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Notes

First Report of *Cucumber mosaic virus* Infecting Pinewood Coneflower (*Rudbeckia bicolor*) in Korea

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A virus isolate causing symptoms of yellow mosaic, fern leaves, malformation and plant necrosis on *Rudbeckia bicolor* was prevalent around Pyeongchang area in Korea. The causal virus was identified as *Cucumber mosaic virus* (CMV) using characteristics from biological, serological and molecular analyses and named as CMV-Rb. CMV-Rb caused mosaic on *Nicotiana benthamiana, N. tabacum, Capsicum annuum,* and *Lycopersicon esculentum*. However, typical local lesions did not develop on inoculated *Pisum sativum*, *Cucurbita moschata, Datura stramonium* and *Tetragonia expansa* plants. Full-length genome sequences of CMV-Rb RNAs 1, 2 and 3 were obtained using 12 primer pairs by RT-PCR analysis. The genome of CMV-Rb RNA segments 1, 2, and 3 consists of 3363nt, 3049nt, and 2214nt in length, respectively. In order to ascertain their taxonomic identity, nucleotide and the deduced amino acid sequence analyses RNAs 1, 2 and 3 of CMV-Rb isolates were conducted with previously reported sequences of CMV strains and/or isolates. CMV-Rb RNAs showed about 90 to 99% sequence identity to those of subgroup I strains suggesting that CMV-Rb is more closely related to CMV isolates belong to subgroup I. To our knowledge, this is the first report of CMV on *Rudbeckia bicolor* in Korea.

Keywords **:** CMV-Rb, phylogenetic analyses, *Rudbeckia bicolor*, subgroup I

Cucumber mosaic virus (CMV), the type member of the genus *Cucumovirus*, infects more than 1,000 species of plants and induces the typical mosaic symptoms in many host plants. Some of the intermediate symptoms, however, include blight, fernleaf, ringspot, and stunt. In Korea, at least 109 natural host plant species has been reported, i.e., 20 in upland crops, 20 in vegetable crops, 28 in floral crops, acacia in wood, 40 in wild plants (List of plant disease in Korea, 2004, The Korean Society of Plant Pathology). Recently, CMV infections were also reported on *Hydrangea macrophylla* (Lee et al., 2001), water chickweed (Choi et al., 2004), *Isodon inflexus* Kudo, *Jeffersonia dubia* Benth, *Phryma leptostachya* var. *asiatica* Hara (Lee et al., 2008). However, no natural outbreaks by CMV have been reported on *Rudbeckia bicolor*, but *Bidens mottle virus* (BiMoV) was only identified (Brunt et al., 1996; Choi, 2001; Jin, 2003; Lee, 1981; Lee et al., 2003; Logan et al., 1984).

The genome of CMV consists of three genomic RNAs (RNA 1, 2 and 3; Roossinck, 2001). In infected host plants, it also produces two subgenomic RNAs (4 and 4A; Palukaitis et al., 1992). Based upon biological, serological, and, molecular biological characteristics of CMV isolates, they were classified into two subgroups, i.e., subgroup I and II (Owen and Palukaitis, 1988). The nucleotide sequence identity between subgroup I and II isolates range from 69 to 77%, while showing above 90% nucleotide sequence identity within each subgroup isolates. More detailed nucleotide sequence analyses, especially with the coat protein (CP) and 5' nontranslated region (NTR) of RNA 3 further suggested subdivision of subgroup I into subgroups IA and IB (Roossinck et al., 1999). This subgrouping was more clearly supported from the analysis of nucleotide sequences of 1a and 3a open reading frames (ORFs) than those of the 2a and CP ORFs (Roossinck, 2002). In this paper, we report the biological and serological characteristics as well as complete nucleotide sequence analyses of CMV-Rb isolate. We believe this is the first report of natural CMV infection on *Rudbeckia bicolor* in Korea.

Pinewood coneflower (*R. bicolor*), family *Asteraceae*, is widely grown as an ornamental crop and a wildflower. In 2005, we could easily observe yellow mosaic, sometimes

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Fig. 1. Yellow mosaic (A) and malformation (B) symptoms on Pinewood coneflower, *Rudbeckia bicolor* caused by *Cucumber mosaic virus* isolate Rb (CMV-Rb) infection.

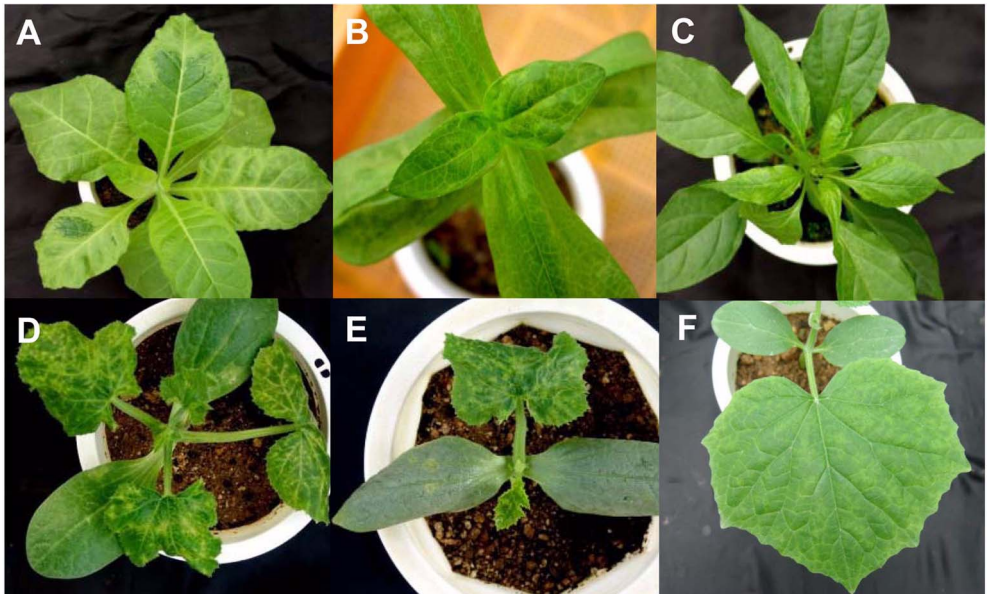

Fig. 2. Symptoms caused by CMV-Rb from *R. bicolor* on tested plants. Typical mosaic symptoms on *Nicotiana tabacum* 'KY-57' (A), *Zinnia elegans* (B), and *Capsicum annuum* (C). CMV-RB had severe virulence causing severe mosaic on the upper leaves after producing chlorotic spots on the inoculated leaves on *Cucurbita pepo* (D) and *Cucumis melo* (E). Mild symptom of chlorotic spots on the upper leaves of *C. sativus* (F) was induced.

fernleaf and malformation symptoms of Pinewood coneflower on the herb plantation in the Pyeongchang area, Korea (Fig. 1). Leaves extracts of the diseased plants reacted positively with CMV polyclonal antibody in double antibody sandwich enzyme linked immune sorbent assay (DAS-ELISA). DAS-ELISA was conducted essentially as described by Clark and Adams (1977) using CMV polyclone antibody (Agdia, USA). Briefly, CMV antibody and conjugated were both diluted 1:200 and all incubations were carried out at 37 °C for 2 h except for the substrate which was incubated for 30 min. Quantitative measurements of p-nitrophenol were made by determining absorbance at 405 nm (A_{405}). Virus isolate was obtained from leaves of *R. bicolor* showing mosaic and necrosis symptoms that were collected from fields around Pyeongchang, Korea. The infected leaf was macerated in 0.01 M sodium phosphate buffer, pH 7.0. The crude sap was rubbed to the leaves of indicator plants including *Chenopodium amaranticlor*. Single lesions were isolated and re-inoculated on healthy plants of *C. quinoa*. This process was repeated three times. This isolate was named as CMV-Rb and propagated in *Nicotiana tabacum* cv. Xanthi nc.

To test host range, at least 30 plants including *Vigna unguiculate* was tested using the virus source in crude sap

Table 1. Symptoms developed on indicator plants that were mechanically inoculated with *Cucumber mosaic virus* isolate Rb (CMV-RB)

Plant species	Symptoms[a] on the leaves	
	inoculated	upper
C. amaranticolor	NL	–
C. quinoa	PP	–
Spinacia oleracea L.	–	M
Brassica peckinensis L.	–	–
Raphanus sativus L.	–	–
Nicotiana tabacum cv. Xanthi-nc	CL	M
N. tabacum cv. KY-57	–	Cl, M
N. glutinosa	–	M
Capsicum annuum	–	VC, M
Lycopersicon esculentum	–	M
Datura stramonium	CL	–
Petunia spp.	–	M, Mal
Solanum melongena	CL	VC, M
Vicia fava	NL	–
Pisum sativum	NL	–
Vigna unguiculate	PP	–
Zinnia elegans	–	M
Tetragonia expensa	CRL	–
Cucurbita pepo 'Taeyang'	CL	SM, Mal
Cuc. pepo 'Bulamhous'	CL	PP, M
Cuc. pepo 'Choigobong'	CL	SM, Mal
Cuc. moschata 'Jinhan Aihobag'	–	–
Cucumis melo 'Best'	–	M
Cu. melo 'Keumssaragi'	CL	M
Cu. sativus L.	CL	M

[a] NL, necrotic local; PP, pin point; M, mosaic; CL, chlorotic local; vc, vein clearing; CRL, cholorotic ring local; Mal, malformation; sM, severe mosaic; –, no symptom.

of *N. tabacum* cv. Xanthi nc. Each test plant were inoculated mechanically at 3-4 leaf stage by rubbing with a piece of sterilized wooden towel dipped into inoculum after scattering 600 mesh Carborundum. Symptoms were visually observed for 3 weeks after mechanical inoculation. CMV-Rb isolate propagated in *N. tabacum,* obtained by single lesion isolation, induce various systemic symptoms, including mosaic, chlorosis, malformation symptoms on *Spinacia oleracea* L., *N. tabacum* cv., *N. glutinosa*, *Capsicum annuum*, *Lycopersicon esculenturm*, *Petunia* spp., *Solanum melongena*, *Zinnia elegans*, *Cucurbita pepo*, *Cucumis melo*, and *Cucurbita sativus* L. (Fig. 2). No symptom was observed on *Brassica peckinensis* L., *Raphanus sativus* L. Local lesions were observed on inoculated leaves of *C. amaranticolor*, *C. quinoa*, *Datura stramonium*, *Vicia fava*, *Pisum sativum*, *Vigna unguiculate, Tetragonia expensa* and *C. moschata*. No significant reaction was observed in the DAS-ELISA with CMV antibody when extracts from their upper leaves were used (Table 1).

Dip preparation were prepared by grinding a small piece of infected leaves with 2~3 drops of 2% phosphotungstic acid (PTA), pH 7.0 for electron microscopy. The extract was mounted on a carbon-stabilized and Formvar-coated grid. The isometric particle approximately 30 nm were observed from symptomatic *N. tabacum* (data not shown). In general, CMV-Rb showed biological and serological characteristics of subgroup I of CMVs. However, the host range of CMV-Rb differed from reported isolates including Mf, Sa, NP, and CARNA5 of subgroup I on *D. stramonium* and *T. expansa* (Choi et al., 1998, 1999, 2001, 2004; Lee et al., 2008). CMV-Rb did not induce systemic symptoms on *D. stramonium* and *T. expansa.* In addition, differences in symptoms were also observed in some indicator plants including *Cu. sativus*, *Ca. annuum*, *N. benthamiana*, and *N. glutinosa*. These results indicate that CMV-Rb might be new distinctive isolate within subgroup I. It is also possible that these different results on tested indicator plants caused by differences in the plant growth stage of indicator plants when they were inoculated and environment conditions after inoculation.

To further clarify these differences of symptoms on tested host plants, we conducted sequence analyses of the CMV-Rb. Complete nucleotide sequences of CMV-Rb RNAs 1, 2 and 3 were obtained by using DNA fragments spanning whole genome regions of RNAs 1, 2, and 3 that were amplified by RT-PCR using each specific sequence primer. Briefly, total RNAs were extracted from the propagated *N. tabacum* leaves by easy-spin™ Total RNA kit (iNtRON, Korea). Specific primers were designed based upon full-length sequences of the previously reported CMV sequences available in GenBank of National Center for Biotechnology (NCBI). Reverse transcription (RT) reaction was carried out at 42°C for 30 min, and was denatured by heating at 95°C for 5 min in 5x RT buffer containing 5l of total RNA, 10 pmoles of the downstream primer, 2.5 mM each of four dNTPs, and 2.5 units AMV reverse transcriptase (Promega Co., USA). The RT reaction mixture was mixed with 1 units LA *Taq* DNA polymerase (Takara Co., Japan), 10× PCR buffer and 2.5 mM $MgCl_2$, and 10 pmoles of the upstream primer were added. Mixtures were then amplified in a Bio-Rad Thermal Cycler (USA). PCR products were analyzed by electrophoresis in 1.0% agarose gel, staining with ethidium bromide, and DNA bands were visualized using a UV transilluminator. The genomes of CMV-Rb RNAs 1, 2 and 3 were found to consist of about 3.3 kb, 3 kb, and 2.2 kb and were deposited at the GenBank under accession codes GU327363, GU327364, and GU327365, respectively. The sequence was then compared with corresponding sequences of CMV isolates from the GenBank database.

Table 2. Amino acid sequence identities (%) between CMV-Rb and the previously reported strains of CMV

Isolate	Strainsa	Amino acid identity (%)				
		RNA1	RNA2		RNA3	
		1a	2a	2b	3a	cp
Rb	CMV-CTL	93.9	93.3	81.8	96.0	97.2
	CMV-Fny	95.3	97.7	97.3	99.3	99.5
	CMV-Leg	95.0	96.6	87.3	99.6	97.7
	CMV-NT9	94.9	93.0	80.7	96.8	98.2
	CMV-Tfn	95.0	92.8	80.7	96.8	98.6
	CMV-Ix	94.3	91.4	78.2	94.3	96.3
	CMV-Y	93.4	98.2	96.4	99.6	96.8
	CMV-Mf	94.8	98.6	99.1	99.3	100
	CMV-Ls	84.5	76.2	54.5	83.2	82.0
	CMV-Ly	84.2	75.6	53.5	83.5	82.5
	CMV-Trk7	84.1	75.8	54.5	83.9	80.6
	CMV-Q	83.9	74.6	54.5	83.2	82.9

[a] The Genbank accession number of the reference CMV isolates : CTL (EF213023, EF213024, EF213025); Fny (D00356, D00355, D10538); Leg(D16403, D16406, D16405); NT9 (D28778, D28779, D28780); Tfn (Y16924, Y16925, Y19626); Ix (Y20220, U20218, U20219); Y (D12537, D12538, D12499); Mf (AJ276479, AJ276480, AJ276481); Ls (F416899, AF416900, AF127976); Ly (AF198101, AF198102, AF198103); Trk7 (AJ007933, AJ007934, L15336); Q (X02733, X00985, M21464). Standard subgroup II CMV isolates are Ls, Ly, Trk7 and Q. Standard subgroup I isolates are CTL, Fny, Leg, NT9, Tfn, Ix, Y and Mf.

All the RNAs have a cap structure at the 5' terminus and 3' portion and also highly conserved in virus-specific manner (Roossinck, 2001). Only the largest three RNAs are required for infectivity; RNA 1 is the only monocistronic RNA which encoding 1a protein that is required for viral replication and contains methyl transferase and helicase motifs (Gal-On et al., 1994; Nitta et al., 1988). RNA 2 encodes the 2a protein, which is the viral RNA dependent RNA polymerase subunit of the CMV replicase, and for the 2b protein, which is translated from a subgenomic RNA (Francki, 1985). The 2b open reading frame (ORF) is over-printed on the carboxy terminal portion of the 2a ORF. The 2b protein of CMV subgroup II was shown to inhibit host post-transcriptional gene silencing (PTGS). In addition, it has been implicated in symptom severity (Shi et al., 2003; Wang et al., 2004). RNA 3 encodes the 3a and capsid protein (CP), but the CP is only expressed from the sub-genomic RNA 4 (Ding et al., 1994). Subgenomic RNA 4A is only encapsidated into virions of CMV strains subgroup II (Ding et al., 1994, 1995, 1995). RNAs 1 and 2 encode components of the viral RNA-dependent RNA polymerase (RdRp) (Hayes and Buck, 1990), while the bicistronic RNA 3 encodes the movement protein (MP) and CP (Gal-On et al., 1996; Owen et al., 1990).

Sequence data was analyzed and compared with those of known CMV strain by the BLAST program at NCBI web-site and the multiple sequence alignment application of

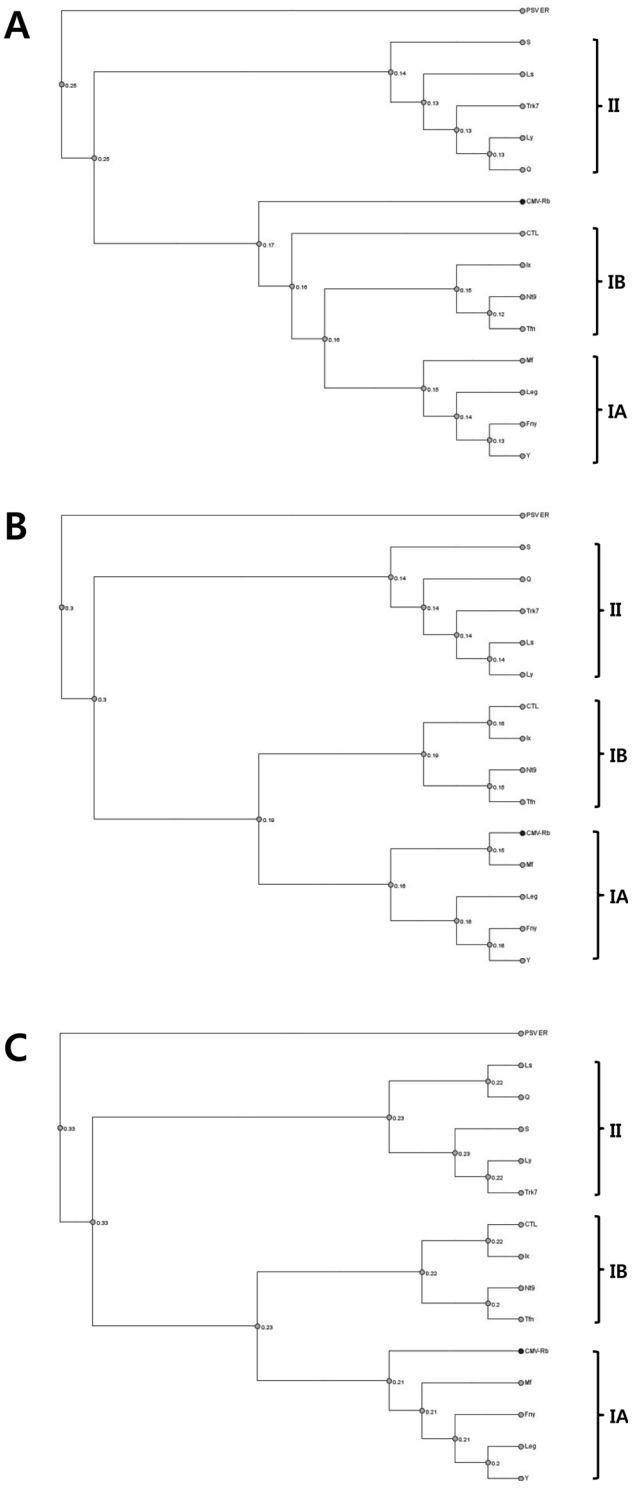

Fig. 3. Neighbor-joining phylogenetic trees base on Jukes-Cantor genetic distances derived from nucleotide sequences of the RNA segments 1, 2 and 3 of CMV-Rb with previously reported CMV isolates (panels A, B, and C, respectively). *Peanut stunt virus* (PSV) was used as an outgroup.

DNAMAN version 4.0 (Lynnon Biosoft, Germany) and Geneious v4.5.4 full optimal sequence alignments. Phylogenetic tree analysis was carried out using neighbor-joining method based on Jukes-Cantor genetic distances with 1000 bootstrap (Geneious 4.5.4) replications. Complete genome sequences of 13 CMV strains in GenBank were used as references and ER strain of PSV was used as an outgroup. Multiple sequence alignment was performed by using Geneious 4.6. Nucleotide sequence analysis between RNA1 of CMV-Rb and subgroup I strain showed over 90% sequence identity and was related to the Fny (USA), Y (Jap), Leg (Jap), and Mf (Kor) strain of CMV. The obtained sequence from RNA segments 2 and 3 revealed 99% and 98% nucleotide sequence identity with the strain Mf, respectively (data not shown). Database comparisons of the deduced amino acid sequences showed 83.9 to 95.3% with 1a ORF gene of CMV. In contrast, the RNA2 ORF (2a and 2b) of CMV strains showed 53.5 to 98.6% sequence identity. The aa sequence identity of RNA3 ORF (MP, CP) between CMV-Rb and other strains were ranged from 80.6 to 100% (Table 2). Nucleotide and amino acid sequence identity between strains of subgroup I (Fny, Mf and Y) revealed above 90%.

CMV-Rb was assigned to subgroup IA based on phylogenetic analysis of the RNA2 and RNA3 but to subgroup IB based on the RNA1 (Fig. 3). Nucleotide sequence analysis of RNA segments 1, 2 and 3 of CMV-Rb isolates and subgroup I strains showed about 90 to 99% sequence identity. Amino acid sequence of the CMV-Rb isolate was compared with the corresponding five ORFs of the other twelve CMV strains. CMV-Rb isolate showed more than 90% of amino acid sequence identity to the subgroup I. Therefore, results of classifications of the RNA1, 2 and 3 molecules suggested that CMV-Rb belongs to subgroup I of CMV. Interestingly, CMV-Rb analysis of RNA segments 2 and 3 showed that strains in IA constitute monophyletic groups, whereas analysis of RNA1 was included in non-monophyletic subgroup IB. This suggests that the subgroups I strains are evolving more rapidly, and three genomic RNAs of CMV has a different evolution history. Results of this study supported the idea that differences in symptomatology observed from different host plants may be at least partly due to the differences between CMV-Rb RNA 1 belonging to subgroup IB and CMV-Rb RNAs 2 and 3 belonging to subgroup IA. A further study is, however, needed like construction of infectious clones to clarify these relations and to characterize the host factors required for CMV infections. Altogether, results of this study indicated that the virus isolate causing symptoms of yellow mosaic, fern leaves, malformation, and necrosis on *R. bicolor* was identified as CMV-Rb belonging to subgroup I based upon biological and molecular analyses.

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