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First Report of *Tobacco mild green mosaic virus* Infecting Pepper in Korea

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A rod-shaped virus was isolated from pepper showing mild mosic during the winter growing seasons of 2001 and 2002 in Korea. Based on its biological reactions, serological relationships, reverse transcription-polymerase chain reaction (RT-PCR) using specific primers, and nucleotide sequence analysis of coat protein (CP) gene, the isolated virus was identified as *Tobacco mild green mosaic virus* (TMGMV) and designated as Korean pepper isolate (TMGMV-KP). Crude sap from infected tissue was mechanically transmitted to various indicator plants, which produced characteristic symptoms of tobamovirus infection. However, no symptom was observed in *Gomphorena globosa*. In RT-PCR assays with specific primers for respective detection of TMGMV, *Tobacco mosaic virus* (TMV), *Pepper mild mottle virus* (PMMoV), and *Tomato mosaic virus* (ToMV), a single strong band of about 500 bp in length was produced from the sample used only with TMGMV primers. The amplified DNA was cloned and the nucleotide sequence was determined. Sequence comparisons with the CP gene of other tobamoviruses indicated that TMGMV-KP shared 99.3% identity with TMGMV Japanese isolate and only 59.1, 58.6, and 58.1% identity with TMV, PMMoV and ToMV, respectively. This is the first report of TMGMV in Korea.

Keywords : identification, pepper, sequence, *Tobacco mild green mosaic virus, Tobamovirus.*

Over 30 viruses are known to cause economic damage on pepper (*Capsicum annuum* L.) in the world (Watterson, 1993). There are about nine families of viruses infecting pepper, namely, *Partitiviridae, Bromoviridae, Comoviridae, Tombusviridae, Luteoviridae, Bunyaviridae, Potyviridae, Geminiviridae, and Rhabdoviridae. Tobacco mild green mosaic virus* (TMGMV) is a member of the genus *Tobamovirus* of RNA-containing rigid particles approximately 300 × 18 nm. Tobamoviruses are readily spread mechanically within the field by handling and mechanical damage to plants, but not by insect or by fungal vectors. The genome structure of *Tobacco mosaic virus* (TMV), the type species of the genus, has been studied more than any other plant viruses. The tobamoviral genome, which is about 6.4 kb long, has an m^7Gppp cap at its 5' terminal and the 3' untranslated sequences can fold in the terminal region to give a tRNA-like structure that accepts histidine (Solis and Garcia-Arenal, 1990). The tobamoviral genome encodes five functional proteins: 126 K and 183 K for polymerase; 54 K readthrough protein of 183 K; 30 K for cell-to-cell movement; and 17.6 K for coat protein (CP) (Kamer and Argos, 1984; Knorr and Dawson, 1988; Leonard and Zaitlin, 1982).

Tobamoviruses infecting pepper worldwide are TMV, TMGMV, *Tomato mosaic virus* (ToMV), and *Pepper mild mottle virus* (PMMoV). TMGMV has not been reported in Korea until now (Choi et al., 1989; Kim et al., 1990). TMGMV was first reported in *Nicotiana gluanca* by McKinney in 1935 and has been found in many solanaceous species (Gibbs, 1988). In 2001, a tobamovirus was isolated from red pepper leaves showing mild mosaic symptoms at forcing-cultured fields in Changyoung, Korea. The virus isolate was identified as TMGMV by bioassay, serological relationship, RT-PCR, and nucleotide sequence determination.

Materials and Methods

Virus source and isolation of TMGMV. Red pepper (*Capsicum annuum* L.) leaves showing mild mosaic symptom were collected from a plastic house in Changyoung, southern region of Korea in 2001 (Fig. 1A). TMGMV-KP as source of virus in this study was isolated from the pepper leaves. After three repetition of single local lesion isolation on *Nicotiana glutinosa,* the biologically pure isolate was maintained in *N. occidentalis.* TMV-U1 and ToMV provided by the Plant Virus GenBank, Seoul Women's University, and PMMoV (data not shown) isolated from pepper deposited in the National Horticultural Research Institute were used as control for the identification of TMGMV.

Infectivity bioassay and observation on electron microscopy.

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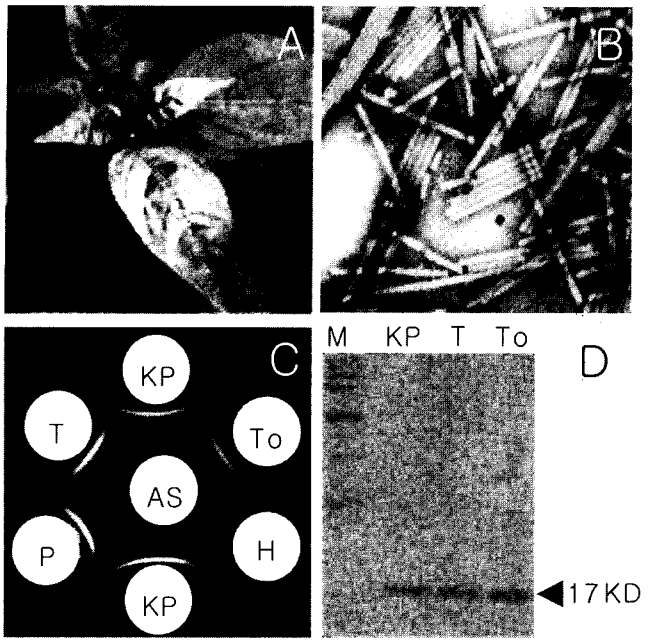

Fig. 1. Symptom on pepper naturally infected with TMGMV-KP (**A**); Electron micrograph of the purified particles (**B**); Immonodiffusion test using antiserum against the virus (**C**); and SDS-PAGE for CP of TMGMV-KP (**D**). Abbreviations for panels C and D: KP = TMGMV-KP; T = TMV-U1; P = PMMoV; To = ToMV; H = sap of healthy *N. occidentalis*; M = molecular weight marker protein (Sigma, MW-SDS-200).

Inoculation for host range tests was conducted in a greenhouse at 25±2°C. Inoculum was extracted from *N. occidentalis* infected with TMV-U1, ToMV, PMMoV, and TMGMV-KP in 0.01 M phosphate buffer (pH 7.0). After mechanical inoculation using carborundum (600 mesh), the six species were maintained and observed for visual inspection of virus symptom development in the greenhouse for at least 3 weeks (Table 1). The particle morphology of TMGMV-KP was observed using transmission electron microscope (Carl Zeiss LEO 905, Germany) operating at 80 kV. The length of the virus particles was determined by measuring the particles from the crude sap of infected leaf tissue negatively stained with 2% uranyl acetate using the leaf dip method.

Virus purification and serological test. TMGMV-KP was purified from the infected leaves of *N. occidentalis* by the method of Gooding and Hebert (1967), followed by an additional 10-40% sucrose density gradient centrifugation for 150 minutes at 22,000 rpm. An antiserum to TMGMV-KP was prepared by muscular-injecting a New Zealand white rabbit with the purified virus (1 mg/ml of virus per injection) four times at weekly intervals (Choi et al., 2001). The immunodiffusion test (Ouchterlony, 1968) was conducted in a 0.7% agar gel medium. Precipitin lines were observed after incubation for 16 hours at room temperature.

Electrophoresis of coat protein. The purified virus (1 mg/ml) was mixed with an equal volume of sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 1% 2-mercaptoethanol, and 0.002% bromophenol blue) and heated for 3 minutes at 100°C. The sample was electrophoresed at 30 mA for 4 hours on 3% and 7.5% discontinuous polyacrylamide slab gel containing 0.1% SDS following the method of Laemmli and Favre (1973). CP bands on the gel were visualized by Coomassie brilliant blue R 250.

RT-PCR detection and treatment of restriction enzyme. Total nucleic acids were extracted from the infected leaves of *N. occidentalis* by the method of Choi et al. (1998). A set of PCR primers for detection of TMGMV-KP was designed based on nucleotide sequence analysis of the reported TMGMV (Solis and Glarcia-Arenal, 1990). The 5'-upstream primer CPTMG-S (5'-TCGAGTACGTTTTAATCAAT-3') and the 3'-downstream primer CPTMG-R (5'-ATTTTAGGAAATCTCACAAC-3') were designed to detect and amplify the CP gene of TMGMV. Reverse trancriptase (RT) reaction was carried out with one cycle at 42°C for 45 minutes and 35 cycles of PCR amplification using the step program (95°C, 45 seconds; 50°C, 60 seconds, and 72°C, 60 seconds), followed by a final extension at 72°C for 10 minutes (Kim et al., 2002). The amplified PCR products were digested with restriction enzyme *Rsa*I (Promega, USA) and analyzed by electrophoresis in 1.5% agarose gel.

Cloning and sequencing of coat protein gene. The amplified cDNA of the CP gene of TMGMV-KP was cloned into the pGEM-T-easy vector (Promega). The nucleotide sequence was determined using a BigDye DNA sequencing kit (Perkin-Elmer Corp., USA) on an ABI 377 DNA sequencer (PE Applied

Table 1. Biological reactions of TMGMV-KP (in this study) and other members of the *Tobamovirus* to different indicator plants

Indicator plant	Host reactions of tobamoviruses[a]			
	TMGMV-KP	PMMoV	TMV-U1	ToMV
Chenopodium amaranticolor	NS/–[b]	NS/–	NS/–	NS/–
Gomphrena globosa	–/–	NS/–	NS/NS	NS/NS
Physalis floridana	CS/M	*l*/YM	*l*/YM	*l*/YM
Nicotiana occidentalis	*l*/M	*l*/M	NS/M	NS/M
N. rustica	NS/–	NS/–	NS/NS	NS/–
N. tabacum cv. Samsun	NS/–	*l*/mM	NS/M	NS/M

[a] TMV-U1 and ToMV were kindly provided from Plant Virus GenBank in Seoul Women's University and PMMoV was isolated from pepper in National Horticultural Research Institute.

[b] Inoculated leaves/upper leaves, NS: necrotic spot, CS: chlorotic spot, M: mosaic, mM: mild mosaic, YM: yellow mosaic, *l*: symptomless, –: no reaction.

Biosystems, USA). All sequences were analyzed using the DNASTAR software package (USA). Homologies of nucleotide and amino acid sequences were performed using a BLAST in the GenBank database.

Results

Host reactions and morphology of the virus. The reactions of the indicator plants, inoculated mechanically with TMGMV-KP isolated from red pepper leaf showing mild mosaic symptom (Fig. 1A), were listed in Table 1. TMGMV-KP systematically infected *Physalis floridana* and *Nicotiana occidentalis. Chenopodium amaranticolor, N. rustica,* and *N. tabaccum* cv. Samsun exhibited necrotic spots only on the inoculated leaves. TMGMV-KP did not infect *Gomphorena globosa,* while PMMoV, TMV, and ToMV were able to infect the plant. Typical rod-shaped particles with a mean length of 312 nm and a mean width of 19 nm were consistently observed in leaf-dip preparations of TMGMV-KP-infected *C. annuum* and the tested plants (Fig. 1B).

Serological relationship and molecular weight of coat protein. An antiserum against TMGMV-KP reacted strongly with the viral antigen, giving a specific precipitin band in the agarose gel with both crude extracts from infected leaves and the purified virus preparation. Crude sap extracts from freshly ground PMMoV-, TMV-, and ToMV-infected tissues gave precipitin bands against TMGMV-KP antiserum. However, the bands formed between TMGMV-KP and the control viruses showed a spur in immunodiffusion test (Fig. 1C). The CP of the purified TMGMV-KP migrated as a single band with molecular weight of about 17 kDa in SDS-PAGE. The molecular weight of TMGMV-KP could not be distinguished from those of TMV and ToMV (Fig. 1D).

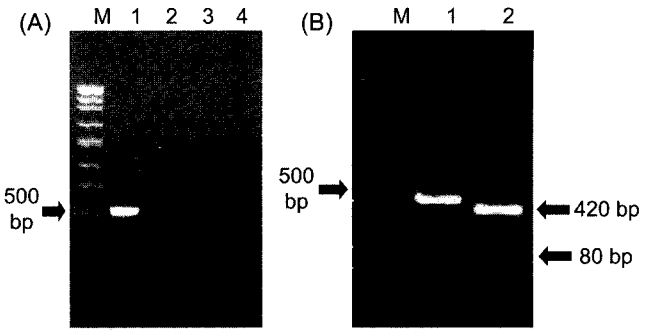

Fig. 2. RT-PCR assay with TMGMV-specific primer. Panel (**A**): Lane 1, TMGMV-KP, Lane 2, TMV; Lane 3, PMMoV, Lane 4, ToMV. Panel (**B**): Lane 1, TMGMV-KP cDNA product; Lane 2, the DNA fragments digested with *Rsa*I.

RT-PCR detection and restriction enzyme digestion. The amplified DNA product of TMGMV-KP corresponded to full-length CP gene approximately 500 bp long (Fig. 2A). However, the PCR primers designed based on the nucleotide sequence analysis of TMGMV did not amplify a DNA of TMV, PMMoV, and ToMV gene. When the DNA product of TMGMV-KP gene was digested with *Rsa*I, two fragments of the DNA product were 420 bp and 80 bp in

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TMGMV-KP    1 MPYTINSPSQFVYLSSAYADPVQLINLCTNALGNQFQTQQARTTVQQQFA 50
TMGMV-J       **************************************************
TMV           *S*S*T******F***VW***IE*L*V***S******************S
ToMV          *S*S*T******F***VW***IE*L*V***S******************S
PMMoV         *A**VS*AN*L***G*VW***LE*Q****S******************S

TMGMV-KP   51 DAWKPVPSITVRFPASDFYVYRYNSTLDPLITALLNSFDTRNRIIEVDNQ 100
TMGMV-J       **************************************************
TMV           EV***F*QS*****GDVYK*****AV*********GA**********E**
ToMV          EV***F*QS*****GDVYK*****AV*********GA**********E**
PMMoV         *V**TI*TA******TG*K*F***AV**S*VS***GA**********E*P

TMGMV-KP  101 PAPNTTEIVNATQRVDDATVAIRASINNLANELVRGTGMFNQAGFETASG 150
TMGMV-J       **************************************************
TMV           QS*T*A*TLD**R**********SA****V********LY**NT**SM**
ToMV          QS*T*A*TLD**R**********SA****V********LY**NT**SM**
PMMoV         QN*T*A*TLD**R**********S**M*********Y***L**S***

TMGMV-KP  151 LVWTTTPAT 159
TMGMV-J       *********
TMV           ****SA**S
ToMV          ****SA**S
PMMoV         *T*A***--
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Fig. 3. Multiple alignment of amino acid sequences of the coat proteins of TMGMV-KP, TMGMV Japanese isolate (GeneBank Database Accession AB078435), TMV (AF103780), ToMV (AY063743), and PMMoV (AB084456). Identical sequences are marked with an asterisk.

molecular size, respectively (Fig. 2B).

Sequence analyses of CP gene. The amplified DNA was cloned and the nucleotide sequence analysis was determined. The TMGMV-KP CP gene was 477 nucleotides long, which encoded 159 amino acid residues. The CP gene of TMGMV-KP shared 99.3% identity in nucleic acid level and 100% in amino acid level with the TMGMV Japanese isolate (GeneBank database accession AB078435). However, sequence comparisons with the CP gene of other tobamoviruses in the GeneBank Database showed 59.1%, 58.6%, and 58.1% identity with TMV (AF103780), PMMoV (AB084456) and ToMV (AY063743), respectively (Fig. 3).

Discussion

Host plants naturally infected with TMGMV were reported in solanaceous species (Gibbs, 1988) and evergreen shrub (Cohen et al., 2002). Results of this study suggest that TMGMV-KP isolated from pepper in Korea has properties common with the genus of *Tobamovirus* (van Regenmortel and Frnkel-Conrat, 1986). These include particle morphology, host ranges, serological relationships, and nucleotide sequence of the CP gene. The host range and symptomatology of TMGMV-KP differed in some respects from those of other tobamovirus isolates reported in pepper cultivating areas in Korea (Choi et al., 1989; Kim et al., 1990). TMGMV-KP induced chlorotic spots on the inoculated leaf and mosaic on the upper leaf of *Physalis floridana*. However, PMMoV, TMV, and ToMV as control viruses induced yellow mosaic only on the upper leaf of the plant. *Gomphorena globosa* inoculated with the three control viruses developed necrotic spot symptom but the test plant inoculated with TMGMV did not have any symptom at all. Some of the differences in host range and symptoms may reflect differences in intrinsic properties of tobamovirus isolates. Satio et al. (1987) described the construction of recombinants between TMV and ToMV, and the viral gene causing the necrotic response lies in the CP gene.

The serological relationships between TMGMV-KP and the three control tobamoviruses were analyzed by immunodiffusion test using TMGMV-KP antiserum. Antiserum to TMGMV-KP gave reactions of partial identity with a spur when the three control tobamoviruses were compared with TMGMV-KP. The results indicate that TMGMV-KP and the control viruses seem to have different antigenic determinants. Specific primers to the known TMGMV, TMV, ToMV, and PMMoV were designed based on nucleotide sequence analyses of those viruses from the GenBank, and each of those primers was amplified only in its viral RNA samples from the infected leaves (data not shown). The amplified DNA product of TMGMV-KP contained a single recognition site of *Rsa*I. The restriction enzyme site of TMGMV-KP was identical with that of a Japanese isolate of TMGMV (GenBank database accession number AB078435). Frail et al. (1996) suggested that TMGMV was genetically stable and was different for the various TMGMV geographical populations based on ribonuclease protection assay and by partial sequence analysis (Frail et al., 1996). The CP gene of TMGMV-KP shared 99.3% identity in nucleotide level and 100% in amino acid level with TMGMV Japanese isolate. Three nucleotide alternations were present between TMGMV-KP and TMGMV Japanese isolate, whereas, isolates among other tobamoviruses (AF103780, AB084456 and AY063743) shared 58.1-59.1% sequence identity.

This is the first report on the occurrence of TMGMV in pepper in Korea based on biological reactions, RT-PCR, and CP gene analyses.

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