Characteristics of *Cucumber mosaic virus* Infecting Zucchini in Korea

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A virus causing stunt, yellowing, severe mosaic, malformation symptoms on leaves and uneven development and malformation on fruits of zucchini was prevalent around Goseong, Gyeongsangnam-do, Korea. A survey conducted (2004) in the Goseong area revealed about 20% virus infection rate. The disease causative identified as *Cucumber mosaic virus* (*CMV-Z1*) was further characterized. The isolate induces mosaic symptoms on *Cucumis sativus*, while severe mosaic, stunt and malformation on *C. pepo*. Thin section analyses have shown that virus inclusions are formed in the cuticle layers as well as epidermal, parenchyma and collenchyma cells in virus-infected *Nicotiana tabacum*. CMV-Z1 isolate induced specific cytoplasmic inclusion bodies such as irregular clumps (IC), crystal (Cr) and irregular chloroplasts (ICh). IC was made up of virus particles interspersed with a darkly stained amorphous material and found both in the cytoplasm and vacuoles, whereas ICh and Cr were rarely found in the vacuoles. The genome of CMV-Z1 RNA-1 consists of 3359 nucleotide (nt) encoding 1a protein of 993 amino acids (aa). The CMV-Z1 RNA-2 was 3050 nt in length containing 2a (857 aa) and 2b (110 aa), while RNA-3 encoding 3a movement protein (279 aa) and coat protein (218 aa) was 2215 nt in length. Phylogenetic analyses of nucleotide sequences of CMV-Z1 isolate appeared it is more closely related to subgroup IA than to subgroup IB or II.

**Keywords**: CMV, complete genome sequence, phylogenetic analysis, zucchini

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*Cucumber mosaic virus* (CMV) occurs world-wide in nature and has the broadest host range among known viruses, infecting more than 1,200 species of plants from monocotyledons to dicotyledons, from herbaceous plants to trees. CMV is transmitted primarily by aphids, and also by seeds, cucumber beetles, parasitic plants, humans, and mechanically (Doolittle, 1916; Edwardson and Christie, 1991; Jagger, 1916). Although the most common symptom incited by CMV is mosaic (Doolittle, 1916; Wellman, 1972), severity of disease may range from no obvious symptom in some crops to death of host species (Van Regenmortel et al., 2000). Some of the intermediate symptoms include blight, fernleaf, ringspot, fruit woodiness and necrosis of bulb and fruit (Wellman, 1972). The genome of CMV consists of three genomic RNAs (1, 2 and 3), as well as the two subgenomic RNAs (4 and 4A). All the RNAs have a cap structure at the 5’ terminus and 3’ portion and also highly conserved in virus-specific manner (Roossinck, 2001; Symons, 1975).

CMV, the type species in the genus *Cucumovirus*, family *Bromoviridae*, is single-stranded, positive-sense RNA virus (Palukaitis and Garcia-Arenal, 2003; Roossinck et al., 1999; Suzuki et al., 1991). Serological data, peptide mapping of coat protein (CP), and nucleic acid hybridization divided CMV strains into three subgroups: IA, IB and II (Chaumpluk et al., 1996; Roossinck et al., 1999). Meanwhile, phylogenetic analyses of individual RNA, gene or even entire genome revealed further subspeciation due to radial evolution (Roossinck, 2002) and, therefore, were used for a complete phylogenetic analysis of the virus.

In Korea, various viruses including CMV, *Watermelon mosaic virus* (WMV), *Zucchini yellow mosaic virus* (ZYMV), *Papaya ringspot virus*, *Kyuri green mottle mosaic virus* and *Zucchini green mottle mosaic virus* were identified from zucchini plants (Choi, 2001; Jin, 2003; Lee, 1981; Lee et al., 2003). In addition, Choi et al. (1998)
reported that eight isolates of CMV were identified and differentiated by dsRNA analysis, RT-PCR assay, MspI restriction mapping, SSCP analysis, serological property, and biological reactions in several hosts. In this study, we isolated CMV isolates from zucchini and compared its characteristics to the other CMV strains using biological, serological, and cytological properties. Additionally, we sequenced the complete genome of CMV RNAs 1, 2, and 3 and analyzed phylogenetic relationship to the previously reported CMV strains.

Materials and Methods

**Virus isolate and host range studies.** A survey of zucchini and *Hemistepta lynata* Bunge virus disease was carried out in Goseong, Gyeongsangnam-do, Korea, in 2004. To isolate pure CMV cultures from possible double infections, virus isolates were inoculated to local lesion host (*Nicotiana tabacum* cv. Xanthi-nc) and reisolated from local lesions at 7-14 days postinoculation at least three times in succession. To determine the infectivity of virus isolates and the symptoms induced on test plants, 5-10 plant seedlings of each of the species listed in Table 2 at the 3-5 leaf stage were mechanically inoculated using sap prepared from infected leaf samples in 0.01 M phosphate buffer, pH 7.0. Inoculated plants were maintained in a pathogen-free greenhouse at 20-25°C. Disease symptoms were checked three times a week for 30 days. Enzyme-linked immunosorbent assay (ELISA) and examination with the naked eye verified both symptomatic and non-symptomatic plants for CMV infection.

**ELISA.** ELISA test kits purchased from Agdia (USA) were used. CMV polyclonal antibodies and conjugate were both diluted 1:500 and all incubations were carried out at 37°C for 2 hr except for the substrate which was incubated for 30 min. Finally, quantitative measurements of generated p-nitrophenol were made by absorbance at 405 nm spectrophotometer (EL312e EIA, Bio-Tek Instruments Inc., USA). Absorbance value higher than twice of negative control was considered as positive.

**Purification.** Virus was purified following Takanami (1981), Lot and Kaper (1976) and Mossop et al. (1976) with modification. CMV-infected tabacco (*Nicotiana tabacum* cv. Xanthi-nc) leaves were homogenized with chilled blender jar with 1:5 (w/v) volume of 0.5 M sodium citrate buffer (SCTB, pH 7.0) containing 5 mM EDTA and 0.5% 2-mercaptoethanol and filtered through several layers of cheesecloth. The extract was strained, stirred with 8% (v/v) chloroform: butanol at 4°C for 30 min and centrifuged at 8,000 rpm at 4°C for 30 min. The supernatant was adjusted to 8% polyethylene glycol (PEG, MW 6,000) and 0.1 M sodium chloride and incubated 1 hr at 4°C with continuous stirring. Pellets saved after centrifugation were resuspended with 100 ml of 0.05 M SCTB containing 5 mM EDTA and 2% Triton X-100. The supernatant was precipitated by ultracentrifugation at 40,000 rpm for 2.5 hrs at 4°C and each pellet was resuspended with 1 ml of 0.05 M SCTB. The resulting solution was centrifuged at 8,000 rpm for 30 min. The supernatant was centrifuged at 40,000 rpm for 2.5 hrs and pellet was resuspended in 0.05 M SCTB. Aliquots were subjected to two cycles of sucrose density gradient centrifugation at 28,000 rpm for 2 hrs. Separated virus layer was dialyzed and centrifuged at 40,000 rpm for 2 hrs. Pellets were dissolved into ddH₂O.

**Electron microscopy (EM).** Dip preparations were prepared by grinding a small piece of infected *N. tabacum* with 2-3 drops of 2% phosphotungstic acid, pH 7.0. The extract was mounted on a carbon-stabilized and Formvar-coated grid. For transmission EM, infected leaves were harvested two weeks after inoculation and immediately cut with a sharp blade into 1-3 mm thick pieces. The pieces were immediately fixed with 2.5% glutaraldehyde in Millonig’s phosphate buffer, pH 7.0 and stored at 4°C for 2 h except for the substrate which was incubated 1 hr at 4°C overnight in 1.0% uranyl acetate at 4°C and then rinsed with distilled water. Stained pieces were dehydrated with 50-100% ethanol series for 50 min and embedded in resin. Embedding blocks were hardened overnight at 60°C and sliced to 80 nm thickness using ultramicrotome. The sections were then stained twice, with 2% uranyl acetate for 20 min and with 0.5% lead citrate for 10 min. For interpretation of results, the sections were observed under electron microscope (LEO 912AB (Carl Zeiss, Germany) at 80 kV.

**Primers.** The primers were designed to sequence full-length genome of each RNAs based on the previously reported sequences of CMV available in GenBank of National Center for Biotechnology (NCBI, USA; Table 1). A modification and reformation of CMV primers previously described was necessary for their use as external primers (Rizos et al., 1992). Genus primers were designed from these alignments for RNA1, 2 and 3 of CMV strains (Table 1). Briefly, sequenced regions of each virus were recovered using the Nucleotide Sequence Search program located in the Entrez Browser program provided by the NCBI (http://www3.ncbi.nlm.nih.gov/Entrez). Conserved regions of each virus were studied using the similarity search tool Advanced BLAST 2.0, with the BLASTN program (http://www3.ncbi.nlm.nih.gov/blast/). The alignment