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FULL RESEARCH PAPER

Sequence analysis within the RNA 3 of seven Spanish tomato isolates of *Parietaria mottle virus* (PMoV-T) reveals important structural differences with the parietaria isolates (PMoV)

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Abstract The genomic regions encoding the putative movement protein (MP), coat protein (CP) and intergenic region (IGR) of seven Spanish isolates of the *Parietaria mottle virus* which infects tomato plants (PMoV-T) were sequenced. Values for the genetic diversity of the PMoV-T isolates were 0.056, 0.047 and 0.013 for the CP, MP genes and IGR, respectively. Nucleotide and amino acid sequence comparison of the seven PMoV-T isolates with those of PMoV revealed significant differences. All of them had a cytosine deletion at position 1366, also confirmed in an Italian tomato isolate, which involves a start codon for the CP gene different from that for the PMoV sequence, resulting in a CP 16 amino acids shorter than the PMoV CP. The certainty of a cytosine deletion only associated to the tomato isolates or the possibility of a mistake in the PMoV published sequence are the two hypotheses that could explain this difference. Structural motifs highly conserved in Ilarviruses were identified in PMoV-T MP and CP. A stable hairpin structure is proposed for IGR, by the initiation site for subgenomic RNA 4 synthesis. Phylogenetic analysis of CP and MP amino acid sequences showed that Spanish PMoV-T isolates form a separate group from PMoV and other members of the *Ilarvirus* genus. Comparative analysis with different PMoV isolates including tomato isolates from other regions and isolates from different hosts are necessary to confirm this differentiation.

Keywords CP · *Ilarvirus* · Phylogenetic analysis · Subgenomic promoter

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Introduction

The analysis of the genetic structure of plant virus populations may provide information on the genetic stability of the majority of plant RNA viruses, in spite of their high potential for variation, and for the control of pathogen-caused diseases (García-Arenal et al. 2001). Analysis of the genetic structure for certain

plant virus species, over most of their geographic range or on a regional scale, has been possible through comparison of nucleotide sequence data (Aparicio et al. 1999; Bateson et al. 2002; Desbiez et al. 2002; Abubakar et al. 2003).

Parietaria mottle virus (PMoV), a member of the genus *Ilarvirus* within the family *Bromoviridae*, was first reported in parietaria plants (*Parietaria officinalis)* showing symptoms of yellow mosaic or mottling (Caciagli et al. 1989). PMoV is a positive-sense RNA plant virus with a tripartite genome. RNA 1 and RNA 2 encode the replicase proteins P1 and P2, respectively (Scott et al. 2006). RNA 3 is bicistronic and encodes the movement protein (MP) and the coat protein (CP) (Ge and Scott 1996). The four open reading frames (ORFs) are flanked by untranslated regions (UTRs) which, on the basis of sequence identity with other Ilarviruses, could be involved in recognition and replication functions (Aparicio et al. 2003; Haasnoot et al. 2003; Vlot and Bol 2003; Scott et al. 2006). A tomato variant of PMoV, later considered a strain of PMoV (PMoV-T) (Lisa et al. 1998; Roggero et al. 2000) was first observed in Piedmont and later in other areas of Italy, such as Apulia, Basilicata, Campania, Lazio, Liguria, Plugia, Sardinia and Sicily (Roggero et al. 2000), and in different regions of other countries including Greece, southern France (Marchoux et al. 1999; Roggero et al. 2000), and the north and south-east of Spain (Aramburu 2001; Janssen et al. 2005). Tomato plants infected with PMoV-T isolates developed a bright necrotic mosaic on apical leaves, progressing to the stem and apex necrosis. New, symptomless shoots appeared 15–30 days after the appearance of the necrotic mosaic. Fruits of infected plants had rings and brown patches with necrotic scars around the edges.

Specific methods for PMoV-T detection based on serology (Roggero et al. 2000), non-isotopic molecular hybridisation and one step RT-PCR (Galipienso et al. 2005) have been developed. To date, only a complete genomic sequence for a PMoV isolate (GenBank Nos AY496068, AY496069 and U35145 for RNAs 1, 2, and 3, respectively) (Ge and Scott 1996; Scott et al. 2003, 2006) and a short, 303-nucleotide sequence, corresponding to the 3′-end of the RNA 1 of PMoV-T isolates (Galipienso et al. 2005) are available. In this paper, we report the first sequencing of RNA 3 of the MP and CP genes and the noncoding intergenic region (IGR) from seven Spanish isolates of PMoV-T. The genetic variability and structural features of these regions were studied and compared with those of PMoV and other Ilarviruses.

Materials and methods

Virus isolates and sample preparation

The Spanish PMoV-T isolates G34H, M31A, SD2, AC1, So7J, JBT1 and RAMS1 were collected from infected tomato plants in a 70 km^2 plot in Catalonia, north-eastern Spain, in 2003–2004. All tomato plants had symptoms of infection similar to those described in a previous report (Roggero et al. 2000; Aramburu 2001). Leaf samples of each isolate were stored at −60°C until use. Plants of *Capsicum annun*, *Cucumis sativus*, *Chenopodium quinoa*, *Datura stramonium*, *Mirabilis jalapa*, *Nicotiana clevelandii*, *N. benthamiana*, *N. glutinosa*, *N. tabacum* cv. Xanthi and *P. officinalis* were mechanically inoculated with the isolates, as previously reported (Galipienso et al. 2005), to determine the host range. Plants of *C. quinoa* were inoculated with the seven original isolates for genetic analysis. Total RNA extracts from infected plants were obtained from approximately 100 mg of fresh tissue using TRIzol® reagent (Invitrogen) following the manufacturer's instructions for samples with a high sugar content.

Primers and RT-PCR amplification

The primers used (Table 1) were designed from the PMoV RNA 3 sequence (GenBank No. U35145) using OLIGO 4.0 software (MBI Inc.). First strand cDNA was synthesized using the H-Superscript II Reverse Transcriptase (Invitrogen) in a 20 µl reaction volume containing 2 µl of total RNA and 100 pmol of each antisense primer. The mix was incubated at 42°C for 1 h, followed by 5 min incubation at 65°C to inactivate the enzyme. PCR was carried out with 5 µl of cDNA in a 50 µl reaction mixture, using 50 pmol of each primer and one unit of AccurePrime *Taq* DNA high fidelity Polymerase (Roche) following the manufacturer's instructions. Amplification was performed in a Perkin-Elmer 9700 thermal cycler (Applied Biosystems) with an initial denaturing cycle of 4 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 55°C and 40 s at 72°C, and a final extension of 7 min at 72°C. PCR products were analysed by electrophoresis in 2% agarose gels in TAE buffer (40 mM Tris–

Table 1 Primers combination used to amplify movement protein (MP), intergenic region (IGR) and coat protein (CP) sequences from RNAs of Spanish tomato isolates of *Parietaria mottle virus* (PMoV-T)

Primer	Location[a]	Genome region[a]	Product size (nt)
PMoV-1F 5′-TGTTGCAGGCTGCTTCAAGG-3′	247–266	MP	712
PMoV-1R 5′-TCTCTTGGAGGCAATATC-3′	941–958		
PMoV-2F 5′-AATCGAGACTTTCGCCAGGA-3′	865–884	MP-IGR-CP	722
PMoV-2R 5′-GGGAAATAGCATCGTTGG-3′	1569–1586		
PMoV-3F 5′-GTTGAAGCTCCCAAACAATC-3′	1508–1527	CP	503
PMoV-3R 5′-CACTCTTTACGTTGGCATCG-3′	1991–2010		
PMoV-4F 5′-**ATG**TATGAACTCTTCAACCATTCAC-3′	1302–1326	CP	663
PMoV-4R 5′-gctagcAAGCTT**TCA**TTGGTTCCCATCCTCC-3′	1946–1964		
PMoV-5F 5′-**ATG**TCTTCGAGTCAAGG-3′	1349–1365	CP	616
PMoV-4R 5′-ctagcAAGCTT**TCA**TTGGTTCCCATCCTCC-3′	1946–1964		

[a] Taken as reference the RNA 3 of PMoV. Putative CP start codons are in bold in PMoV-4F and PMoV-5F primers. PMoV-4R primer contains a *Hin*dIII restriction site (underlined), non-viral sequences (lowercase letters) and the putative CP stop codon (in bold).
F Forward, *R* reverse

acetate pH 8.0, 1 mM EDTA) and visualized on a UV-transiluminator by ethidium bromide staining.

Cloning, sequencing and phylogenetic analysis

PCR-amplified products obtained using the PMoV-1F/PMoV-1R, PMoV-2F/PMoV-2R and PMoV-3F/PMoV-3R primer pairs were cloned in the pGEM-T vector (Promega), and sequenced using a 3730 DNA sequencer (Applied Biosystems). The complete nucleotide sequence of the MP, IGR and CP genomic regions from PMoV-T isolates were obtained from five overlapping clones with two sequence readings for each nucleotide position. Sequences of the PMoV-T isolates and PMoV were aligned with the CLUSTAL W programme (Thomsom et al. 1994). The MEGA programme (Kumar et al. 2001), applying the Kimura two-parameter method (Kimura 1980), was used to estimate nucleotide distances between pairs of sequences. The DOTPLOT programme, using the Blosum 62 similarity matrix, was used for amino acid sequence comparison. Tree topology was inferred from these amino acid sequences using the Neighbour Joining method (Saitou and Nei 1987) and bootstrap statistical analysis was carried out with 1,000 replicates. The RNA secondary structure and the protein hydrophobic patterns were obtained using the MFOLD and PEPTIDESTRUCTURE programme respectively. The protein secondary structure and transmembrane regions were predicted using the PREDICTPROTEIN programme (accessible via e-mail: predictprotein@embl-heidelberg.de) in the EMBL remote server (Rost et al. 1995).

The RNA 3 nucleotide sequence data corresponding to the MP and CP genes and the IGR from G34H, M31A, SD2, AC1, So7J, JBT1 and RAMS1 of PMoV-T Spanish isolates have been deposited in the GenBank database under the accession numbers: AM182744, AM182749, AM182745, AM182743, AM182746, AM182747 and AM182748, respectively. The RNA 3 accession numbers of viruses belonging to different Ilarvirus subgroups used for sequence analysis were PMoV (U35145), *Tobacco streak virus* (TSV, NC003845), *Strawberry necrotic shock virus* (SNSV, AY363228), *Elm mottle virus* (EMoV, NC003570), HdMV (AF172965), *Spinach latent virus* (SpLV, NC003810), *Liliac ring mottle virus* (LiRMoV, U17391), *Apple mosaic virus* (ApMV, U15608), *Prunus necrotic ringspot virus* (PNRSV, AF013287), *Prune dwarf virus* (PDV, L28145) and *American plum line pattern virus* (APLPV, NC003453).

Cloning and expression of the putative PMoV-T CP gene

To find the start of the putative PMoV-T CP gene, two different proteins, fused to the maltose binding protein (MBP), were synthesized in *Escherichia coli* strain DH5α. To fuse the putative PMoV CP gene (nt 1302–1964) (Ge and Scott 1996) to the *malE* gene of plasmid pMal-c_2 (New England Biolabs), the corresponding cDNA fragment of the PMoV-T AC1 isolate was PCR amplified with *Pwo* high fidelity DNA polymerase (Roche) using the PMoV-4F/PMoV-4R primers (Table 1). To fuse the putative PMoV-T CP

gene (nt 1349–1964), the corresponding cDNA fragment of the same PMoV-T AC1 isolate was PCR amplified using the primer PMoV-5F and the reverse primer PMoV-4R (Table 1). This reverse primer contains a *Hin*dIII restriction site and nonviral sequences to facilitate cloning and digestion of the PCR amplified fragments. After *Hin*dIII treatment, the PCR-amplified fragments were ligated in the bacterial expression vector pMal-c_2 and digested with *Asp*700 and *Hin*dIII, to generate the recombinant plasmids pMal-CP1302 and pMal-CP1349. These recombinant plasmids were then used to transform *E. coli* cells. Cultures of the bacterial cells transformed with pMal-CP1302 or pMal-CP1349 plasmids were incubated at 37°C in LB medium containing ampicillin (100 μg ml^{-1}) to an OD_{600} of 0.5. Protein expression was then induced by adding 0.3 mM isopropylthio-β-D-galactoside and growing the cells for 3 h at 37°C. Cells were collected by centrifugation and resuspension in TE buffer. Aliquots of total proteins were analyzed by SDS-PAGE in 10% gels and stained with Coomassie brilliant blue R.

Results

There were no differences between the Spanish PMoV-T isolates on assaying in different indicator hosts, but there were differences when compared to the results reported for the Italian PMoV-T isolates (Lisa et al. 1998). Our PMoV-T isolates induced local lesions in *C. sativus* and systemic infection in *N. benthamiana*, *N. clevelandii* and *C. quinoa*. They did not infect *N. glutinosa, N. tabacum* cv. Xanthi or *D. stramonium*. In addition, they neither infect *C. annum,* a new host for PMoV isolates detected in the south of Spain (Janssen et al. 2005), nor *M. jalapa* or the natural host, *P. officinalis*, from which host PMoV was first isolated (Parrella 2002).

Analysis of the movement protein gene

The complete nucleotide sequence of the MP gene for all Spanish PMoV-T isolates was obtained from overlapping products amplified using the PMoV-1F/PMoV-1R and PMoV-2F/PMoV-2R primers pairs (Table 1). Nucleotide identity among the PMoV-T isolates ranged from 93.3% (SD2 and JBT1) to 99.6% (SD2 and M31A or M31A and G34H), whereas nucleotide identity with regard to PMoV ranged from 92.4% (JBT1) to 93.3% (SD2 and M31A). Genetic diversity (average genetic distance between genetic variants selected randomly) among PMoV-T isolates was 0.047. A comparison of the MP nucleotide sequences of the PMoV-T isolates with all known Ilarvirus MP sequences revealed the highest identity with TSV (65%, data not shown).

Translation of the nucleotide sequence of the MPs encoded by the seven PMoV-T isolates into amino acid sequences showed an extra amino acid at position 283 compared to the MP gene of PMoV (Fig. 1). Amino acid identity within the PMoV-T isolates ranged from 96 to 100%, while this value ranged from 94% (So7J) to 96% (SD2, M13A, G34H and AC1) when compared to the PMoV MP. Most differences were in the C-terminal region, where identity of the last 40 amino acids ranged from 82 to 100% between PMoV-T isolates and from 76 to 80% with the PMoV sequence (Fig. 1). None of the PMoV-T isolates conserved the last three amino acids present in the PMoV sequence; four maintained the last two amino acids, and three PMoV-T isolates only conserved the penultimate amino acid. A phylogenetic tree was generated from amino acid sequences obtained from PMoV-T isolates, PMoV and other Ilarviruses, belonging to different subgroups, available in the GenBank database (Fig. 2a). All PMoV-T isolates were grouped in one cluster, separated from PMoV with bootstrap values of 100.

The analysis of conserved motifs, proposed by Mushegian and Koonin (1993) for MPs of plant viruses from different genera, revealed two different motifs/domains in the PMoV-T isolates, which are highly conserved in Ilarviruses (Sanchez-Navarro and Pallás 1997). One of these domains was rich in arginine and/or lysine basic amino acids (aa), with an amphipatic α-helix structure (from aa 59 to 75), showing a high surface probability. This basic motif has been shown to have RNA binding properties in other related Ilarviruses (Herranz and Pallás 2004; Herranz et al. 2005). The other domain was a region with β-sheet structure (from aa 83 to 95) (Fig. 1).

Analysis of the coat protein gene

The complete CP gene nucleotide sequence for all PMoV-T isolates was obtained from overlapping products amplified using the PMoV-2F/PMoV-2R and PMoV-3F/PMoV-3R primers pairs (Table 1). Nucleotide identity of the PMoV-T isolates ranged from

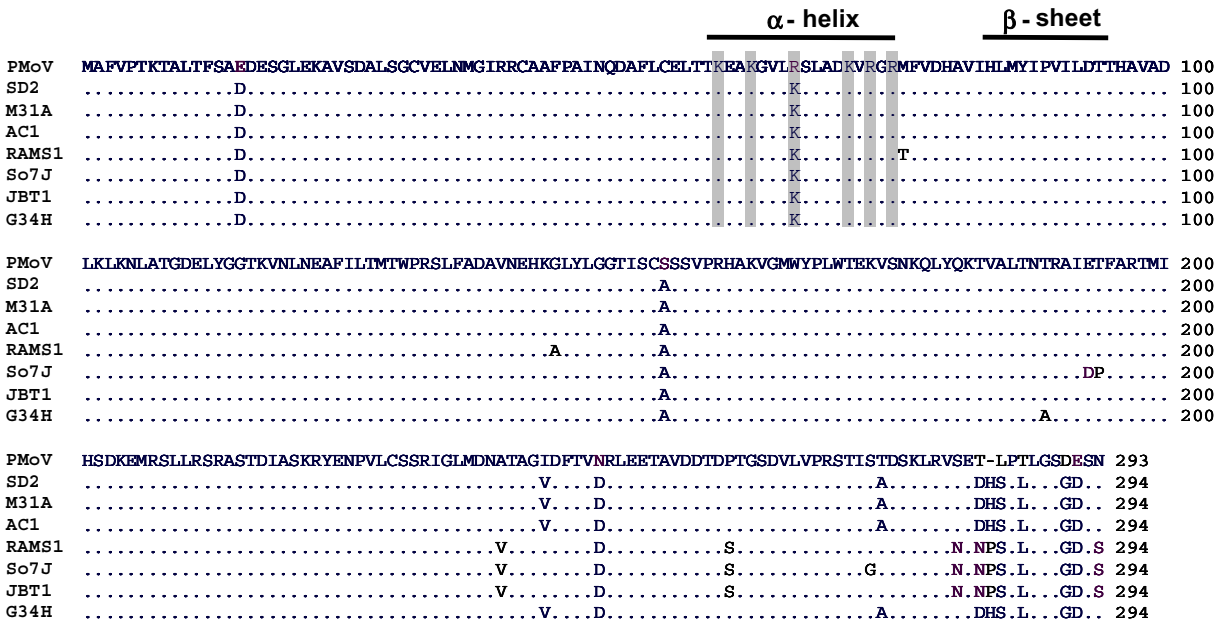


Fig. 1 Alignment of the putative movement protein of the *Parietaria mottle virus* (PMoV) with those of the Spanish tomato isolates (SD2, M31A, AC1, RAMS1, So7J, JBT1 and G34H). *Dots* indicate identical residues and *dashes* denote gaps in the sequence. The *grey boxes* indicate the conserved basic amino acids lysine (K) or arginine (R) and the secondary structures (α-helix or β-sheet) are signalled with black lines

91.6% (AC1 and JBT1) to 98.8% (SD2 or M31A and G34H), whereas nucleotide identity with regard to PMoV ranged from 84% (JBT1) to 84.6% (SD2 and M31A). Genetic diversity of the PMoV-T isolates was estimated to be 0.056. When compared to other members of the genus *Ilarvirus*, TSV again was most similar to the PMoV-T CP (46–48% nucleotide identity). Amino acid identity among all PMoV-T isolates ranged from 94% to 100%, whereas that with the PMoV CP ranged from 81% (JBT1) to 85% (G34H and AC1).

Phylogenetic analysis of CP amino acid sequences of all PMoV-T isolates, PMoV and other Ilarviruses belonging to different subgroups were carried out (Fig. 2b). The topology of the phylogenetic tree was similar to that obtained for MP. All PMoV-T isolates were grouped in one cluster, separated from PMoV by bootstrap values of 100. The M31A, SD2, G34H and AC1 isolates formed a subgroup with a common ancestor by CP sequence analyses, with bootstrap values higher than 90. Interestingly, this subgroup is the same as the one identified by MP sequence analyses (Fig. 2a).

Examination of the N-terminal region of the CP revealed an Arg-rich motif with an α-helix secondary structure, from aa 14 to 34 (Fig. 3), as previously described for other Ilarviruses (Sanchez-Navarro and Pallás 1997), and with putative RNA-binding properties (Aparicio et al. 2003). The PMoV-T isolates had a cytosine deletion at position 1366 compared to the PMoV sequence. It is also present in the Italian PMoV-T T-32 isolate (kindly provided by Dr. A.M. Vaira, IVV-CNR, Torino, Italy) (data not shown). This deletion was confirmed by sequencing up to thirty clones of the 5′-terminal region of the G34H isolate CP gene, obtained using the PMoV-2F/PMoV-2R primer pair. Maintaining the start site described for PMoV CP would give rise to a frame shift that would render the CP nonviable. Assuming that the CP of the PMoV-T Spanish isolates could start 48 nt downstream from the start site proposed for PMoV CP, it should be 16 amino acids shorter (Fig. 3).

To obtain biological evidence of this alternative PMoV-T CP gene start site, the cDNA was cloned, as well as that corresponding to the complete CP-ORF of PMoV (Ge and Scott 1996), into the *E. coli* expression vector pMal-c_2 to generate fusion proteins of the *E. coli* maltose binding protein and the putative CP. Analysis by SDS-PAGE revealed the accumulation of

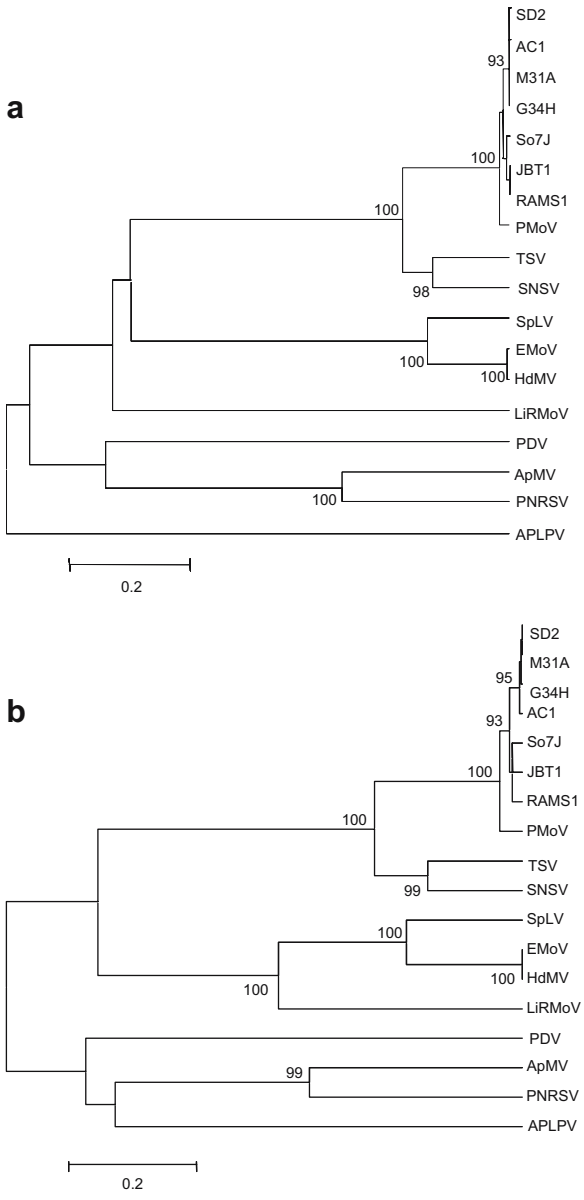


Fig. 2 Neighbour-joining unrooted phylogenetic trees based on amino acid sequences of putative movement (**a**) and coat (**b**) proteins of *Parietaria mottle virus* (PMoV), Spanish tomato isolates of PMoV (SD2, M31A, AC1, RAMS1, So7J, JBT1 and G34H) and the ilarvirus; *Tobacco streak virus* (TSV) and *Strawberry necrotic shock virus* (SNSV) (*subgroup 1*); *Spinach latent virus* (SpLV), *Elm mottle virus* (EMoV), *Hydrangea mosaic virus* (HdMV) and *Liliac ring mottle virus* (LiRMoV) (*subgroup 2*); *Apple mosaic virus* (ApMV) and *Prunus necrotic ringspot virus* (PNRSV) (*subgroup 3*); *Prune dwarf virus* (PDV) (*subgroup 4*); and *American plum line pattern* (APLPV) (*subgroup 5*). Bootstrap values exceeding 90 are shown

a protein with the mobility expected for the fusion product of the *malE* gene of the plasmid pMal-c_2 to which nts 1349–1964 of PMoV-T were fused in frame. By the contrary, *E. coli* cells expressing the *malE* gene of the plasmid pMal-c_2 fused to nts 1302–1964 of PMoV-T, and then containing the putative frameshift, gave rise to a fusion product not consistent with the expected size (data not shown). This result agrees with the cytosine deletion at position 1366 proposed for PMoV-T isolates, which should generate a stop codon at position 1510–1512 giving rise to a non-viable CP and strongly supports the notion that the start site for the CPs of PMoV-T isolates must be located 48 nt downstream from the start site described for PMoV-CP, giving a CP 16 amino acids smaller.

Analysis of RNA secondary structure of the intergenic region

A stable secondary structure of the core promoter for the synthesis of subgenomic (sg) RNA 4 has been proposed in the IGR sequence of RNA 3 in different members of the *Ilarvirus* (Jaspars 1998; Aparicio and Pallás 2002). The stem-loop structure of this core promoter site for PMoV (Jaspars 1998) was compared with those predicted for PMoV-T isolates (Fig. 4). The putative initiation site for PMoV RNA 4 synthesis, proposed by Jaspars (1998), coincides with the initiation codon for synthesis of CP and has a positive value of free energy (ΔG +2 Kcal mol^{-1}). These features make a stem-loop structure very unlikely (Fig. 4a, right). The stem-loop structure we propose for PMoV-T isolates resolves these two problems, with negative free energy values (ΔG −2.7 Kcal mol^{-1}) and a leader region preceding the CP start codon (Fig. 4a, left). Conserved nucleotides in promoter regions of different Ilarviruses were also identified in PMoV-T isolates. The nucleotides of this hairpin structure are highly conserved in all PMoV-T isolates and only one change, (A→G nt 138) on RAMS1 and JBT1 isolates was observed (Fig. 4b). There was a GAGU deletion at position 90 in AC1, M31A, So7J, G34H and SD2 isolates but not in RAMS1 and JBT1 isolates or PMoV. In addition, there was a C→U change at position 112 in all PMoV-T isolates. The nucleotide identity of the IGRs ranged from 94.8 to 100% for the PMoV-T isolates, and from 65.3 to 70.6% when compared to the PMoV sequence. Genetic diversity of the PMoV-T isolates was estimated to be 0.013.

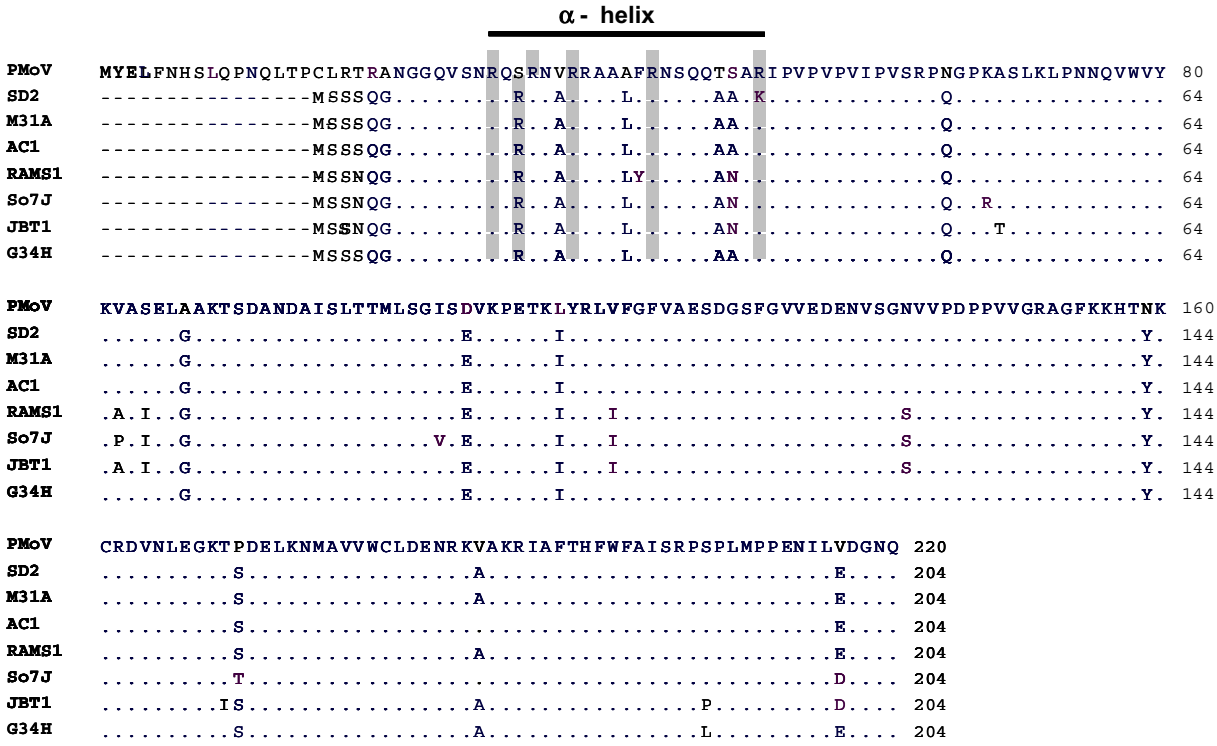


Fig. 3 Alignment of the putative coat protein of the *Parietaria mottle virus* (PMoV) with those of the Spanish tomato isolates (SD2, M31A, AC1, RAMS1, So7J, JBT1 and G34H). *Dots* indicate identical residues and *dashes* denote gaps in the sequence. The *grey boxes* indicate the conserved basic amino acids lysine (K) or arginine (R) and the secondary structure (α-helix) is signalled with a *black line*

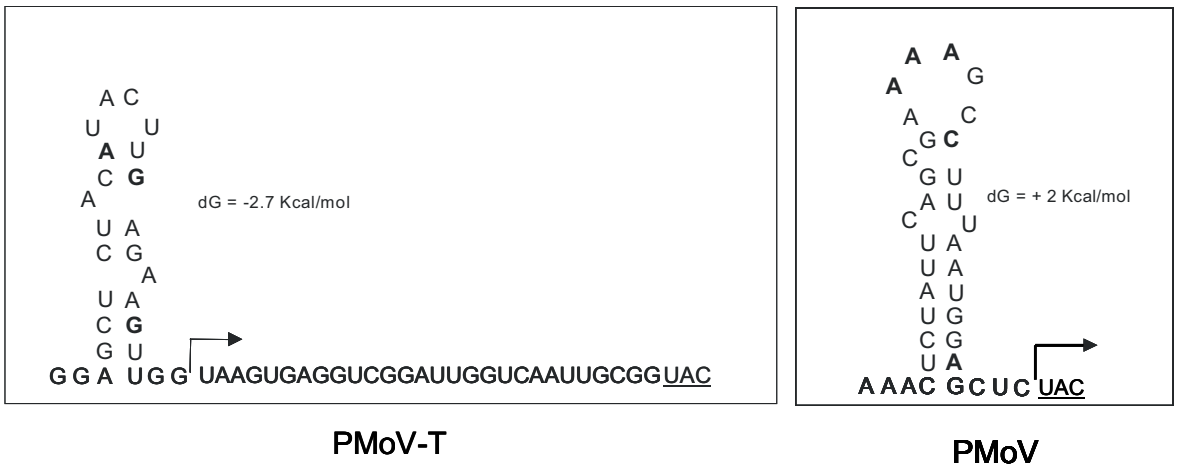
Discussion

RNA viruses have a high potential for genetic variation. Research on these viruses has a practical implication for plant virus control, since strategies based on monogenic host resistance are dependent on the genetic variation of the virus. In this work, genetic variation among Spanish PMoV-T isolates infecting tomato was analysed and compared with the sequence of the PMoV isolated from *P. officinalis*. The structural properties of putative MP and CP and IGR of the RNA 3 from Spanish PMoV-T isolates were also analysed.

Genetic diversity among PMoV-T isolates was 0.056, 0.047 and 0.013 for MP and CP genes and the IGR sequence, respectively. The values obtained for MP and CP genes are relatively high compared with those of other plant viruses (García-Arenal et al. 2001), even that PMoV-T isolates were only obtained from one host-species in a small area. By contrast, the IGR was the less variable, in spite of this region of RNA 3 shows the highest variability in other Ilarviruses, such as PNRSV, (Aparicio and Pallás 2002). Phylogenetic analysis based on the amino acid sequences of CP and MP showed that the PMoV-T isolates are grouped in one cluster, separate from PMoV, and some (M31A, SD2, G34H and AC1) have a common ancestor. The PMoV-T cluster is next to PMoV and TSV viruses, both belonging to the subgroup I of *Ilarvirus* (Fig. 2). This agrees with serological results, where cross-reaction with a TSV extract against PMoV-T polyclonal antiserum has been found (Galipienso et al. 2005), and with recent results obtained by comparison of P1 and P2 proteins with PMoV and other Ilarviruses (Scott et al. 2006).

Ilarvirus MPs belong to the 30 K superfamily, a grouping of proteins related in sequence and structure (Melcher 2000). Their members are characterized by a motif of 30 highly conserved amino acids, which may include a hydrophobic interaction domain (Mushegian and Koonin 1993). Structural analysis of the PMoV-T MP revealed, in all of them, two different motifs/domains that were highly conserved in the *Ilarvirus* genus (Sanchez-Navarro and Pallás 1997) (Fig. 1): (1) an amphipatic α-helix structure probably involved in

a



b

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PMoV     UAUUCGUGAAUGUCCACUAAGACAUGGGGGGGGGUUUGGAAGAGGUUAUUAGUCUCUCGCUGGUAUGCUGAAUUUAGCUGA 80
SD2      .U..........U...C.............................C.....C....U..................... 80
M31A     .G..........U...C.............................C.....C....U..................... 80
AC1      .G..........U...C.............................C.....C....U..................... 80
RAMS 1   ..............................................C.....C....U..................... 80
So7J     .G..........U...C.............................C.....C....U..................... 80
JBT 1    ..............................................C.....C....U..................... 80
G34H     .U..........U...C.............................C.....C....U..................... 80

PMoV     UGAAUUCUUGAGUUUGAGAUAAGUCGCUUUUCGGAAAUUACCUCGAG-------------------------------- 127
SD2      ..........----------.....U.....AUGUAUGAACUCUUCAACCAUUCACUCCAGCCU 156
M31A     ..........----------.....U.....AUGUAUGAACUCUUCAACCAUUCACUCCAGCCU 156
AC1      ..........----------.....U.....AUGUAUGAACUCUUCAACCAUUCACUCCAGCCU 156
RAMS 1   .........A..........U.....AUGUAUGAACUCUUCGACCAUUCACUCCAGCCU 160
So7J     ..........----------.....U.....AUGUAUGAACUCUUCAACCAUUCACUCCAGCCU 156
JBT 1    .........A..........U.....AUGUAUGAACUCUUCGACCAUUCACUCCAGCCU 160
G34H     ..........----------.....U.....AUGUAUGAACUCUUCAACCAUUCACUCCAGCCU 156

PMoV     -------------- 127
SD2      AACCAGUUAACGCC 170
M31A     AACCAGUUAACGCC 170
AC1      AACCAGUUAACGCC 170
RAMS 1   AACCAGUUAACGCC 174
So7J     AACCAGUUAACGCC 170
JBT 1    AACCAGUUAACGCC 174
G34H     AACCAGUUAACGCC 170
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Fig. 4 **a** Predicted stem-loop structures at expected subgenomic RNA 4 core promoter sites on RNA 3 minus strand of Spanish tomato isolates of *Parietaria mottle virus* (PMoV-T) (*left*) and PMoV (*right*). Nucleotides in *bold* are conserved at the promoter region of other ilarviruses. The *arrows in full line* indicate the putative initiation sites of RNA 4 synthesis and the *underlined nucleotides* correspond to CP initiation codons. **b** Alignment of nucleotide sequences of the non-translated intergenic regions of PMoV and PMoV-T isolates (SD2, M31A, AC1, RAMS1, So7J, JBT1 and G34H). *Dots* indicate identical nucleotides and *dashes* denote gaps in the sequence. *Non-shaded boxes* indicate the nucleotide sequence of subgenomic RNA 4 core promoter sites of PMoV-T and PMoV isolates

the RNA-binding properties of the PMoV MP, by analogy with the results obtained for PNRSV (Schoumacher et al. 1994; Herranz and Pallás 2004; Herranz et al. 2005) and (2) a predicted β-sheet structure that may correspond to a transmembrane domain involved in MP association with the cell wall. Interestingly, most of the amino acid differences between the PMoV-T MPs and PMoV were located at the C-terminus of the protein; however, the nucleotide mutations corresponding to the last three encoded amino acids are conserved. This is of interest as this part of the protein may be involved in specific interaction with its cognate CP,

as has been reported for the *Alfalfa mosaic virus* (AMV) (Sánchez-Navarro et al. 2006). In addition, it has been also reported that a deletion of the C-terminal three amino acids of the AMV MP rendered this protein nonfunctional in cell-to-cell movement (Van der Vossen et al. 1994) and could be related to host specificity.

All the Spanish PMoV-T isolates and the Italian PMoV-T T-32 isolate lack the C_{1336} present in PMoV. Taking into account the PMoV start codon, this deletion would cause a frame shift in the ORF giving a nonviable CP. These differences could be explained as: (1) the C additional nucleotide in the reported sequence of PMoV could be an artefact as consequence of a cloning or sequencing error, and (2) the difference could be associated with the host-species from which the isolate was obtained. As the PMoV isolate from *P. officinalis* could not be used in the present study for expression of the putative CP gene, for PMoV-T isolates we propose an alternative start site 48 nucleotides downstream from the AUG described for the PMoV CP, giving a protein 16 amino acids shorter than that of the PMoV CP previously described. Several observations support this proposal at least for the tomato isolates: (1) expression in bacteria of the two alternative CP cistrons revealed that only those rendering the shorter CP was functional; (2) the largest version of the CP could not be synthesized in the seven isolates characterized here nor in the Italian PMoV-T T-32 isolate, and (3) the thirty clones corresponding to the G34H isolate had the same cytosine deletion at position 1366. Besides, these differences are at the very end N-terminus, whereas the rest of the molecule shares the important features characteristics of the Ilarvirus CPs: e.g. all PMoV-T isolates and PMoV had the Arg-rich motif with an α-helix secondary structure within the N-terminus of the CP, from aa 14 to 34 (Fig. 3). It has been demonstrated that this Arg-rich motif is involved in the interaction with viral RNA in other Ilarviruses (Aparicio et al. 2003).

The IGR nucleotide sequence of the seven isolates of PMoV-T characterized in this work ranged from 170 to 174 nt, being the less variable region of the RNA 3. Subgenomic promoter sequences have been studied in different viruses. Using deletion mutants, sequences 20–30 nt upstream of the *Brome mosaic virus* (BMV) sgRNA 4 initiation site have been identified (Marsh et al. 1988), which are sufficient for a basal level of transcription. The hypothesis that a hairpin structure is required for subgenomic activity has been proposed (Jaspars 1998). We propose a new stem-loop structure with a putative core promoter function located in nucleotide −2 upstream of the initiation site for sgRNA 4 synthesis (Fig. 4a, left). This structure is more stable than the stem-loop previously predicted (Fig. 4a, right). According to Jaspars (1998) predictions, the initiation site of sgRNA 4 synthesis was in the initiation codon of CP without leader sequence. We propose that the start of the sgRNA 4 transcription site and the CP initiation codon is separated by 28 nucleotides.

Mutational analysis of the BMV core promoter showed that at least the four residues G, A, C, and G at positions −17, −14, −13 and −11, respectively, relative to the subgenomic initiation site (+1), are essential for subgenomic activity, probably due to their implication in direct interaction with the replicase (Adkins and Kao 1998). Jaspars (1998) found conserved nucleotides in different Ilarviruses at positions −17, −13, −11 and −5, and two at positions −18 and −16. These nucleotides were essential for the stem-loop secondary structure of the core promoter. The positions of these conserved nucleotides in the promoter sequence of PMoV-T isolates and PMoV are shown (Fig. 4a), taking into account any displacement by insertions or deletions with respect to the BMV core promoter sequence (Jaspars 1998). There is an A→G change in position −4 in JBT1 and RAMS1 isolates, but this does not alter the predicted stem-loop structure. Only one stable hairpin was predicted for IGR in all PMoV-T isolates, as for the other four genera of the Bromoviridae family and other Ilarviruses, except PNRSV and ApMV for which two stable hairpin structures have been predicted (Jaspars 1998; Aparicio and Pallás 2002).

On the basis of biological comparative assays other authors suggested that PMoV-T isolates should be considered as a tomato strain of PMoV (Lisa et al. 1998). This situation would have important implications on the biological and epidemiological characteristics of this virus, which infects an economically important crop such as tomato. The biological differences between Spanish PMoV-T isolates and PMoV shown here also favoured this proposal. In addition, the phylogenetic analysis of Spanish PMoV-T isolates gave a cluster separated from the PMoV based on important genetic differences. These differences could be associated to the host, but they could also be a conse-

quence of the restricted area where the Spanish PMoV-T isolates were obtained. However, the host effect in the biological behaviour of PMoV isolates seems to be the determinant, since PMoV-T isolates did not infect pepper plants, whereas PMoV isolates from pepper in the south of Spain infect *Nicotiana* sp. but did not infect *Chenopodium* sp. (Janssen et al. 2005) as tomato isolates did. The completion of the nucleotide sequence of PMoV from pepper or PMoV-T isolates from different countries as well as the research on the genetic and epidemiological characteristics of this virus in tomato and pepper crops are in progress. These studies could shed light on this intriguing situation.

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