



# Molecular analysis of a Spanish isolate of chili pepper mild mottle virus and evaluation of seed transmission and resistance genes

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**Abstract** An isolate of chili pepper mild mottle virus (CPMMV-Sp; GenBank OQ920979) with a 99% identity to CPMMV (GenBank MN164455.1) was found in symptomatic pepper plants in Spain. RACE analysis, performed using a stem-loop primer developed in this study to prime at the end of the introduced poly(A)/(U) tail, revealed the presence of an extra 22 nt at the 5' end, starting with a cytosine, which were essential to generate infectious clones. However, the 5' terminal cytosine was dispensable for initiating the infection. The design of two specific digoxigenin riboprobes targeting the more divergent area of CPMMV-Sp,

compared to the closely related bell pepper mottle virus (BPeMV) (identity percentage of 80.6% and 75.8%, respectively), showed that both probes specifically detected CPMMV-Sp when the hybridization was performed at 68°C and 60°C, respectively. However, the BPeMV probe, targeting a region with an 89.4% identity percentage to CPMMV-Sp, showed cross-hybridization at 60°C but not at 68°C. The comparison of the detection limits between molecular hybridization and RT-PCR techniques revealed that the former was 125 times less sensitive than RT-PCR. The analysis of the vertical transmission of CPMMV-Sp using seeds from naturally or mechanically infected pepper plants revealed a transmission percentage ranging from 0.9% to 8.5%. Finally, the analysis of the resistance of capsicum species carrying different alleles of the L gene (L1, L2, L3, and L4) revealed that varieties with the L1 gene were infected by CPMMV-Sp (20–40% of

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Clara Ontañón and Mikel Ojinaga Both authors have contributed equally to the study and are considered as co-first authors independently.

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inoculated plants), while varieties with the L2, L3, and L4 genes were resistant.

**Keywords** Tobamovirus · Non-radioactive molecular hybridization · Seed transmission · Infectious clones · L resistance genes

## Introduction

Hot and sweet peppers (*Capsicum spp.*) are important spice and vegetable crops worldwide (Gniffke et al., 2013), currently with a production of thirty-eight million tonnes (<http://www.fao.org/faostat/en/?#data/QC/visualize>). There are five domesticated species of the genus *Capsicum* (*C. annum*, *C. frutescens*, *C. chinense*, *C. baccatum*, *C. pubescens*) which are widely cultivated commercially worldwide (Kenyon et al., 2014). Some of the main threats to pepper production are pests and diseases that compromise the yield and quality of cultivated vegetables. Diseases caused by viruses are the main limiting factor in pepper cultivation in the world, affecting plant growth and fructification (Kenyon et al., 2014; Moury & Verdin, 2012). At least forty-nine species of viruses capable of infecting *C. annum* have been described, among them twenty can cause damage to these crops (Moury & Verdin, 2012). Insect-transmitted viruses include those from the *Potyvirus*es, *Cucumovirus*es, *Orthophotospovirus*es, and *Begomovirus*es genera. However, the most prevalent viruses that infect pepper are those transmitted mechanically and by pollen such as those belonging to the *Tobamovirus* genus (Gullino et al., 2020).

The genus *Tobamovirus* (*Virgaviridae* family), currently encompasses 37 accepted species, including the type species *Tobacco mosaic virus* (TMV) (Walker et al., 2022). Their genome consists of a linear positive -sense single stranded RNA of 6.3–6.6 kb in size with a 5'-terminal cap and 3'-tRNA-like structures (Ishibashi & Ishikawa, 2016; Moury & Verdin, 2012). The genomic RNA encodes four known proteins: two non-structural proteins involved in viral RNA replication, a movement protein (MP) required for cell-to-cell and long-distance movement, and the only structural protein or coat protein (CP), which is not required for cell-to-cell movement, but plays a role in vascular tissue dependent virus accumulation. Both the MP and CP are expressed from individual 3' co-terminal subgenomic mRNAs.

Tobamoviruses infect vegetable crops mostly solanaceous plants such as *Solanum lycopersicum* and *Capsicum spp.*, also cucurbitaceous plants, ornamental plants, weeds, and medicinal plants (Ghodoum & Keshavarz-Tohid, 2020; Lee et al., 2020; Reingold et al., 2016; Salem et al., 2022). Among species of tobamoviruses that infect peppers, tobacco mosaic virus (TMV), tomato mosaic virus (ToMV), paprika mild mottle virus (PaMMV) and pepper mild mottle virus (PMMoV) predominantly affect pepper crops in South Europe (Gniffke et al., 2013), with infections reaching up to 100% of the plants and causing a drastic reduction in the yield of marketable fruit (Gullino et al., 2020). Tobamoviruses induce leaf chlorotic mosaic or mottling, leaf distortion and surface reduction, and irregular shapes and colors associated with a reduction of fruit size. Necrosis can also be observed on leaves and fruits (Moury & Verdin, 2012). However, sometime symptoms may not be visually detected due to asymptomatic infections or when plants exhibit virus-like symptoms as a response to adverse environmental conditions, nutritional imbalances, infection by other types of pathogens, damage caused by pests or abiotic agents and others (Jeong et al., 2014; Van Der Want & Dijkstra, 2006). Tobamovirus are seed-borne and mechanically transmitted stable viruses.

Approximately 18–20% of plant viruses are transmitted by seed (Hull, 2002; Singh & Mathur, 2004). Seed transmission of viruses can be classified into two main types. The first one is external contamination, as seen in tobamoviruses, which primarily infect external tissues of the seed such as seed coat, perisperm, or endothelium, allowing seedling infection during the germination process, but they do not usually infect the embryo or endosperm (Genda et al., 2011; Dombrovsky et al., 2017). The Second one is internal transmission, which occurs mainly through embryos infected by the paternal or maternal pathways, transmitting viruses to the offspring very efficiently (Dombrovsky et al., 2017; Sastry, 2013). Therefore, only a few of the seed-borne viruses may be carried as surface contaminants, as tobamoviruses, while the remainder are carried through embryo considered as true seed-borne viruses (Bhat and Rao, 2020). Seed transmission is an effective survival strategy for plant viruses, especially for those viruses with narrow host ranges and infecting annual plant species (Sandra et al., 2020). These viruses can be dispersed over long distances with seed trade (Genda et al., 2005; Hull, 2002) and survive within the seed as long as they remain viable (Sastry, 2013). Different groups

of viruses show different rates of seed transmission. Tobamoviruses usually show seed transmission rates of 1–20%, while potyviruses have reported seed transmissions of 1–80%. Although the rate of transmission from seed to seedling is usually low in tobamoviruses, these sources of infection are sufficient to subsequently spread by mechanical contact and generate an epidemic in the crop (Dombrovsky et al., 2017).

Resistance to tobamoviruses is regulated by a single monogenic resistance gene in pepper (Di Dato et al., 2015). Within the genus *Capsicum*, there are several species and varieties that carry the L genes, which confer resistance to tobamovirus through four allelic genes (L1, L2, L3 and L4) (Tomita et al., 2008, 2011). There are various tobamovirus pathotypes (P0, P1, P1,2 or P1,2,3), which are classified on their capacity to overcome the resistance conferred by the corresponding L gene. Consequently, viruses with the P0 pathotype cannot infect plants carrying any L gene, meanwhile viruses with the P1, P1,2 or P1,2,3 pathotypes can infect plants with the L1 gene, L1 and L2 genes, and L1, L2 and L3 genes, respectively (Genda et al., 2007).

The starting point of the present work was the presence of symptomatic pepper leaves that rendered negative ELISA results to the main viruses affecting pepper crop but a positive result by RT-PCR using universal tobamovirus primers. Determination of full-length nucleotide (nt) sequence showed that the new virus should be considered an isolate (99% nt identity) of the recently identified chili pepper mild mottle virus (GenBank n<sup>o</sup>: MN164455.1), except for the presence of 22 nucleotides (nt) extra at the 5' termini. In the present study, we have analyzed different biological properties of this new virus including the generation of infectious clones and the role of the extra nt at the 5' termini, the seed transmission rate, and the capacity to infect pepper plants harboring different resistance L alleles. Also, we developed a molecular detection method based on molecular hybridization and analyzed its capacity to discriminate between viruses showing identity percentages higher than 89%.

## Materials and methods

### Plant material, RNA extraction and purification of double strand RNA

Samples of plants with symptoms of dwarfism, light green mosaic and leaf blistering, as well as shortened

and deformed fruits were collected in 2018 and 2019 from pepper plants (cv. Padrón and Gernika) cultivated in Granada and Bizkaia (Spain), respectively. Bell pepper mottle virus (BPemV) infected *Nicotiana clevelandii* tissue was obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (DSMZ).

For total RNA extraction, 0.1 g of leaf was frozen with nitrogen, ground in a mortar and subjected to RNA extraction using 1 ml of TRIzol reagent (ThermoFisher Scientific Inc.) following the manufacturer's instructions. The extracted RNA was then resuspended in 50 µl of sterile water and stored at -80°C until use. Alternatively, samples were extracted using a fast extraction protocol in which 1 g of tissue was homogenized with 3 volumes of fast extraction buffer and applied (1 µl) directly onto nylon membranes (Boehringer Mannheim, Mannheim, Germany) (Sánchez-Navarro et al., 1999).

For the extraction of dsRNA, 3.5 g of leaf samples were immediately frozen in liquid nitrogen and mixed for 30 min with 10 ml buffer STE 1X (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH: 8.0), 1 ml SDS 10%, 500 µl of bentonite 2% and 9 ml phenol-STE1 X. The solution was centrifuged and the aqueous phase was separated and precipitated with ethanol 96% and 2.5% CF-11 cellulose. Following agitation and centrifugation, the precipitate was washed with 20 ml of STE 1X (16% ethanol) and resuspended in nuclease-free water and incubated 70°C 4 min. The supernatant obtained was stored at -80 °C.

### Amplification of new tobamovirus fragments by RT-PCR

cDNA was synthesized by Reverse Transcription Polymerase chain reaction (RT-PCR) assay, using the kit SuperScript ® III HiFi OneStep RT-PCR System-Platinum Taq DNA polymerase (Invitrogen Thermo Fisher Scientific) following the manufacturer's instructions. Molecular detection of tobamovirus was carried out with universal primer pairs (Li et al., 2018). RT-PCR parameters for the primers used in this study were as follows: initial cDNA synthesis of 50°C for 30 min, followed by one cycle of 94°C for 2 min for denaturation RT enzyme and polymerase activation. Subsequently, 35 cycles of 94°C for 15s, 55°C for 15s and 68°C for 1 min/kb were included. The cDNA product generated by

RT-PCR was used as template for a second PCR using PrimeSTAR (Takara Bio Inc.) polymerase.

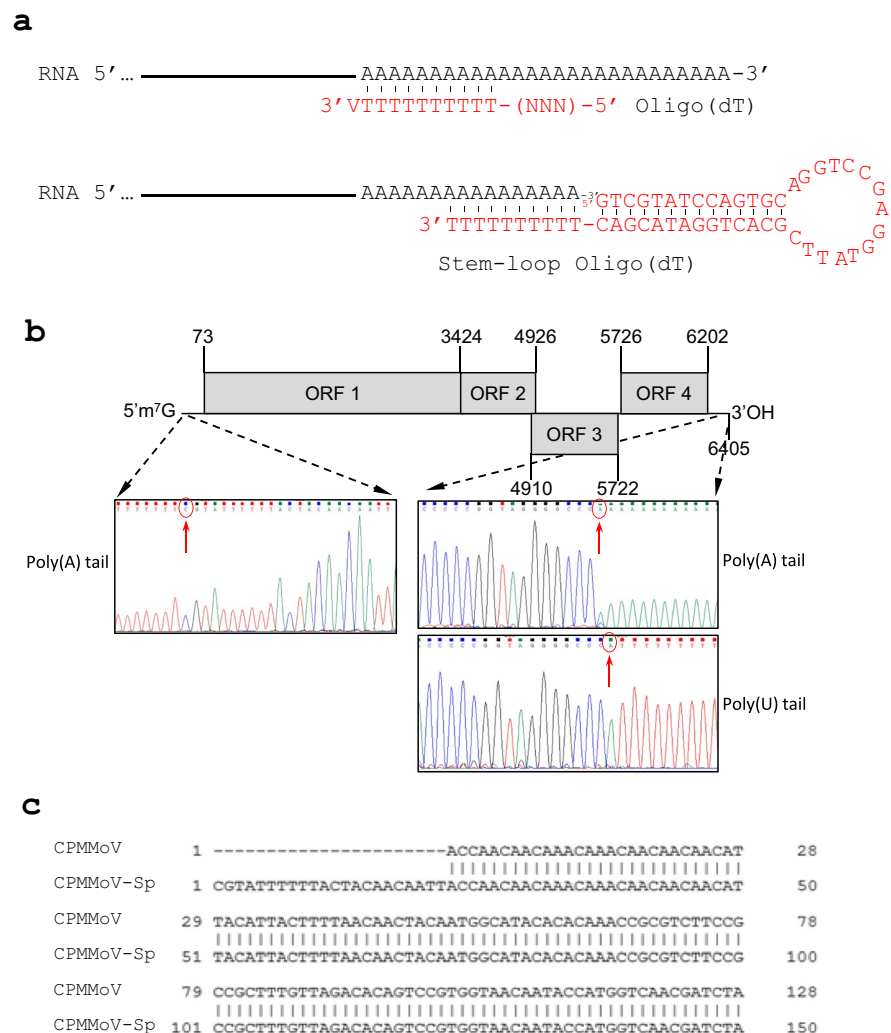
### Amplification of 3' and 5' ends of the viral genome

The 5' and 3' nucleotide sequence ends of the viral genome were determined by RACE (Yeku & Frohman, 2011) using modified primers designed to hybridize at the end of the poly(A) or poly(U) tails, respectively (Fig. 1a). The standard RACE primers have a 3' degenerate nucleotide to allow the hybridization at the first nt of the tail, including the first (5' end) or the last (3' end) viral nucleotide. First, dsRNA samples were incubated in two different reactions in order to introduce a poly(A) or a poly(U) tails, by using the polymerase polyA (polyA Polymerase,

Yeast -Affymetrix-). To determine the nucleotide sequence of the 3' end, the RNAs carrying the poly(A) or poly(U) tails, were then subjected to two RT-PCR amplifications using the sense primer 3337 and the antisense primers 2721-T or 2721-A (Supplementary Table S1). The 2721-T and 2721-A primers contains a secondary structure designed to hybridize at the 3' termini of the poly(A) or poly(T) RNA tails, respectively (Fig. 1a). Amplification of the 5' end was carried out using a similar approach designed for the 3' end but only poli adenylated RNAs were used as template. The RT-PCR and subsequent PCR reactions were performed with the 2721-T/3345 and 2721-T/3346 pair of primers, respectively.

PCR Amplified DNA fragments were eluted from the agarose gels by using the commercial Kit Thermo

**Fig. 1** Genomic organization of CPMMoV-Sp (GenBank: OQ920979) and characterization of the 5' and 3' terminal sequences. **a)** Schematic representation of the Oligo(dT) primer used in the standard RACE analysis, which carries a degenerate 'V' nucleotide (C, A, or G) at the 3' terminus, and the stem-loop Oligo(dT) designed in this study which anneals at the end of the poly(A) tail. **b)** CPMMoV-Sp genome organization. Numbers indicate the first and last nucleotide of each Open Reading frame (ORF) and the complete genome. Chromatogram obtained during the RACE analysis of the 5' and 3' termini using a poly(A) or poly(U) tails are shown. **c)** Alignment of the 150 nt 5' termini obtained for the CPMMoV-Sp isolate and the CPMMoV present at the database (GenBank no: MN164455.1)



Scientific GeneJET Gel Extraction Kit (Thermo Fisher Scientific), and sequenced directly by the Sequencing Service of IBMCP (CSIC-UPV) using specific primers and Sanger method.

#### Generation full-length infectious clones

Total RNA extracted from infected tissue was subjected to RT-PCR reaction using SuperScript III RT/Platinum® Tag HiFi enzyme Mix and primers 3585 and 3586, targeting the 5' and 3' end of viral sequence. The sense primer 3585 contains the T7 promoter sequence preceding the 5' terminal viral nucleotide sequence. The resultant PCR product was digested with the *SacI* and *XhoI* restriction enzymes and introduced in the pUC18 plasmid, previously digested with the same enzymes. The resultant pUC18T7CPMMoV construct was used as template to amplify the full virus genome carrying different 5' end termini sequences, using the PrimeStar HS DNA polymerase and the corresponding primers (Supplementary Table S1). The PCR products were treated with *DpnI* restriction enzyme to eliminate any rest of the plasmid template and subjected to phenol extraction and ethanol precipitation. The PCR products were then used for transcription reactions.

To generate the infectious clones under the control of the 35S promoter of cauliflower mosaic virus (CaMV) and the inhibitor II terminator from the potato proteinase (PopIt), we used an approach based on the use of the *BsaI* restriction enzyme which digests the DNA outside of the specific sequence site. First, the double 35S promoter and PopIt terminator sequences were amplified with the 3533/3519 and 3670/3534 pairs of primers, respectively, using as template the pSK+35S-MPTMV:HA construct (Peiro et al., 2014). The *BsaI* digestion of the amplified 35S promoter fragment generated a 5' termini compatible with the *HindIII* site meanwhile the 3' contained the 4-terminal nucleotide of the 35S promoter ready for ligation with compatible fragments. The *BsaI* digestion of the amplified PopIt terminator fragment generated a 5' termini carrying the first 4 nt of the PopIt terminator ready for ligation with compatible fragments and a 3' termini compatible with the *EcoRI* site. The CPMMoV full genome was amplified with the 3672, 3673 and 3674 sense primers (each one contains different 5' termini sequences of CPMMoV) and the 3671 antisense primer, using as template the pUC18T7CPMMoV construct. The

sense and antisense primers contain the *BsaI* restriction site and 6 extra nt which, after the *BsaI* digestion, generate DNA ends compatible with the 3' termini of the 35S promoter (sense primers) or the 5' termini of the PopIt terminator (antisense primer). The three *BsaI* digested fragments (35S, PopIt and full length CPMMoV) together with the pMOG800 binary plasmid, previously digested with *HindIII* and *EcoRI* restriction enzymes, were mixed in the same ligation reaction following the manufacturer's instructions (Promega Inc.). The resultant constructs were sequenced to confirm the correct 5' and 3' termini of the viral genome and introduced in the C58 *Agrobacterium* strain for agroinfiltration experiments.

#### Generation of cDNA clones, synthesis of the digoxigenin-labelled riboprobes and hybridization procedure

In order to avoid cross-hybridization between CPMMoV and the closely related BPeMV (GenBank NC\_009642.1), sequences with the lowest identity percentage between the two tobamoviruses were identified through a BLAST search. The results obtained revealed the presence of two regions of 143 nt (between nt 1501–1622) and 352 nt (between nt 5449–5800) (GenBank MN164455) with an identity percentage with respect to BPeMV (GenBank NC\_009642.1) of 80.6% and 75.8%, respectively (Fig. 4a). PCR reactions were carried out as described previously (Herranz et al., 2005a, 2005b) using specific primers targeting two regions of the CPMMoV genome (Supplementary Table S1) and the pUC18T7CPMMoV construct as template. The PCR fragments encompassing nt 1501–1643 and 5449–5800 (GenBank OQ920979), were introduced in the pSK+plasmid, previously digested with *XhoI* enzyme and dephosphorylated, to generate the pSK+Probe#1 and pSK+Probe#2 constructs, respectively. To generate the pSK+Poly2 construct, the PCR fragment carrying the nt 1501–1643, was introduced in the pSK+Probe#2 plasmid, digested with *XhoI* enzyme and dephosphorylated. Together with the CPMMoV probes, a specific BPeMV probe of 291 nt (from nt 5873 until nt 6164; GenBank DQ355023) showing an identity percentage of 89.4% with CPMMoV, was generated. All plasmids were sequenced to confirm the presence of the introduced CPMMoV fragments.



Synthesis of the digoxigenin-labelled probes was performed as described previously (Peiró et al., 2012). Prehybridizations and hybridizations with the single probe or the polyprobes were conducted as described previously (Pallás et al., 1998a, 1998b; Sanchez-Navarro et al., 1999). The hybridizations were performed at 68°C and 60°C for the Probe#1 and Probe#2 probes and at 55°C for the Poly2 probe. Chemiluminescent detection using CSPD reagent as substrate was performed as recommended by the manufacturer (Roche Inc.). Films were exposed for 30 min.

#### Northern blot analysis

Total RNA was denatured by formaldehyde treatment and analyzed by northern blot hybridization as described previously (Pallás et al., 1998a, 1998b) using a DIG-ribo probe (Roche Diagnostics GmbH, Mannheim, Germany) complementary to the 5449–5800 region of CPMMoV-Sp (probe #2). The membranes were exposed with Kodak X-Omat AR film for approximately 15 min. Alternatively, the chemiluminescent signal was detected using the LAS 3000 Imaging System from Fuji digital system.

#### Inoculation of *Nicotiana benthamiana* plants

*Nicotina benthamiana* plants were inoculated using T7 derived transcripts or by agroinfiltration. Synthesis of infectious CPMMoV transcripts were generated through the use of amplified full length CPMMoV fragments. The PCR reactions were performed using the 3666, 3667 and 3668 sense primers (carrying different 5' end termini), the 3339 antisense primer (Supplementary Table S1), the pUC18T7CPMMoV plasmid as template and the PrimeStar HS DNA polymerase (Takara Inc.). The amplified PCR fragments were then incubated with the *DpnI* restriction enzyme during 60 min at 37°C to digest the plasmid template and subjected to phenol extraction and ethanol precipitation. Amplified PCR products (200 ng) were used as template for the T7 transcription reaction (Roche Inc.) in a 20 µl final volume. After the phenol extraction and ethanol precipitation, 7 µg of the obtained transcripts were capped using the Scriptcap m7G capping system (CellsScript Inc.) and directly inoculated on the leaves by using carborundum.

Inoculation of plants by agroinfiltration was performed using *Agrobacterium tumefaciens* (strain C58)

cultures (OD<sub>600</sub>=0,4) transformed with the corresponding binary plasmids pMOG800. The *Nicotiana benthamiana* plants were infiltrated as previously described (Herranz et al., 2005a, 2005b). The plants were kept at 24°C day/18°C night, with a 16h day/8h night photoperiod.

#### Alignment and phylogeny analysis

All sequences generated in this study were assembled using Geneious 2019.2.3 software (<http://www.geneious.com>) (Kearse et al., 2012). Contigs were analyzed using BLASTx (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) by calculating nucleotide (nt) and deduced amino acid (aa) identities using the ClustalX2 program (Thompson et al., 1994) and MEGA X 10.1 BETA software (Kumar et al., 2018). Phylogenetic trees were constructed using the Neighbor-joining (Saitou & Nei, 1987), with 10,000 bootstrap replicates implemented in MEGAX based on complete sequences of tobamovirus available at the database. The tree was rooted using the sequence of potato virus X (PVX) as the outgroup, since this virus has a similar genome organization, except for the movement protein (MP). In this case, the closely related MP of alfalfa mosaic virus was used.

#### CPMMoV seed to plant transmission bioassay

Two different sources of CPMMoV-Sp contaminated pepper seeds were used in the seed to plant transmission bioassay: (i) seeds collected from naturally infected plants in a commercial greenhouse and (ii) seeds from fruits collected from infected plants that were mechanically inoculated with CPMMoV-Sp isolate and grown in a climatic chamber. Seeds used in the transmission assays were not treated to eliminate any virus located at the seed surface. In the first seed source (i), pepper plants (Cv. Derio) showing symptoms of viral disease were identified in a commercial pepper greenhouse in Bizkaia (northern Spain). These plants were analyzed in the laboratory by Molecular Hybridization (MH) for CPMMoV (specific probe#2). Three plants infected with this virus were detected. From these plants, fruits were harvested, and their seeds were individually extracted, resulting in three lots of virus-infected seeds. Each lot of seeds was sown in 8 trays, with 28 plants in each tray (for a total of 224 seeds per lot), using disinfected

peat substrate. These trays were kept under glass-greenhouse conditions (25–28°C/18–20°C day/night). Symptoms were recorded for each individual plant. All plants were analyzed at 52 days after sowing.

In the second source of seed, 30 pepper plants (Cv. Celta) were mechanically inoculated at a stage when they had four fully developed leaves. Three CPM-MoV-Sp isolates from Granada and Bizkaia were used for inoculation, with 10 plants per isolate. Additionally, 10 pepper plants were mock-inoculated and kept as CPMMoV-free control. Plants were grown in a growing chamber under controlled conditions (25°C/18°C day/night, 12 h day/night and 70% RH) for three months. After 20 days post-inoculation (dpi), all plants were analyzed by MH (CPMMoV Probe#2) to confirm the presence or the absence of the virus in CPMMoV or mock-inoculated plants, respectively. Fruits were collected, segregated into separate trays, and allowed to dry. Subsequently, seeds were extracted from the dried fruits, resulting in the establishment of three lots of seeds contaminated with the virus. Seeds obtained from non-infected fruits were also extracted and constituted the non-infected seed lot. A total of 80 seeds per lot were sown on disinfected peat substrate and grown under controlled conditions in a growing chamber. Symptoms were recorded periodically, and plants were individually sampled at 35 days after sowing. Plants of all assays were individually analyzed to detect the presence of CPMMoV by MH (probe#2).

CPMMoV pathotype determination bioassay in pepper varieties with different resistance genotype

Finally, the pathotype determination of CPMMoV-Sp isolate from Bizkaia (Basque Country) was performed through a bioassay involving various pepper varieties carrying different alleles of the 'L' resistance gene to tobamoviruses, as well some varieties lacking the resistance. A total of twenty-three pepper varieties with different L resistant genes to Tobamovirus pathotypes were inoculated: “Garbino F1”, “2168” and “2171” (*C. annuum* L. with L1 gen; resistant to pathotype P0), “BGHZ 3789” and “BGHZ 3353” (*C. baccatum* L. var. *pendulum* with L2 gen; resistant to pathotypes P0 and P1), “Salvatore F1”, “104,216” and “2166” (*C. annuum* L. with L3 gen; resistant to pathotypes P0, P1 and P1.2) and “Medulas F1”, “El Lobo”, “Sanakka Ichnigo” and “10,255” (*C. annuum*

L. with L4 gen; resistant to pathotypes P0, P1, P1.2 and P1.2.3). Additionally, pepper varieties lacking any resistant genes (*C. annuum* L.; susceptible to all tobamoviruses pathotypes) were also included, such as the commercial hybrids “Gaitanes F1” and “Celta F1”, as well as local varieties from the Basque Country such as “Iker”, “Sima-334”, “Maddiper”, “Izartxo”, “Leuna”, “Luzea”, “Cor-01”, and “Loiola19.1”. A virus inoculum suspension was prepared using frozen infected fresh leaves tissue of CPM-MoV-Sp isolate, homogenized in 50 mM phosphate buffer (NaH<sub>2</sub>PO<sub>4</sub>-Na<sub>2</sub>HPO<sub>4</sub>) at pH 7.5 in a 1:8 (w/v) ratio, with carborundum used as abrasive. The inoculation was performed by rubbing the suspension onto the two true basal leaves of 20 plants from each variety when they were at the six true leaves stage. After inoculation, plants were kept under glass-greenhouse or growing chamber conditions (25–28°C/18–20°C day/night). Symptoms were recorded for each individual inoculated plant at 7, 13, 20, and 27 dpi. MH test (probe#2) was used to determine the presence or absence of CPMMoV in non-inoculated leaves of each individual plant at 34 dpi. Additionally, 7 plants carrying either the L3 or L4 resistance genes, were also analyzed by RT-PCR using the tobamovirus general primers described by Li et al. (2018).

## Results

Survey, collection of samples and first analysis

During a field survey conducted in 2019 across different pepper-growing areas of Bizkaia and Granada Spanish regions, tobamovirus-like symptoms such as leaf yellow and light green chlorotic mosaic, greenish yellow wide bands in the entire length of the fruit, surface reduction and irregular shapes and colors in the leaves associated with a reduction of fruit size, were observed. Serological tests for the main viruses affecting pepper crops, such as paprika mild mottle virus, alfalfa mosaic virus, broad bean wilt virus, tobacco mild green mosaic virus or Tomato brown rugose fruit virus, yielded negative results. However, RT-PCR analysis using universal tobamovirus primers (Li et al., 2018) amplified a specific band of 880 nt. BLAST analysis of the nucleotide sequence showed 81% nt identity with bell pepper mottle virus (BPeMV) (GenBank: NC\_009642.1). Taken together,

the preliminary results suggested either the presence of a new tobamovirus or a Spanish isolate of BPeMV.

#### Characterization of the complete genome of the new tobamovirus and phylogenetic analysis

RACE analysis using the modified stem-loop primer allowed the characterization of the 5' and 3' terminal nucleotide sequences, including a cytosine at the 5' terminus and an adenine at the 3' terminus, respectively (Fig. 1a). The complete genome sequence of the new virus was found to be 6405 nt long (GenBank: OQ920979) and showed 99.58% sequence identity with a reference genome recently deposited in the GenBank database, named chili pepper mild mottle virus (CPMMoV) (GenBank: MN164455.1). The new isolate should be considered an isolate of CPMMoV specie in the genus Tobamovirus, rather than a member of BPeMV species which showed an 84.42% sequence identity. Thereafter, we will refer to the new isolate virus as chili pepper mild mottle virus Sp (Spain-Granada isolate, CPMMoV-Sp). The nucleotide sequence of the new virus showed the typical genomic organization of tobamoviruses: the 5' and 3' non-coding regions of 72 nt and 203 nt, respectively; two 5'-proximal ORFs (ORF 1 and ORF 2) of 3351 nt and 4853 nt coding for the two subunits of the RNA polymerase of 128 kDa and 187 kDa, respectively; an ORF 3 of 812 nt coding for the movement protein (MP) of 27.8 kDa; and, finally, the 3' proximal ORF 4 of 476 nt coding for the capsid protein (CP) of 19.2 kDa (Fig. 1b). Blast analysis between CPMMoV-Sp and CPMMoV (GenBank: MN164455.1), revealed that the isolate in the database lacked 22 nt at the 5' termini, suggesting a possible partial sequencing of this region (Fig. 1c).

Phylogenetic analysis was performed using the complete genome nucleotide sequence of CPMMoV-Sp and 37 tobamoviruses sequences available in the GenBank database (Fig. 2). The phylogenetic tree reveals the presence of three subgroups (I, II, III) according to the historical classification based on natural host range, genomic organization and phylogenetic clustering (Salgado-Ortíz et al., 2020). CPMMoV-Sp was placed in subgroup I and showed close relatedness to BPeMV (GenBank: NC\_009642.1) and CPMMoV (GenBank: MN164455.1), with a bootstrap value of 100%. Similar results were obtained when phylogenetic analysis of amino acids sequence

of movement protein and coat protein were performed (Supplemental Fig. S1), supporting the inclusion of CPMMoV-Sp in subgroup I among the three observed phylogenetic groups.

#### Infectious clone of CPMMoV-Sp

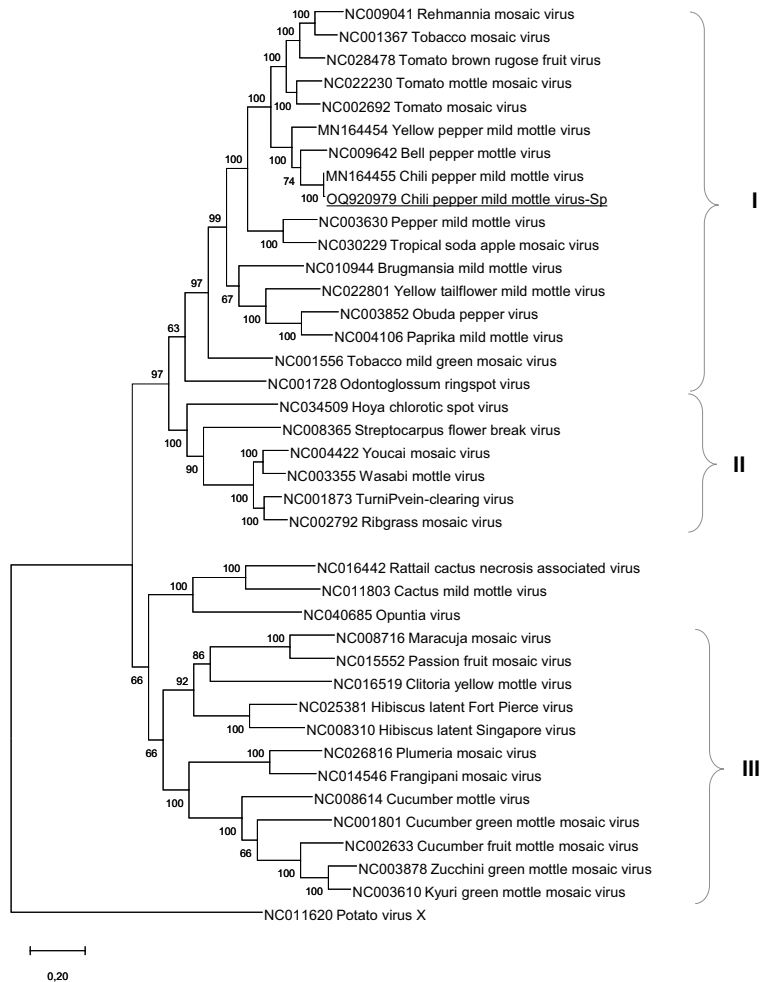
In the next step, we decided to generate infectious clones of CPMMoV-Sp in order to analyze the effect of the extra cytosine residue characterized herein by using the modified RACE protocol but also to see the requirement or not of the 5' terminal 22 nt identified in the CPMMoV-Sp when compared with the CPMMoV present at the database. The infectious clones were generated by two different approaches. First, by cloning the complete viral genome in a binary plasmid under the control of the 35S promoter and the PopIt terminator and subsequent agroinfiltration of the plant and second, by using the T7 promoter and generating the corresponding transcripts that were directly inoculated onto leaves using carborundum after undergoing capping reaction. In both approaches, three different constructs were generated, differing in the starting nucleotide at the 5' termini: nt+1 (the cytosine identified herein), nt+2 (a guanine, like the 95% of the tobamovirus in the database) or nt+23 (the starting point of CPMMoV present in the database) (Fig. 3a). Similar results were obtained when the plants were inoculated either by agroinfiltration or by direct inoculation of the T7-derived capped transcripts. Northern blot analysis of the inoculated (5 dpi) and systemic (10 dpi) leaves showed clear viral RNA accumulation of the CPMMoV-Sp constructs starting at nt+1 or nt+2, meanwhile no signal was observed for the constructs starting at nt+23 (Fig. 3b), indicating that the 5' terminal 22 nucleotides of CPMMoV-Sp were necessary for viral infection.

#### Development of a sensitive detection technique by non-radioactive molecular hybridization and comparison to RT-PCR technique

In the next step, a detection method based on non-radioactive molecular hybridization using digoxigenin-labeled RNA probes, was developed for the specific detection of CPMMoV. Two individual riboprobes (Probe#1 and Probe#2), targeting the more divergent regions between CPMMoV and BPeMV (identity percentage of 80.6% and 75.8%,

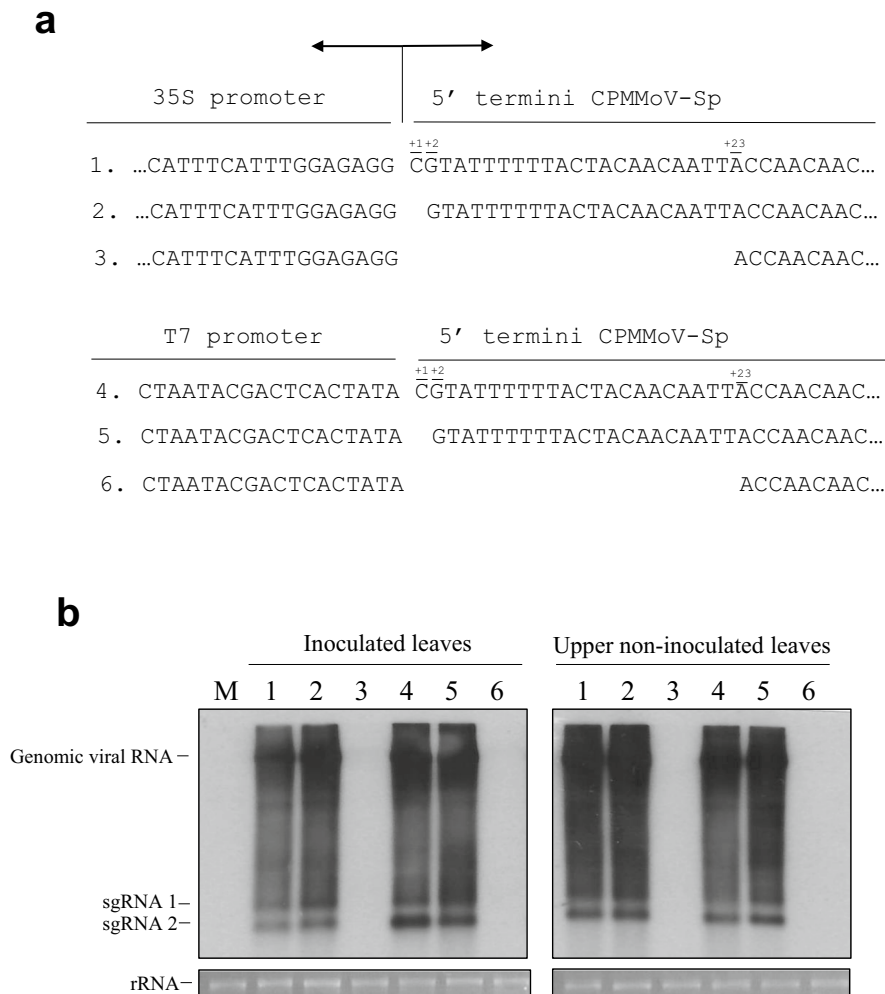


**Fig. 2** Phylogenetic tree based on the complete genomic nucleotide sequence of 37 viruses of the genus *Tobamovirus* available in the GenBank database plus the new virus CPMMoV-Sp. Bootstrap analysis was performed with 1000 replicates and numbers indicate the bootstrap percentage value for each node. I, II and III are subgroups according to historical classification based on host-plant range, genomic organization and phylogenetic relationship. The complete genomic sequence of potato virus X (PVX) was used as an outgroup. Scale bars correspond to substitutions per nucleotide site



respectively), together with a polyprobe (Poly2) carrying the two individual probes fused in tandem, were analyzed. Together with the CPMMoV probes, a specific BPeMV probe of 291 nt showing an identity percentage of 89.4% with CPMMoV, was evaluated. The sensitivity and specificity of each individual probe and polyprobe was evaluated using healthy (pepper) and CPMMoV-Sp (pepper) or BPeMV (*Nicotiana clevelandii*) infected tissue. Total RNA (25 nanograms) was serially diluted in TE buffer ( $5^{-1}$ ) and applied on nylon membranes. Replicas of the same membrane were hybridized with the three individual probes at 68°C and 60°C, to evaluate how the temperature could influence the specificity. The results obtained at 68°C, revealed that the three probes were specific, showing no cross-hybridization between the CPMMoV and BPeMV infected tissue and no signal in the healthy tissue. The CPMMoV probe#2,

complementary to part of the MP and CP genes, rendered positive hybridization signal up to the dilution  $5^{-3}$ , a detection limit 25 higher to that observed for the CPMMoV probe#1 ( $5^{-1}$ ), targeting only the replicase gene. When replicas of the same membranes were hybridized with the individual probes at 60°C we observed clear cross-hybridization signal with the BPeMV meanwhile no cross-hybridization was observed with the CPMMoV probe#1 and only a smooth hybridization signal was observed with the BPeMV undiluted sample (25 ng/μl) using the CPMMoV probe#2 (Fig. 4a). In the next step, we evaluated the detection limit and the specificity of poly2. The hybridization was performed at 55°C, since no hybridization signal was observed in the healthy tissue. The obtained results revealed a hybridization signal up to the dilution  $5^{-3}$ , similar to that obtained with probe#2, indicating that the fusion of two probes



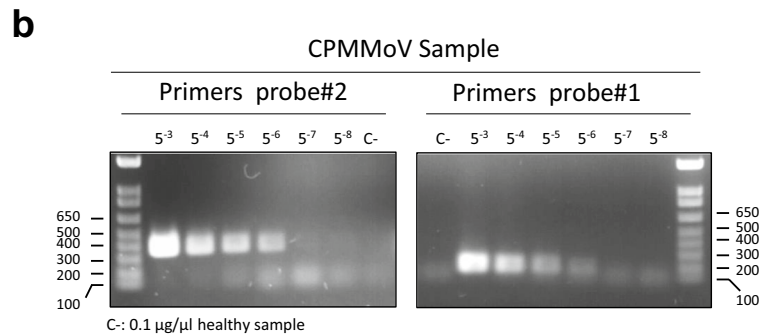
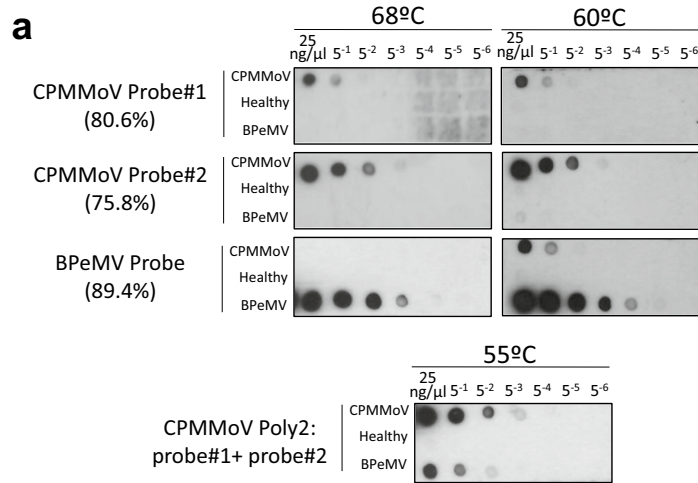
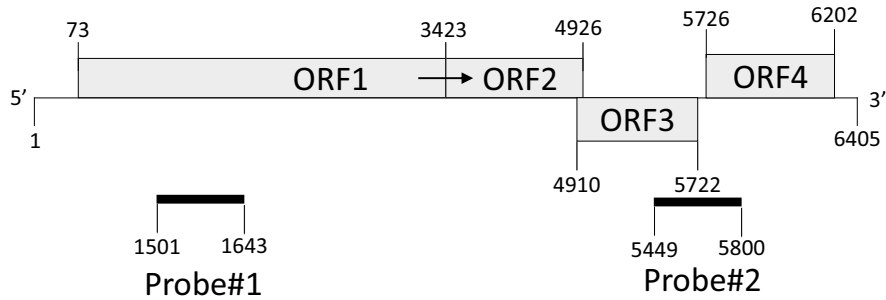
**Fig. 3** Analysis of CPMMoV-Sp infectious clones that carry different 5' termini sequences. **a)** scheme of the 5' termini of the infectious clones that were generated under the control of either the 35S promoter (clones 1, 2, and 3) or the T7 promoter (clones 4, 5, and 6). These clones carry the full 5' termini sequence identified in this study with (clones 1 and 4) or without the cytosine residue (clones 2 and 5) or without the extra 22 nucleotides when compared to the CPMMoV sequence deposited in the GenBank data-

base (accession number MN164455.1). **b)** Northern blot analysis of *Nicotiana benthamiana* plants inoculated with the constructs indicated in a. One microgram of total RNA extracted from inoculated (at 5 days post-inoculation) or upper (at 10 days post-inoculation) leaves was hybridized with a digoxigenin-labelled RNA probe#2. The films were exposed for 30 min, and the positions of the genomic and subgenomic viral RNAs are indicated. M denotes non-inoculated plants

of the same virus does not increment the detection limit. The poly2 also rendered cross-hybridization signal with the BPeMV infected tissue, indicating that probes cross-hybridize with sequences showing 75–80% identity when the hybridization is performed at 55°C.

Finally, we decided to compare the detection limit between the Molecular Hybridization technique used in the present study for the detection of CPMMoV

and the RT-PCR. In this sense, the same dilutions of total RNA extracted from CPMMoV-Sp infected tissue analyzed by molecular hybridization, were analyzed by RT-PCR using the two set of primers used to amplify the two CPMMoV probes. The results showed that specific amplicons of 144 nt (using primers of probe#1) or 350 nt (using primers of probe#2) were detected until a dilution of 5<sup>-6</sup>, representing a detection limit 125 times higher than that observed



**Fig. 4** Detection of CPMMoV by molecular hybridization and RT-PCR and evaluation of cross hybridization between CPMMoV and BPeMV infected tissue. **a**) Total RNA (25 ng/µl) extracted from CPMMoV and BPeMV pepper plants was serially diluted five times in water and applied to nylon membranes. Healthy pepper plants were used as a control. Replicas of the same membrane were hybridized with two CPMMoV probes (indicated in the scheme) and the BPeMV probe at 68°C and 60°C, respectively while the poly2 CPMMoV probe, which carries the two CPMMoV probes fused in tandem, was hybridized at 55°C. The

films were exposed for 30 min. The percentage below each probe indicates its identity with either the BPeMV (CPMMoV probes) or CPMMoV (BPeMV probe) viral sequences. **b**) RT-PCR analysis of the same CPMMoV dilutions analyzed by molecular hybridization using the specific set of primer for probe#1 and probe#2. The last positive dilution corresponded to 5<sup>-6</sup> for both set of primers, 125 times more sensitive than the molecular hybridization assay (5<sup>-3</sup>). C denotes total RNA extracted from healthy pepper tissue. The numbers at the right or left borders of the gels indicate the DNA size in base pairs

with molecular hybridization ( $5^{-3}$ ), meanwhile no PCR product was observed in the healthy tissue (Figs. 4b).

#### CPMMoV seed to plant transmission rates in pepper

In the next step, the vertical CPMMoV-Sp transmission (seed-to-plant transmission) was analyzed using six batches of seeds obtained from naturally infected plants (Cv. Derio) in a commercial greenhouse or from pepper plants (Cv. Celta) mechanically infected with CPMMoV-Sp. The analysis of all plants by molecular hybridization using the specific probe#2, revealed a percentage of transmission that ranged between 0.9 and 8.5% (Table 1). Both sources of seeds, extracted from naturally infected plants or from plants mechanically infected with CPMMoV-Sp, showed seed transmission to plant, except for one seed batch (Table 1; seed lot 5). Some of the infected plants that resulted from the transmission trial using naturally infected seeds exhibited symptoms such as yellowing and mosaic patterns (Supplementary Fig. S2). All together clearly indicate that CPMMoV is seed transmitted although the transmission percentage might be influenced by the cultivar and/or the growth conditions.

#### CPMMoV pathotype determination in pepper varieties with different resistance genes to tobamoviruses

Finally, the CPMMoV-Sp pathotype was determined by inoculation of various pepper varieties carrying distinct alleles of the 'L' resistance gene. All pepper varieties carrying 'L' resistance genes rendered a hypersensitive response in the inoculated leaves at

7 dpi, a response that was not observed in the non-resistant varieties (Table 2). However, viral symptoms in upper non-inoculated leaves were observed in the cultivars without the resistance gene and only in the varieties carrying the L1 and L2 resistant alleles (Supplementary Fig. S3). Molecular hybridization (probe#2) analysis for the presence of CPMMoV-Sp in the upper leaves revealed that the varieties with L2, L3 and L4 genes were resistant (0% of positive samples) meanwhile in the varieties with L1 allele some plants were infected (20–40% of plants testing positives for CPMMoV-Sp). The varieties without any resistance genes showed high susceptibility to CPMMoV-Sp, with over 87% of plants testing positive. To ensure the absence of virus infection in plants carrying the L3 (cv. 2166) and L4 (cvs. Sanakka Ichnigo, 10,255 F1) genes, systemic leaves from 7 plants of each variety were collected at 34 dpi and analyzed by RT-PCR using tobamovirus general primers described by Li et al. (2018). A slight band was only amplified in all systemic leaves collected from L3 plants cv. 2166, which were sequenced, showing a 100% nt identity with CPMMoV-Sp.

#### Discussion

In the present study we have identified a new Spanish isolate of the CPMMoV. The sequence presented an identity percentage of 99.58% with CPMMoV present in the database. However, the main differences were located at the 5' termini with an extension of 22 residues. The characterization of the 5' terminal sequence was performed by using a variant of the RACE protocol in which a stem-loop primer was developed to prime at the end of the introduced

**Table 1** Seed-to-plant virus transmission rates in naturally (Cv. Derio) or mechanically (Cv. Celta) CPMMoV-Sp infected pepper plants

| Seed source                                | Seed lot | Total plants | Infected Plants | Virus transmission (%) |
|--|----------|--------------|-----------------|------------------------|
| Naturally infected plants (Cv. Derio)      | 1        | 224          | 19              | 8.5%                   |
|  | 2        | 224          | 9               | 4.0%                   |
|  | 3        | 224          | 2               | 0.9%                   |
| Mechanically inoculated plants (Cv. Celta) | 4        | 68           | 2               | 2.9%                   |
|  | 5        | 68           | 0               | 0%                     |
|  | 6        | 68           | 1               | 1.4%                   |
|  | 7        | 68           | 0               | 0%                     |
| Mock inoculated plants (Cv. Celta)         | 7        | 68           | 0               | 0%                     |

**Table 2** CPMMoV pathotype determination in inoculation bioassay with a set of pepper varieties with different resistance genes to tobamoviruses

| Pepper variety<br>(tobamoviruses resistance gene) | Days post inoculation (dpi) |                         |                 |
|---|-----------------------------|-------------------------|-----------------|
|   | 13                          | 27                      | 34              |
| Gaitanes F1                                       | _ <sup>a</sup> / _          | _ / D, Chl, N           | 95 <sup>b</sup> |
| Celta F1  | _ / M, N                    | _ / M, N, W             | 100             |
| Iker  | NL / M, N                   | NL / D, N, M, Chl, AN   | 87              |
| Sima-334  | Y / Chl                     | Y / Chl, B              | 100             |
| Maddiper  | NL / MM, N                  | NL / MM, D, N           | 100             |
| Izartxo   | Y / Mo, MM                  | Y / Mo, D, B, MM, N     | 100             |
| Leuna   | NL, Y / M, Chl              | NL, Y / M, D, B, Chl    | 94              |
| Luzea   | Y / Chl, Mo, M              | Y / Chl, D, M, Mo, B, Y | 100             |
| Cor01   | NL / M                      | NL / D, M, N, B         | 93              |
| Loiola 19.1                                       | Y / M, B                    | Y / M, B, D             | 100             |
| Garbino F1 (L1)                                   | HR, A / _                   | HR, A / N, D, Chl       | 35 <sup>b</sup> |
| 2168 (L1)   | HR / MM                     | HR / MM, B              | 40              |
| 2171 (L1)   | _ / MM                      | _ / MM, VN              | 20              |
| BGHZ 3789 (L2)                                    | A / _                       | A / N, Chl              | 0               |
| BGHZ 3353 (L2)                                    | A / _                       | A / N, Chl              | 0               |
| Salvatore F1 (L3)                                 | HR, A / _                   | HR, A / _               | 0               |
| 10,416 (L3)                                       | HR / _                      | HR / _                  | 0               |
| 2166 (L3)   | HR / _                      | HR / _                  | 0               |
| Medulas (L4)                                      | A / _                       | A / _                   | 0               |
| El Lobo (L4)                                      | HR / _                      | HR / _                  | 0               |
| 10,258 (L4)                                       | HR / _                      | HR, Y / _               | 0               |
| Sanakka Ichnigo (L4)                              | HR / _                      | HR, D / _               | 0               |
| 10,255 F1 (L4)                                    | HR / _                      | HR / _                  | 0               |

The CPMMoV-Sp isolate collected in the survey was inoculated in twenty plants of each variety in the two true basal leaves. <sup>a</sup>Symptoms were registered in inoculated and non-inoculated leaves (left and right side of slash respectively) at different days post inoculation (dpi). Type of symptoms: (HR) Hypersensitive response, (A) Abscission, (AN) Apical necrosis, (B) Bubbling, (Chl) Chlorosis, (D) Distortion, (MM) Mild Mosaic, (M) Mosaic, (N) Necrosis, (NL) Necrotic lesion, (Mo) Mottling, (VN) Veinal necrosis, (W) Wilting, (Y) Yellowing. <sup>b</sup>Percentage of inoculated plants with positive result to CPMMoV analyzed in the non-inoculated leaves by MH at 34 dpi

poly(A) tail, without modifying any residue of the viral sequence. The modified RACE protocol also revealed that the first 5' terminal residue was a cytosine instead of the guanine present in the 97.3% (37 out 38) of viruses assigned to the Tobamovirus genus which the complete genome was deposited in the database. Only plumeria mosaic virus (Database NC\_026816.1) presented a cytosine at the 5' termini. The open question is if the cytosine residue identified in the CPMMoV-Sp is an exception in the tobamovirus genus or it is a consequence to use the modified RACE protocol used herein. In this sense, the use of the modified RACE protocol also identified a cytosine at the 5' termini of the RNA 3 of prunus necrotic

ringspot virus and alfalfa mosaic virus which was not present in all sequences of both viruses deposited in the database (data not shown). In any case, the presence or the absence of the cytosine residue did not affect the infectivity of the infectious clones generated by either 35S promoter and PopIt terminator or by the T7 promoter. However, the extension of 22 nt identified herein at the 5' termini, was critical for the virus infection suggesting that the CPMMoV deposited in the database correspond to a partial sequence.

The use of molecular hybridization has been proved to be a powerful technique for the routine diagnosis of plant viruses (Pallas et al., 2018; Sanchez-Navarro et al., 2018; Sánchez-Navarro et al.,



2019). In the present study we have analyzed how the molecular hybridization could discriminate between CPMMoV-Sp and BPeMV, two viruses with an 84.4% identity, by design three different probes sharing identity percentages of 75.8% and 80.6% (CPMMoV probes) or 89.4% (BPeMV probe). The results revealed that the molecular hybridization could discriminate between two pathogens sharing an identity percentage up to 89.4% when the hybridization was performed at 68°C. However, when the temperature was reduced by 8°C, cross hybridization occurred with regions having identity percentages higher than 80.6%. Similar observations were made with probes targeting conserved regions of potyviruses, where cross hybridization occurred at 60°C with regions showing up to 72.5% identity (Sanchez-Navarro et al., 2018). We have also explored the possibility to increment the detection limit of the molecular hybridization technique by fusing different probes of the same virus in tandem. However, the results obtained revealed that the fusion of two probes did not improve the detection limit. Further analysis will be addressed to see if the fusion of additional fragments could have an impact on this aspect. Finally, the comparison of the detection limits between molecular hybridization and RT-PCR for the analysis of CPMMoV revealed that the RT-PCR was 125 times more sensitive than molecular hybridization, being in the range of previous studies performed with other viruses (Sanchez-Navarro et al., 1996, 1998).

Seed transmission of tobamovirus is highly relevant in pepper, tomato and cucumber crops (Genda et al., 2005). Due to the high stability of tobamovirus viral particles (Genda et al., 2005), these viruses usually have a high capacity to infect seedlings through mechanical contact with infected seeds or tissues (Genda et al., 2011). In the present study, the rates of virus seed transmission varied between 0.9–8.5% or 0–2.9% in untreated seed lots coming from naturally or mechanically inoculated plants, respectively. Similar fluctuation in the rate of seeds transmission has been observed for other tobamovirus. For instance, cucumber green mottle mosaic virus (CGMMV) presented rates of seed transmission of 0.08–2.83% depending on the cucurbit species (Al-Tamimi et al., 2010; HuiJie et al., 2011), while tomato brown rugose fruit virus (ToBRFV) presented a seed transmission rates of 0.08–1.8% (Davino et al., 2020; Salem et al., 2022). In *Capsicum* species, seed transmission rates

of tobamoviruses from 0 to 65.3% were also observed (Demski, 1981; McKinney, 1952; Nagai, 1981; Tosić et al., 1980). This indicates that the tobamovirus seed transmission could be influenced not only by the virus and/or host species, but also by environment conditions. In this sense, factors affecting virus seed transmission rates include host cultivar, virus isolates, environmental conditions, the timing of infection and/or even viral synergism phenomena (Montes & Pagán, 2019; Simmons & Munkvold, 2014). The seeds utilized in this study were directly extracted from infected fruits, and no enzymatic or chemical seed treatment was applied before sowing. Disinfection treatments used by seed companies could diminish the transmission rate in commercial seeds; however, specific tests are needed to determine which disinfection method is the most effective in preventing CPMMoV seed transmission.

Some *Capsicum* species express a resistance hypersensitive response (HR) against tobamovirus, which is conferred by alleles of the L gene: L1, L2, L3, and L4 (Tomita et al., 2008, 2011). In the present study, the varieties with L2, L3 and L4 resistance genes were resistant to the CPMMoV-Sp, while some plants (20–40%) of the three varieties with the L1 gene were infected. The fact that only 20–40% of the L1 plants were infected could suggest that the L1 resistance partially inhibits virus infection although we cannot discard other factors such as the concentration of the virus in the inoculum, the penetrance of the L1 gene, climate conditions, etc. (Gallois et al., 2018). Our results contrast with a previous study by Vélez-Olmedo et al. (2021) that reported that the entire L1-L4 allelic series was resistant to CPMMoV, opening the possibility that the CPMMoV-Sp isolate specifically overcomes the L1 resistance. In the analysis performed by Vélez-Olmedo et al. (2021), an atypical diffuse HR of weak local necrotic lesions and necrosis in the veins of the inoculated leaves of plants with the L1 resistance gene was observed. Similar diffuse HR without vein necrosis was observed in the inoculated leaves of L1 varieties in this work (Supplementary Fig. S3) but also in the upper non inoculated leaves of varieties carrying the L1 and L2 resistances, although positive results by MH were only observed in the L1 variety. The positive phenotypic results observed in the upper non inoculated leaves of the variety carrying the L3 were correlated with a low viral titer (virus detected only by RT-PCR), raising the question of

whether the observed virus accumulation could be enough to transmit CPMMoV mechanically. Further analysis will be addressed to answer this question.

Finally, surveys were carried out using the molecular hybridization technique established here over several years to determine the prevalence and impact of CPMMoV-Sp in pepper and chili pepper crops in the Basque Country. In 2019, CPMMoV-Sp was detected in 10 out of 128 surveyed plots, indicating a prevalence of 7.8%. In random samplings carried out in 2021 in four plots of chili pepper farmers in open-field conditions, CPMMoV-Sp was detected in one of the plots. The virus incidence in this plot was 52.5% (21 out of 40) at the end of the crop cycle. Coinfections with CMV were also detected in some of these samples. In samplings carried out in the same plot in 2022, CPMMoV-Sp incidences of 16.6% were detected, with 5 positive plants out of 30 randomly sampled, in the sensitive cultivar "Ibarroria". However, the incidence was zero (55 randomly sampled plants) in the resistant cultivar "Iribarra" carrying the L3 resistance gene, indicating that this resistance effectively controls CPMMoV-Sp under field conditions. Recently, CPMMoV has been also isolated in solanaceous ornamental calibrachoa (*Calibrachoa* spp.) plants in the United States (Groth-Helms et al., 2022), representing an epidemiological risk for other horticultural and ornamental crops of the Solanaceae family, but also confirming the presence of this virus in regions beyond its initial identification in Peru (Vélez-Olmedo et al., 2021). Consequently, it is advisable to carry out surveys in crops and nearby adventitious flora, as well as seed analysis, in order to prevent the further spread of CPMMoV and mitigate its impact on agricultural and ornamental plant species.

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## Declarations

**Conflict of interest** The authors declare that they have no conflicts of interest.

**Ethical approval** This article does not contain any studies with human participants or animals requiring ethical approval.

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