Variation in the Pathogenicity of Lily Isolates of Cucumber mosaic virus

Jin-A Lee1, Seung Kook Choi2, Ju Yeon Yoon1, Jin Sung Hong3, Ki Hyun Ryu3, Sang Yong Lee1 and Jang Kyung Choi1

1Department of Applied Biology, Kangwon National University, Chunchon 200-701, Korea
2National Institute of Agricultural Biotechnology, RDA, Siwon 441-707, Korea
3Division of Environment & Life Sciences, Seoul Women's University, Seoul 139-774, Korea
4Department of Forest Resources Protection, Kangwon National University, Chunchon 200-701, Korea

(Received on August 6, 2007; Accepted on October 6, 2007)

Two isolates of Cucumber mosaic virus (CMV) originated from lily plants, named Ly2-CMV and Ly8-CMV, were compared with their pathological features in several host plants. Ly2-CMV and Ly8-CMV could induce systemic mosaic symptom in Nicotiana benthamiana, but Ly2-CMV could not symptomically infect tomato and cucumber plants that have been used for CMV-propagative hosts. While Fny-CMV used as a control infected symptomically the same host plants, producing typical CMV symptoms. Ly8-CMV could infect symptomically two species of tobacco (N. tabacum cv. Xanthi-nc and N. glutinosa) and zucchini squash (Cucurbita pepo), but Ly2 failed systemic infection on these plants. As resulted from tissue-print immunoblot assay, different kinetics of systemic movement between Ly2-CMV and Ly8-CMV were crucial for systemic infection in tobacco (cv. Xanthi-nc). Sequence analysis of full-length genome of two lily isolates showed Ly2 and Ly8 belonged to subgroup IA of CMV. The lily isolates shared overall 98% sequence identity in their genomes. Coat protein, 3a protein, and 2b protein involved in virus movement was highly conserved in genomes of the isolates Ly2 and Ly8. Although there is the low frequency of recombinants and reassortants in natural CMV population, phylogenetic analysis of each viral protein among a number of CMV isolates suggested that genetic variation in a defined population of CMV lily isolates was stochastically produced.

Keywords: Cucumber mosaic virus, host reaction, sequence analysis, systemic symptom, virus pathogenicity

Genetic variations of plant viruses, including mutation, play an important role in virus evolution in nature. Genetic analysis of plant virus families have been showed various degrees of genetic exchange can occur by recombination of genome regions that are swapped between nucleotide strands, or by reassortment of complete genome segments in viruses with segmented genomes (Domingo et al., 1996, 1997; Roossinck, 1997). These genetic exchange or mutations generated naturally in the replication process of its genomes resulted in novel genetic combinations that could acquire important phenotypic effects (García-Arenal et al., 2001; Gibbs et al., 1997). Such phenotypic effects are associated with host range expansion, with host switches, or with increased pathogenicity, resulting in epidemic of viral disease on crops.

Cucumber mosaic virus (CMV), genus Cucumovirus, family Bromoviridae, is a positive-sense RNA plant virus with a tripartite genome (Palukaitis and Garcia-Arenal, 2003; Palukaitis et al., 1992). CMV has an extremely broad host range (approximately 1,000 species). By serological data, nucleic acid hybridization, peptide mapping and sequence analysis of the coat proteins (CP) of CMV strains can be divided into three subgroups, designated IA, IB and II (Roossinck et al., 1999). RNAs 1 and 2 encode the nonstructural proteins involved in viral replication. Another open reading frame (ORF), the 2b ORF, that has been studied extensively, is also encoded on RNA 2. RNA 3 encodes a 3a protein (movement protein, MP) and the CP, which is translated from a subgenomic RNA 4.

Numerous strains of CMV have been characterized in aspects of symptomatology and host ranges (Choi et al., 2005; Huppert et al., 2002; Palukaitis and Garcia-Arenal, 2003; Palukaitis et al., 1992; Shimata and Palukaitis, 1992; Suzuki et al., 1995; Szilassy et al., 1999; Takeshita et al., 2001). One of major pathogenicity determinants in phenotypes of such CMV strains has been implicated in virus movement and symptom severity (Choi et al., 2005; Gal-On et al., 1994; Takeshita et al., 2001; Zhang et al., 1994). Although several isolates of CMV from lily plants has been reported to cause systemic infection in lily plants (Choi et al., 2004; Hagita et al., 1989; Jung et al., 2000; Ryu et al., 2002), most CMV lily isolates seemed to have a narrow host range which they could infect only a small number of host plants, unlike typical CMV isolates. These unique
biological properties of lily isolates are speculated that the lily isolates have been evolutionarily adapted to lily plants (Jung et al., 2000; Masuta et al., 2002). In this report, we examined pathological properties of two CMV lily isolates on several species of host plants and further analyzed kinetics of systemic infection between Ly2-CMV and Ly8-CMV on tobacco as well. We also determined full-length genome sequences of the two CMV isolates and analyzed multiple alignment and phylogenetic tree with a representative strain of CMV. These results suggested that lily isolates of CMV have consisted of a unique pathological population in nature.

**Materials and Methods**

**Virus isolates.** Two lily isolates were used in this study investigation. Briefly, Ly2-CMV isolated from easter lily (*Lilium longiflorum*) plant was described previously (Jung et al., 2000). Ly8-CMV was originated from a different field-grown *L. longiflorum* that had been shown yellow mosaic symptom at Chunchon, Korea in 2001 (Fig. 1). Fny-CMV was used as a control (Rizzo and Palukaitis, 1990). Each isolate was inoculated onto *N. benthamiana* by mechanical inoculation in 10 mM phosphate buffer (pH 7.0) containing 0.01 M sodium diethylthiocarbamate. The inoculated tobacco plants were grown in a greenhouse at 20-30°C. For host range test, five or more plants of each species or cultivar of host plants were inoculated mechanically with sap from each CMV isolate, respectively. The inoculum was prepared by grinding Ly2-CMV or Ly8-CMV infected leaves of *N. benthamiana* in a mortar with a pestle in 10 mM phosphate buffer. The inoculated plants were observed symptom development for 4 weeks, and then the plants tested were repeatedly back-inoculated to *N. benthamiana* in order to determine whether the virus was present, or whether there were any changes in pathological properties of isolates Ly2 and Ly8.

**RT-PCR analysis.** Total RNA was extracted from inoculated and healthy plants, as described previously (Choi et al., 1998). PCR fragment containing the CP gene of CMV was amplified using one-tube RT-PCR mixture kit (Promega) with Cucumovirus-specific primers as described by Choi et al. (1999). Briefly, total RNAs purified from infected plants or mock-inoculated plants were denatured at 70°C for 5 min and were added to the RT-PCR mixture. The conditions of RT-PCR is as follows: one cycle of RT reaction at 42°C for 45 min, 30 cycles of PCR amplification using the programmed steps (94°C, 30 sec; 40°C, 1 min; 72°C, 1 min), and one cycle of final extension at 72°C for 10 min. Ten microliters of the amplified DNA fragments were separated by electrophoresis on a 1.2% agarose gel in 1×TAE buffer and stained with ethidium bromide.

**Tissue-printing immunoblot assay.** The inoculated and upper leaves of tobacco plants were pressed onto nitrocellulose membranes (Schleicher and Schuell) using a roller. The membranes were blocked with 1× TBSB [20 mM Tris-HCl (pH 7.5), 150 mM NaCl and 3% BSA] for 30 min. Subsequently, the membrane was incubated with a solution of primary polyclonal antibody against CMV (Agdia) that was diluted 1:2000 in 1× TBSB buffer for 2 hr. Then, the membrane was washed three times with 1× TBSB buffer and incubated with anti-rabbit IgG-alkaline phophatase (Roche) diluted 1:1000 in 1× TBSB for 60 min. Membranes were further washed three times with 1× TBSB, and then incubated with a solution of NBT/BCIP for color development, according to manufacturer’s instruction (Roche).

**Cloning of full-length cDNAs and DNA sequencing.** The cDNA clones of RNA1, RNA2 and RNA3 of CMV lily isolates were synthesized with proper primer sets specific to viral genomic RNAs using *Taq* DNA polymerase, as described by McGarvey et al. (1995). The information of primer sequences will be available on request. The cDNA inserts were analyzed by digestion with restriction enzymes. To determine nucleotide sequences of cDNAs, recombinant cDNAs of the CMV isolates with restriction enzymes were subcloned into appropriate plasmid vectors digested with the same enzymes. Sequences of full-length cDNA of the isolates were determined by the dideoxynucleotide chain termination method (Sanger et al., 1977) using big-dye terminator kit (version 3.0) and automatic DNA sequencer (Model 377, ABI). Assembled nucleotide sequences and the deduced amino acid sequences were analyzed using the DNASTAR software package (Madison, USA).

**Results**

**Characterization of pathological properties of CMV lily isolates.** Ly2-CMV and Ly8-CMV induced similar mosaic
Table 1. Reaction from indicator plants inoculated mechanically with Ly2-CMV, Ly8-CMV and Fny-CMV

<table>
<thead>
<tr>
<th>Host plants</th>
<th>Symptom* (RT-PCR detection)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ly2</td>
</tr>
<tr>
<td><em>Nicotiana benthamiana</em></td>
<td>SL/M</td>
</tr>
<tr>
<td><em>N. glutinosa</em></td>
<td>--/ --</td>
</tr>
<tr>
<td><em>N. tabacum</em> cv. Xanthi-nc</td>
<td>NR/ --</td>
</tr>
<tr>
<td><em>Lycopersicon esculentum</em></td>
<td>--/ --</td>
</tr>
<tr>
<td><em>Cucumis sativus</em></td>
<td>--/ --</td>
</tr>
<tr>
<td><em>Chenopodium amaranticolor</em></td>
<td>SL/ --</td>
</tr>
<tr>
<td><em>Cucurbita pepo</em> cv. Black Beauty</td>
<td>--/ (--)</td>
</tr>
<tr>
<td><em>Vigna unguiculata</em></td>
<td>L/ --</td>
</tr>
</tbody>
</table>

*Abbreviations: M, mosaic; NR, necrotic ringspots; L, large size of necrotic local legions; sL, small size of necrotic local legions; SL, symptomless and --, uninfected.

*Inoculated leaves/systemic leaves.

Based on RT-PCR analysis, positive reaction (+) and negative reaction (--) were indicated in parenthesis.

and yellow-streak symptoms in easter lily plants which were their natural hosts (Fig. 1). To characterize further pathological properties between the two lily isolates, two isolates purified were mechanically inoculated onto 8 species of indicator plants. For direct comparison of their pathological properties, CMV strain Fny (Fny-CMV) that has been well studied was used as a control. Host range and symptoms of Ly2-CMV and Ly8-CMV were summarized in Table 1. Although the difference of induction time and severity of symptoms was observed somehow, two lily isolates showed systemic infection on *N. benthamiana*, and local infection on cowpea and *C. amaranticolor*, similar to Fny-CMV. However, Ly2 and Ly8 did not infect systemically tomato and cucumber that are natural CMV hosts, compared to Fny. Interestingly, Ly8-CMV induced mild mosaic symptom on un-inoculated upper leaves of *N. glutinosa* and zucchini squash (*Cucurbita pepo*) and ringspot symptom on both inoculated and systemic leaves of *N. tabacum* cv. Xanthi-nc, respectively (Table 1 and Fig. 2). Ly2-CMV showed successful local infections on inoculated leaves of Xanthi-nc, but failed to produce systemic symptoms on these plants.

To determine systemic symptom productions in tested plants were caused by Ly8-CMV, Ly8-CMV detection was analyzed by RT-PCR using Cucumovirus-specific primers, as described previously (Choi et al., 1999). Result from RT-PCR analysis clearly demonstrated the detection of genomic RNA of Ly8-CMV in symptom-induced leaf tissues of the tobacco and squash plants (data not shown). As expected, any RT-PCR product from Ly2-CMV was not detected in the systemic leaves of plants inoculated. These results confirmed systemic infection of Ly8-CMV on tobacco and zucchini squash.

To know whether mutations on the genome of Ly8-CMV or of Ly2-CMV was generated, Ly8-inoculum from systemically infected tobacco leaves or Ly2-inoculum from locally infected tobacco leaves was back-inoculated onto *N. benthamiana* as well as several host plants. We observed indistinguishable pathogenicity of Ly8-CMV and Ly2-

![Fig. 2. Symptoms of host plants infected with Ly2-CMV (A, C, E) or Ly8-CMV (B, D, F). Symptom on inoculated leaf (a) and symptom on un-inoculated upper leaf (b) were indicated at the bottom of photos.](image-url)
CMV on the basis of host responses. Additionally, the analysis of the CP genes from the back-inoculated plants showed no sequence changes (data not shown). Although two lily isolates showed less infectivity than Fny-CMV, the pathogenicity of Ly8-CMV is likely to be higher than that of Ly2-CMV on the basis of infectivity data. These results suggested that pathologic diversity had been existed in a population of lily isolates of CMV, affecting that the lily isolates retained a distinct niche in CMV population.

Analysis of systemic infection between Ly2-CMV and Ly8-CMV. As demonstrated above, Ly8-CMV was able to infect systemically tobacco and squash plants, while Ly2-CMV could not.

To determine whether the different infectivity between Ly2-CMV and Ly8-CMV affected rate of local and systemic movements in those plants, we chose tobacco (cv. Xanthi-nc) plants that showed distinguishable systemic symptom. Sap from N. benthamiana infected by Ly2 or Ly8 was inoculated onto young leaves of tobacco. Inoculated leaves (5 dpi) or systemic leaves (10dpi) were detached and blotted onto nitrocellulose membrane by physical press, respectively. The membrane blotted with inoculated tobacco leaves showed there were little difference of local movement between Ly8 and Ly2 (data not shown). However, it was clearly shown that systemic spreading of Ly8-CMV was detected on throughout systemic leaves of tobacco along with veins, suggesting that Ly8-CMV was extensive-ly accumulated in systemic leaves of the tobacco. While, accumulation of Ly2-CMV on systemic tobacco leaves was not detectable until 14-20 dpi (Fig. 3 and data not shown). It is coincident with RT-PCR result which confirmed the systemic infection of Ly8-CMV in tobacco. It is possible that host factors involved in a resistance that was specific to prevention of long-distance movement of Ly2-CMV, but not Ly8-CMV. In absence of specific proteins involved in such resistance, it came from virus in itself devoid of the ability of systemic infection.

Sequence analysis of Ly2-CMV and Ly8-CMV. To know whether the alterations of genomic sequences determined the rate of systemic invasion also affected movement of the corresponding viruses, genome sequences of Ly2-CMV and Ly8-CMV were determined by dye-termination protocol, as standard protocol (Sambrook et al., 1989). The determined nucleotide sequences were assembled by using DNASTAR program. The genome sequence of Fny-CMV was used as a control for sequence comparisons. Overall, the sequence identity (approx. 97%) of genome between Ly2 and Ly8 was higher than that of Fny-Ly2 or of Fny-Ly8 (Table 2). The isolates Ly8 and Ly2 belonged to CMV subgroup IA, according to CP analysis (data not shown). There was high sequence identity ranged from 96 to 98% in 1a protein or 2a protein among lily isolates and Fny strain (Fig. 4). Many variation of aa was found in the central region of 1a protein and the N-terminal region of 2a protein. Although we could not find any specific amino acids/sequences conferring unique phenotypes of Ly2-CMV and Ly8-CMV, the amino acids of 3a protein, CP and 2b protein responsible for virus movement and resistance breakage were higher conservation than 1a and 2a proteins (Fig. 4).

Discussion

CMV is one of the commonest plant viruses in many plant species, including pepper, tobacco, cucurbits, ornamentals, vegetables, and legumes, in Korea. In this paper, we charac-terized biological properties and genome comparison of two lily isolates of CMV. Different isolates of CMV from
lily species have been previously reported and characterized (Chen et al., 2001; Choi et al., 2004; Jung et al., 2000; Masuta et al., 2002; Ryu et al., 2002). In general, most CMV lily isolates showed different host reactions from CMV isolates, which they induced mild systemic symptoms in many indicator plants. Furthermore, CMV lily isolates had a relatively narrow host range, compared to other CMV strains. There were reported that most CMV isolates collected from various plants infected well many species of tobacco and cucurbit plants that have been used for CMV propagation as good hosts (Palukaitis et al., 1992). Although it was reported that CMV isolate Li (Li-CMV) was able to infect tobacco (cv. Xanthi-nc) systemically (Ryu et al., 2002), many CMV lily isolates failed systemic infection, or failed even local infection in many species of tobacco and cucurbit plants. Similarly, pathogenicity of Ly8-CMV was dramatically different from that of Ly2-CMV in tobacco and zucchini squash. We demonstrated that both viruses could infect locally Xanthi-nc plant, but systemic infection of Ly8-CMV was only observed (Table 1 and Fig. 2). Systemic symptom of tobacco induced by Ly8-CMV was distinguished from that of tobacco induced by Fny-CMV (necrotic ringspot vs. mosaic). Furthermore, we clearly showed that Ly2 failed local and systemic infections on N. glutinosa, but Ly8 successfully infected the same plants (Table 1 and Fig. 2). Interestingly, Li-CMV which was able to infect Xanthi-nc plant systemically failed even local infection of N. glutinosa (Ryu et al., 2002).

Although isolates Ly2 and Ly8 used in this study shared high sequence identity each other or high sequence identity with Fny that is able to infect tobacco. It is plausible that some amino acids of 1a protein and 2a protein are more important for the difference of phenotypes between Ly2 and Ly8 than 2b, 3a protein and CP.

Since a pseudorecombinant between Fny-CMV and LKCMV (designated F1P2L3-CMV) could move systemically in Xanthi-nc (S.K. Choi, unpublished data) and changed its systemic symptom on zucchini squash (Choi et
Fig. 4. Multiple alignment analysis of coding proteins among Ly2-CMV, Ly8-CMV and Fny-CMV. The dash bar means that the same amino acids residue. The aa position was indicated on the left side of sequence panel.
2b protein

3a protein

coat protein

Fig. 4. Continued.

al., 2004). In case of Fny-Sny, additionally, RNA1 controlled induction time of systemic symptoms in zucchini squash (Roossinck and Palukaitis, 1990). In radish and zucchini squash, RNA2 itself or RNA2 and RNA3 was involved in viral systemic spread in radish plant (Choi et al., 2003, 2005; Takeshita et al., 1998). Results from pseudo-recombinant and chimeric viruses between Y-CMV and HL-CMV demonstrated that RNA1 of HL-CMV was a determinant in systemic infection of lily plants (Yamaguchi et al., 2005).

Regarding 2a protein, the mutation of amino acid (aa) 631 and 641 of Fny 2a protein allowed host reactions from hypersensitive reaction to systemic mosaic symptom in cowpea (Kim and Palukaitis, 1997). Similarly, previous studies with Pf-CMV had high sequence identity (approx. 98%) with Fny-CMV have demonstrated that the alteration of aa 267 of 2a affected their phenotypes in zucchini squash (Choi et al., 2005). Further delimitation of genomic sequences responsible for pathogenicity determination is to be remained.

In view sides of host plant, tomato and cucumber appeared to have high resistance against isolates Ly2 and Ly8, but not strain Fny. Although we did not have an obvious evidence, it is assumed that this resistance was involved in a block in cell-to-cell movement of Ly2-CMV and Ly8-CMV, because any CP genes from tomato and cucumber inoculated were not detected by RT-PCR. As shown similarly the case of extreme resistance for M-CMV (subgroup IA), RNA3 of M-CMV that share high sequence identity (99%) with RNA3 of Fny-CMV failed to infect maize (Ryu et al., 1998). Otherwise, another possible hypothesis is that genomic RNAs of lily isolates by itself are not able to replicate efficiently in single cell of tomato or cucumber.

Detail explanation for this resistance mechanism specific to CMV lily isolates is to be remained.

According to this resistance concept, we observed that one tobacco species (cv. Xanthi-nc) had resistance specific to Ly2-CMV, but not to Ly8-CMV. Leaf-press blot assay clearly showed the resistance specific to Ly2 is associated with prevention of uploading into phloem tissues (Fig. 3). The CP of CMV plays extensive roles in long-distance movement between different tissue types (Blackman et al., 1998; Taliansky and Garcia-Arenal, 1995). So far, the understanding of systemic movement of viruses on the phloem is limited, both regarding necessity of plant factors and the viral structures that move through sieve tubes (Scholthof, 2005; Wagemann et al., 2004).

Complete nucleotide sequence data would be sufficient to establish relationships between strains. Small changes in nucleotide sequence could give very different phenotypic affects. Substantial variations of CMV lily isolates may occur by exchanges between parts of a multi-partite genome.
during viral replication under selection pressure. As a result, the adapted CMV strain has different host ranges that often produce different disease symptoms on some common host (Roossinck, 1997). In these views, systematic study of symptoms for the fitness of several host species or varieties under standard conditions may help considerably delineate strains among large numbers of field isolates of plant viruses, such as CMV, Tobacco mosaic virus, Potato virus Y and Alfalfa mosaic virus (J.K. Choi, unpublished data).

Clearly, data of this study and previous studies show that infections of host plants as well as many other cultivars included in this study, depend on CMV isolates, which in turn indicates biological variation between CMV isolates.

Acknowledgements

This study was supported by a Research Grant (2006) from Kangwon National University.

References


74:5463-5467.