Characterization of a Novel *Cucumber mosaic virus* Isolated from *Petunia hybrida*

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(Received on April 9, 2012; Revised on May 16, 2012; Accepted on May 16, 2012)

Petunia hybrida is commonly used in landscapes and interiors for its attractive flower. Virus-like foliar symptoms, including a mosaic with dark green islands surrounding the veins and chlorosis on the leaf margins, were observed on a petunia plant from Icheon, Gyeonggi-do, Korea. *Cucumber mosaic virus* (CMV) was identified in the symptomatic petunia by serological testing for the presence of CMV coat protein (CP) with a direct antibody-sandwich-enzyme-linked immunosorbent assay. An agent was mechanically transmitted to indicator plant species including *Chenopodium quinoa*. Examination of the inoculated plant leaves by RT-PCR analysis and electron microscopy revealed the presence of specifically amplified CP products and spherical virions of approximately 28 nm in diameter, respectively, providing confirmation of a CMV infection. Analysis of CP sequences showed that CMV petunia isolate (CMV-YJC) shared 82.5–100% amino acid sequence identity with CPs of representative CMV strains. Phylogenetic analysis of CPs supports that CMV-YJC is a member of CMV subgroup IA (CMV-IA) and has biological properties of CMV-IA on host species. To our knowledge, this is the first report of CMV from *P. hybrida* in Korea.

Keywords : coat protein, *Cucumber mosaic virus*, petunia, RT-PCR, sequence analysis, symptom

Petunia hybrida is a perennial plant, known as the “king of parterre flowers”. *Petunia hybrida* is one of six members of the *Solanaceae* grown primarily as an ornamental plant, and the most economically important due to its horticultural value (Sink, 1984). Petunia is a popular bedding plant, the third most valuable after geranium and impatiens (Hamrick, 1997) and it is also grown in pots, hanging baskets, and

balcony/window boxes (Lesemann, 1996). Petunia plants are propagated by seeds in nature, but new species of vegetatively propagated petunia have been recently cultivated as garden ornamentals in the world (Dai and Bao, 2004). Plant viruses are responsible for severe diseases in many crops, ornamental plants, and various plants, resulting in major economic losses (Hull, 2002). Virus symptoms usually vary from local discoloration to severe perturbation of growth and development, or even death of the plant. In most relationships between virus and susceptible host, the induction of disease symptoms results from colonization of plant virus in which the agent modifies the metabolism of its host at many different levels. In turn, host plant shows various responses to virus, depending upon genetic backgrounds, development stage and surrounding environment (Hull, 2002). Several viruses have been reported as naturally infecting petunia plants (Bellardi et al., 1996; Mavric et al., 1996; Pirone, 1978), and many viruses capable of infecting petunia plants have been identified using artificial inoculation methods since petunia plants are frequently used as indicator hosts for virus diagnostic assays (Brunt et al., 1996; Lane, 1992). The virus most often detected in petunia plants is *Tobacco mosaic virus* (TMV) and other commonly detected viruses are *Chrysanthemum virus B* (CVB), *Cucumber mosaic virus* (CMV), *Tobacco ringspot virus* (TRSV), *Tobacco streak virus* (TSV), *Tomato ringspot virus* (ToRSV), and *Tomato spotted wilt virus* (TSWV) (Brunt et al., 1996; Lawson and Hus, 1994; Sanchez-Cuevas and Nameth, 2002). Among viruses infecting *Petunia hybrida*, CMV is a prevalent plant pathogen all over the world and has one of the widest host ranges of any plant virus, infecting over 885 plant species in 65 families (Palukaitis et al., 1992). CMV is multi-component single stranded virus, with three positive-sense RNA's (RNA1, RNA2 and RNA3) and an additional subgenomic RNA (RNA4) derived from RNA3 (Habibi and Francki, 1974; Peden and Symons, 1973). RNA2 codes for the 2a protein,

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which is an RNA-dependent RNA polymerase of replication complex, whereas, RNA 1 codes for the 1a protein, another subunit of CMV replicase complex (Hayes and Buck, 1990). RNA3 encodes for two proteins involved in viral movement and encapsidation (Canto et al., 1997; Kaplan et al., 1997). In several genetic studies, the determinants of the various phenotypes of CMV have been mapped to one or more of genomic RNAs (Gal-On et al., 1994; Huppert et al., 2002; Palukaitis and Garcia-Árenal, 2003; Rao and Francki, 1982; Shintaku et al., 1992; Suzuki et al., 1995; Szilassy et al., 1999; Takeshita et al., 2001).

The purpose of this study was to identify the virus infecting a petunia plant in commercial greenhouse in Korea and to investigate the nature and genetic basis of the differences between a novel CMV isolate and the better-characterized CMV strain Fny (CMV-Fny) (Roossinck and Palukaitis, 1990; Choi et al., 2005).

A petunia plant displaying virus-like symptom was collected from a commercial greenhouse in Icheon, Gyeonggi-do in 2011. The plant was transplanted and maintained under insect-free greenhouse conditions to minimize cross-contamination. For virus isolation, sap from the diseased petunia was inoculated mechanically to indicator plant species including *Chenopodium quinoa*. At least four times, a single local lesion produced on *C. quinoa* was excised and extracted with 10 mM phosphate buffer (pH 7.0). The virus recovered from the fifth passage through *C. quinoa* was inoculated mechanically to tobacco plants (*N. tabacum* cv. Samsun NN and *N. benthamiana*) using Carborundum® (Fisher, USA) for virus propagation. CMV-Fny reported previously was used as a control to compare host responses (Choi et al., 2007; Lee et al., 2007). The inoculated plants were maintained in a greenhouse and the symptoms were evaluated until 5 weeks. Virus particles were observed using a transmission electron microscope (Model No. Carl Zeiss LEO 905, Germany) operating at 80 kV. All experiments were performed at least three times. Leaf tissue (approximately 0.2 g) harvested from the plant was tested by direct antibody-sandwich (DAS)-ELISA, as described previously (Clark and Adams, 1977; Yoon et al., 2011). In brief, leaf tissues were homogenized in 0.1 M phosphate buffer containing 0.02% NaN₃, 0.1% Tween 20, and 0.1% non-fat milk (pH 7.4) at a sample-to-buffer ratio of 1:3 (w:v), and 100 µL of extracted sap was loaded in duplicate on micro-titer plates. The primary antibodies specific to CMV, CVB, TAV, TMV, ToRSV, TRSV, TSV, and TSWV using polyclonal antisera produced in rabbit (Agdia, USA; Choi et al., 2005) was diluted to 1:100 in 1X carbonate coating buffer (0.159% sodium carbonate, 0.293% sodium bicarbonate and 0.02% sodium azide, pH 9.6). Subsequently, goat anti-rabbit IgG-conjugated alkaline phosphatase was used as a secondary antibody, according to manu-

facturer's instruction (Promega, USA). Absorbance values (optical density at 405 nm) of four times of the healthy control reading were used as the positive threshold. Total RNA was extracted from leaf tissues of the infected petunia plant using TRIzol reagent and phenol/chloroform (25:24, v/v) according to the manufacturer's instructions (Invitrogen, USA). Total RNAs were precipitated with 0.7 volume of 2-propanol, followed by centrifugation and further purification by ethanol precipitation, DNase I treatment, and lithium chloride precipitation. The RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific, USA), and RNA quality was assessed by electrophoresis in 1.5% agarose gels containing 6% formaldehyde. The total RNA extracted was used as a template for RT-PCR analysis. Briefly, cDNA synthesis from total RNA (500 ng) was performed in a 20-µL volume of 1x SuperScript II reaction buffer (Invitrogen, USA) containing 20 pmol gene-specific reverse primer (5'-tcagactgggagcactcca gatgtggg; complementary to nt 1887–1913 of CMV-Fny RNA3; Genbank accession no. NC_001440), 0.5 mM dNTP mix, 5 mM DTT, 40 U RNaseOut, and 200 U of SuperScript II reverse transcriptase (Invitrogen, USA) under the following conditions: 60 min at 50°C followed by 20 min at 70°C. One-tenth volume of the RT reaction was used for PCR amplification with a gene-specific forward primer (5'-atggacaaatctgaatcaaccagtgt; corresponding to nt 1257–1283 of CMV-Fny; Genbank accession no. NC_001440) and the reverse primer. PCR amplification was performed in 50 µL of 1x Platinum *Taq* DNA polymerase reaction buffer containing 1 mM MgSO₄, 0.2 mM dNTP, 10 pmole of each primer and 1 U of Platinum *Taq* DNA polymerase, according to manufacturer's instructions (Invitrogen, USA). The thermal cycles were as follows: 2 min at 94°C (1 cycle); 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C (35 cycles). Amplified RT-PCR products were separated on a 1.5% agarose gel and stained in ethidium bromide solution. All RT-PCR experiments were performed in duplicate.

RT-PCR fragment was eluted using a QIAquick PCR Purification Kit, according to the manufacturer's protocol (Qiagen, Germany). The RT-PCR products were cloned into pCR-XL-TOPO and used to transform TOP10 competent *E. coli* cells according to the manufacturer's instructions (Invitrogen, USA). Colonies were randomly selected and screened for cDNA inserts. The selected clones were prepared for automated sequencing following the instructions of the BigDye terminator cycle sequencing kit (Applied Biosystems, USA). The sequences of the CP genes were determined twice to avoid errors. Sequencing was performed on an ABI Prism 377 DNA sequencer. The sequences obtained were analyzed using a BLAST search, MEGA4 and DNASTAR software (USA).

Fig. 1. Symptoms of petunia infected by cucumber mosaic virus strain YJC (CMV-YJC). CMV-YJC induces mottling, yellowing, and vein-clearing and mosaic symptoms in petunia leaves (A), compared to mock inoculation (B), Blue arrows indicate small necrotic spot symptoms on petunia flowers infected at late growth stage (C).

One petunia plant collected from a commercial greenhouse showed virus-like symptoms in leaves such as mottling, yellowing, and vein-clearing and mosaic (Fig. 1A). The petunia plant continued to express the same symptoms initially observed at planting. Flowers also expressed necrotic

spot symptoms, but the flowers did not show color breaking and flower deformation that are frequently observed from the virus-infected petunia plants (Fig. 1C). To identify a causal virus, leaf tissues were subjected to DAS-ELISA analysis. The diseased petunia plant was negative for CVB,

Table 1. Reaction of indicator plant species inoculated mechanically with CMV-YJC and CMV-Fny

Plant Species	CMV-YJC		CMV-Fny	
	Symptom	RT-PCR	Symptom	RT-PCR
<i>Capsicum annuum</i>	–/M ^a	(+/-) ^b	–/M	(+/+)
<i>Chenopodium amaranticola</i>	NLL/–	(+/-)	NLL/–	(+/-)
<i>C. quinoa</i>	CLL/–	(+/-)	CLL/–	(+/-)
<i>Cucumis sativus</i>	–/CS	(+/+)	–/M	(+/+)
<i>Cucurbita pepo</i>	–/–	(+/-)	–/M	(+/+)
<i>Datura stramonium</i>	–/mM	(+/+)	–/M	(+/+)
<i>Gomphrena globosa</i>	–/M	(+/+)	–/M	(+/+)
<i>Nicotiana benthamiana</i>	–/M	(+/+)	–/M	(+/+)
<i>N. clevelandii</i>	–/M	(+/+)	–/M	(+/+)
<i>N. glutinosa</i>	–/mM	(+/+)	–/M	(+/+)
<i>N. occidentalis</i>	–/M	(+/+)	–/M	(+/+)
<i>N. rustica</i>	–/mM	(+/+)	–/M	(+/+)
<i>N. tabacum</i> cv. Burley 21	–/M	(+/+)	–/M	(+/+)
<i>N. tabacum</i> cv. Ky57	–/M	(+/+)	–/M	(+/+)
<i>N. tabacum</i> cv. Samsun NN	–/M	(+/+)	–/M	(+/+)
<i>N. tabacum</i> cv. White Burley	–/M	(+/+)	–/M	(+/+)
<i>N. tabacum</i> cv. Xanthi nc	–/M	(+/+)	–/M	(+/+)
<i>Physalis floridana</i>	–/M	(+/+)	–/M	(+/+)
<i>Petunia hybrida</i>	–/Ch, VC, M	(+/+)	–/M	(+/+)
<i>Solanum lycopersicum</i>	–/mM	(+/+)	–/M	(+/+)
<i>Tetragonia tetragonioides</i>	–/M	(+/+)	–/M	(+/+)
<i>Vigna unguiculata</i>	NLL/–	(+/-)	NLL/–	(+/-)

^aInoculated leaves/upper leaves. Symptoms were assessed by observations in the inoculated and upper leaves until 21 days after inoculation. The symptoms were briefly indicated as follows: Ch, chlorosis; CS, chlorotic spot; CLL, chlorotic local lesion; mM, mild mosaic; M, mosaic; NLL, necrotic local lesion; VC, vein clearing; –, symptomless.

^bRT-PCR was performed from total nucleic acids extracted from indicator plant species 14 days after inoculation as described by Choi et al. (2005). Plus symbol (+) indicates synthesis of RT-PCR fragment and negative symbol (–) indicates no synthesis of RT-PCR product.

TAV, TMV, ToRSV, TRSV, TSV, and TSWV, but a positive reaction was observed using the CMV antibody in the petunia (supplementary Table 1). Transmission electron microscopy of infected petunia leaves revealed presence of an icosahedral virus particle of ~28–29 nm diameter (data not shown). The shape and size of the virus particle were identical with those of CMV particle obtained from other sources. These results indicate that the virus causing the petunia disease is an isolate of CMV. Thus, the isolate was named CMV-YJC. To characterize further pathological properties of CMV-YJC, the virus was serially inoculated to *C. quinoa* plants and a single local lesion was excised from leaves of *C. quinoa* after fifth passage. Then, CMV-YJC isolated from a single local lesion on leaves of *C. quinoa* was inoculated mechanically to 22 species of indicator plants. For direct comparison of its pathological properties, CMV-Fny that has been well studied was used as a control. Host range and symptoms of CMV-YJC were summarized in Table 1. Although the difference of induction time and severity of symptoms was observed in a few indicator plant species, symptomatology of CMV-YJC is similar to that of CMV-Fny in most plant species tested. It is noteworthy that

CMV-YJC induced systemic chlorosis, vein clearing and mosaic symptoms in petunia plants inoculated, similar to those in the original source petunia plant (Table 1). In addition, CMV-YJC induced necrotic local lesions similar to those induced by CMV-Fny on the inoculated leaves of *C. amaranticolor* and *V. unguiculata*. These results suggest that CMV-YJC has pathological properties of CMV subgroup IA, similar to CMV-Fny.

To further molecularly characterize CMV-YJC, total RNA extracted from indicator plant species including *P. hybrida* was subjected to RT-PCR analysis using primers specific to CMV coat protein (CP) gene. The electrophoresis of RT-PCR products in 1.5% agarose gel revealed presence of the expected size band (657 bp) in naturally infected *P. hybrida* as well as 22 plant species inoculated (Fig. 2A). In contrast, RT-PCR products were not synthesized in healthy plant samples, thereby confirming infections of CMV-YJC on plant samples. The 657 bp RT-PCR product covering full-length CP gene was cloned into pCR-XL-TOPO vector and sequenced from both directions. The consensus sequence information obtained independently from two cDNA clones has been deposited to the GenBank database (accession

Fig. 2. RT-PCR analysis and phylogenetic analysis of cucumber mosaic virus YJC isolate and other isolates based on deduced coat protein (CP) sequences. (A) Approximately 100 ng of total RNA from infected plant species were subjected to RT-PCR for 40 cycles with primers specific to the CP gene. RT-PCR products were analyzed in 1.5% agarose gel and then photograph was taken after ethidium bromide staining. Lane M contains a 100bp DNA size marker. DNA size is indicated on the left and an arrow indicates the expected RT-PCR products of the CP. (B) Unweighted neighbor-joining (NJ) tree constructed from the sequence alignment of CPs from this study and the Genbank database. Multiple sequence alignments were generated by using the MEGA4.0, and phylogenetic tree was constructed by the NJ algorithm, based on calculations from pair-wise amino acid sequence distances for amino acid analysis of CPs derived from the multiple-alignment format. Bootstrap analysis was done with 1,000 replicates of the starting tree. Sequence similarity is indicated on the top. The CP sequences of CMV strains analyzed are as follows; CMV-YJC (AB698449), CMV-As (AF013291), CMV-K (AF127977), Gs (AB290915), Mf (AJ276481), Pa (AB290152), Ph (AB290153), Rs (AB290154), Rsk1 (AB290155), Sa1 (AB290208), Fny (NC_001440), Ga (AB290210), Lc (AB290209), LK3 (AJ495841), Tai'an (EF409974), Lucknow (JF798578), ND2 (EU414786), Pf (AJ237850), LS (AF127976), and Q (M21464). The CP sequence of tomato aspermy virus strain KC (TAV-KC; accession no. 237849), which represents the species whose members are most closely related to CMV was used as the out-group, but the exact scale is not indicated.

Table 2. Sequence analysis of coat proteins between CMV-YJC and other CMV isolates

Isolate name	CP		Accession number	Subgroup
	Nucleotide	Amino acid		
YJC	— ^a	—	AB698449	IA
As	94.5 ^b	97.2	AF013291	IB
K	95.3	96.5	AF127977	IB
Gs	98.6	99.5	AB290915	IA
Mf	98.5	99.5	AJ276481	IA
Pa	95.6	98.6	AB290152	IA
Ph	98.6	99.5	AB290153	IA
Rs	96.5	98.2	AB290154	IA
Rsk1	96.3	98.2	AB290155	IA
Sa1	96.3	98.2	AB290208	IA
Fny	94.2	99.1	NC_001440	IA
Ga	91.5	94.0	AB290210	IA
Lc	94.4	87.6	AB290209	IA
LK3	97.0	98.6	AJ495841	IA
Tai'an	98.2	99.5	EF409974	IA
Lucknow	99.3	99.5	JF798578	IA
ND2	99.3	100	EU414786	IA
Pf	94.2	99.1	AJ237850	IA
LS	76.9	82.5	AF127976	II
Q	77.2	82.9	M21464	II
TAV-KC ^c	48.8	64.2	AJ237849	— ^d

^a—: not presented due to CMV-YJC sequences in itself.

^bSequence identity (percent) of CP genes between CMV-YJC and other CMV isolates was presented.

^cTAV-KC was used as the outgroup for multiple sequence alignments and phylogenetic analysis.

^dNot applicable.

number AB698449; see also Table 2).

Sequence analysis of CP gene revealed that the sequenced region of the CP gene contained a single open reading frame, comprised of 657 nucleotides with initiation and termination codon. Sequences of CMV-YJC CP were compared with CP sequences of available petunia-infecting CMV isolates, CMV Korean isolates and a few representative isolates from different part of the world. The sequence analysis revealed that CMV-YJC shared 76.9% to 99.3% and 82.5% to 100% sequence identities at the nucleotide and amino acid sequence levels, respectively (Table 2). Interestingly, the sequence identity between CMV-YJC and other CMV strains isolated from petunia was higher at both nucleotide (98.2–99.3%) and amino acid (99.5–100%) levels. Multiple alignment studies for nucleotide and amino acid sequences of CMV-YJC with other reported isolates of CMV from Korea as well as different sites of the world revealed 91.5–99.3% and 87.6–100% identity with CMV isolates that belong to subgroup IA. Meanwhile, CMV-YJC CP sequences had 76.9–77.2% and 82.5–82.9% identity in

nucleotide and amino acid sequences with the members of subgroup II (strains LS and Q), respectively (Table 2). These results confirm that CMV-YJC is a member of subgroup IA. In general, N-terminal region of the CMV CP was more conserved than the C-terminal region (data not shown). However, there were no distinct regions showing remarkable variability in the CPs. Phylogenetic analysis of the CP sequences showed that most CMV isolates used for analysis were placed in subgroup IA and subgroup IB (As and K), except for strains LS and Q (subgroup II). Close relationships were observed among petunia-infecting CMV isolates (Fig. 2), suggesting that petunia was the original host species for CMV-YJC.

Considering that by electron microscopy, host range test, ELISA, RT-PCR and CP sequencing used CMV-specific primers, data from those assays clearly support the conclusion that the petunia plant was infected only with CMV. Furthermore, its involvement in the etiology of the disease was hypothesized and confirmed by fulfilling Koch's postulates. Symptoms induced by these viruses in infected petunia plants are variable, as they are influenced by environmental conditions, virus strains, mixed infections with other viruses, genetic diversity of petunia cultivars, and cultural conditions (Hull, 2002). The symptoms associated with virus diseases in petunia cultivars are stunting and deformation of the foliage, light-green streaks, disclosed and malformed flowers, irregular vein-yellowing, mottling, color breaking, and a reduction in the number and size of the flowers (Edwardson and Christie, 1997; Lesemann, 1996; Lindgren, 1993). Mixed virus infections obviously aggravate symptoms and some infected plants remain symptomless (Brunt et al., 1996; Lesemann, 1996). Virus diseases are detrimental to the production and quality of the petunia crop (Sikron et al., 1995). CMV infection in trailing petunias therefore represents a clear risk to a number of commercially important pot and bedding plant species. In addition this spread of a damaging isolate of CMV in ornamental hosts is a clear risk to pepper, tomato, and even solanaceous crops in areas where these crops are grown in close vicinity and ornamentals can clearly act as a source of inoculum for the virus (Le Romancer and Nedellec, 1997). Petunia-virus diseases became widespread when asexually propagated hybrids were introduced without strict virus-indexing schemes. It is not surprising that sudden and widespread occurrence of CMV in petunias was caused largely by the distribution of infected vegetatively propagated material (Lesemann, 1996). However, CMV is readily transmitted by aphids and in sap, hence it is desirable that growers start with virus free plants, if available, and observe strict hygiene and aphid control practices to minimize the risk of virus spread to other crops, both in and outside of the glass- or green-house (Palukaitis and Garcia-

Árenal, 2003). In general, since a great number of CMV isolates are commonly found from cultivation fields surrounding many weed species, the overall importance of this virus in petunias may be much greater than an accidental infection, thus requiring particular attention. The positive identification of CMV reported in this paper suggests that this virus could be the unidentified pathogen frequently detected in single or double flower petunia hybrids. To the best of our knowledge, it is the first report of CMV in petunia plant in Korea.

Acknowledgements

This work was supported by grants from Basic Research Program of National Institute of Horticultural and Herbal Science (NIHHS), RDA and 2012 Postdoctoral Fellowship Program of NIHHS, RDA, Republic of Korea.

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