

International Journal of **Virology**

ISSN 1816-4900



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Cloning and Sequencing of a cDNA Encoding the Coat Protein of an Egyptian Isolate of Pepper mild mottle virus

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Abstract: A *Tobamovirus* isolated from pepper plants grown in Egypt has been characterized. Pepper mild mottle Tobamovirus (PMMoV) was isolated from naturally infected pepper plants grown in Kafr El-sheikh Governorate. Reverse Transcription Polymerase Chain Reaction (RT-PCR) using a specific non-degenerate primer pair for the PMMoV coat protein gene (PMM-F and PMM-R) revealed 470 bp amplified product. Dot blot hybridization was used to establish the authenticity and specificity to the RT-PCR amplified products of PMMoV. The coat protein gene of an Egyptian isolate of PMMoV was cloned and sequenced. The sequence contained a full-length ORF coding for the viral CP. It comprises 473 nt and a polypeptide chain of 157 amino acids with a M(r) of 17.27. The comparison of the CP ORF nucleotide sequence of Egyptian isolate (PMMoV-E2) with those of another isolates revealed that it was very close to the Brazilian and Japanese isolates (99 and 98%, respectively). Whereas the CP of PMMoV-E2 isolate was closely related to TMV-CP (99 and 97%) of Japan and Korea isolates, respectively. In the meantime it appeared far from Tropical Soda Apple Mosaic Virus-CP (TSAMV-CP) Florida isolate (USA) of *Tobamovirus*.

Key words: Pepper mild mottle virus, RT-PCR, hybridization, nucleotide sequencing

INTRODUCTION

Peppers (*Capsicum* sp.) are very important vegetables world wide, they provide spice and color to foods, while providing essential vitamins and minerals. Five species of Tobamovirus, Tobacco mosaic virus (TMV, P0), Tomato mosaic virus (ToMV, P0), Tobacco mild green mosaic virus (TMGMV, P0), Paprika mild mottle virus (PaMMV, P1), and Pepper mild mottle virus (PMMoV, P1,2 or P1,2,3), have been reported as pathogens of *C. annuum* in Japan (Hamada *et al.*, 2002, 2003; Nagai, 1981; Nagai *et al.*, 1981; Tsuda *et al.*, 1998, respectively). These viruses can cause serious economic losses in both field and greenhouse grown crops.

Pepper mild mottle virus (PMMoV) is one of the major viral pathogens of cultivated peppers; it causes mosaic symptoms and malformations on the leaves and fruit of green peppers, reducing the yield of commercial products worldwide (Nagai, 1981; Alonso *et al.*, 1989). Pepper mild mottle virus (PMMoV) consists of a rod-shaped particle with a length of about 300 nm and a width of 18 nm (Wetter and Conti, 1988) in which a positive-sense linear single-stranded RNA (6.3 kb) is encapsidated (Alonso *et al.*, 1991; Kirita *et al.*, 1997). The deduced CP amino acid sequences in some of these isolates differed not only from that of PMMoV-J, P1,z phenotype, but also from those

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reported for PMMoV-I and PMMoV-Ij, P1,z,3 phenotypes. Pepper mild mottle virus has only recently been identified in commercial bell pepper fields in Florida, in Italy, Southwest Florida and then in Southeast Florida (Lamb *et al.*, 2001).

Reverse Transcription Polymerase Chain Reaction (RT-PCR) method is becoming increasingly popular for rapid analysis of gene transcripts, primarily because of its high sensitivity and specificity. The use of Reverse Transcription-Polymerase Chain Reaction (RT-PCR) (Letschert *et al.*, 2002) or Restriction Fragment Length Polymorphism (RFLP) analysis following RT-PCR (Velasco *et al.*, 2002) successfully discriminated between the P0 pathotypes (TMV, ToMV, and TMGMV), the P1,2 pathotype of PMMoV and the Italian type of P1, 2, 3 PMMoV (Wetter *et al.*, 1984; Berzal-Herranz *et al.*, 1995). However, the P1 pathotype of PaMMV could not be distinguished by these methods. In addition to these PMMoVs, two other types of P1, 2, 3 PMMoV, distinct from the Italian type, have been reported in Japan (Hamada *et al.*, 2002; Tsuda *et al.*, 1998). Tests based on nucleic acid hybridization can be used for diagnosing plant viruses (Jones and Torrance, 1986). Until now, radioactive labeled probes have been commonly used for nucleic acid hybridization. However, such techniques have some disadvantages, in concern about the environmental impact, safety and short lifetime and cost using radioactive labels. Therefore, alternative hybridization methods that employ non-radioactive labeled probes have been developed and proving to be much more practical (Leary *et al.*, 1983; Forster *et al.*, 1985).

Here, we report the sequence of the coat protein gene of an Egyptian PMMoV RNA and compare it with those of other *Tobamoviruses*.

MATERIALS AND METHODS

Virus Source

Pepper mild mottle Tobamovirus (PMMoV) was isolated previously from naturally infected pepper plants collected from Kafr El-Sheikh Governorate under plastic conditions and identified on the basis of host range, stability in sap, modes of transmission, inclusion bodies, serological detection and electron microscopy (El-Khewey *et al.*, 2007).

Isolation of Viral Genomic RNA from Purified Virus Suspension

The viral genomic RNA was isolated from purified PMMoV (El-Kady *et al.*, 2007) according to Wilcockson and Hull (1974) using the phenol-SDS method. It was released from the purified virus by using approximately 100 µL of purified suspension and extracted in one volumes of 1x extraction buffer (200 mM Tris base; 100 mM NaCl; 10 mM EDTA, pH 8.0) and 0.2 volume of 10% SDS solution followed by vortexing for 2 min. Two volumes of phenol: chloroform: isoamyl alcohol mixture (PCI) were added and shaken vigorously for 10 min. The aqueous phase of each sample was recovered by low-speed centrifugation at 10,000 Xg for 15 min at 4°C (Sigma 2K15 laborzentrifugen). The RNA was precipitated by the addition of 1/10 volume of 3 M Na. acetate mixed with 2.2 volume of ice cold absolute ethanol and incubated at -20°C overnight. The RNA pellets were obtained by centrifugation at 10,000Xg for 15 min at 4°C. The RNA pellets were washed with ice cold 75% ethanol and centrifuged as above, dried under vacuum desiccator for 15- 20 min and resuspended in 50 µL of RNase-free water. The viral RNA was determined by analysis on 1% agarose gel electrophoresis (Maniatis *et al.*, 1982).

Oligonucleotide Primers

Newly designed specific non-degenerate primers were used in molecular studies for coat protein gene amplification as shown in Table 1. These primers should be amplifying a DNA copy of approx. 470 base pairs of PMMoV virus genome. The virus sense primer (PMM-F) corresponds to nucleotides (nt) positions 5685 to 5704 was designed according to PMMoV complete sequence (Wang *et al.*, 2006), while the complementary (antisense) primer (PMM-R) corresponds to (nt) positions 6135 to 6155 of PMMoV coat protein sequence (Table 1). The primers were synthesized at Operon Co., USA.

Table 1: Oligonucleotide sequences of the used primers

Primer	Primer size	Sequence (mers) 5'-----3'	Prod. size bp ^a
FCP	30	ATGTCTTACA GTATCACTAC TCCATCTCAG	500
PCP	18	TGGGCCCCCTACCGGGGGT	500
PMM-F	28	GGGCTAGA-ATGGCTTACACAGTTTCCAG	470
PMM- R	28	AGGTGATCCTAAGGAGTTGTAGCCCAGGT	470

^aThe approximate product size of the primers pair

cDNA Synthesis Using Random Primers

The PMMoV-cDNA was synthesized and amplified from purified viral RNA using random primers. A 10-50 ng of viral RNA was used as template for the Reverse Transcription reaction (RT). The RT reaction was prepared as follows; 0.625 ng of the random primer, 1X first strand cDNA buffer (5X buffer: 250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂, 50 mM DTT) 0.2 mM dNTPs, 0.1 µL of RNAase inhibitor (40 units µL, invitrogen), 0.1 µL of the superscript II reverse transcriptase enzyme (10 µ/µL invitrogen) were added to above mixture, the deionized water was added to final volume 25 µL. Finally, the reaction mixture was incubated at 42°C for 1 h and the resulting mixture was heated for 5 min at 94°C and chilled in ice for 2 min.

PCR Amplification Using PMMoV-Specific Primers

The PCR reaction mixture using specific PMMoV-CP primers was prepared by using 5 µL of the resulting cDNA. The PMMoV cDNA was transferred to a tube containing 45 µL of 1X polymerase chain reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.01% gelatine), 1.5 mM MgCl₂, 200 µM dNTPs, 10 pmol of PMM-F and PMM-R of forward and reverse primer respectively, 2.5 µ of Taq DNA polymerase (BioLab in rate of 5 µ µL⁻¹). The amplification proceeded in the thermocycler (Uno II Biometra) at 94°C for 3 min and through 30 cycles of 92°C for 30 sec and 60°C for 30 sec and 72°C for 1 min, with a final step at 72°C for 10 min.

Virus Detection by Nucleic acid Hybridization Technique

DNA Probe Labeling

The Dig-Labeled TMV-cDNA probe was synthesized using PCR-Dig labeling technique according to Roche, Boehringer Mannheim corp., Indianapolis, IN, USA, protocol. The DNA-PCR product of TMV virus was fractionated in 1% agarose gel and subjected to gene cleaning by Qiaquick gel extraction kit (Qiagen) The purified TMV DNA was used as a template for the polymerase chain reaction using PCR Dig Labeled dNTPs mixture. The PCR reaction was performed using degenerate primers (kindly provided by Dr. Shoman S. Microbiology Departmen, Faculty of Science, Ain Shams University) specific for detection of the TMV strains. The virus sense primer (Fcp) and the complementary (antisense) primer (Bcp) were according to Shoman (2004) as shown in Table 1.

The PCR cocktail was performed in 50 µL total volume containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.01% (w/v) gelatin, 4 mM MgCl₂, 200 µM dNTPs, 0.2 µM each primer, the virus sense (FCP) and antisense primer (BCP) and 2.5 units of the thermostable Taq DNA polymerase (Promega). The amplification proceeded in the thermocycler (Uno II Biometra) at 94°C for 3 min and through 40 cycles of 92°C for 30 sec and 60°C for 45 sec and 72°C for 2 min, with a final step at 72 °C for 10 min.

Dot Blot Hybridization Assays

The preparation of tissue extracts for dot blots was carried out according to Loebenstein *et al.* (1997) with some modifications. The leaf tissue (0.5 g) was ground in 2.5 mL of denatured solution containing [8X standard saline citrate (SSC) (1X SSC is 150 mM NaCl, 15 mM Na. acetate, pH 7.0) plus 10% formaldehyde] and then heated to 60°C for 15 min and kept on ice. Five microliter of the

denatured viral RNA and the supernatant of the plant extracts was spotted onto presaturated nitrocellulose membrane (Boehringer Mannheim corp.) with 20X SSC. The membrane was then crosslinked by the U.V irradiation for 2 min, followed by nucleic acid hybridization.

Cloning of PMMoV- Coat Protein Gene in Topo-T easy Vector

The PMMoV-Cp PCR amplified product was directly cloned in the linearized and thymidylated Topo-T-easy plasmid. The construct was transformed into DH5 α *E. coli* competent cells according to Sambrook *et al.* (1989). Blue/white colonies had screened up to select the bacterial colonies transformed with recombinant Topo plasmids using the PCR technique. Clones from transformed cells were purified using the Wizard minipreps DNA purification system (Promega Corporation MD) and sequenced directly.

Nucleotide Sequence Analysis

The DNA sequence of PMMoV-isolate was confirmed by direct sequencing the resulted clones briefly. The PMMoV coat protein gene region (470 bp) was sequenced in one direction using the M₁₃-21 forward primer. The sequence was carried out using ABI PRISM model 310 versions 3.4 semi-adaptive version 3.2 at gene analysis unit, UCDavis sequences facilities, (California, Davis, USA). Sequence analysis was performed using Bio Edit program version 5.0.6.

RESULTS AND DISCUSSION

Virus Detection by RT-PCR Amplification

Detection of PMMoV by reverse transcriptase-polymerase Chain Reaction (RT-PCR) was performed on purified viral RNAs extracted using the phenol-SDS method. Figure 1 show the viral genomic RNA integrity of the TMV (as positive control) and the PMMoV. The PCR using PMMoV specific primers gave a 470 bp amplified product corresponding to the coat protein gene of PMMoV as shown in Fig. 2 (lane 2) whereas no amplification was detected with the healthy pepper plant (Fig. 2, line 3).

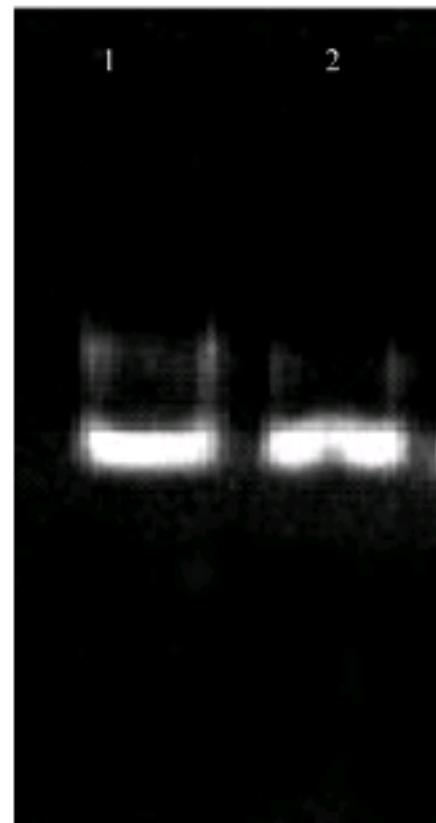


Fig. 1: One percent agarose gel electrophoresis for the RNA extraction from purified Viruses. Lane 1: RNA extraction from purified TMV virus, Lane 2: RNA extraction from purified PMMoV

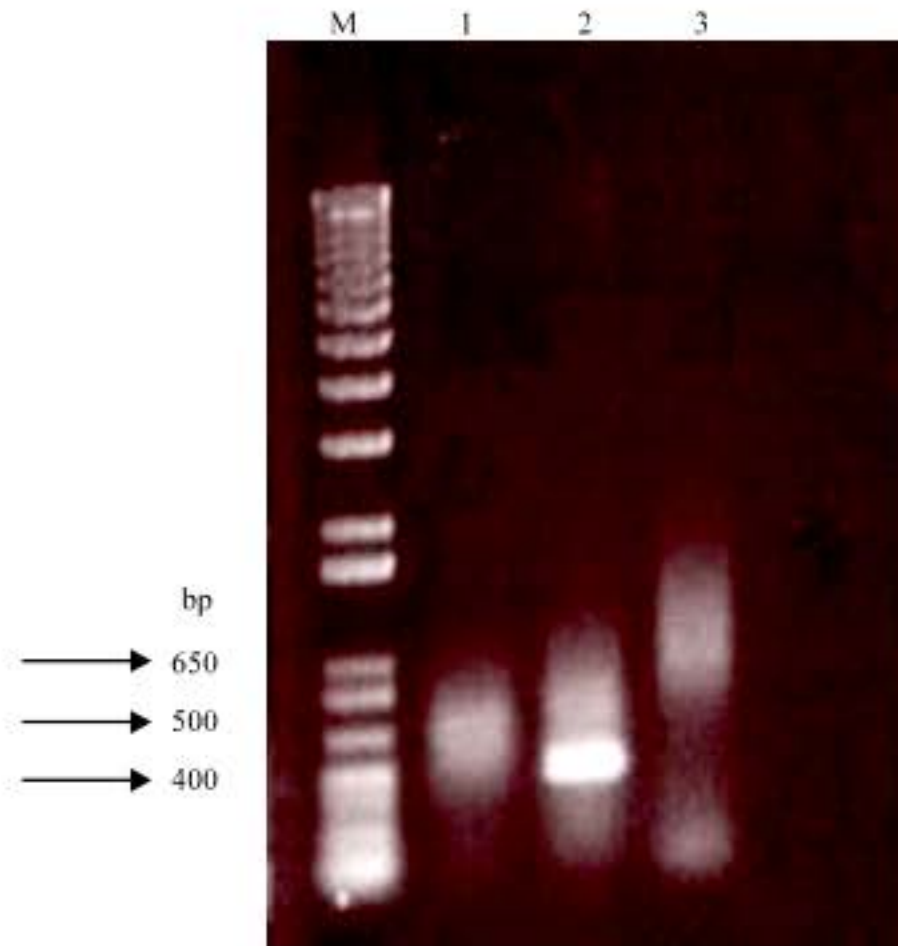


Fig. 2: Two percent agarose gel electrophoresis analysis of the amplified product of PMMoV coat protein gene using PMM-1 and PMM-2 specific primers. M:1 kb ladder, Lane 1: RT-PCR from PMMoV- virus purified from pepper infected plant, Lane 2: RT-PCR amplified product from PMMoV virus purified from *Nicotiana clevelandii* plant, Lane 3: RT-PCR from water PCR negative control

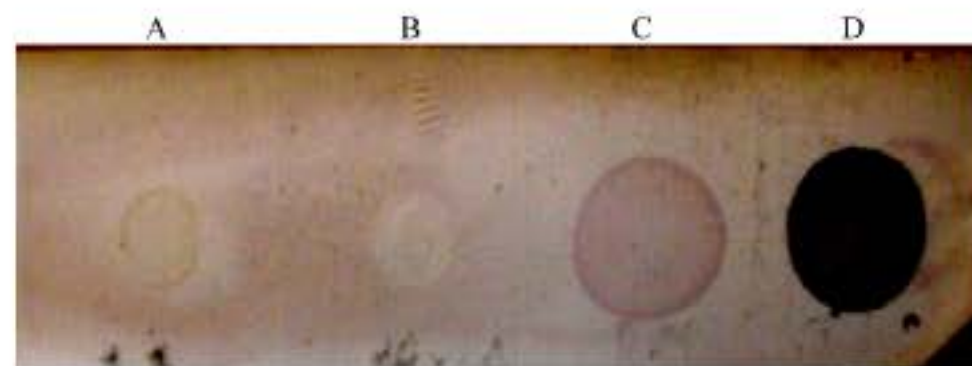


Fig. 3: DNA dot blot hybridization on nitrocellulose membrane. The membrane hybridized with specific non-radioactive TMV –DNA –Dig labeled probe. Track A: Uninfected *N. clevelandii* dot blot extract. Track B: Denatured PMMoV viral RNA dot blot. Track C: PCR product of PMMoV RNA. Track D: PCR product dot blot from the TMV RNA

Dot Blot Hybridization Technique

To detect the authenticity of the amplified product and its correlation to TMV virus previously detected and sequenced; the purified TMV DNA amplified product (TMV control) was used for creation of dig labeled- cDNA probe, specific for the TMV coat protein gene. The non-radioactive-Dig labeled probe was used for dot blot hybridization and clearly hybridized with our PCR products.

The TMV-cDNA probe hybridized and showed signals with high intensity with amplified product of TMV (positive control) (Fig. 3, track D).

As well as, the cDNA Dig labeled probe showed signals with the denatured PMMoV viral RNA and the amplified PCR product of PMMoV as showed in dot blot tracks B and C, respectively. No signals have been observed with uninfected *N. clevelandii* dot blot extract as shown in

track A. The virus RNA could be detected directly with high sensitivity and specificity from their natural host tissues by dot blot hybridization technique.

Nucleotide Sequence Analysis

One of this research objectives was to clone and sequence the coat protein gene of PMMoV. The 470 bp amplified CP gene product of the isolate PMMoV-E2 was ligated into the Topo-T-easy vector and cloned. Only 6 clones of PMMoV-E2 isolate was sequenced revealing 473 base fragments as expected as shown in Fig. 4 with deduced coat protein consisted of 157 amino acids with a M(r) of 17.27. A multiple alignment was done along with sequences previously obtained which have already been used as reference sequences in other studies. The following sequences were used in the comparisons: Brazil (PMMoV-SaltoBR12 and Itapetininga BR17) with isolate accession No. AM411434 and AM411438, PMMoV-Japanese according to Hamada *et al.* (2001) (PMMoV-Ge 1 and Tosa with accession No. AB062049 and AB062053 respectively), TMV-isolate (CP) Japanese isolate (Kirita *et al.*, 1997) with accession No. L35074 and the Korean from the accession No. AF103778, AB084456 (TMV and PMMoV-P2 isolate) and PMMoV-P91 and PMMoV-CN isolate from Germany and China, respectively (Letschert *et al.*, 2002; Wang *et al.*, 2006) were also included in the comparisons.

The nucleotide sequence alignment in Fig. 4 showed 99-97% similarity with (PMMoV-SaltoBR12 and PMMoV-Ge 1) isolates belongs to Brazil and Japan, respectively. In the meantime the Egyptian isolate (PMMoV-E2 isolate) showed 98, 97, 96 and 94% similarities with Itapetininga BR17, (PMMoV-CN, PMMoV-P2) and (Tosa, PMMoV-Italian and PMMoV-P91 isolate), respectively and correspond to Brazil, China, Korea, Japan, Italy and Germany isolates. However, the phylogenetic homology tree based on multiple sequence alignments (Fig. 4, 5) of the Egyptian present isolate (PMMoV-E2 isolate), revealed that the (CP) of the PMMoV-E2 isolate was closely related to TMV-CP (99 and 97%) of Japan and Korea, respectively.

These results, revealed that the PMMoV-E2 present isolate appeared far from Tropical Soda Apple Mosaic Virus-CP (TSAMV-CP) Florida isolate (USA) of *Tobamovirus* which showed 81%.

The viral sequence obtained in this study was deposited in the National Center for Biotechnology Information Gene Bank with accession No. EU380719 for PMMoV Coat Protein (CP) gene sequence.

The cDNA of PMMoV was synthesized using random primers and used as a template for PCR amplification of the full length of the viral coat protein gene approximately 470 bp amplified product using a specific non-degenerate primer pair for the PMMoV coat protein gene (PMM-F and PMM-R). This size was in agreement with those expected from the nucleotide sequence of the PMMoV-CP gene (Wang *et al.*, 2006).

The RT-PCR technique is highly sensitive, simple and useful in overcoming many difficulties encountered with serological methods, such as low antigen titer, availability of antibodies and cross reactivity of antibodies with heterologous antigens (Matthews *et al.*, 1997). It seems also that, RT-PCR is faster than traditional methods and other methods such as squash blot and dot blot hybridization. It can be concluded from our study that the RT-PCR assay has a great sensitivity that enables the detection of TMV and its strains in infected samples; this result was in agreement with Tenllado *et al.* (1994) who stated that a procedure based on polymerase chain reaction was developed allowing the rapid detection and molecular differentiation of the distinct pathotypes of the tobamoviruses infecting TMV resistance genotypes of pepper in RNA-enriched fractions from infected plants. Letschert *et al.* (2002). Also, developed the same technique involving RT-following PCR using single primer pair for detection and differentiation of the five *Tobamovirus* species which are related serologically using the same PCR conditions and primer sets.

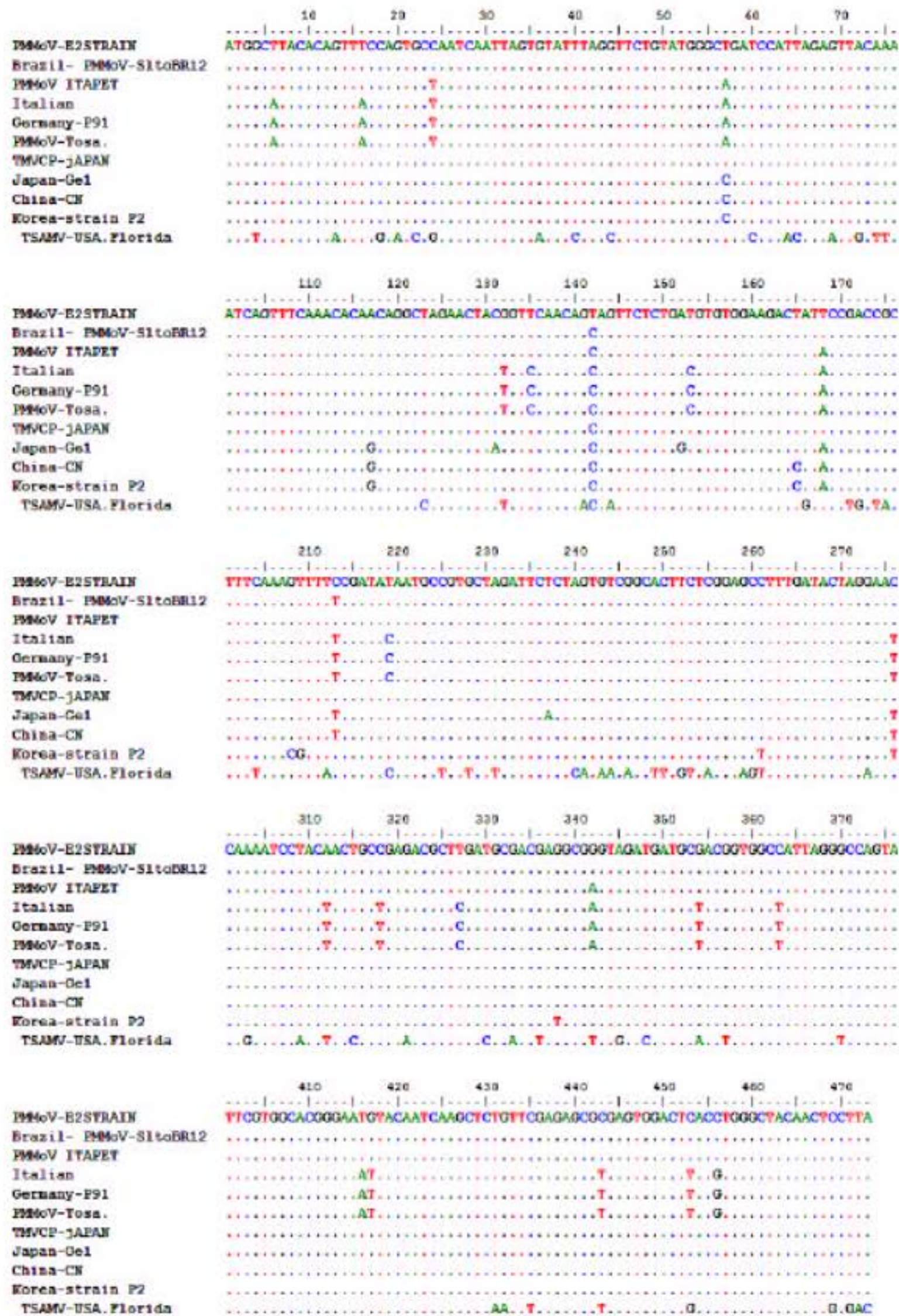


Fig. 4: Nucleotide sequence alignment of cloned PMMoV-E2 coat protein gene with another published sequences

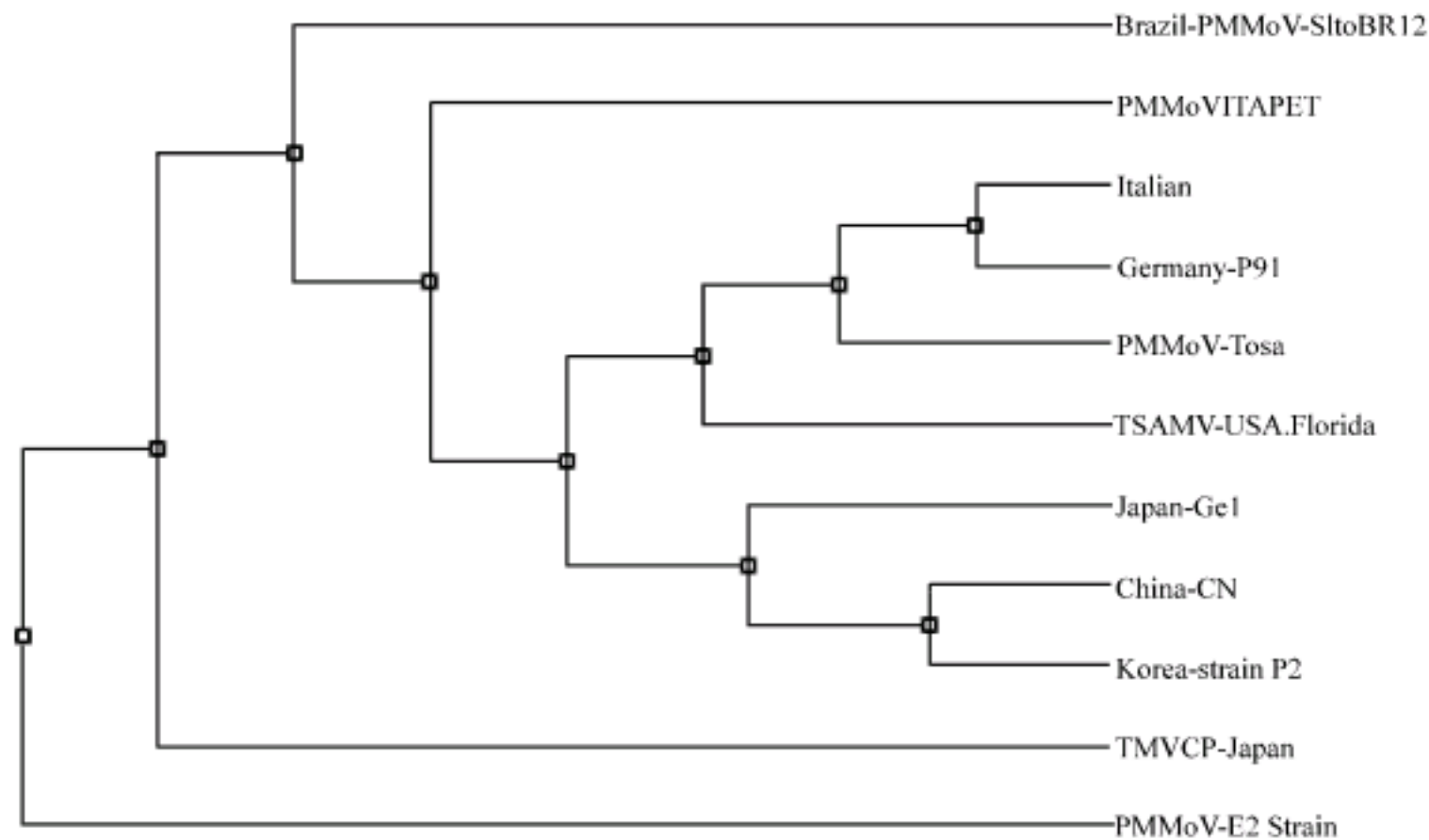


Fig. 5: The phylogenetic homology tree based on multiple sequence alignments of the PMMoV-E2-Egyptian isolate compared to previously sequenced isolates

This also has intense interest to use this simple technique (RT-PCR) to detect the viral spread earlier. These results in accordance with Pereda *et al.* (2000) who stated that the Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) was very sensitive for detecting viral spread earlier and TMV-Cg in *Arabidopsis thaliana*, but less sensitive for TMV-U1 detection. In addition Tobacco mosaic virus (TMV) was used in previous study as a positive control, therefore, it could be used for addition to Reverse Transcription Polymerase Chain Reaction (RT-PCR) assays as an internal control specially incase of the duplex RT-PCR (Torok and Randles, 2001). The non-radioactive DIG-labelled TMV cDNA probe, amplified from the full length coat protein gene using Fcp and Bcp degenerate primers, was used for detection of the PMMoV via., dot blot hybridization technique. The TMV-cDNA specific probe and dot blot hybridization were used to establish the authenticity and specificity of the RT-PCR products. In addition, the virus RNA could be detected directly with high sensitivity and specificity from their natural host tissues by dot blot hybridization technique.

The nucleotide sequence of the coat protein gene of PMMoV was determined. The deduced coat protein consisted of 157 amino acids and this in agreement with Garcia-Luque *et al.* (1993). The composition of the CP ORF nucleotide sequence of Egyptian isolate (PMMoV-E2) with those of another isolates revealed that it was very close to the Brazilian and Japanese isolates (99 and 98%). Whereas the PMMoV-E2 isolate, revealed that the CP of the PMMoV-E2 isolate was closely related to TMV-CP of Japan and Korea (99 and 97%), respectively in the meantime it appeared far from Tropical soda apple mosaic virus-CP (TSAMV-CP) Florida isolate (USA) of *Tobamovirus*.

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