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Characterization of *Cucumber mosaic virus* Isolated from Water Chickweed (*Stellaria aquatica*)

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A strain of *Cucumber mosaic virus* (CMV) was isolated from a weed, water chickweed (*Stellaria aquatica*), growing in the pepper field in Chunchon, Korea. This isolate, CMV-Sa, was differentiated from other CMVs based on biological properties and nucleotide sequence analysis of the coat protein (CP) gene. CMV-Sa showed different reactions to all the tested plants, except *Capsicum annuum* and *Cucumis sativus*, when compared with those of CMV-Mf (subgroup I) and CMV-PaFM (subgroup II). Remarkably, in *Nicotiana tabacum* cvs. Samsun, Xanthi-nc and Ky-57, CMV-Sa induced local necrotic ring spots on the inoculated leaves and venal wave pattern and mosaic on the upper leaves. RNA analysis, serology, and RT-PCR of CP gene showed that CMV-Sa belonged to subgroup I of CMV. However, restriction enzyme analysis of the cDNA using *Alu*I, *Hha*I, *Hinc*II, *Hind*III, *Hinf*I and *Msp*I showed that CMV-Sa was distinct from that of CMV-Mf. Based on comparison of the nucleotide of CP gene and deduced amino acid sequences between other CMV strains, CMV-Sa was closely related to CMV-Mf with 93.7% and 97.2% identity, respectively.

Keywords : *Cucumber mosaic virus* (CMV), *Stellaria aquatica*, subgroup, water chickweed

Cucumber mosaic virus (CMV) is a member of the genus *Cucumovirus* (family *Bromoviridae*). It is one of the most common plant viruses of substantial agricultural significance. CMV infects more than 1,000 species of plants, shrubs, and trees and both monocots and dicots (Roossinck, 1999). The genomic RNAs of CMV are designated as RNAs 1, 2, and 3 by diminishing size (Palukaitis et al., 1992). All the RNAs have a cap structure at the 5' terminus. The 3' portion of all the RNAs is also highly conserved in virus-specific manner and can form a tRNA-like structure that can be amino-acylated with tyrosine.

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RNAs 1 and 2, encoding the 1a and 2a proteins; respectively, are involved in viral replication (Hayes et al., 1990; Nitta et al., 1988). CMV RNA 2 encodes a second protein, 2b, which is translated from a subgenomic RNA, RNA 4, and plays a role in systemic spread of the virus and virulence determination, possibly by suppressing a host RNA silencing mechanism (Brigneti et al. 1998; Ding et al., 1995). RNA 3 encodes two proteins dispensable for viral replication in protoplasts. The 5'-proximal open reading frame (ORF) on RNA 3 is for the 3a protein, the cell-to-cell movement protein of CMV (Ding et al., 1995). The 3'-proximal ORF is for the coat protein that is translated from a subgenomic RNA 4 (Boccard & Baulcombe, 1993). CMV isolates are classified into two subgroups, I and II, according to various converging criteria which include symptomatology, serology, and nucleic acid homology (Palukaitis et al., 1992).

Recently, a new grouping for the subgroup S-I, designated as S-IB (Asian isolates) and S-IA (all other isolates), has been proposed on the basis of genomic sequences data (Roossinck et al., 1999).

In this study, the biological, serological, and partial nucleotide sequence analyses of CMV-Sa isolated from water chickweed were investigated. Some characteristics of CMV-Sa, different from the other isolates of CMV, are reported and discussed.

Materials and Methods

Virus source and isolation. A virus, CMV-Sa, was isolated from water chickweed showing mosaic symptom (Fig. 1). After three repetition of single local lesion transfer on *Chenopodium quinoa*, biologically pure isolate was maintained in *Nicotiana tabacum* cv. Xanthi-nc. Two typical isolates of CMV-Mf in subgroup I (Choi et al., 1998) and CMV-PaFM in subgroup II (Kim et al., 2002) were used as control.

Host range and symptomatology. Inoculations for host range test were conducted in a glasshouse at 25 ± 2°C. Inoculum was extracted from *N. tabacum* cv. Xanthi-nc and infected with CMV-Sa in 0.01 M phosphate buffer (pH 7.0). After mechanical

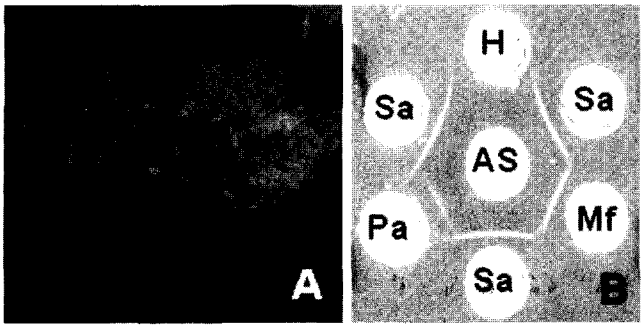

Fig. 1. Mosaic symptom on water chickweed infected naturally with CMV-Sa (A). Immunodiffusion test conducted with antigens of CMV-Sa (Sa), CMV-Mf (Mf), CMV-PaFM (Pa), and healthy sap of tobacco (H) using antiserum against CMV-Sa (AS) (B).

inoculation, the 15 species of plants were maintained in the glasshouse for at least 3 weeks to observe the virus symptoms.

Virus purification and viral RNA preparation. For purification, CMV-Sa was propagated in *N. tabacum* cv. Xanthi-nc for 10 days post inoculation. The virus was purified as described by Takanami (1981). Further purification was done by conducting 10-40% sucrose density-gradient centrifugation for 180 minutes at 22,000 rpm (SW-28, Beckman).

The viral genomic RNA was extracted from virus particles purified by using Nuclisens™ isolation kit, followed by precipitating it with ethanol (Sambrook et al., 1989). The resulting precipitates of viral RNA were dissolved in RNase-free water and used for subsequent experiments.

Polyclonal antiserum and serological test. New Zealand white rabbit was immunized with purified virus particles. The rabbit was injected subcutaneously at weekly intervals with 1.0 mg/mL virus in Freund's complete adjuvant for 3 weeks, and bled 1 week after the last injection. The antiserum was tested by agar gel immuno diffusion test. Subsequent booster injection of 1.0 mg/mL was administered into external marginal vein of the ear of the rabbit. Immuno diffusion test was conducted in the 1% agarose gel medium with CMV-Sa antiserum. Precipitin line was observed and recorded after incubation for 48 h at room temperature.

Viral RNA analysis. Purified viral RNAs from the virions were separated in a formaldehyde-denatured 1.2% agarose gel under denatured condition in MOPS buffer (Sambrook et al., 1989) at 90 volt for 40 min. The RNA bands were visualized on the UV transilluminator after gel staining with ethidium bromide solution.

RT-PCR and RFLP analysis. The pair of PCR primer (CTPALL-3 and CPTALL-5) for CMV coat protein gene was used following the same sequence as described by Choi et al. (1999). For the differentiation of amplified PCR products, these were digested with restriction enzymes, *Alu*I *Eco*RI, *Eco*RV, *Hha*I *Hin*cII *Hin*dIII, *Hin*fI, *Msp*I, *Sal*I, *Sau*3AI, and *Xho*I (TaKaRa Co.). Analysis by electrophresis in 4% agarose gel was done.

Cloning and sequencing of coat protein gene. The cDNA of the 3' half region of CMV-Sa RNA 3 was cloned into the pGEM-T-Easy vector. The plasmid that contained cDNA inserts was selected for nucleotide sequencing. Sequencing reactions using ABI Prism™ 377 DNA sequencer with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Forster City, CA, USA) were performed. The analysis of the nucleotide and the deduced amino acid sequences were performed with DNASTAR software package (Madison, WI). Homologies of nucleotide and amino acid sequences were performed by using a BLAST and in the GenBank database (Table 2).

Table 1. Reactions of Cucumber mosaic virus (CMV) isolates to different indicator plants

Indicator plants	Host reactions of CMV isolates[a]		
	Mf	PaFM	Sa
Chenopodium amaranticolor	NL/-[b]	NL/-	NL/-
C. murale	NL/-	NL/-	NL/-
Gomphorena globosa	-/-	-/-	NS/M
Datura stramonium	-/M	-/M	NS/NS
Physalis floridana	-/M	-/-	VN/M
Tetragonia expensa	-/M	-/-	NS/M
Nicotiana benthamiana	-/M	-/MM	NS/M
N. occidentalis	-/M	-/M	RS/M
N. rustica	-/M	-/-	RS/M
N. tabacum cv. Samsun	-/M	-/-	RS/WP, M
N. tabacum cv. Ky-57	-/M	-/MM	RS/WP, M
N. tabacum cv. Xanthi-nc	-/M	-/-	RS/WP, M
Capsicum annuum	-/M	-/MM	-/M
Cucumis sativus 'Baegbong'	-/M	-/MM	-/M
Cucubita pepo 'Taeyang'	-/M	-/MM	CS/CS, M

[a] Mf-CMV (Choi et al., 1998) isolate was kindly provided by Dr. Choi J. K. (Kangwon National University) and PaFM-CMV (Kim et al., 2002), used as control.

[b] Inoculated leaves/upper leaves, NL: necrotic local, M: mosaic, MM: mild mosaic, RS: necrotic ring spot, WP: wave pattern, CS: chlorotic spot, −: no reaction.

Results and Discussion

Biological properties of CMV-Sa isolate. The reactions of the indicator plants inoculated mechanically with CMV-Sa

Table 2. *Cucumber mosaic virus* (CMV), *Peanut stunt virus* (PSV), and *Tomato aspermy virus* (TAV) and their Genbank accession number used for sequence analysis in this study

Virus	Accession no.	Subgroup
Mf-CMV	AJ276481	IA
Fny-CMV	D10538	IA
As-CMV	AF013291	IB
Ly2-CMV	AJ296154	IB
PaFM-CMV	AB109908	II
Kin-CMV	Z12818	II
LS-CMV	AF127976	II
ER-PSV	U15730	
KC-TAV	AJ237849	

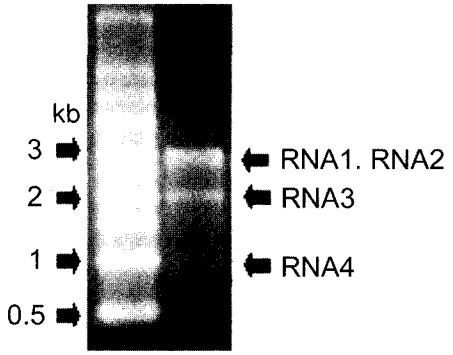

Fig. 2. Electrophoresis of genomic RNA of CMV-Sa in 1.2% agarose gel. Lane M: RNA ladder (BMA), 1: CMV-Sa genomic RNAs purified from virions.

are listed in Table 1. The host range and symptomatology of CMV-Sa differed in some respect from those of other CMV isolates reported. Interestingly, in *N. tabacum* cvs. Samsun, Xanthi-nc, and Ky-57, CMV-Sa induced local necrotic ring spot on the inoculated leaves and wave pattern and mosaic on the upper leaves, but CMV-Mf and PaFM did not produce any symptom on the inoculated leaves.

The inoculated leaves of *D. stramonium* with CMV-Sa showed necrotic spot, however the inoculated leaves with CMV-Mf or PaFM did not produce the symptoms. The upper leaves showed mosaic.

Serological relationship. Antiserum against CMV-Sa showed positive reaction serologically with CMV-Mf and CMV-PaFM in agarose gel double diffusion test. Both antigen CMV-Sa and CMV-Mf (subgroup I) formed homologous one precipitin line, however CMV-Sa and CMV-PaFM (subgroup II) formed a spur line (Fig. 1-B).

Viral RNA analysis. When purified viral RNA preparation was separated in formaldehyde-denatured MOSP agarose gel, four ethidium bromide-stained RNA bands were observed; however, the satellite RNA was not contained (Fig. 2). Sizes were 3.4 kb for RNA1, 3.1 kb for RNA2, 2.2. kb for RNA3, and 1.0 kb for RNA4 of CMV-Sa.

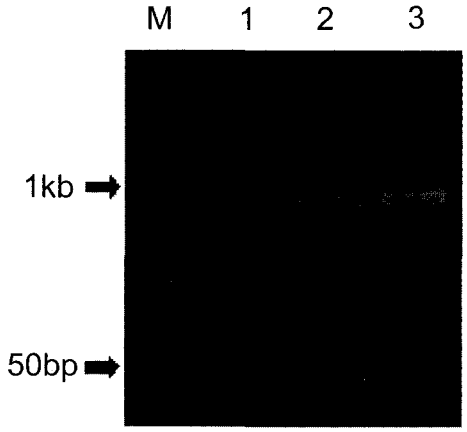

Fig. 3. Agarose gel electrophresis (1.2% agrose gel) of the amplified producted by the RT-PCR with a single pair of primers, CTPALL-3 and CTPALL-5, to detected isolates of CMV. Lane M: 1 kb DNA ladder, 1: CMV-Sa, 2: CMV-Mf and 3: CMV-PaFM.

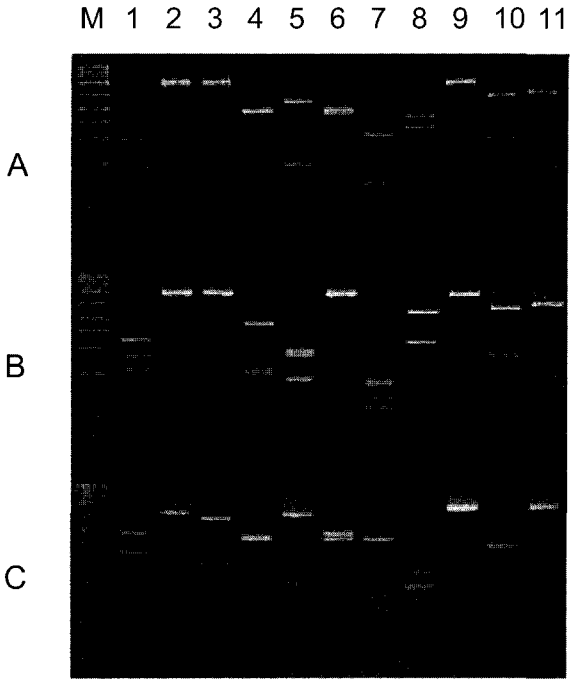

Fig. 4. RFLP analysis of RT-PCR amplified products of strains of CMV-Sa, CMV-Mf and CMV-PaFM with a set of the genus *Cucumovirus*-specific primer. Digested restriction fragments were separated on a 4% agarose gel and stained with ethidium bromide. A: CMV-Sa, B: CMV-Mf (subgroup I) and C: CMV-PaFM (subgroup II). Lane M: 1 kb DNA lader (BMA), 1: *Alu*I, 2: *Eco*RI, 3: *Eco*RV, 4: *Hha*I, 5: *Hin*cII, 6: *Hin*dIII, 7: *Hin*fI, 8: *Msp*I, 9: *Sal*I, 10: *Sau*3AI and 11: *Xho*I digestions.

RT-PCR and RFLP analysis. To identify the isolate of CMV-Sa, RT-PCR was conducted with a pair of primer (CTPALL-3 and CTPALL-5), and to the target size, approximately 960 bp long (Fig. 3). Fig. 4 shows the RFLP analysis of PCR products for the CP genes of CMV-Sa, CMV-Mf, and CMV-PaFM. RT-PCR of CP gene showed that CMV-Sa belonged to subgroup I of CMV.

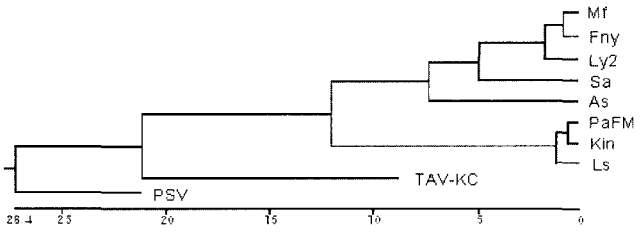

Fig. 5. Relationships of coat protein gene sequences between CMV-Sa and other CMV strains. The degree of relatedness is indicated by horizonal distance. The dendogram was constructed from aligned CP gene sequences using DNASTAR software.

However, restriction enzyme analysis of the cDNA using *Alu*I, *Hha*I, *Hinc*II, *Hin*dIII, *Hin*fI and *Msp*I showed that CMV-Sa was distinct from that of CMV-Mf. These RFLP results were matched with computer-generated estimated pattern of the target gene.

Sequence analysis of coat protein gene. The CP gene of CMV-Sa contains 657 nucleotides and the nucleotide sequence is similar to that of CMV-Mf. There is also a high degree of conservation between their putative gene products in CMV-Sa and CMV-Mf, with seven amino acid changes among the 218 amino acids of the CPs (Fig. 5).

Results based on serological and molecular properties show that CMV-Sa belongs to CMV subgroup I. Further studies on molecular characteristics such as pseudo-recombinant analyses and full-length genomic RNA sequences will be required to define different pathological characteristics of the CMV-Sa.

Acknowledgments

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