Virus Genes (2010) 41:111–117
DOI 10.1007/s11262-010-0480-9

Immunodetection and subcellular localization of *Mal de Río Cuarto virus* P9-1 protein in infected plant and insect host cells

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Received: 20 December 2009 / Accepted: 2 April 2010 / Published online: 24 April 2010

Abstract *Mal de Río Cuarto virus* (MRCV), a member of the genus *Fijivirus*, family *Reoviridae*, has a genome consisting of 10 dsRNA segments. The segment 9 (S9) possesses two non-overlapping open reading frames (ORF-1 and ORF-2) encoding two putative proteins, MRCV P9-1 and MRCV P9-2, both of unknown function. The MRCV S9 ORF-1 was RT-PCR amplified, expressed in pET-15b vector, and the recombinant protein produced was used to raise an antiserum in rabbit. Western blot with the specific MRCV P9-1 antiserum detected a protein of about 39 kDa molecular weight present in crude protein extracts from infected plants and insects. However, no reaction was observed when this antiserum was tested against purified virus. In contrast, only virus particles were detected by a MRCV-coat antiserum used as a validation control. These results suggest that MRCV S9 ORF-1 encodes a non-structural protein of MRCV. Immunoelectron microscopy assays confirmed these results, and localized the MRCV P9-1 protein exclusively in electron-dense granular viroplasms within the cytoplasm of infected plants and insects cells. As viroplasms are believed to be the replication sites of reoviruses, the intracellular location of MRCV P9-1 protein suggests that it might be involved in the assembly process of MRCV particles.

Keywords MRCV · Fijivirus · Non-structural virus protein · Viroplasm

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Introduction

Mal de Río Cuarto virus (MRCV), member of the genus *Fijivirus*, family *Reoviridae* [1], causes the most important maize disease in Argentina. MRCV replicates in phloem cells of monocotyledonous plants such as maize, oat, wheat, and other cultivated and wild species in the families *Poaceae* and *Cyperaceae* [2–4], which are natural reservoirs of its main vector *Delphacodes kuscheli* Fennah (Hemiptera: *Delphacidae*) [5, 6]. Typical symptoms of MRCV in maize include general stunting, internode shortening, general deformation, leaf enations, panicle atrophy, and multiple ears per node with defective grain formation [4].

MRCV genome consists of 10 linear segments of dsRNA, eight of which are monocistronic while segments 7 (S7) and 9 (S9) show two non-overlapping open reading frames (ORF-1 and ORF-2) [7]. This organization is similar to other members of the genus, and it has been suggested as a possible strategy to regulate gene expression [8]. The complete MRCV genome has been sequenced [1, 9–11] and the function proposed for some of the predicted proteins. MRCV S1 encoded protein has a putative RNA polymerase motif. MRCV S2 or MRCV S4 codes for a presumably B-spike protein. MRCV S8 protein contains an NTP-binding motif site that is present in a number of proteins including helicases. MRCV S10 encodes for the major outer capsid protein. The functions of MRCV S5–S7

and S9-encoded proteins are still unknown [1, 9–11]. Nevertheless, PROSITE and Pfam-HMMs analysis of MRCV S7 and S9-encoded putative proteins allowed their classification within the group of non-structural fijivirus proteins [11], as suggested for *Rice black streaked dwarf virus* (RBSDV) S7 and S9, and *Fiji disease virus* (FDV) S9 ORF-2 [12, 13]. With the exception of the major core capsid protein [14], encoded by MRCV S3, for which the subcellular localization has been experimentally demonstrated, function and cellular localization of proteins encoded by MRCV have not been elucidated. Consequently, the molecular mechanisms that govern MRCV replication and movement remain unknown.

Many plant viruses form cytoplasmic structures during the infection process. The nature of these structures is under intensive study to better understand their function during virus infection and host response. The reovirus cytopathology is similar among its members. Typical alterations include the presence of inclusions commonly called viral factories or viroplasms, tubular structures that frequently enclose viral particles inside and, occasionally, other kind of inclusions called crystal arrangements [15, 16].

Viroplasms are mainly composed of viral dsRNA, viral proteins, partially and fully assembled viral particles, microtubules, and thinner "kinky" filaments suggested to be intermediate filaments. Although the formation mechanism of these inclusions is largely unknown, they are thought to play an important role in viral infections because they are probable sites of viral genome replication, protein synthesis, and virus assembly [12, 16–18]. This study focused on the MRCV P9-1 protein encoded by MRCV S9 ORF-1. We demonstrated that MRCV P9-1 protein accumulates in intracellular viroplasms in infected plant and insect host cells.

Materials and methods

Source of virus and purification of dsRNA

A greenhouse-maintained MRCV isolate propagated in sorghum (*Sorghum bicolor* (L.) Moench) was used as the virus source. The dsRNA was purified from fresh leaves or roots (8 g) by differential adsorption to CF11 cellulose as described by Dodds [19]. The extractions were analyzed by 10% SDS-PAGE to confirm the standard mobility pattern of the 10 genome segments and to determine purity and quality.

Cloning of MRCV S9 ORF-1

In order to amplify the complete sequence of MRCV S9 ORF-1, two primers were designed: MRCV S9-1-Fw (5′-ACGCATATGGCAGACCTAGAAC-3′, nt 52-67) and MRCV S9-1-Rv (5′-ACG**GGATCC**GAGTATAATAATCA AAC-3′, nt 1076-1060) with a *Nde*I (underlined) and *Bam*HI (in bold) restriction enzyme recognition sites, respectively. These primers were based on the MRCV S9 sequence previously reported (Acc. No. DQ023312) [11]. RT-PCR was carried out by the Access RT-PCR System Kit (Promega Biotech, USA) using 100–150 ng of genomic MRCV dsRNA as template. The amplified product was run in 1% preparative agarose gel and purified by *Geneclean* II System (BIO 101 Inc., USA). The RT-PCR product was cloned into pBluescript II (SK^+) vector (Stratagene, USA) in the multiple cloning site digested with *Sma*I, using the DNA Ligation Kit (TaKaRa Shuzo Co LTD, Japan) according to the manufacturer's instructions. Finally, MRCV S9 ORF-1 was purified after digestion with *Nde*I and *Bam*HI and subcloned into pET-15b expression vector (Novagen, Madison, USA).

Expression and purification of recombinant MRCV P9-1 protein

E. coli BL21(DE3) cells were transformed [20] with the pET-15b-MRCV S9 ORF-1 construct to express full-length MRCV P9-1 protein as a His-tagged protein. In order to induce recombinant MRCV P9-1 protein, a 20-µl culture aliquot was diluted in 30 ml of fresh LB medium containing ampicillin (100 µg/ ml) and grown until 0.7 OD_{600}. Isopropylthio-β-D-galactoside (IPTG) 1 mM final concentration was added and cells were incubated for additional 3 h. The protein expression was examined by 12% SDS-PAGE and stained with coomassie brilliant blue (Bio-Rad Laboratories, USA).

The recombinant MRCV P9-1 protein was purified following Sambrook et al. [20] [adapted from 21]. Briefly, the cell cultures were pelleted and resuspended in lysis buffer (50 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0, and 100 mM NaCl), 1 mM phenylmethylsulphonyl fluoride (PMSF), and 0.2 mg/ ml lysozyme. The insoluble fraction was pelleted and washed in lysis buffer containing 0.5% Triton X-100 and 10 mM EDTA pH 8.0. Finally, the inclusion bodies were solubilized in 8 M urea and the supernatant dialyzed overnight at 4°C against 10 mM sodium citrate buffer. Total bacterial protein extracts were separated on a preparative electrophoresis gel (12% SDS-PAGE) and the band corresponding to recombinant MRCV P9-1 protein was excised from the gel and electroeluted in dialysis tubes. The purified protein was run on 12% SDS-PAGE and stained with coomassie brilliant blue to analyze its purity. The concentration was determined spectrophotometrically (OD_{280}) and by comparison with a quantitative ladder (SDS-PAGE molecular weight standards, Sigma Chemical Co., USA).

Antiserum production

In order to obtain the MRCV P9-1 antiserum, a rabbit was immunized with the recombinant MRCV P9-1 protein. In the first application, approximately 300 μg of recombinant MRCV P9-1 protein (emulsified with complete Freund's adjuvant) was injected intradermally as described by Vaitukaitis [22]. Then, an intramuscular (300 μg of recombinant MRCV P9-1 protein emulsified with incomplete Freund's adjuvant) and two intravenous (100 μg each) protein injections were administered at 20 days interval. The antiserum titer was tested before each immunization by indirect enzyme-linked immunosorbent assay (PTA-ELISA) [23] in dilutions from 1:1,000 to 1:512,000 using purified recombinant MRCV P9-1 protein and *E. coli* BL21(DE3) total protein extract.

Purification of MRCV particles from infected maize plants

The purification of MRCV particles was performed as rapidly as possible on ice because of the instability of the particles. The MRCV particles were purified from infected maize plants following the protocol described by Giménez Pecci et al. [24]. About 50 g of roots were ground with a meat grinder and resuspended in 200 ml of phosphate buffer (0.2 M $NaKPO_4$, 0.5 mM EDTA, and 10 mM $NaSO_3$ pH 7.0). The homogenate was further extracted by squeezing through a double layer of medical gauze. After addition of 200 ml of CCl_4, the mixture was stirred for 30 min at 4°C and centrifuged at 10,000×*g* for 10 min at 4°C. The supernatant was centrifuged at 64,000×*g* for 2 h at 4°C. The pellet was gently resuspended in 6 ml of phosphate buffer. After addition of 6 ml of CCl_4, the mixture was stirred for 10 min at 4°C and then centrifuged at 12,100×*g* for 10 min at 4°C. The supernatant was centrifuged at 113,000×*g* for 1 h at 4°C. The pellet was gently resuspended in 500 μl of 0.2 M $NaKPO_4$ buffer. The supernatant was then loaded onto a 20–50% Cs_2SO_4 gradient and centrifuged at 91,100×*g* for 160 min at 8°C. The virus zone in the middle of the tube was collected, dialyzed against phosphate buffer (0.1 M $NaKPO_4$, 0.5 mM EDTA, and 10 mM $NaSO_3$ pH 7.0) overnight and then centrifuged at 137,000×*g* for 1 h at 4°C. The pellet was gently resuspended in 100 μl of 0.05 M $NaKPO_4$, 2.5 mM EDTA, and 5 mM $NaSO_3$ buffer.

Transmission assay

Delphacodes kuscheli were allowed to feed on two MRCV-infected wheat plants during a 4-day acquisition period. Laboratory-reared first instar nymphs were used based on previous experiments [25]. After a 12-day latent period, the insects were recovered and individually tested for infectivity through experimental transmissions to select the viruliferous specimens [25, 26]. Two 2-day serial transmissions were performed with each insect to healthy wheat seedlings. The plants were kept in a greenhouse and examined for virus symptoms.

Western blots

The total plant protein extracts were prepared from 1 g of MRCV-infected and healthy maize plants, macerated in 1 ml extraction buffer (1X TBS, 0.01% (w/v) DIECA, and 0.01% (w/v) EDTA). Selected insects (18 males and 18 females) were separately homogenized in 30 μl of TBS pH 7.5 (1:10 w/v). Plant and insect protein samples, purified recombinant MRCV P9-1 protein, and *E. coli* BL21(DE3) total protein extracts were analyzed by SDS-PAGE [27]. Acrylamide concentration was appropriately adjusted according to the expected protein size. Following electrophoresis, the fractionated proteins were transferred onto a nitrocellulose membrane (Hybond^{TM}-C Amersham, UK) at 60 V during 90 min using a Mini Trans-Blot cell (Bio-Rad Laboratories, USA) by standard procedures [20]. The blotted proteins were detected using proper dilutions of MRCV P9-1 or MRCV-coat antiserum. Bound antiserum was developed with phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad, USA) 1:3,000 [28].

Immunoelectron microscopy

MRCV symptomatic wheat leaves infected by experimental transmission with *D. kuscheli* [26, 29] were taken 2 months after inoculation. MRCV-infected insects were obtained as previously described in the transmission assay. An MRCV isolate obtained from the disease endemic area was used as inoculum source in both cases.

Wheat leaf portions and insect heads were embedded in Unicryl® (British Biocell International, UK) after fixation in glutaraldehyde/paraformaldehyde and dehydratation in ethanol series: 50% (30 min at 4°C), 70%, 90%, and 100% (1 h at −20°C each). Ultrathin 90–100 nm-thick sections were mounted on nickel grids, incubated in block solution (PBS pH 8.5 containing 1% BSA, 0.1% Tween 20, and 1% preimmune serum) and then transferred to 1:100 dilution of specific MRCV P9-1 antiserum. Immunogold labeling was performed employing goat anti-rabbit antibodies conjugated to 10 ± 2 nm colloidal gold (anti-rabbit IgG-gold, Sigma Chemical Co., USA) at 1:100 dilution [30]. Sections were contrasted with 2% uranyl acetate and lead citrate and observed with a JEOL 1200 transmission electron microscope. MRCV particles were also immunolabeled using an antiserum against purified MRCV particles at 1:1,000 dilution, according to the protocol described above. In all

cases, healthy leaves and insects were included as negative controls.

Results and discussion

Obtainment of recombinant MRCV P9-1 protein and production of a specific antiserum

The full-length MRCV S9 ORF-1 gene (1,014 bp, 337 amino acids) was found to encode a 39-kDa protein. The sequence was amplified using the specific primers, cloned into pET-15b vector and sequenced. Sequence analysis showed that the complete fusion sequence (His-tag sequence fused in frame to the 5′-terminal end of the cloned gene MRCV S9 ORF-1) encodes a His-tagged recombinant protein of 353 amino acids with a calculated molecular mass of 40.82 kDa. The presence of the recombinant MRCV P9-1 protein was confirmed by SDS-PAGE (Fig. 1) after induction with IPTG of the expression vector pET-15b-MRCV S9 ORF-1 in *E. coli* BL21(DE3). A band of the expected size (apparent molecular mass 41 kDa) was observed in the induced cell lysates (Fig. 1, compare lane 2 and 3). On the other hand, no bands were differentially detected in the cell lysates containing an empty plasmid (Fig. 1, lane 1). When the *E. coli* cultures harboring recombinant plasmid were induced, over expressed protein was located in the pellet and not in the supernatant after centrifugation. The pellet was solubilized using the protocol described for inclusion bodies, confirming that the recombinant MRCV P9-1 protein forms inclusion bodies when is over expressed in *E. coli* [31]. Dissolved protein was then refolded by gradual removal of the denaturing reagents by dialysis and successfully recovered from the gel (Fig. 1, lane 4), avoiding the use of His-tag affinity column. An antiserum was obtained by rabbit immunization with the recombinant MRCV P9-1 protein.

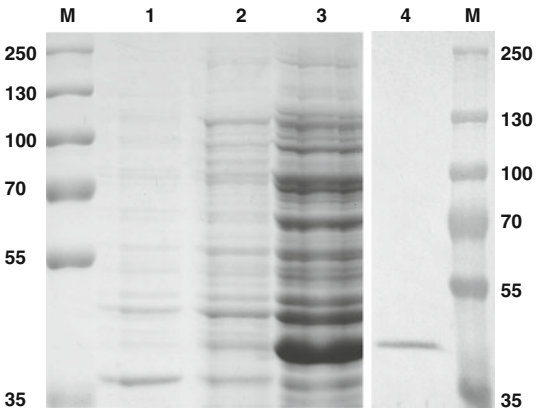

Fig. 1 Expression of recombinant MRCV P9-1 protein in bacterial cells visualized by coomassie brilliant blue staining. *M* Molecular mass markers, *1 E. coli* BL21(DE3) containing a pET-15b empty plasmid cultured in the presence of IPTG to induce expression, *2 E. coli* BL21(DE3) containing a pET-15b-MRCV S9 ORF-1 plasmid cultured in the absence of IPTG, *3 E. coli* BL21(DE3) containing a pET-15b-MRCV S9 ORF-1 plasmid cultured in the presence of IPTG to induce expression, *4* purified recombinant MRCV P9-1 protein

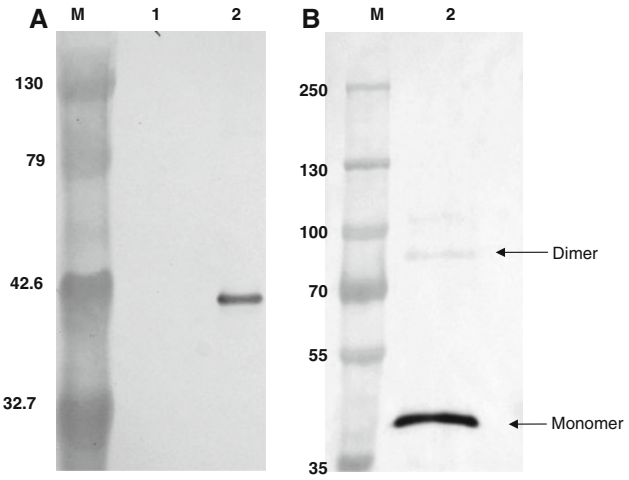

Fig. 2 **a** Detection of recombinant MRCV P9-1 protein by Western blot using specific antiserum (1:500 dilution). *M* Molecular mass markers, *1* total protein extract of *E. coli* BL21(DE3) transformed with empty plasmid, *2* purified recombinant MRCV P9-1 protein, **b** Detection of a dimer band when the purified recombinant MRCV P9-1 protein was not heated before SDS-PAGE

The raised MRCV P9-1 antiserum detected the recombinant protein by PTA-ELISA at all the dilutions tested (up to 1:512,000) while no reaction was observed against *E. coli* BL21(DE3) protein-purified extract (data not shown). The titer increased from the first to the third bleeding of rabbit, thereafter, no increasing signal was observed. By western blot, the antiserum against the complete purified recombinant MRCV P9-1 protein recognized the protein expressed in *E. coli* and did not show reaction with *E. coli* BL21(DE3) total protein extract (Fig. 2a). Furthermore, a band at approximately 80 kDa (twice the apparent molecular mass of MRCV P9-1 protein) was detected when the protein was not heated before SDS-PAGE, indicating that the protein might form dimers in in vitro as previously mentioned for RBSDV [32] (Fig. 2b).

Evaluation of the structural or non-structural status of MRCV P9-1

In order to elucidate the nature of the MRCV S9 ORF-1 encoded protein, the recombinant MRCV P9-1 protein and purified MRCV particles were separated by 8% SDS-PAGE and blotted on nitrocellulose membranes. Western blot analysis revealed that the antiserum against MRCV

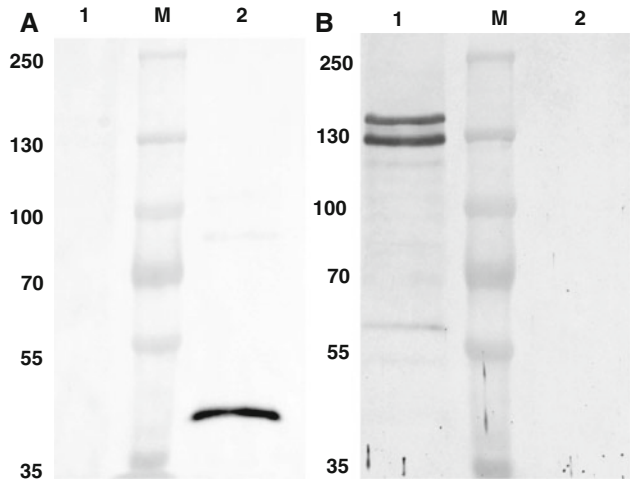

Fig. 3 Western blot analysis of the structural or non-structural status of MRCV P9-1 protein. **a** Antiserum against MRCV P9-1 protein (1:500). **b** MRCV-coat antiserum (1:250). *1* purified MRCV particles, *2* recombinant MRCV P9-1 protein, *M* molecular mass markers

P9-1 protein recognized only the recombinant MRCV P9-1 protein. No reaction was observed with proteins from purified MRCV particles. Conversely, the MRCV-coat antiserum (obtained through virus purification and rabbit immunization by Dr. M. Paz Giménez Pecci, IFFIVE-INTA) reacted with purified MRCV particles, but did not recognize recombinant MRCV P9-1 protein purified from *E. coli* (Fig. 3a, b).

MRCV has, according to del Vas et al. [14], five structural proteins, 170, 140, 130, 66, and 62 kDa. The comparison of the calculated molecular masses of the MRCV-predicted proteins coded by the different genome segments indicated that S1 codes for the largest protein (170 kDa) and S3 codes for a 140-kDa protein. Of the remaining genome segments, only MRCV S2 and S4 had coding capacity close to 130 kDa protein (134.39 and 131.67 kDa, respectively) while S8 and S10 code for a 66- and 62-kDa protein, respectively [14]. These observations suggest that the protein coded by MRCV S9 ORF-1 is smaller than the smallest structural protein (62 kDa) estimated for MRCV.

The MRCV-coat antiserum detected three of the five structural proteins reported by del Vas et al. [14] for MRCV, corresponding to 140, 130, and 62 kDa. In addition, this antiserum detected a forth structural protein of approximately 120 kDa that did not match any previously reported structural protein.

The antiserum against MRCV P9-1 protein recognized the recombinant MRCV P9-1 protein (40.82 kDa) and no reaction was observed against structural proteins of the virus. These results suggest that MRCV S9 ORF-1 encode a non-structural protein.

Detection of MRCV P9-1 in crude infected plant and insect extracts

The western blot analysis performed with the MRCV P9-1 antiserum recognized a 39-kDa protein (in accordance with the expected putative MRCV S9 ORF-1 coded protein) present in crude protein extracts from infected maize plants and viruliferous *D. kuscheli* insects (Fig. 4). No reaction was observed with proteins from healthy plant and insect controls (Fig. 4), thereby confirming that the MRCV P9-1 antiserum was able to specifically recognize the MRCV P9-1 protein.

Correlation between infected wheat plants and *D. kuscheli* insects

The transmission assay showed the presence of MRCV P9-1 protein in 16 of the 36 *D. kuscheli* specimens analyzed (7/18 males and 9/18 females). All the plants exposed to MRCV P9-1-positive insects developed MRCV symptoms about 1 month after inoculation while no symptoms were visible in plants exposed to insects that resulted negative for MRCV P9-1 protein. This total correlation reveals that the MRCV P9-1 protein normally occurs in infective insects.

Subcellular localization of MRCV P9-1 in plants and insects

As previously described by Arneodo et al. [2], the observation of ultrathin sections of infected wheat leaves showed electron-dense viroplasms associated with complete or incomplete virus particles and typical tubular

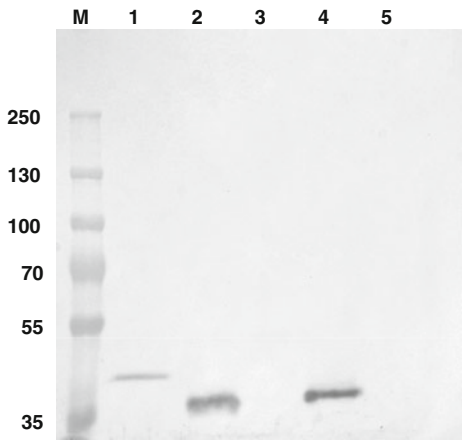

Fig. 4 Western blot detection of MRCV P9-1 protein in crude infected plant and insect extracts using specific antiserum (1:500 dilution). *M* molecular mass markers, *1* recombinant MRCV P9-1 protein (40.82 kDa), *2* MRCV-infected maize plant, *3* healthy maize plant, *4* MRCV-infected *D. kuscheli, 5* healthy *D. kuscheli*

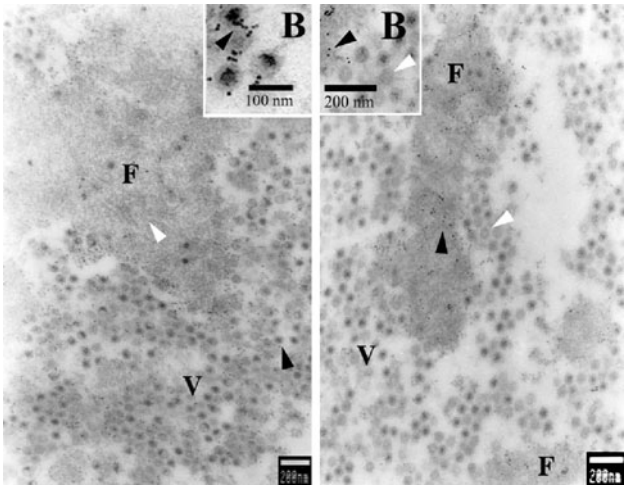

Fig. 5 Immunogold electron micrographs of ultrathin sections of MRCV-infected wheat cells probed with MRCV-coat antiserum (*left*) and antiserum against MRCV P9-1 protein (*right*). On the left, MRCV-coat antiserum is labeling only virus particles (V) (*black arrows*), while viroplasm fibrilar areas (F) remain unlabeled (*white arrow*). On the right, antiserum against MRCV P9-1 protein is showing the ultrastructural localization of MRCV P9-1 protein in viroplasm fibrilar areas (*black arrows*) within MRCV-infected phloem cells of wheat leaves. No gold labeling of virus particles is observed (*white arrow*). **b** Insets show reaction in more detail

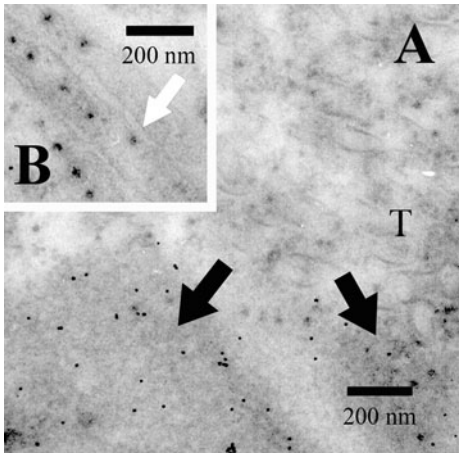

Fig. 6 **a** Immunogold electron micrograph showing the ultrastructural localization of MRCV P9-1 protein in electron-dense granular aggregates (*black arrows*) within a MRCV-infected *D. kuscheli* salivary gland cell. No labeling is observed in the surrounding transversally sectioned tubular structures (T). **b** Inset shows tubules in longitudinal section. Note the presence of virus particles inside, also unlabeled (*white arrow*)

structures of reovirus, exclusively found in the cytoplasm of phloem cells. Complete particles about 60–70 nm in diameter have a dark core surrounded by a less contrasted region while incomplete particles are nucleic acid-free inner capsids [2, 4].

Immunogold labeling of MRCV-infected phloem cells of wheat leaves with MRCV P9-1 antiserum localized the MRCV P9-1 protein associated exclusively to the electron-dense granular viroplasms and no gold labeling of virus particles was observed, also in the photo is possible to evaluate antisera reactions with the specific target and the background too (Fig. 5, right). Absence of labeling occurred in healthy plant cells (data not shown).

Viruliferous *D. kuscheli* head sections showed the presence of viroplasms, tubules, and viral particles (frequently accumulated in paracristalline arrays) inside the insect salivary glands. The presence of viroplasms, at least in the infected salivary gland cells, supports the statement of MRCV replication in *D. kuscheli*, in agreement with earlier approaches revealing an increase in viral concentration some days after virus ingestion [26]. Immunolabeling with MRCV P9-1 antiserum localized the MRCV P9-1 protein in MRCV-infected *D. kuscheli* salivary glands associated with the electron-dense granular viroplasms (Fig. 6). The antiserum did not react with healthy insect cells (data not shown).

On the other hand, the MRCV-coat antiserum reacted only with virus particles scattered through the cytoplasm of plant and insect cells, while viroplasm fibrilar areas remain unlabeled (Fig. 5, left).

In previous studies, Zhang et al. [32] reported that RBSDV P9-1 is a thermostable α-helical protein and has an intrinsic ability to self-interact and form homodimers in vitro and in vivo. Furthermore, it is the minimal viral component required for viroplasm formation and plays an important role in the early stages of the virus life cycle by forming intracellular viroplasms. Our assays have shown the localization of MRCV P9-1 protein exclusively in electron-dense granular viroplasms, within the cytoplasm of infected plants and insects cells suggesting that this protein, like some non-structural proteins of other viruses from the family *Reoviridae*, might be involved in the replication and particles assembly process. In addition, the putative protein encoded by MRCV S9 ORF-1 has several phosphorylation susceptible residues [11]. Protein phosphorylation is an important mechanism that controls a large number of intracellular processes. In the case of RNA viruses, protein phosphorylation has been shown to regulate virus transcription and replication, RNA binding activity, viroplasm formation, and virus assembly [16], as previously mentioned for *Rice dwarf virus* (RDV) [33] and *Bluetongue virus* (BTV) [34]. Therefore, it is essential to elucidate the functions of all MRCV viral proteins, either structural or non-structural, to gain insights into the molecular biology of this virus, and thus, control the disease.

Acknowledgments We are very grateful to Dr. Claudia Nome for helpful advice in immunoelectron microscopy, and Dr. Liliana Di Feo for guide in virus purification. We also thank Claudio Nikolaus who

collaborated in UV chamber construction. This study was supported by the projects FONCyT 08-04416; CABBIO 00006 and Agencia Córdoba Ciencia SE. JDA is career member of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). ABSP hold a Doctoral Fellowship of CONICET and Ministerio de Ciencia y Tecnología de Córdoba, Argentina. AVL is professional member of CONICET.

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