Plant Pathol. J. 21(4) : 361-368 (2005)

The Plant Pathology Journal
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Molecular Characterization and Infectious cDNA Clone of a Korean Isolate of *Pepper mild mottle virus* from Pepper

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(Received on September 21, 2005; Accepted on November 15, 2005)

A Korean isolate of *Pepper mild mottle virus* (PMMoV-Kr) was isolated from a diseased hot pepper plant and its biological and molecular properties were compared to that of PMMoV-J and PMMoV–S. The genomic RNA of PMMoV-Kr consists of 6,356 nucleotides. The nucleotide and amino acid sequences identities of four viral proteins and two noncoding regions among PMMoV-Kr, PMMoV-S and PMMoV-J were 96.9% to 100.0% and 97.5% to 98.6%, respectively. Full-length cDNA amplicon of PMMoV-Kr was directly amplified by RT-PCR with a set of 5'-end primer anchoring T7 RNA promoter sequence and 3'-end virus-specific primer. Capped transcript RNAs from the full-length cDNA clone were highly infectious and caused characteristic symptoms of wild type PMMoV when mechanically inoculated to systemic host plants such as *Nicotiana benthamiana* and pepper plants.

Keywords : genome, infectious cDNA clone, pepper, *Pepper mild mottle virus*, sequence, *Tobamovirus*

Pepper mild mottle virus (PMMoV) is a member of the genus *Tobamovirus* and occurs worldwide in fields-grown bell pepper, hot pepper and ornamental pepper species (Garcia-Luque et al., 1990; Kirita et al., 1997). Its genome consists of single strand positive RNA of approximately 6,357 nucleotides. It contains five open reading frames; a 126-kDa protein with methyl-transferase (MT) and RNA helicase (HEL) motifs and a read-through 183-kDa protein containing RNA replicase motifs, both of which are necessary for viral replication. A 30-kDa movement protein for cell-to-cell movement, and 17.5-kDa coat protein are encoded in tobamoviral genome RNA (Garcia-Luque et al., 1990; Grdzelishvili et al., 2000; Lewandowski and Dawson, 2000; Tenllado et al., 1996).

The chronic viral diseases caused by *Broad bean wilt virus* (BBWV), *Cucumber mosaic virus* (CMV), *Pepper mottle virus* (PepMoV), PMMoV, *Tomato mosaic virus* (ToMV), *Tobacco mild green mosaic virus* (TMGMV) and *Tobacco mosaic virus* (TMV) have occurred in pepper crops in Korea (Choi et al., 2005; Im et al., 1991). Moreover, most of diseases revealed as mixed infection with two or three viruses of them resulted in severe yield losses annually. Several Tobamoviruses including TMV, PMMoV, ToMV and TMGMV, have been identified as major agents of disease in pepper crops in the world and they were originally designated as pepper strains of the tobamoviruses. Among them, PMMoV is the major viral disease agents in pepper plants in Spain and Japan as well as in Korea, even though it causes mild mosaic or sometimes symptomless (Alonso et al., 1989; Kirita et al., 1997). To date, two isolates, namely an Italian isolate (PMMoV-I) and a Japanese isolate (PMMoV-Ij) have been described which are able to overcome L^3 resistance. In both isolates, changes in the amino acid sequence of the viral CP are responsible for this resistance-breaking pathotype (Garcia- Luque et al., 1993; Tsuda et al., 1998). PMMoV-S and PMMoV-J have been categorized to $P_{1,2}$ by their responses against four allelic tobamoviral resistance genes, L^1, L^2, L^3 and L^4 in *Capsicum* spp. (Boukema, 1984).

In this study, virus-infected pepper cultivars that were growing under open field were surveyed every three or four weeks after seedling in the Gyeonggi province of Korea. Among them, 147 diseased leaves showing various viral symptoms were collected and analyzed by RT-PCR with specific primer sets of CMV, PepMoV, BBWV and PMMoV. A Korean isolate of PMMoV was analyzed for its biological, molecular and serological properties. To obtain a system for developing resistance screening against this virus as well as to study the function of the virus-pepper host interaction, an infectious full-length clone of PMMoV-Kr was constructed and its infectivity for systemic host plants such as *Nicotiana benthamiana* and pepper plants was assessed by RT-PCR and Western blot analysis.

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Materials and Methods

Virus source and survey of pepper-infecting viruses in Korea. Virus-infected pepper cultivars grown under open field with or without virus-like symptoms were surveyed in the Gyeonggi province of Korea. Since the samples were checked every three or four weeks, 147 diseased leaves showing viral symptoms were collected on 20 weeks after seedling. To detect pepper-infecting viruses, specific primer sets of CMV, BBWV, PepMoV and PMMoV were designed in this study (Table 1). PMMoV-Kr was obtained from three consecutive serial passages of single lesion from *N. tabacum* cv. Xanthi-nc and propagated in *N. tabacum* cv. Samsun (Fukuda et al., 1980). PMMoV-J kindly provided by Dr. Shinya Tsuda was used as a positive control in this study. All plants for virus purification were maintained in a growth chamber or greenhouse at 28°C with a 16h light/8h dark photoperiod.

Plant culture and virus inoculation. Green pepper plant (*C. annuum* L. var. *angulosum* Mill.) was used as the host plant in all of the experiments. Pepper seeds were sown in a greenhouse maintained at 22°C to 28°C with a 16h light/8h dark photoperiod, and plants were used approximately four weeks after sowing. Crude sap extracted from progeny virus of PMMoV in inoculation buffer (0.01M potassium phosphate buffer, pH 7.6) was used to mechanically inoculate healthy green pepper and *N. benthamiana* plants.

RT-PCR and full-length cDNA cloning. The viral RNA from leaf tissue (0.1 g) was extracted with RNA extraction buffer and phenol/chloroform (1:1 v/v) twice, and total nucleic acids were ethanol-precipitated by standard protocol (Sambrook et al., 1989). cDNAs from viral RNA was synthesized using GeneAmp RNA PCR Core kit (Applied Biosystems, Roche). PCR condition for PMMoV CP were 94°C for 2 min, 35 cycles of 94°C for 30 sec, 53°C for 30 sec and 72°C for 1 min, followed by 72°C for 5 min extension. PCR for CMV, TMV and PepMoV was conducted as above except for the annealing temperatures adjusted to its specific primer sequences.

Full-length cDNA was synthesized by Expand long template system with specific primers corresponding to 5'- and 3'-end of PMMoV. The 5'-end primer, 5PMMoVT7 (5'-GAGA*GAATTC*TAATACGACTCACTATAGTAAATTTT TCACAATTTAACA-3'), contains *Eco*RI site (italic character) and T7 promoter sequences (underlined) and the 3'-end primer, 3PMMoVXb (5'-GAGA*AGATCT*TGGGC-CGCTACCCGCGGTTC-3'), contains *Xba*I site (italic character) for cloning. PCR was performed as described previously (Yoon et al., 2001). First cDNAs from RNAs were synthesized using Superscript II RNase H^- reverse transcriptase (Invitrogen) and 3PMMoVXb downstream primer at 42°C for 60 min. Second PCR was performed with 5PMMoVT7 and 3PMMoVXb primer by Expand long template DNA polymerase (Roche) in an i-cycler (Bio-Rad, USA). PCR cycles were 2 min at 94°C, followed by three different step cycling. First step was 5 cycles of 20 sec at 94°C, 30 sec at 55°C and 7 min at 68°C, followed by 10 cycles of 20 sec at 94°C, 30 sec at 57°C and 8 min at 68°C. Final step was 10 cycles of 20 sec at 94°C, 30 sec at 59°C and 9 min at 68°C. The reaction was terminated by a 10 min elongation at 72°C. An aliquot of the final RT-PCR products was analyzed by 1.0% agarose gel electrophoresis. Amplified RT-PCR products were digested with *Eco*RI and *Xba*I restriction endonucleases and cloned into the pUC18 plasmid cut by the same enzymes. Full-length cDNA clone was used as template DNAs for *in vitro* transcription reaction.

Full-length sequencing of PMMoV-Kr. Primers for full-length sequencing of PMMoV-Kr were designed corresponding to PMMoV-J and primer positions are addressed every 600bp of PMMoV-J sequence (data not shown). Full-length cDNAs were sequenced by using ABI PRISM BigDye™ Terminator v 3.0 Cycle Sequencing Kits (Roche) with 12 sets of PMMoV-specific designed primers (data not shown). Sequencing reactions were run on a fluorescent

Table 1. RT-PCR primers for detection of pepper-infecting major RNA viruses used in this study

Virus	Primer	Sequences	Product size
CMV	CPTALL-5[a]	5'-YASYTTTDRGGTTCAATTCC-3'	940bp
	CPTALL-3	5'-GACTGACCATTTTAGCCG-3'	
PepMoV	PepCP-UP	5'-AGCAGCTCAAGATCAGACACAT-3'	818bp
	PepCP-DN	5'-CATTTCCCTGACCCCAAGCAA-3'	
PMMoV	PMMCP-UP	5'-GAGGATCCATGGCTTACACAGTCTCC-3'	474bp
	PMMCP-DN	5'-GAGAATTCTTAAGGAGTTGTAGCCCAG-3'	
BBWV	BBWCP-UP	5'-AATGAAGTGGTGCTCAACTACACA-3'	653bp
	BBWCP-DN	5'-TTTTGGAGCATTCAACCATTTGGA-3'	

[a] Choi et al.. 1999

DNA sequencer ABI3100 (Perkin-Elmer). Sequences were analyzed using the Sequencer program (Gene Codes Corporation, USA).

Sequence analysis. Analysis of nucleotide and the deduced amino acid sequences of PMMoV were performed by using the DNAStar (Madison, USA) and DNAMAN Sequence Analysis Program (Version 5.1, Lynnon Biosoft Co., Canada). The nucleotide and amino acid sequences of PMMoV-S and –J and those of other viruses of tobamovirus group were surveyed using GenBank site (http:// www. ncbi.nlm.nih.gov). Secondary structure analysis of the 3'NCR of PMMoV sequence was predicted by RNA folding program (http://www.bioinfo.rpi.edu/applications).

***In vitro* transcription for inoculation of host plants.** The full-length cDNA clone of PMMoV, pPMFEX1 used as templates for transcription was digested with *Xba*I for run-off transcription. After linearization with *Xba*I, the DNA was extracted with phenol:chloroform and then precipitated with ethanol. Transcription reaction was performed in cap analog ($m^7G[5']ppp[5']G$) using T7 RNA polymerase according to the methods described previously (Yoon et al., 2001). RNA transcripts were analyzed on 1.0% agarose gel and each plant was directly inoculated with 2.0 to 3.0 mg of transcripts per leaf. The synthesized transcripts were directly inoculated onto Carborundum-dusted leaves of *N. benthamiana* and pepper plants at the four-to-five leaf stage. Virus symptoms on the transcripts RNA-inoculated plants were compared to those of wild type virus in the greenhouse.

Western blot analysis. Total proteins were extracted from diseased systemic leaves on any plants, as previously described by Yoon et al. (2001). Subsequently, the purified virus or total proteins were mixed with 2 x protein sample buffer, denatured by boiling for 3 min and chilled on ice. The sample was subjected to 12% of SDS-polyacrylamide gel electrophoresis described by Laemmli (1970). For Western blot assay, the gel was incubated with transfer buffer (39 mM glycine, 48 mM Tris, 0.037% SDS and 20% methanol) and electro-blotting onto nitrocellulose membrane was performed as a protocol by manufacturer's instruction (Bio-Rad) and standard protocol (Sambrook et al., 1989). The blotted proteins were immunoprobed with the antiserum against PMMoV CP diluted in 1:1000 (v/v). Subsequently, the membranes were incubated with the solution of goat anti-rabbit IgG diluted in 1:7500 (Promega) conjugated with alkaline phosphatase as the secondary antibody. Color reaction was developed with Western Blue Stabilizer Substrate (Promega, USA).

Results and Discussion

Survey of virus diseases in pepper crops in Korea. Incidence of virus from diseased pepper crops grown in open field was surveyed in this study. The diseased plants produced mosaic, mottling, yellow mosaic, yellowing, vein necrosis, vein clearing or vein banding or mixed symptoms. The infection rate of pepper crops to the viruses was extremely high ranging from 8.8 to 90.6% of the crop plants in that field (data not shown). Very recently, Choi et al. (2005) reported the results of distribution of viruses from cultivated pepper plants, and occurrence of virus infection and kinds of viruses were similar to those of our study. Among them, 147 samples with symptoms were confirmed by RT-PCR (Table 2). Most of diseased pepper crops were infected by single or mixed viruses. PMMoV and CMV were major pathogens from the collected pepper plants in this study, whereas BBWV was not detected (Table 2). PMMoV was found to be the major disease agent in pepper field of Korea. A Korean isolate of PMMoV, denoted as

Table 2. Percentage of symptom incidence by pepper infecting viruses in open field

Virus	Symptoms incidence by viruses in pepper plants (%)									
	M[a]	Mo	YM	Y	VN	Vc	Vb	Y, Vb	M, Ro	YM, Ro
PMMoV	38(56.7)	18(85.7)	4(30.8)	1(50.0)	1(50.0)	0(0.0)	1(50.0)	0(0.0)	4(17.4)	8(53.2)
CMV	16(23.8)	2(9.5)	5(38.4)	1(50.0)	1(50.0)	2(100)	0(0.0)	0(0.0)	2(8.7)	1(6.7)
PepMoV	5(7.5)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(50.0)	0(0.0)	8(34.8)	1(6.7)
BBWV	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
PMMoV, CMV	6(9.0)	1(4.8)	2(15.4)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(100)	2(8.7)	1(6.7)
PMMoV, PepMoV	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	2(8.7)	3(20.0)
PepMoV, CMV	2(3.0)	0(0.0)	2(15.4)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	4(17.4)	1(6.7)
PMMoV, PepMoV, CMV	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(4.3)	0(0.0)
Total (147)	67	21	13	2	2	2	2	1	22	15

[a]M, mosaic; Mo, mottling; Ro, rosette; Vb, vein banding; Vc, vein clearing; VN, vein necrosis; Y, yellowing; YM, yellow mosaic

PMMoV-Kr, was isolated from one of diseased leaves by successive single local transfer on *N. tabacum* cv. Xanthi-nc for genome sequence analysis.

Genomic sequence of PMMoV-Kr. Sequences of the entire genomic RNA of PMMoV-Kr were determined with a fluorescent DNA sequencer (ABI3100, Roche) using 12 sets of PMMoV-specific primers and a long contig was made by DNAStar computer program. The complete genome of PMMoV-Kr is 6,356 nt long and contains four ORFs which encode a 126-kDa protein (70 to 3423nt) and 183-kDa protein (nt 70 to 4908), a 30-kDa movement protein (MP, nt 4909 to 5682) and a 17.5-kDa coat protein (CP, nt 5685 to 6158). The 5' non-coding region (NCR) contains 69 nucleotides and there is no G residue upstream AUG codon, whereas the 3'NCR consisted of 198 nucleotides. This has none of ORFs overlap. The complete nucleotide and predicted amino acid sequences of PMMoV were submitted to the DDBJ/EMBL/GenBank database (accession number AB126003).

Both of the nucleotide and predicted amino acid sequences were compared with those of PMMoV-J, –S and fifteen other tobamoviruses (Table 3). As shown in Table 3, PMMoV-Kr showed the highest degree of nucleotide and amino acid sequence identity with PMMoV-J and –S. When compared with complete genome sequences of PMMoV-J and –S, PMMoV-Kr was found substituted or silent differences of 18 to 20 amino acids and 167 to 172 nucleotides. A comparison of PMMoV-J and –S revealed only seven nucleotide sequence differences in spite of geographical distance between Japan and Spain, so that it suggested to be derived from same origin by import or seed transmission (Kirita et al., 1997). The 126-kDa protein showed 47.5% to 97.3% and 39.9% to 99.2% identities in the nucleotide and amino acid levels, respectively whereas the 183-kDa protein showed 50.0% to 97.2% (nucleotide) and 43.8% to 99.0% (amino acid) sequence identities. PMMoV-Kr MP revealed nucleotide sequence identities of 38.9% to 97.7% and amino acid sequence identities of 22.6% to 97.3% with those of PMMoV-J and –S and other tobamoviruses. The CP gene of the PMMoV-Kr showed 44.8% to 98.1% nucleotide sequence identities and 35.3% and 100% amino acid identities with those of PMMoV-J and –S and other tobamoviruses. The 5'- and 3'-non coding regions showed 37.3% to 98.6% and 43.7% to 98.0% sequence identities, respectively.

The eighteen tobamoviruses were classified into four main subgroups based on a phylogenetic tree analysis on the 126 and 183 kDa, MP and CP amino acid. *Solanaceae*-infecting tobamoviruses could be grouped into one based on phylogenic tree of amino acid sequence and PMMoVs formed a subgroup together with ORSV, TMV and ToMV.

Table 3. Percentages of nucleotide (nt) and amino acid sequence (aa) identities of the PMMoV-Kr and other tobamoviruses

	5'NCR	126-kDa		183-kDa		MP		CP		3'NCR
		nt	aa	nt	aa	nt	aa	nt	aa	
PMMoV-S[a]	98.6	97.3	99.2	97.2	98.9	97.5	96.9	97.9	100	97.5
PMMoV-J	98.6	97.3	99.2	97.2	99.0	97.7	97.3	98.1	100	98.0
TMV	66.7	68.0	73.1	69.5	75.1	63.2	64.2	64.8	72.0	70.1
ToMV	66.2	68.8	74.5	70.3	76.7	64.8	62.9	67.6	72.0	68.2
TMGMV	66.7	61.7	61.8	64.0	65.0	61.2	64.4	65.2	70.1	50.0
PaMMoV	70.6	62.4	65.1	64.7	68.2	61.6	61.8	66.0	70.1	55.4
ORSV	50.0	57.8	57.1	61.3	62.2	63.3	66.9	63.6	68.6	43.7
ObPV	72.1	62.8	65.4	64.4	68.0	61.3	61.4	66.8	69.4	56.4
Cr-TMV	62.1	56.9	56.2	60.3	61.5	48.2	37.9	52.7	45.8	46.4
RMV	62.1	58.3	58.4	61.4	62.8	47.2	38.2	54.3	50.3	46.4
YoMV	62.1	58.3	58.1	61.4	62.7	47.2	38.2	54.3	50.3	45.7
TVCV	63.1	57.4	56.5	60.4	61.6	48.4	38.3	53.5	48.4	47.1
CGMMV	58.6	47.8	40.4	50.9	46.1	42.4	30.0	48.1	35.9	55.4
KGMMV	38.9	49.1	40.9	52.2	45.4	41.4	31.6	45.3	36.5	59.7
ZGMMV	37.5	48.6	40.9	51.7	45.7	40.3	33.2	44.8	35.3	60.8
CFMMV	37.3	49.0	40.4	51.6	44.8	43.3	33.2	45.3	35.9	56.6
SHMV	37.9	47.5	39.9	50.0	43.8	38.9	22.6	46.3	40.8	47.6

[a]The following sequences were obtained from NCBI GenBank database. PMMoV-S [M81413], PMMoV-J [AB000709], TMV [V01408], ToMV [AF332868], TMGMV [M34077], PaMMoV [AB089381], ORSV [X82130], ObPV [D13438], cr-TMV [Z29370], RMV [AF254924], YoMV [U30944], TVCV [U003387], CGMMV-SH [D12505], KGMMV-C1 [AJ295948], ZGMMV [AJ295949], CFMMV [AF321057], SHMV [J02413].

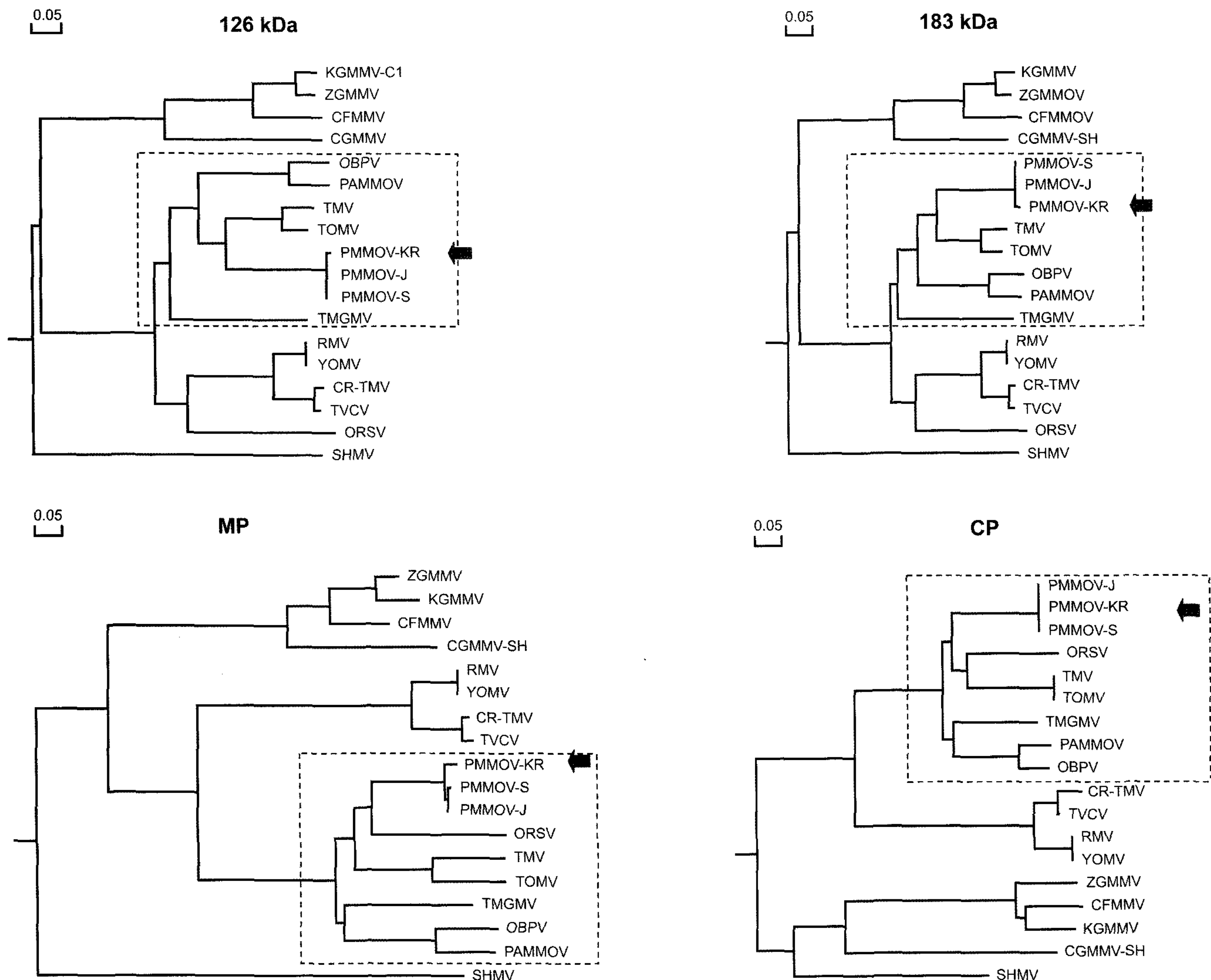

Fig. 1. Phylogenic relationship of amino acid sequence of the 126 (A) and 183 (B) kDa protein, MP (C) and CP (D) among viruses of tobamovirus genus. DNAman software program was used for the analysis of the alignment. Data from PMMoV-S [M81413], PMMoV-J [AB000709], TMV [V01408], ToMV [AF332868], TMGMV [M34077], PaMMoV [AB089381], ORSV [X82130], ObPV [D13438], cr-TMV [Z29370], RMV [AF254924], YoMV [U30944], TVCV [U003387], CGMMV-SH [D12505], KGMMV-C1 [AJ295948], ZGMMV [AJ295949], CFMMV [AF321057], SHMV [J02413].

(Fig. 1)

Infectious full-length cDNA cloning and infectivity test for PMMoV-Kr. Full-length cDNA molecules of PMMoV-Kr were directly amplified by RT-PCR with 5'- and 3'-end primers and cloned into pUC18 cut by *Eco*RI and *Xba*I (Fig. 2A and 2C). Full-length cDNA construct was designed to make full-length in vitro transcript using the T7 RNA promoter in the vector (Fig. 2A and 2D). Full-length cDNAs clone of PMMoV, pPMFEX1, was linearized with *Xba*I and transcribed with T7 RNA polymerase. The pPMFEX1 and pTPW1 (a full-length cDNA clone of PMMoV-J as a positive control) were about 6.4kb long, the same size as the wild-type RNA genome obtained from virus particles (Fig. 2D). PMMoV-Ia, –I and –Ij (pathotype $P_{1,2,3}$) could overcome the resistance conferred by the L^3 genes in pepper plants but PMMoV-Kr, –J and –S (pathotype $P_{1,2}$) could not. The L^3 gene resistance is elicited by the tobamovirus CP (Berzal-Herranz et al., 1995). By changing a single amino acid from Asn in PMMoV-I to Met in PMMoV-S at position 138 on the CP, elicitor activity and the ability to contain the virus within necrotic lesions were conferred to peppers carrying the L^3 gene (Berzal-Herranz et al., 1995). In addition, substitutions at both amino acids 43 and 50 differed between PMMoV-J and –Ij would enable to PMMoV-J to overcome L^3 resistance (Tsuda et al., 1998) (Fig. 2B). Capped transcripts developed typical mosaic symptom in *N. benthamiana* and

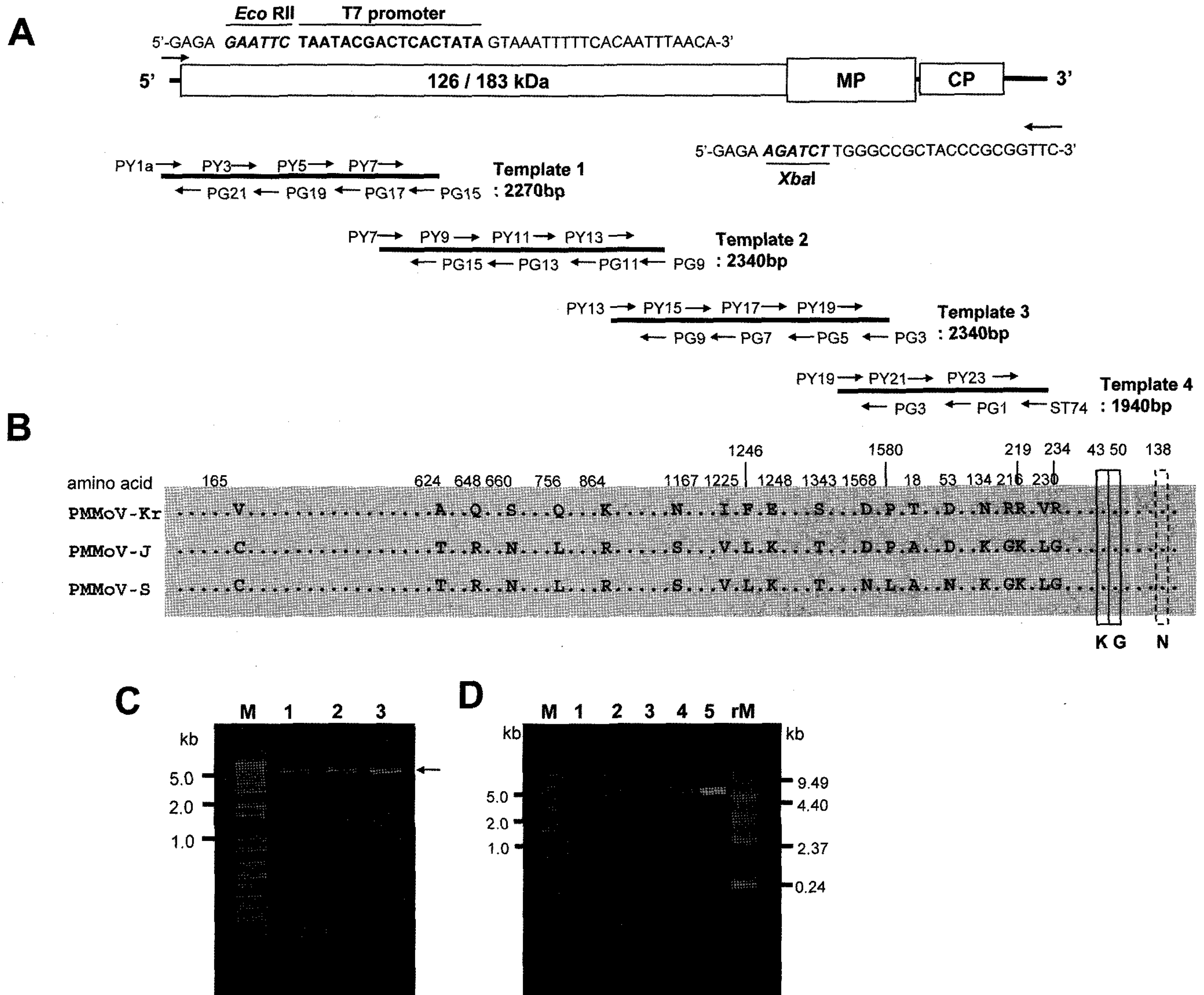

Fig. 2. Schematic representation (A) and comparison of predicted amino acid sequences (B) and RT-PCR products (C) for full-length cDNA synthesis of PMMoV-Kr and *in vitro* transcripts from PMMoV clones (D). **A.** Primer set for full-length cDNA synthesis of PMMoV-Kr was represented. Restirction enzyme sites for cloning and T7 promoter site were represented with italic and bold character, repectively. **B.** Comparison of predicted amino acid sequences of the full-length PMMoV-Kr gene with PMMoV-J and –S. PMMoV belong to subgroup 1b of tobamovirus group. Open boxes represent amino acid of PMMoV-Ij (black lined box) and PMMoV-I (dot lined box) CP of $P_{1,2,3}$ as a elicitor of L3 gene-mediated resistance. **C.** Full-length cDNAs of PMMoV-Kr were synthesized directly by RT-PCR with specific primers corresponding to 5'- and 3'-end of PMMoV. Lane 1, viral RNA (50 ng) of PMMoV-Kr; lane 2, viral RNA (100 ng) of PMMoV-Kr; lane 3, viral RNA (100 ng) of PMMoV-J. Arrowhead indicates PCR products, approximately 6.4 kb. **D.** Transcripts from PMMoV-Kr and –J full-length cDNAs, pPMFEX1 and pTPW1, respectively. Lane 1, linearized pPMFEX1 by *Xba*I; lane 2, transcripts from pPMFEX1; lane 3, linearized pTPW1 by *Xba*I; lane 4, transcripts from pTPW1; lane 5, PMMoV-Kr viral RNA. Lane M is 1 kb plus DNA ladder (Invitrogen) and rM is RNA ladder (Invitrogen). Viral RNA and full-length clone (pTPW1) of PMMoV-J were used as a positive control in this study.

pepper plants and the symptoms were similar to those generated by wild-type PMMoV (Fig. 3).

Infectivity of cDNA transcripts of PMMoV in inoculated and upper leaves of *N. benthamiana* plants was verified by RT-PCR and western blot analyses (Fig. 4A). Progeny virus from plants infected with transcripts RNA could be passaged to healthy plants, and gave the same symptoms. Progeny virus derived from infectious *in vitro* transcripts was efficiently transmitted by sap inoculation on its host plants, and its physical and biochemical properties were the same with wild type virus. The CP level of PMMoV-Kr and –J was undistinguishable in plants inoculated with RNA transcripts or viral RNA and the progeny virus also reacted positively with homologous antiserum (Fig. 4B). To deter-

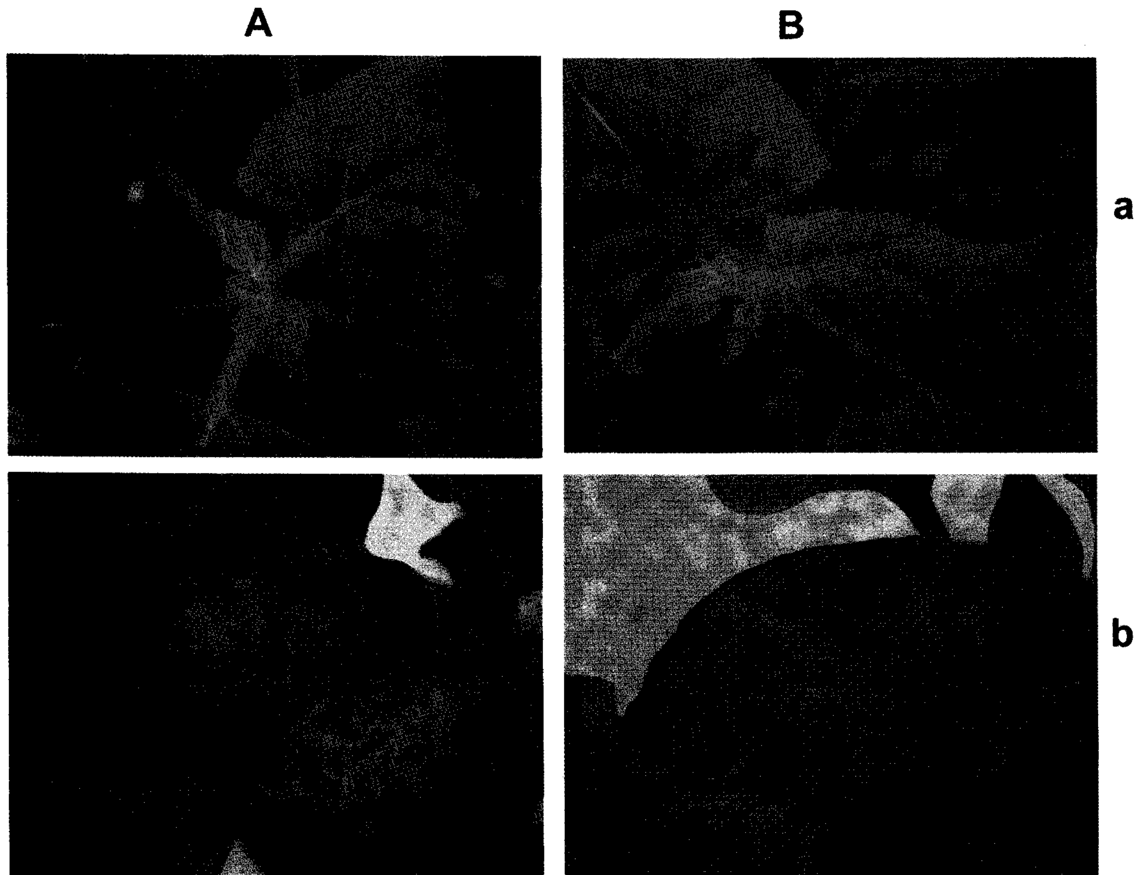

Fig. 3. Symptoms by progeny viruses of pPMFEX1 (A) and pTPW1(positive control) (B) transcripts on the *Nicotiana benthamiana* (a) and *Capsicum annuum* 'Early Calwonder' (L+/L+) (b) plants. Mosaic symptom on upper leaves of *N. benthamiana* and *C. annuum* 'Early Calwonder' is appeared in 7 and 10 days post-inoculation.

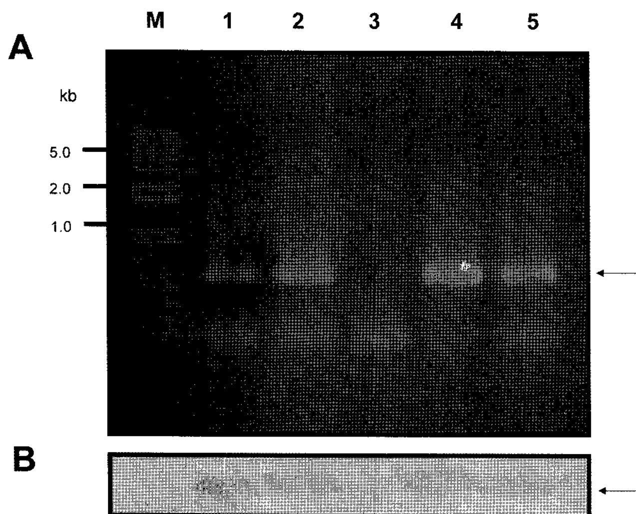

Fig. 4. Infectivity test of RNA transcripts on upper leaves of *N. benthamiana* plants by RT-PCR (a) with CP specific primers and western blotting (b) with antiserum against PMMoV-CP. Lane 1, from pPMFEX1; lane 2, from pTPW1; lane 3, Mock; lane 4, wild type virus; lane 5, viral RNA. Lane M is 1 kb + DNA ladder (Invitrogen).

mine the pathogenicity domain and to develop engineered attenuated mutants of PMMoV, this infectious cDNA clone can be useful and further studies will be needed for practical cross protection in pepper crops.

Acknowledgments

The authors thank Dr. S. Tsuda for proving plasmid pTPW1 for control. This study was supported by a grant from the BioGreen 21 Program, from the Rural Development Administration in Korea, and in part by a grant from the Agricultural Research Promotion Center, the Ministry of Agriculture and Forestry in Korea.

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