$_{16}$ is a good candidate for the NoLS of OuMV CP. In order to verify the importance of this region in targeting the fusion protein to the nucleolus, we developed two sets of mutants: one carrying different deletions in the CP region and one obtained through alanine scanning mutagenesis to replace charged amino acids in the putative NoLS with alanine residues (Table 1 and Fig. 1).

A previously described eGFP-CP fusion clone, named pGC-GFP-CP [3], was used to generate the deletion mutant clones described in Fig. 1. Deletions were produced by PCR mutagenesis with Phusion high-fidelity polymerase (New England Biolabs) as detailed before [3]: we used forward and reverse oligonucleotides that amplified the full-length clone, excluding the cDNA region that we wanted to delete. All clones assembled in this study were checked by full-length sequencing of the promoter and viral cDNA region in order to confirm that only the expected mutations were present. Agroinfiltration experiments were carried out as detailed previously [16]. Leaves were examined 3 days post-inoculation (dpi), since this has been shown to be the day on which expression of the fusion proteins reaches a maximum under the conditions tested [3].

Since eGFP localizes in the cytosol and nucleus when expressed as a free protein, we first of all wanted to verify that the putative NoLS of the CP was sufficient for transport of the eGFP to the nucleolus (Fig. 2C). To this end, a cDNA clone encoding an altered protein consisting of only the first 17 amino acids of CP fused with eGFP, named eGFP-N(1-17), was constructed, and its intracellular localization was observed using confocal laser scanning microscopy (CLSM). The localization of eGFP-N(1-17) in the nucleolus confirmed the presence of an NoLS function in the first 17 aa of the CP (Fig. 2G). Further deletion of a cDNA region encoding 5 amino acids of the putative NoLS, resulting in the eGFP-N(1-11) construct, did not interfere with

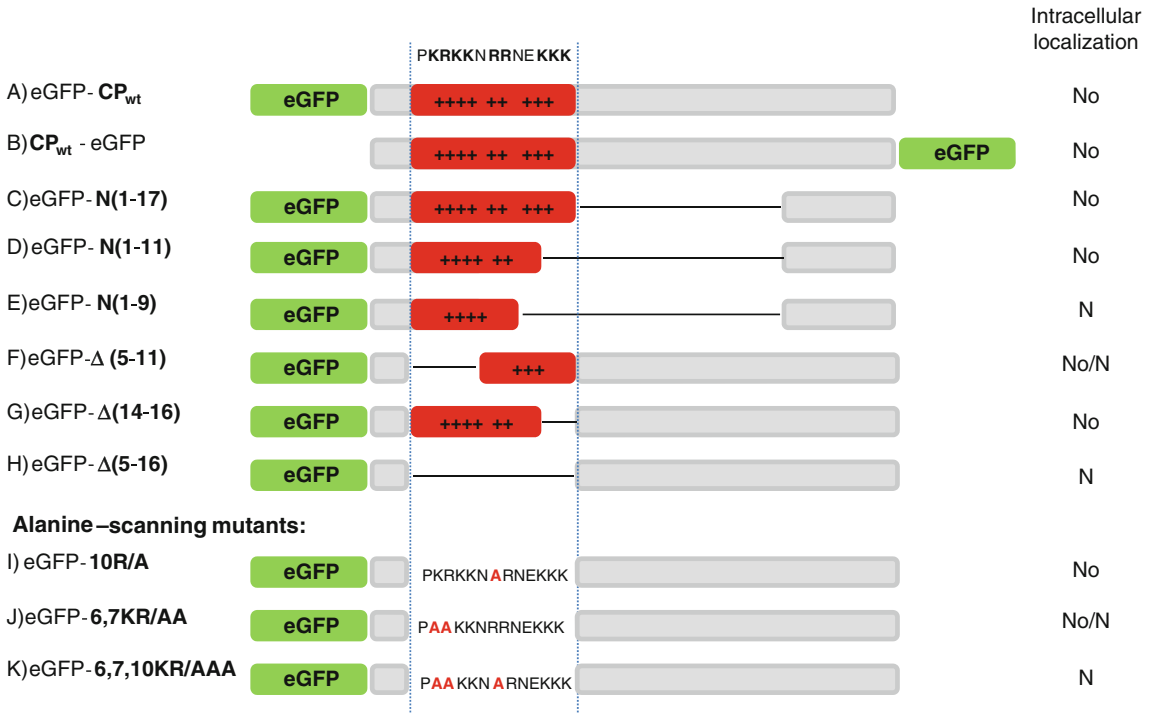

Fig. 1 Schematic representation of the constructs used in this study and their intracellular distribution. The green boxes correspond to the eGFP sequence, whereas the CP sequence is represented by grey boxes. The boxes in red highlight the protein region containing the NoLS, and the symbol "+" correspond to a positively charged amino acid. No = the fusion protein is preferentially accumulated into the nucleolus; N = the fusion protein can enter into the nucleus but not into the nucleolus; No/N = the fusion protein can enter into the nucleolus, but it is not preferentially accumulated in this compartment

nucleolar localization (Fig. 2I). Only the removal of two extra nucleotide triplets in the cDNA construct eGFP-N(1-9) led to a reduction in accumulation of eGFP in the nucleolus (Fig. 2K). These results confirmed our hypothesis that a NoLS is present at the N-terminus of CP. In particular, the first 11 amino acids are necessary and sufficient to drive the chimeric protein into the nucleolus.

In order to confirm these results and detect possible interactions with other regions of the CP that might prevent the exposure of the NoLS signal in the context of the full-length CP, we progressively removed the cDNA region coding for the basic amino acid stretch from the full-length CP fused to eGFP and observed the intracellular distribution of each construct. Deletion of the cDNA region encoding the entire putative signal resulted in a complete exclusion of eGFP- Δ(5-16) from the nucleolus (Fig. 2O). A partial deletion of the cDNA encoding the putative NoLS, resulting in the clone eGFP- Δ(5-11), led to limited accumulation of the protein in the nucleolus (Fig. 2M). Finally, deletion of the cDNA coding for three sequential K residues (eGFP-Δ(14-16)) did not affect nucleolar accumulation of the fusion protein (Fig. 2Q).

With the caveat that even short deletions may dramatically alter protein conformation and functionality, especially in such small proteins as OuMV CP, we generated cDNA encoding specific amino acid substitutions by alanine scanning mutagenesis using the QuikChange II Site-Direct Mutagenesis Kit (Agilent). The template for mutagenesis was a full-length RNA3 cDNA clone, pGC-GFP-CP, which was used previously for agroinfiltration and expression of CP [3]. A single alanine substitution at position 10 (eGFP-10-R/A) did not influence targeting of CP (Fig. 2S), but when we replaced the amino acids at positions 6 and 7 (eGFP-6,7-KR/AA), we observed an accumulation of the mutant CP at the periphery of the nucleolus and a drastic reduction in protein accumulation in the nucleolus (Fig. 2U). The triple substitution of the mutant eGFP-6,7,10-KRR/AAA resulted in the complete loss of targeting of the CP to the nucleolus (Fig. 2W). This strongly confirmed the role of these basic amino acids in nucleolar localization of OuMV CP.

In brief, our results indicate that the CP NoLS is functional in the full-length protein. Both of our approaches, based on partial deletions and targeted amino acid substitutions, demonstrated that the nucleolar localization of the eGFP-CP fusion protein is strongly dependent on the presence of three charged amino acids within the identified NoLS.

The nuclear transport mechanisms in mammalian, yeast and plant cells are highly conserved. Nuclear localization

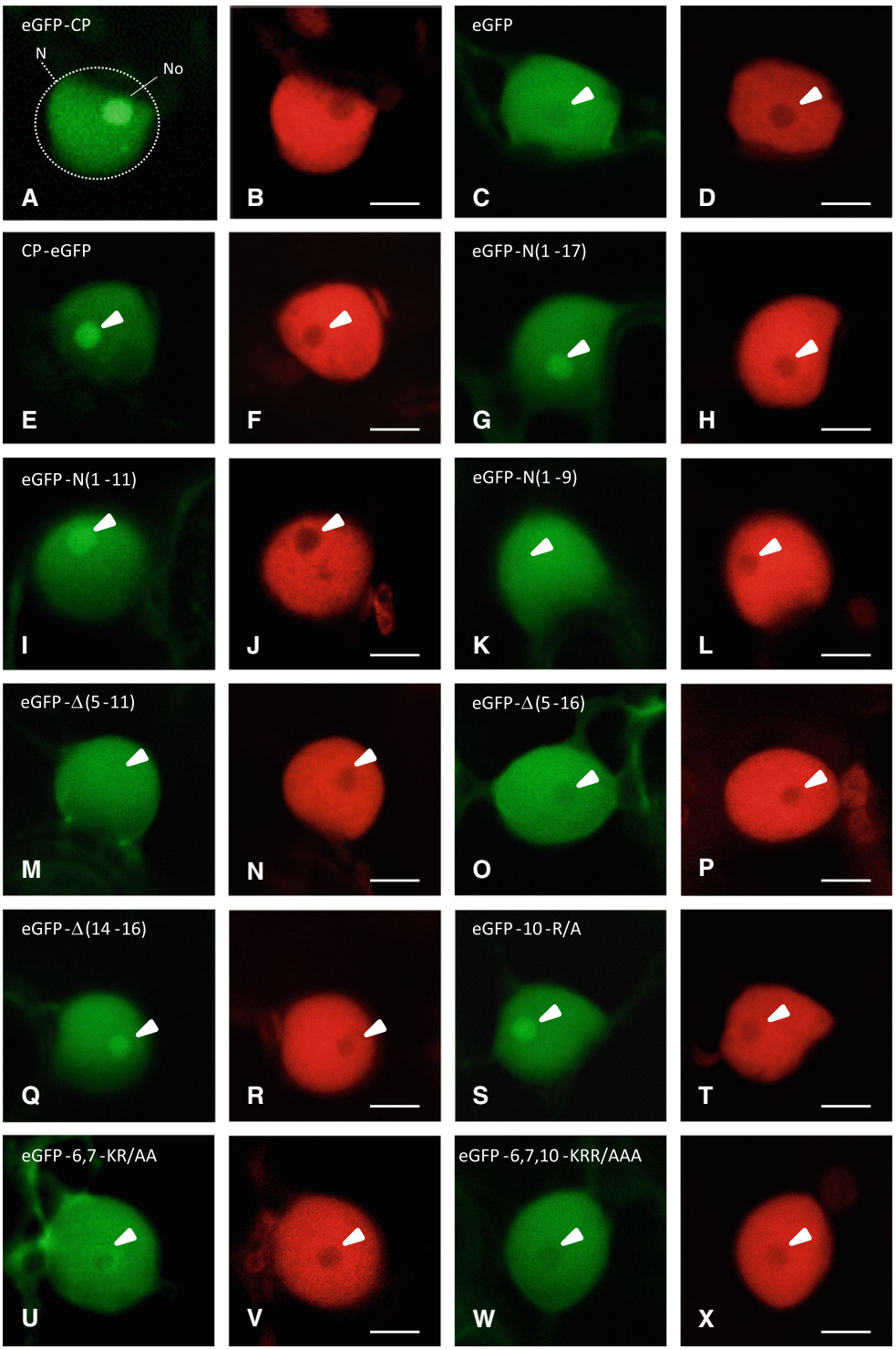

Fig. 2 Nuclear and nucleolar distribution of CP derivatives fused to GFP. Confocal laser scanning microscopy (CLSM) images are shown of single nuclei of epidermal cells of transgenic *N. benthamiana* expressing the nuclear marker H2B-RFP (panels B, D, F, H, J, L, N, P, R, T, V, X) three days after agroinfiltration with constructs expressing eGFP-CP(A), eGFP (C), CP-eGFP (E), eGFP-N(1-17) (G), eGFP-N(1-11) (I), eGFP-N(1-9) (K), eGFP-Δ(5-11) (M), eGFP-Δ(5-16) (O), eGFP-Δ(14-16) (Q), eGFP-10-R/A (S), eGFP-6,7-KR/AA (U), and eGFP-6,7,10-KRR/AAA (W). The nucleolus is indicated by a white arrowhead. CLSM was performed using a Leica TCS-SP2 microscope (Leica Microsystems). For GFP imaging, excitation at 488 nm and collection between 500 nm and 525 nm were used. RFP was excited at 544 nm and imaged at 550 to 620 nm. N = nucleus; No = nucleolus. Bars equal 5 μm

signals (NLSs) are well known and characterized. They generally consist of a single or double stretch of basic amino acids. Commonly, the NLS is not cleaved from the protein after nuclear import and its activity is independent of its position within the protein [13]. Nuclear export signals (NESs) have also been identified as consisting of a leucine-rich stretch of ~11-13 amino acids [13]. Analysis of the OuMV CP sequence revealed a putative NES at the C-terminus of the protein ($_{194}$LGELSLTI$_{201}$). A deletion mutant of the putative NES was constructed and named eGFP Δ(194-201). Even though this mutation did not affect protein stability when not fused to GFP, as confirmed by western blot (Supplementary Fig. S1), GFP fluorescence of the fusion construct could not be detected.

More limited information is available for NoLSs, and a unique and conserved consensus signal has not been identified yet. The putative NoLSs that have been characterized so far are rich in arginine and lysine residues and can be grouped in two categories: single- and multiple-motif signals [4, 6, 9]. Our results show that only a single nucleolar localization motif is present in OuMV CP. Only a few cases of plant viral proteins targeted to the nucleolus have been reported, and the NoLSs that have been characterized so far are rich in positively charged amino acids [2, 7, 8, 11, 19, 20, 23–26]. Alfalfa mosaic virus (AMV), for instance, has a short NoLS that is rich in lysine and arginine residues at the N-terminus of the CP, which, in addition to its role as a sorting signal, acts as modulator of RNA binding and it is responsible for cell-to-cell and long-distance movement of AMV [8]. Chiba and co-workers [2] showed that the presence of the NoLS in p14 of beet necrotic yellow vein virus (BNYVV) is crucial for maintaining p14 stability and its silencing suppression activity. These examples show that a NoLSs can have multiple roles during the viral life cycle.

In this study, we have identified a short positively charged region at the N-terminus of OuMV CP that is necessary and sufficient to target eGFP-CP to the nucleolus. Nucleolar accumulation is unexpected for a viral structural protein such as the CP. This finding suggests as yet unknown functional roles for OuMV CP during viral infection other than encapsidation.

Compared to NLSs, which are targeting sequences, NoLSs are defined as retention signals. In fact, they are usually binding domains for nucleolar proteins, rRNA, and other nucleolar components [9]. The interaction between these elements and NoLSs guarantees protein retention in the nucleolus [28]. The constructs that we have developed for the present study will be useful tools for identifying possible interactors responsible for CP accumulation in the nucleolus and for understanding the possible biological role of the CP-nucleolus interaction in ourmiaviruses.

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