Plant Pathol. J. 16(6) : 306-311 (2000)

The Plant Pathology Journal
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A Novel Strain of *Cucumber mosaic virus* Isolated from *Lilium longiflorum*

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(Received on October 18, 2000)

A new strain of *Cucumber mosaic virus* (CMV) from easter lily (*Lilium longiflorum*), Ly2-CMV, was identified and compared to the well-characterized Mf-CMV (subgroup I) and LS-CMV (subgroup II) by host reaction in several indicator plants, dsRNA analysis, serological property, RT-PCR analysis, restriction enzyme profile of the PCR products and nucleotide sequence of coat protein (CP) gene. Remarkable differences in symptoms of Ly2-CMV were found between Mf-CMV or LS-CMV in tobacco plants and *Datura stramoinium*. Ly2-CMV induced small necrotic ringspots on the inoculated leaves of *Nicotiana tabacum* cvs. Xanthi nc and Burley 21 and *D. stramonium*, and failed to infect these species systemically. Of the indicator plants tested, *N. benthamiana* only reacted with systemic infection by inoculation of Ly2-CMV. In experiments of dsRNA analysis, serology and RT-PCR of CP gene, Ly2-CMV was come within subgroup I CMV. However, restriction enzyme analysis of the PCR products using *Msp*I showed that Ly2-CMV was distinct to Mf-CMV. The CP gene of Ly2-CMV contains 657 nucleotides, and the nucleotide sequence is similar to that of Mf-CMV. There is also a high degree of conservation between their putative gene products in Ly2-CMV and Mf-CMV, with five amino acid changes in the 218 amino acids of the CPs.

Keywords **:** *Cucumber mosaic virus* (CMV), *Lilium longiflorum*, host reaction, coat protein, nucleotide sequence.

Cucumber mosaic virus (CMV) is a positive-sense, tripartite RNA plant virus, with a broad host range and worldwide distribution (Kaper and Waterworth, 1981). RNAs 1 and 2 of CMV are associated with the replication of the viral genome (Nitta et al., 1988), whereas RNA 3 contains both the viral coat protein (CP) gene and the 3a gene, which encodes a protein thought to be involved in cell-to-cell movement of virus particles or genomic RNAs (Davies and Symons, 1988). There are many strains of CMV which differ in host range and pathogenicity (Kaper and Waterworth, 1981; Palukaitis et al., 1992). Differences in various biological properties between such strains have been determined to specific RNAs by pseudorecombination and sequencing. For example, RNA 3 of the M strain of CMV (M-CMV), which encodes the viral coat protein, is associated with a loss of aphid transmissibility (Mossop and Francki, 1977), an inability to infect some cultivars of squash (Shintaku and Palukaitis, 1990), an altered particle stability (Mossop et al., 1976) and the induction of systemic chlorosis in tobacco (Rao and Francki, 1982).

Easter lily (*Lilium longiflorum*) originated in Asia and has grown in Korea from an ancient time. In 1999, we isolated a strain of CMV, Ly2-CMV, from *L. longiflorum* plants of a farmer's greenhouse at Youngwol, Kangwondo in Korea, and the virus has been isolated from diseased lily which seemed different from the strains of characterized CMVs. We report some characteristics of the virus which strongly suggest that it is a novel strain of CMV which is distinct from other CMVs reported.

Materials and Methods

Virus Source and propagation. Virus was isolated from a *L. longiflorum* showing yellow mosaic and streak symptoms and which had been shown a putative CMV by dsRNA analysis and inoculating the indicator hosts cowpea (*Vigna unguiculata* cv. Kegonnodaki) to be free of any of the viruses known to infect lily. Single local lesions were isolated from the cowpea and then propagated to *Nicotiana benthamiana*. Mf-CMV (subgroup I) (Choi et al., 1998) and LS-CMV (subgroup II) (Wahyuni et al., 1992) were used as a control CMVs.

Host range and symptomatology. Host range and symptomathology of Ly2-CMV were determined by sap inoculation of systemically infected *N. benthamiana* leaves. The inoculation was done by using the extract in 0.01 M phosphate buffer to mechanically inoculate Carborundum-dusted leaves of at least five each of the following test plants: *N. benthamiana, N. glutinosa, N. tabacum* cvs. Xanthi nc and Burley 21, *Datura stramonium, Lycopersicon esculentum, Cucumis sativus, Cucurbita pepo, Chenopodium amaranticolor, V. unguiculata, Zea mays, L. longiflorum, L.*

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oriental hybrid cv. Casa Blanca and *Phaseolus vulgaris*. After inoculation, plants were kept in the greenhouse at 25 to 30°C for up to 3 weeks.

DsRNA analysis. Double stranded RNA (dsRNA) was purified from 7 g of leaf tissues of *N. benthamiana* infected with the Ly2-CMV by the procedure of Morris and Dodds (1979). DsRNA was analysed by electrophoresis through 6% polyacrylamide gel containing 1 x TAE (40 mM Tris, 40 mM acetic acid, 2 mM EDTA, pH 7.8). The gel was stained with ethidium bromide and visualized under UV illuminator.

Serological test. Agar gel double diffusion test was used to establish the relationship of the viruses with antiserum to the Mf-CMV (Choi et al., 1998). Diffusion gel contained 1% (w/v) agarose in 0.01 M phosphate buffer saline (PBS), pH 7.0. Leaf extracts in PBS from *N. benthamiana* inoculated with the CMV strains were used as antigens. Precipitin lines were observed after incubation for 12 hr at room temperature.

Virus purification. The Ly2-CMV was purified after 7-10 days post inoculation from systemically infected leaves of *N. benthamiana* by the procedure described for Y-CMV (Takanami, 1981). The virus was further purified by sucrose density gradient centrifugation at 28,000 rpm for 2 hr in a swinging-bucket 28.1 rotor. Virus concentration was estimated using an assumed extinction coefficient of 5.0 (Francki et al., 1966).

RT-PCR assay. Total nucleic acids were extracted from infected and healthy *N. benthamiana* leaves using a method previously described (Choi, et al., 1998). PCR primers for CMV coat protein (CP) gene were used the sequence described by Choi et al. (1999). Total RNAs extracted from infected plant tissues were denatured at 70°C for 5 min and amplified with 0.5 units of MMLV reverse transcriptase (Promega), 2.5 units of *Taq* DNA polymerase (Promega), 0.2 mM dNTP and 0.3 uM of each primer per 100 ul reaction. RT-PCR was carried out with one cycle of RT reaction at 42°C for 45 min and 30 cycles of PCR amplification using the step programme (94°C, 30 sec; 40°C, 1 min; 72°C, 1 min) followed by a 7 min final extension at 72°C. Ten microliters of the amplified DNA fragments were separated by electrophoresis on a 1.2% agarose gel in 1 x TAE buffer and stained with ethidium bromide. PCR products were digested with restriction enzymes, *Msp*I and *Eco*RI (Promega), using 6 ul amplified DNA fragments diluted twofold in distilled water, 2 ul of 10 x reaction buffer, and 1 ul each of enzyme. Digestion was done at 37°C for 2 hr and analysed by agarose gel electrophoresis.

Cloning and sequencing of CP cDNA. The cDNA to 3' half region of Ly2-CMV RNA 3 was constructed by RT-PCR as described above. The PCR product was analysed by 1% agarose gel electrophoresis, the cDNA band was isolated using DEAE cellulose membrane (NA-45, Schleicher & Schuell), purified using Bioclean kit (USB) and cloned in pUC18 by standard procedures (Sambrook et al., 1989). The nucleotide sequence of the 3' half region of Ly2-CMV RNA 3 was determined using the cDNA fragments cloned into pUC18 and using M13 primers for both strands of the cDNA clone. The nucleotide sequence was analysed and compared with published data using programs of the DNA sequence analysis computer packages for PC (DNASTAR, Madison, Wis., USA).

Table 1. Reaction of indicator plants by mechanical inoculation of the Ly2-CMV, Mf-CMV and LS-CMV

Host plants	Symptoms (RT-PCR)		
	Ly2	Mf	LS
Nicotiana benthamiana	–/M[a](+/+)[b]	–/M	–/M
N. glutinosa	–/–(–/–)	–/M	–/M
N. tabacum cv. Xanthi nc	NR/–(+/–)	–/M	–/M
N. tabacum cv. Burley 21	NR/–(+/–)	–/M	–/M
Datura stramonium	NR/–(+/–)	–/M	–/M
Lycopersicon esculentum	–/–(–/–)	–/M	–/M
Cucumis sativus	–/–(–/–)	–/M	–/M
Cucurbita pepo	–/–(–/–)	–/M	–/M
Chenopodium amaranticolor	sL/–(+/–)	L/–	L/–
Vigna unguiculata	L/–(+/–)	L/–	L/–
Phaseolus vulgaris	–/–(–/–)	–/–	–/–
Zea mays	–/–(–/–)	–/M	–/–
Lilium longiflorum	–/–(+/+)	–/–(+/+)	–/–(+/+)
L. oriental hybrid cv. Casa Blanca	–/–(+/+)	–/–(+/+)	–/–(+/+)

[a] Inoculated leaves/upper leaves, : mosaic, NR: necrotic ringspots, L: necrotic local lesions, sL: necrotic local lesions of small size. –; sysptomless or not infected.

[b] RT-PCR was performed from total nucleic acids extracted from indicator plants 10 days after inoculation using a method previously described by Choi et al. (1998). +: positive assay, –: negative assay.

Results

Host reaction. The host range and symptomathology of Ly2-CMV are given in Table 1. Of the indicator plants tested, Ly2-CMV only caused systemic symptoms to upper leaves of inoculated *N. benthamiana*. Mf-CMV and LS-CMV used as control virus in this work infected tobacco plants, *N. glutinosa*, *D. stramonium*, *C. sativus* and *C. pepo* and developed mosaic symptoms, while Ly2-CMV did not infect these plants systemically. However, Ly2-CMV could locally infect to inoculated leaves of tobacco plants and *D. stramonium* which are expressed with small necrotic ringspots. In addition, Ly2-CMV induced necrotic local lesions similar to those induced by Mf-CMV or LS-CMV on the inoculated leaves of *C. amaranticolor* and *V. unguiculata*.

DsRNA analysis. The sizes and numbers of the dsRNA species produced during infection of the Ly2-CMV in *N. benthamiana* compared to those of Mf-CMV and LS-CMV are shown in Fig. 1. All CMVs revealed four dsRNA species with estimated molecular sizes of 3.4, 3.2, 2.2 and 1.0 kbp and the mobility of the species of dsRNA was similar for all CMVs.

Serological relationship. Crude sap extracted from *N. benthamiana* infected with the Ly2-CMV was tested by agar gel diffusion test against Mf-CMV antiserum. The antigens of Ly2-CMV reacted positively with the antiserum, giving a single precipitin line closely fused with that

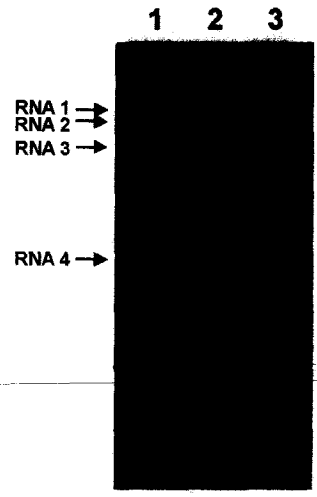

Fig. 1. Double stranded RNA (dsRNA) profiles of the CMV strains extracted from infected *Nicotiana benthamiana* plants. Electrophoresis was performed in 6% polyacrylamide gel and the gel was stained with ethidium bromide. Lane 1: dsRNA of Ly2-CMV; lane 2: dsRNA of Mf-CMV; lane 3: dsRNA of LS-CMV.

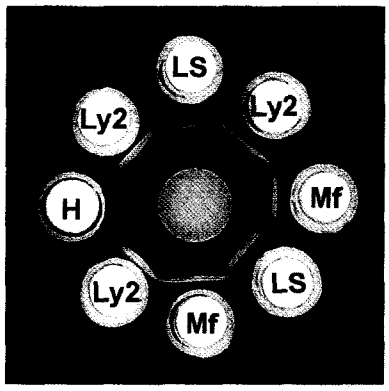

Fig. 2. Immunodiffusion test using an antiserum to Mf-CMV (central well). The peripheral wells contain crude sap from the CMV strains- infected *Nicotiana benthamiana* plants.

of Mf-CMV (Fig. 2).

Purification. Purified preparations of Ly2-CMV was infectious and appeared to be relatively free from contamination as evidenced by electron microscopic observation (data not shown). In routine purification for the Ly2-CMV, about 17-25 mg of virus could be recovered from 100 g of infected *N. benthamiana* leaves.

RT-PCR and restriction enzyme analysis. For Ly2-CMV, RT-PCR products corresponding to 3' half region of RNA 3 covering full-length CP gene amplified expected size, approximately 950 bp long (Fig. 3A). Digestion of the PCR amplified CMV cDNAs with *Msp*I or *Eco*RI produces one of two restriction patterns, which are corresponding to CMV subgroup I and II differed by serology (Rizos et al., 1992; Singh et al., 1995). The cDNA of Ly2-CMV was not digested by *Eco*RI, and this digestion pattern obtained was identical to that from Mf-CMV, which is belong to subgroup I. However, *Msp*I digestion did not show the pattern of subgroup I or subgroup II (Fig. 3B). The PCR product from Mf-CMV yielded two bands of 595 and 349 bp by *Msp*I digestion, while Ly2-CMV yielded two visible bands of 418 and 193 bp.

Sequence analysis of CP gene. In the RT-PCR products corresponding to 954 nucleotide sequences of the 3' half region of Ly2-CMV RNA 3, open reading frame (ORF) of CP gene contains 657 nucleotides (Fig. 4). A comparison with the nucleotide sequences of Mf-CMV CP gene and LS-CMV CP gene showed they share 96.2% and 76.4% identities with that of Ly2-CMV, respectively. This result indicates that sequence of Ly2-CMV CP gene is similar to that of Mf-CMV which has been reported with a subgroup I CMV (Choi et al., 1998). The nucleotide differences between CP genes of Ly2-CMV and Mf-CMV include 26 nucleotide changes in 657 nucleotides. The putative amino

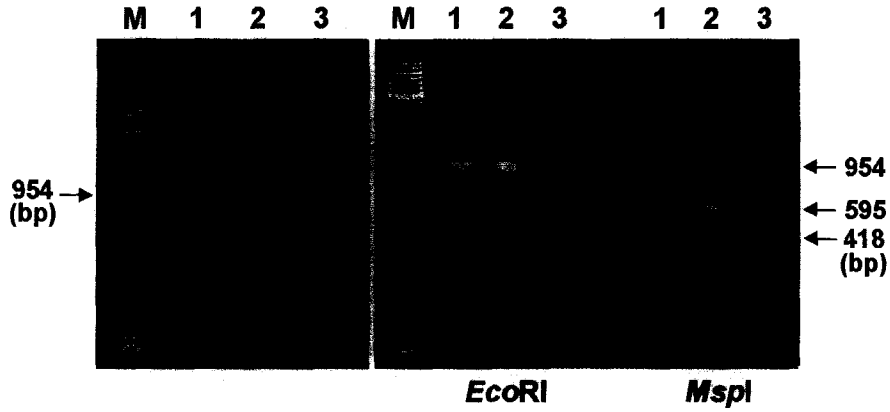

Fig. 3. Reverse transcription-polymerase chain reaction (RT-PCR) assay (A) and restriction analysis of the PCR products (B) for the 3' half region of RNA 3 covering full-length coat protein gene from the CMV strains. Electrophoresis was performed in 1.2% agarose gel and the gel was stained with ethidium bromide. Lane 1: Ly2-CMV; lane 2: Mf-CMV; lane 3: LS-CMV; lane M: 1kb DNA ladder as a size marker.

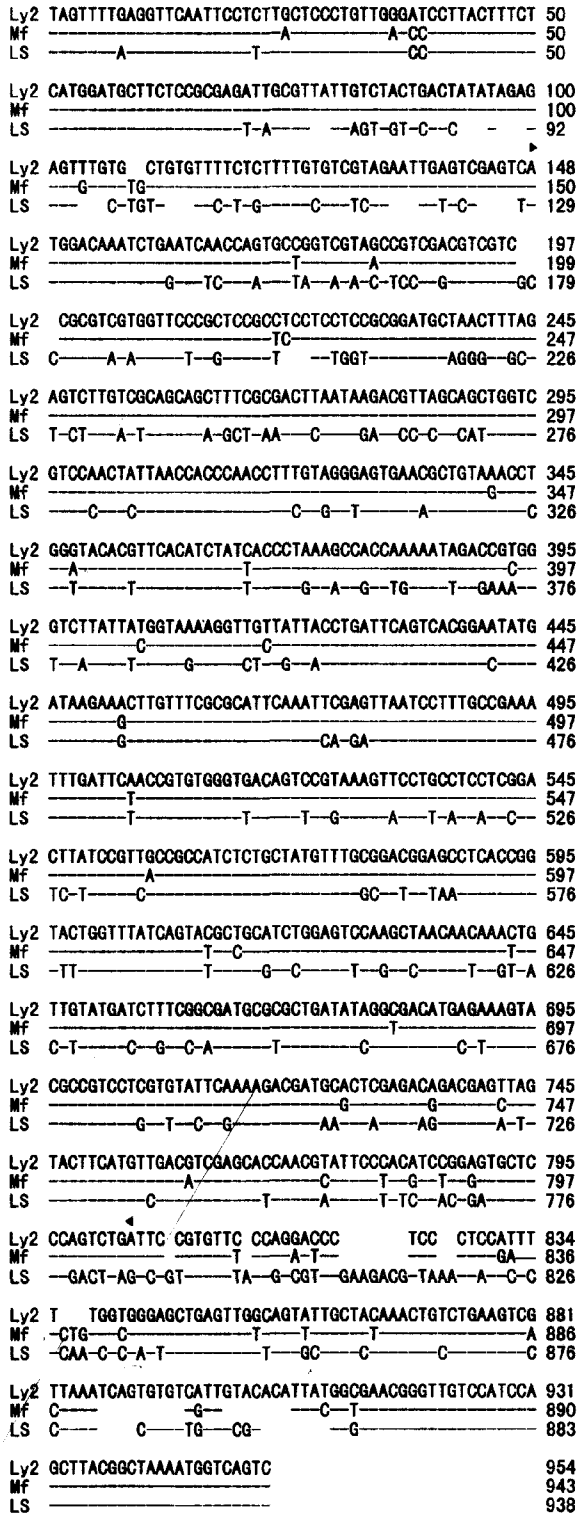

Fig. 4. Nucleotide sequences of the 3' half region of Ly2-CMV RNA 3 containing the coat protein gene. The differences between the nucleotide sequences of Ly2-CMV, Mf-CMV (GenBank accession no. AJ276481; Choi et al., 2000) and LS-CMV (GenBank accession no. AF127976; Roossinck et al., 1999) are indicated; dashed lines indicate an identical sequence. Codons of start and stop of the coat protein gene are presented as arrow heads.

acid sequence of CP is presented in Fig. 5. It is apparent that there also is a high degree of conservation between their putative gene products in Ly2-CMV and Mf-CMV, with five amino acid changes in the 218 amino acids of the CPs.

Discussion

Based on reactions in immunodiffusion test, CMV strains can be separated into two serotypes, DTL and ToRS, which are equivalent to subgroups I and II by some molecular properties (Owen and Palukaitis, 1988). Furthermore, sequence analysis of a representative strain from each subgroup verified the designations, and recent analysis of the CP genes and/or 5' non-translated region (5'NTR) of RNA 3 of several subgroup I CMV strains suggested that they can be further divided into two groups, IA and IB (Chaumpluk et al., 1996; Roossinck, et al., 1999). In this paper, we reported the isolation of a new strain of CMV, Ly2-CMV, from diseased easter lily in Korea. The virus had some of the main biological, serological and molecular properties characteristic of subgroup IA CMV. However, the host range and symptoms of Ly2-CMV differ in some respects from those reported for other IA CMVs. Ly2-CMV induced systemic mosaic in *N. benthamiana* only but did not induce systemic symptoms in several host plants for CMV diagnosis examined such as *N. tabacum* cvs. Xanthi nc and Burley 21 and *D. stramonium*. Thus, considerable interests of Ly2-CMV are the differences between the host reactions to the well-known CMV strains, and some of the differences in host range and symptoms may reflect differences in the intrinsic properties of the Ly2-CMV.

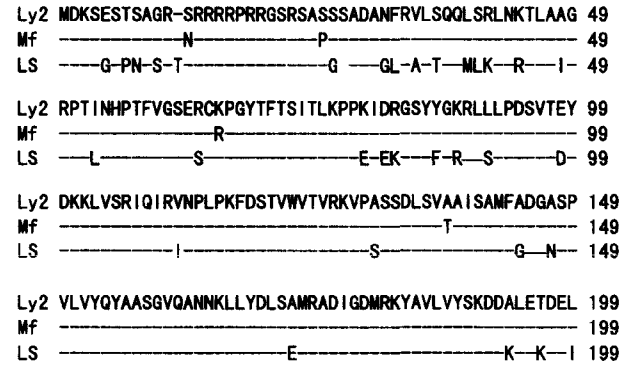

Fig. 5. Alignment of the putative amino acid sequences of the coat proteins of Ly2-CMV, Mf-CMV (GenBank accession no. AJ276481; Choi et al., 2000) and LS-CMV (GenBank accession no. AF127976; Roossinck et al., 1999). Identical sequences are indicated by dashed lines.

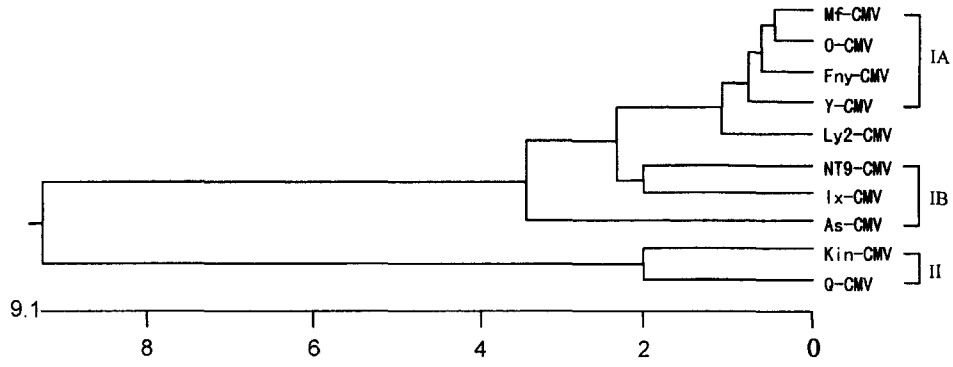

Fig. 6. Phylogenetic estimation of the CMV strains based on the coat protein gene, derived from 100 bootstrap replicates, using the heuristic search method of neighbour-joining.

Restriction analysis of PCR products of CP gene and 3' terminal region of the RNA 3 provides a simple routine method to determine the CMV subgroup present (Singh et al., 1995). When the cDNA of Ly2-CMV CP gene was digested with *Eco*RI, the fragment pattern obtained was identical to that from Mf-CMV, which is belong to subgroup I, but *Msp*I digestion did not show the subgroup I profile (Fig. 3B). The nucleotide differences between CP genes of Ly2-CMV and Mf-CMV include 26 nucleotide changes in 657 nucleotides. The putative amino acids of the respective CP of Ly2-CMV and Mf-CMV show a similar sequences, with between five changes in 218 amino acids. However, in an estimation of phylogenetic relationships of the CP with several CMV strains well characterized, Ly2-CMV formed a slightly distinct cluster from the subgroup IA CMVs (Fig. 6).

The major function of the CP is believed to be to protect the viral RNA within the infected cell and to permit transmission of the viral genome from plant to plant, either mechanically or by vectors. On the other hand, for most plant viruses CP is required for systemic movement of the virus from leaf-to-leaf through the vascular system of the plant (Carrington et al., 1996). In the case of CMV, the coat protein is required for local movement (Suzuki et al., 1991) and also for systemic movement (Taliansky and Garcia-Arenal, 1995), and specific regions of the CP also have been delimited that affect systemic infection by CMV (Shintaku and Palukaitis, 1990). Previously, Ryu et al. (1998) have been reported that a host range determinant for infection of maize by CMV strains Fny and M are due to two amino acid substitution at positions 129 and 162 of the CP. Interestingly, the Ly2-CMV and Mf-CMV in this work both have in Pro at position 129 and in Ala at position 162 of the CP, which are involved in the maize-infecting Fny-CMV strain, but Ly2-CMV did not infect maize plants. In addition, five amino acid substitution of Ly2-CMV at positions 12 (Asn → Ser), 25 (Pro → Ser), 65 (Arg → Lys), 137 (Thr → Ala) and 205 (Ile → Val) between the Mf-CMV are detected at the CPs. We do not know, however, whether these amino acid changes in CP are related with their different phenotypes in several test plants. Moreover, it also is not known whether any of the Ly2-CMV shows variation in host range that could be attributed to the putative function of the CP, i.e. promoting the long distance movement of the virus. Further detail molecular characteristics such as pseudorecombinat analyses or full-length genomic RNA sequences will be required in order to define different pathological characteristics of the Ly2-CMV.

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