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Mal de Río Cuarto virus (MRCV) genomic segment S3 codes for the major core capsid protein

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Abstract *Mal de Río Cuarto virus* (MRCV) is a newly described species of the genus *Fijivirus*, family *Reoviridae*. Compared with other plant-infecting genus of the family, the function and localization of MRCV and other *Fijivirus* proteins are poorly understood. Through analysis of viral particle purifications, we positively identified five structural proteins of approximately 170, 140, 130, 66, and 62 kDa. The protein encoded by MRCV S3 genomic segment was expressed as a fusion protein in *Escherichia coli*, purified and used for rabbit immunization. The resulting antiserum reacted with the 140 kDa structural protein and with incomplete virus particles in immunoelectron microscopy assays, suggesting that MRCV S3 codes for the major core capsid protein.

Keywords MRCV · *Fijivirus* · Maize · Structural virus proteins · Core protein

Introduction

Mal de Río Cuarto virus (MRCV) is a member of the genus *Fijivirus* within the family *Reoviridae* that causes the most important maize disease in Argentina [1] which is the second largest worldwide maize exporter. Other *Fijivirus* are also important maize pathogens in several Mediterranean countries and in Far East Asia [1]. MRCV is transmitted by the planthopper *Delphacodes kuscheli* [2] and is able to multiply in phloem tissue nucleated cells of several monocotyledonous plants [3, 4]. *Fijivirus* are classified into five groups [1]. MRCV is included into Group 2 along with *Rice black streaked dwarf virus* (RBSDV) and *Maize rough dwarf virus* (MRDV). The *Reoviridae* family includes two other genera able to infect plants and delphacids besides *Fijivirus*: *Phytoreovirus* and *Oryzavirus*. *Rice dwarf virus* (RDV) is the *Phytoreovirus* type member and is the most extensively studied plant virus within this family [5–10]. *Fijivirus* form icosahedral double-shelled particles containing 10 genomic double-stranded RNA segments (S1-S10) and RNA-dependent RNA polymerase. The outer shell and short surface A-spikes easily break down giving rise to an inner capsid (core) bearing 12 type B-spikes [11]. Twelve putatively encoded proteins (named P1-P6, P7.1, P7.2, P8, P9.1, P9.2 and P10) have been deduced from MRCV S1-S10 nucleotide sequences [12–15]. Electron microscopy from MRCV-infected maize, wheat, and barley leaves revealed electron-dense viroplasms associated with complete or incomplete virus particles in the phloem tissue. Complete particles are about 65 nm in diameter and have a dark core surrounded by a less contrasted region. Incomplete particles (or "empty" inner capsids not filled with nucleic acids) form large masses [16, 17]. Unlike plant-infecting viruses of other genus of the *Reoviridae* family, very little is known about the function and localization of *Fijivirus* structural and nonstructural proteins [18–24]. This paper reports the identification of the MRCV structural proteins, the assignment of one of them to MRCV S3 and the localization of MRCV P3 to incomplete virus particles.

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Materials and methods

Cloning of MRCV S3 complete ORF

MRCV dsRNA genome was extracted directly from infected maize plants (3 g of roots) by differential adsorption to CF11 cellulose [25]. cDNA was synthesized by reverse transcription of 3 μg of total purified dsRNA using random primers (Superscript II, Invitrogen). Amplification of the entire MRCV S3 ORF was done using Platinum Pfx DNA Polymerase (Invitrogen, USA) and primers pS3 up (5′-CGGAGGATAATCGGAAAAAAGAA-3′, nt 54-76) and pS3 low (5′-TTAAATCAGAGACGAAACTCTAATGT-3′, nt 3727-3702), based on sequence Acc AF499928. The amplified product was ligated into pGem-T easy vector (Invitrogen, USA), entirely sequenced using an ABI 3730 XL automated sequencer and finally subcloned into pRSET expression vector (Invitrogen, USA).

Expression and purification of pRSET S3 fusion protein

MRCV P3 fusion protein was expressed from a pRSET construct in *E. coli* BL21 pLys cells by using the procedures described by Sambrook et al. [26]. Expressed protein was purified with ProBond^{TM} nickel resin (Invitrogen, USA) according to the manufacturer's recommendations. Briefly, cell lysates were prepared under denaturing conditions using Tris-phosphate buffer containing 8 M urea and recombinant MRCV P3 protein was purified by Ni-affinity column chromatography and eluted with 200 mM imidazole.

Antiserum preparation

Polyclonal antibodies to the complete MRCV P3 fusion protein were raised in a 12-week-old Japanese female white rabbit. One milliliter of the purified protein (1 mg/ml) was emulsified with an equal volume of complete (for the first injection) or incomplete (for the subsequent four injections) Freund's adjuvant and used for intramuscular and subcutaneous injection. The injections were applied 2 weeks apart from each other. Antiserum was collected 10 days after the final inoculation and tested by western blot analysis during the immunization scheme.

Purification of MRCV particles from infected maize plants

The MRCV particles were purified from MRCV-infected roots using the method described by Isogai et al. [22] with minor modifications. All procedures were performed in a cold room at 4°C or in ice. About 80 g of fresh infected maize plants were ground with a mortar and pestle in liquid nitrogen and resuspended in 35 ml phosphate buffer (0.1 M Na_2HPO_4, 0.1 M KH_2P0_4 pH 5.8). The homogenate was further centrifuged at 1,500×*g* for 5 min and filtered through filter paper. After addition of Triton X-100 to a final concentration of 3% (v/v), the mixture was stirred for 1 h and then centrifuged at 1,500×*g* for 30 min. The supernatant was centrifuged at 75,000×*g* for 90 min through 40% sucrose (w/v). The pellet was gently resuspended in 5 ml phosphate buffer and centrifuged at 3,500×*g* for 5 min. The supernatant was loaded onto a 10–50% sucrose density gradient and centrifuged at 87,000×*g* for 90 min. The gradient was fractionated using a vacuum pump and MRCV particles were concentrated by centrifugation at 100,000×*g* for 2 h and resuspended in 80 μl of PBS.

Structural MRCV proteins were analyzed by 7.5% SDS-PAGE [27] and stained with silver [26]. The proteins molecular masses were calculated using Gel Compar, Version 4.1 software (Applied Maths, Belgium).

Preparation of plant protein samples

Leaves from healthy and MRCV-infected maize plants were homogenized in liquid nitrogen and proteins were extracted in a buffer containing 50 mM Tris–HCL, pH 8, 0.02% SDS, 1% 2-mercaptoethanol, and 2 mM phenylmethylsulphonylfluoride (PMSF) (2 ml/per gram of tissue). Total proteins were quantified by Bradford's method [28] using a Bio-Rad protein assay kit (Bio-Rad Laboratories, USA).

Western blot analysis

All samples were analyzed by SDS-PAGE [27]. Acrylamide concentration was appropriately adjusted according to the protein size. Following electrophoresis, proteins were transferred to nitrocellulose membranes using a mini trans-blot cell (Bio-Rad Laboratories, USA) in buffer containing 25 mM Tris pH 8.3, 192 mM glycine, 20% methanol, and 0.1% SDS. The blotted proteins were developed using proper dilutions of anti-Xpress^{TM} (Invitrogen, USA) or polyclonal antibodies against recombinant MRCV P3 protein. Bound antibodies were detected with phosphatase conjugated goat anti-rabbit IgG and anti-mouse IgG (Accurate, USA) 1/3000 as described by Blake et al. [29].

Immunoelectron microscopy

Leaves from healthy and MRCV-infected maize plants were fixed in a mixture of 1.5% glutaraldehyde, 2.5% paraformaldehyde in 0.5 M phosphate buffer, pH 7 for 2 h. Each fixed sample was dehydrated in a graded series of

ethanol (from 50 to 100%) and embedded in LR White™/gold™ resin (SPI Supplies®, USA) at 4°C for 12 h. Ultrathin sections were cut with a diamond knife and mounted on meshed nickel grids (Electron Microscopy Science, USA). Specific antiserum against MRCV P3 was pre-absorbed with total leaves-protein extracts from healthy maize plants (5 µg). Samples were incubated with antiserum and immunolabelled with gold (10 nm)-conjugated protein A (Sigma-Aldrich, USA) at a dilution of 1:20. Samples were subsequently negatively stained with 2% uranyl acetate for observation in a Zeiss EM10 transmission electron microscope.

Results

Expression of full-length MRCV P3 in bacteria and obtention of a polyclonal antiserum

MRCV S3 (3826 bp) contains a single ORF that theoretically codes for a highly conserved putative 141.66 kDa protein [13]. To study and assign the MRCV protein coded by S3, the entire MRCV S3 ORF was cloned into pRSET A. Following IPTG induction of *E. coli* BL21 pLys cells, cell lysates were resolved on a 6% SDS-PAGE and stained with Coomassie brilliant blue (Fig. 1a). A band corresponding to the expected size was observed in the induced cell lysates (Fig. 1a, compare lanes 2 and 3). On the other hand, no bands were differentially detected in cell lysates containing an empty plasmid (Fig. 1a, lane 1). Next, recombinant MRCV P3 protein was successfully purified using ProBond™ nickel resin (Fig. 1a, lane 4) and this band was recognized by commercial anti-Xpress antiserum (Fig. 1a, lane 5). Taken together these results show that full-length MRCV P3 protein was expressed as a bacterially His-tagged protein.

Polyclonal antibodies against complete purified MRCV P3 recombinant protein, obtained in Japanese white rabbit and probed against the same purified MRCV P3 protein, recognized the His-tagged protein expressed in *E. coli* while the pre-immune antiserum did not show cross reaction (Fig. 1b, lanes 2 and 1, respectably).

Purification and assignment of MRCV structural proteins

In order to determine the number and size of MRCV structural proteins, MRCV particles were purified from MRCV-infected roots. A low pH buffer was used to favor particle stabilization [22]. Proteins were separated by 7.5% SDS-PAGE [27] and stained with silver. Bands of apparent molecular masses of 170, 140, 130, 66, and 62 kDa were detected (Fig. 2a, lane 1), thus suggesting that the virions

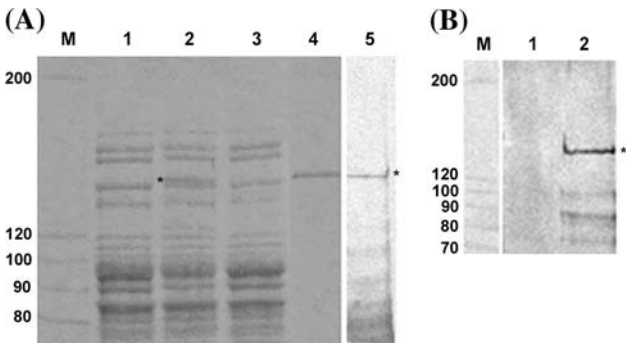

Fig. 1 **a** Bacterial expression of full-length recombinant MRCV P3 protein. *E. coli* BL21 pLys cells harboring pRSET-P3 were cultured in the presence (*lane 2*) or absence (*lane 3*) of IPTG to induce expression. Cell lysates were analyzed by 6% SDS-PAGE followed by Coomassie blue staining. Cell lysates transformed with a pRSET empty plasmid were used as a negative control (*lane 1*). Recombinant MRCV P3 protein was purified by Ni-affinity column chromatography. The fraction containing MRCV P3 protein was analyzed by SDS-PAGE and visualized by Coomassie Brilliant Blue staining (*lane 4*) or by western blot analysis with anti-Xpress antibody (1/1000 dilution) (*lane 5*). **b** IPTG-induced cell lysates expressing pRSET-P3 from **a** were resolved by 6% SDS-PAGE followed by western blot analysis using polyclonal anti-MRCV P3 antibodies (1:250 dilution) as a primary label (*lane 2*) or pre-immune antiserum at a dilution of 1:100 (*lane 1*). In **a** and **b**, M: The numbers indicate the location of molecular mass markers in kDa. The position of MRCV P3 is denoted by an asterisk

were formed by at least five structural proteins. To discard possible contaminations with maize proteins, an identical procedure was applied to roots from healthy maize plants. No proteins were detected in this case after silver staining (Fig. 2a, lane 2).

Immunological detection of MRCV P3 in purified virus particles and infected maize plants

Polyclonal antibodies against complete recombinant MRCV P3 were probed against MRCV-purified particles in western blot analysis. Antibodies to MRCV P3 reacted with the 140 kDa structural protein (Fig. 2a, lane 4). This is in accordance with the expected putative MRCV S3 coded protein [13]. No reaction was observed after incubation with pre-immune antiserum as a primary label (Fig. 2a, lane 3).

Next, total protein extracts from healthy and MRCV-infected maize plants were extracted, separated in polyacrylamide gels and blotted on nitrocellulose membranes. Western blot analysis revealed that the antiserum against MRCV P3 recognized a 140-kDa protein specifically present in infected maize plants (Fig. 2b, lane 2). No reaction was observed with proteins from a control healthy plant (Fig. 2b, lane 1) thereby confirming that the polyclonal anti-MRCV P3 antiserum was able to specifically recognize the viral protein. This polyclonal antiserum was

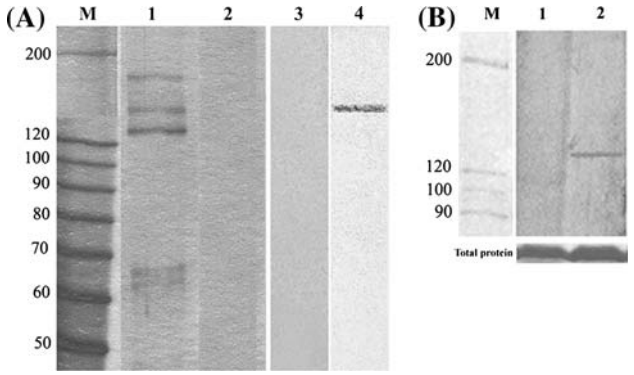

Fig. 2 Detection of MRCV structural proteins by silver staining and western blotting. **a** Structural MRCV proteins obtained after sucrose density gradient ultracentrifugation from infected plants were separated by 7.5% SDS-PAGE and stained with silver (*lane 1*) or electroblotted on nitrocellulose membranes (*lanes 3 and 4*). Lane 2, total proteins obtained after the same purification procedure from roots of healthy maize plants stained with silver. Lanes 3 and 4, immunodetection of MRCV structural proteins using polyclonal antiserum to MRCV P3 as a primary label at a dilution of 1:250 (*lane 4*) or pre-immune antiserum as a primary label at a dilution of 1:100 (*lane 3*). **b** 5 μg of total proteins extracted from maize healthy (*lane 1*) or MRCV-infected (*lane 2*) leaves were resolved in 6% SDS-PAGE, electroblotted on nitrocellulose membrane and probed with antiserum against MRCV P3 (1:250 dilution). Total proteins stained with Ponceau S after electroblotting are shown below. M: The numbers indicate the location of molecular mass markers in kDa. The molecular masses of MRCV structural proteins were calculated using Gel Compar, Version 4.1 software (Applied Maths, Belgium)

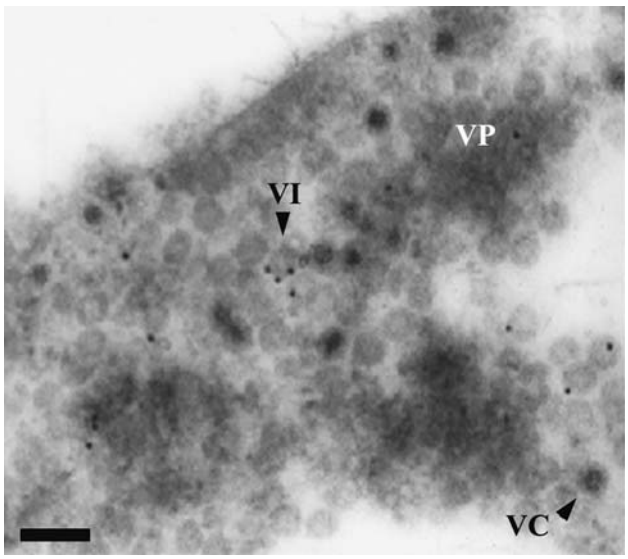

Fig. 3 Immunogold labelling of MRCV P3 in sections of MRCV-infected phloem parenchyma cells of maize leaves. *VP* viroplasm, *VC* complete virion, *VI* incomplete virion; Bar, 100 nm. Specific antiserum to MRCV P3 was pre-absorbed with 5 μg of total leaves-protein extracts from healthy maize plants. Antiserum to MRCV P3 was used at a dilution of 1:10. Immunolabelling was done with gold (10 nm)-conjugated protein A (Sigma-Aldrich, USA) at a dilution of 1:20. No gold labelling was observed in cells from healthy plants or in controls without MRCV-specific antiserum (data not shown)

subsequently used in immunolectron microscopy analysis for the intracellular localization of P3 in MRCV-infected maize cells.

Immunogold labelling of MRCV P3 in infected maize cells

The subcellular localization of MRCV P3 was examined by immunogold labelling of infected maize leaf sections. We observed a great amount of incomplete particles (Fig. 3). Antiserum to MRCV P3 solely reacted with incomplete virions (Fig. 3), suggesting that MRCV P3 was the major core protein. No labelling was detected in cells from healthy plants after incubation with MRCV P3 antiserum and then immunolabelled, and in healthy or infected plant cells after incubation with gold-conjugated protein A without a primary antiserum (data not shown).

Discussion

Unlike plant-infecting viruses of other genus of the *Reoviridae* family, very little is known about the function and localization of *Fijivirus*-coded proteins. Characterization of MRCV proteins could help to understand not only their function in this particular virus species but also in other *Fijivirus*, and compare them with those described for well-known members of other *Reoviridae* genus such as RDV (*Phytoreovirus*).

In this report, we were able to successfully express the full-length MRCV P3 as a His-tag fusion protein and use it for the production of a polyclonal antiserum. This enabled us to ascertain the presence and precise localization of MRCV P3 in infected plants and purified virus particles by western blot analysis and inmunoelectron microscopy (Figs. 2a and 3).

Upon virus purification and SDS-PAGE followed by silver staining, we detected five MRCV structural proteins of approximately 170, 140, 130, 66, and 62 kDa. The observed pattern resembled that of RBSDV structural proteins (148, 130, 120, 65, and 56 kDa) [22], and slightly differed from that of *Nilaparvata lugens reovirus* (NLRV, *Fijivirus*) (three major structural proteins of 140, 135, and 65 kDa, three intermediate of 160, 110, and 75 kDa and one minor of 120 kDa) [20]. RBSDV is closely related to MRCV. When aligning both genomes MRCV P3 is the most conserved protein and has 84.5% homology to RBSDV S3 coded protein [12–15]. The 140 kDa protein was recognized by an antiserum raised against MRCV P3 in virus purification preparations (Fig. 2a, lane 4) or in infected maize plants (Fig. 2b, lane 2) and detected on incomplete virus particles (Fig. 3), suggesting that MRCV

P3 is a component of the core since it is not exposed in complete virus particles [11]. Accordingly, MRCV P3 is 22.4% homologous to NLRV P3 major core structural protein [12, 20] and capable of dimerizing in bacteria (Distéfano, unpublished result) probably due to the presence of a coiled-coil motif [12]. Taken together, these data indicated that MRCV P3 is the major core capsid protein. While performing immunolocalizations in MRCV-infected maize leaves, we observed a great amount of incomplete particles ("empty"). This was also observed in MRDV and *Pangola stunt virus* (PaSV, *Fijivirus, Reoviridae*)-infected plants, and could be due to an over production of inner capsids (cores) that do not mature but remain within the viroplasm or to breakdown products of the virions present in older infections [30, 31].

Based on the molecular masses of the deduced MRCV proteins and the particle structure of *Fijivirus*, the 170 kDa protein (Fig. 2a, lane 1) was assigned to segment S1 (coding capacity of 168.4 kDa) because no other segment has such a large coding capacity. We have previously shown that deduced MRCV P1 contains RNA-dependent RNA polymerases (RdRps) distinctive sequence motifs [13]. MRCV S2 and S4 have coding capacities of 134.39 and 131.67 kDa, respectively. As a consequence, theoretically any of them could code for the 130 kDa structural protein since no other segment has such a high coding capacity. Most probably the 130 kDa protein corresponds to the B-spike since these types of spikes are relatively more stable than A-spikes which are readily lost upon purification [11]. Interestingly, MRCV P4 showed 20.74% identity with S4-encoded VP3 protein of *Bombix mori cytoplasmic polyhedrosis virus* (BmCPV, *Cypovirus* genus) [13] which is an outer component of the virion [32]. Presumably the 66 and 62 kDa structural proteins (Fig. 2a, lane 1) correspond to a minor component of the capsid coded by MRCV S8 (coding capacity of 68.26 kDa) and the major outer capsid protein coded by MRCV S10 (coding capacity of 63.5 kDa), respectively. The differences between expected and observed molecular masses could be attributed to the amount of SDS bound to the proteins [33]. The relatively low amount of the 62 kDa protein in the purified MRCV-particle preparations probably reflects the fact that purified virus particles are unstable and easily break down giving rise to subviral particles (SVPs) without the outer capsid [11, 34].

In conclusion, our work characterized MRCV structural proteins, and assigned one of them to the S3 genomic segment. Immunoelectron microscopy localization suggested that MRCV P3 is a component of the inner capsid, presumably the major core protein. The data generated provide a starting point for further study on the functions of MRCV-coded proteins and to establish the molecular mechanisms underling important biological process such as viral replication, packaging, and movement within the plant.

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