Plant Pathol. J. 20(3) : 212-219 (2004)

Symptom Determinant as RNA3 of Lily Isolates of *Cucumber mosaic virus* on Zucchini Squash

Seung Kook Choi[1], Hong Il Ahn[1], Minjea Kim[2], Jang Kyung Choi[3] and Ki Hyun Ryu[2]*

[1]Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea
[2]Plant Virus GenBank, PVGABC, Division of Life and Environmental Sciences, Seoul Womens University, Seoul 139-774, Korea
[3]Division of Biological Environment, Kangwon National University, Chunchon 200-701, Korea

(Received on June 20, 2004; Accepted on August 11, 2004)

Three isolates of *Cucumber mosaic virus* (CMV) from lily plants showing mosaic and distortion symptoms were detected by reverse-transcriptase polymerase chain reaction (RT-PCR) using primers specific to *Cucumovirus* genus namely, LK-CMV, LK4-CMV, and LK5-CMV. Restriction enzymes patterns of the RT-PCR products revealed that the lily isolates belonged to subgroup IA of CMV. In terms of biological properties, the lily isolates have highly similar but distinct pathogenicity as reported in other lily strains and ordinary strains of CMV. To characterize the molecular properties, cDNAs containing coat protein (CP) gene and 3' non-coding region (NCR) of RNA3 for the isolates were cloned and their nucleotide sequences were determined. The CP similarity (218 amino acids) was highly homologous (>97%) with that of subgroup I CMV strains. However, an additional 20-nulcleotide long segment was only present in 3' NCR of lily isolates, which form an additional stem-loop RNA structure. By using chimeric construct exchange cDNA containing 3'NCR of LK-CMV into the full-length cDNA clone of RNA3 of Fny-CMV, this additional segment may prove to be significant in the identification and fitness of the virus in lily plants. The pathology of zucchini squash infected by F1F2L3-CMV, a pseudorecombinant virus was showed to change drastically the severe mosaic and stunting symptom into a mild chlorotic spot on systemic leave, compared with Fny-CMV. To delimit the sequence of RNA3 affected the pathology, various RNA3 chimeras were constructed between two strains of CMV. The symptom determinants of F1F2L3-CMV were mapped to the positions amino acid 234, 239, and 250 in 3a movement protein (MP). RNA3 chimeras changed the sequences encoding three amino acids were resulted in alteration of systemic symptom.

Keywords **:** *Cucumber mosaic virus*, evolution; lily, pathogenicity, symptom determinant

*Corresponding author.
Phone) +82-2-970-5618, FAX) +82-2-970-5610
E-mail) ryu@swu.ac.kr

Cucumber mosaic virus (CMV), a type species of the genus *Cucumovirus* in the family *Bromoviridae*, is known to have the largest host range among a number of plant viruses, causing damage to more than 1,000 species of plants in 365 genera of 85 families (Palukaitis et al., 1992). CMV causes disease in a wide variety of economically important crops, such as ornamentals, worldwide because of its extensive host range and mode of transmission (Palukaitis et al., 1992; Tomlinson, 1987). In particular, CMV poses serious damage to cultivated lilies singly or in combination with other viruses, such as *Lily symptomless virus* (LSV) and *Lily latent virus* (LiLV), in Korea and in other countries as well (Loebenstein et al., 1995; Ryu et al., 2000; Ryu et al., 2002).

CMV contains a tripartite genome of messenger-sense single-stranded RNAs, designated RNA1, RNA2 and RNA3 in order of decreasing sizes, those are encapsidated in isometric particles of about 28 nm in diameter (Peden and Symons, 1973). The RNAs1 and 2, which encode 1a and 2a proteins, respectively, are required for replication. A 2b protein, a new overlapping small protein, is most likely expressed through subgenomic RNA4A (Ding et al., 1994) and is involved in the long-distance movement of the virus, as well as, in suppressing RNA silencing (Ding et al., 1995; Li et al., 2002). Di-cistronic RNA3 encodes a movement protein shown to be involved in cell-to-cell movement and a coat protein expressed from subgenomic RNA4 (Palukaitis et al., 1992). The 3'- terminal 270-nucleotide long CMV RNA is highly conserved, with only 7-10% sequence differences throughout this non-coding region (NCR) between any two RNAs (Itaya et al., 2002). These sequences form a tRNA-like structure that provides the initial binding site for viral RNA polymerases, which are necessary to synthesize (−) RNA strand (Boccard and Baulcombe, 1993). The precise mechanism that regulates the level of RNA accumulation has yet to be identified, but the sequences of the 3 NCR could be involved (Duggal et al., 1992).

To date, a few reports on lily isolates of CMV have shown that most of these isolates were quite different in terms of host reactions by various indicator plants from that of

typical CMV strains, e.g., some lily isolates only re-infect lily plants (Chen et al., 2001; Choi et al., 2003b; Jung et al., 2000; Ryu et al., 2002). In this paper we report the biological and molecular properties of CMV isolates from each lily source, compared with that of the typical CMV strains.

Materials and Methods

Virus source and host range test. Three lily plants, *Lilium* Oriental hybrid cv. Marcopolo, *Lilium* cv. Casa Blanca, and *Lilium lancitoium*, showing severe mosaic and distortion symptoms were used for isolation of viruses. Detection of CMV from these plants was performed by RT-PCR with *Cucumovirus*-genus specific primers (CPTALL-3/-5) (Choi et al., 1999). Fny-CMV was obtained from the Plant Virus GenBank (Seoul, Korea) and used as a control. Three lily isolates of CMV (LK-CMV from *Lilium* Oriental hybrid cv. Marcopolo, LK4-CMV from *Lilium* cv. Casa Blanca, and LK5-CMV from *Lilium lancitoium*) were biologically isolated from the plants and maintained in *Nicotiana benthamiana* by mechanical inoculation. Eleven plants were used for the host range test of each virus as listed in Table 1. The plants inoculated with the viruses were grown in a greenhouse at 26°C and symptom assessment was conducted for 4 weeks.

Virus purification and analysis of CP and 3'NCR. Virion particles of lily isolates of CMV were purified from systemically infected leaves of *N. benthamiana* according to the Choi et al. (1999).

RT-PCR for LK-, LK4-, and LK5-CMV was performed for molecular characterization by the combination of a forward primer (CPTALL-5) (Choi et al., 1999) and a reverse primer (5'-GATC**CTGCAG**TGGTCTCCTTTTRGAGGCC-3') with *Pst*I site (in bold) and 19 nts (designated as CMV3ALL) or 10 nts (named as 3ALL) identical to those at 3' end of Fny-CMV using Titan one-tube RT-PCR system according to the manufacturers instruction (Roche) (Choi et al., 2002a; Yoon et al., 2002). The synthesized RT-PCR product was directly cloned to pGEM-T easy vector (Promega), generating pLK-3NCR, pLK4-3NCR, and pLK5-3NCR, respectively. Using these clones, nucleotide sequencing of CP and 3'NCR was determined by dye-termination-PCR based method (Sanger et al., 1977). The determined nucleotides and deduced amino acids of CP and 3'NCR for lily isolates of CMV were analyzed with the type-strain, Fny-CMV (subgroup IA), As-CMV (subgroup IB), Q-CMV (subgroup II), and other previously reported Korean (Ly2-CMV and Li-CMV) and foreign lily strains (LiCK3-, LiNB-, and LiTW-CMV (Chen et al., 2001) by MagAlign CLUSTAL V in DNASTAR package (Madison, US) and Genetic Computer Group sequence analysis software.

Construction and pathogenicity assay of chimeric RNA3. To characterize biological property of LK-CMV in molecular level, the full-length cDNAs of LK-CMV RNA3 was constructed from purified viral RNA by previously described (Choi et al., 2002b; Rizzo and Palukaitis, 1990). Briefly, the cDNA for RNA3 of LK-CMV was synthesized at 42°C for 60 min using First-Strand synthesis kit (BRL) in a 50 μL reaction mixture containing 100 ng of purified viral RNA with CMV3ALL primer (5'-AATT-**CTGCAG**TGGTCTCCTTTTRGAGGCC-3') containing *Pst*I site (in bold) described above. For PCR, the oligonucleotide (5'-AATC**CTGCAG**TAATACGACTCACTATAGTAATCTTACCAC TGTGTGT-3' with *Bam*HI (in bold), T7 RNA polymerase promoter (underline), and 20 nt identical to those at 5' end of Fny RNA3 were used with reverse primer. The PCR condition was as follow: 94°C, 2 min (1 cycle); 94°C, 20 s, 55°C, 30 s, 68°C, 3 min (5 cycles); 94°C, 20 s, 57°C, 30 s, 68°C, 3 min (25 cycles); 68°C, 20 min (1 cycle).

Table 1. Reactions of indicator plants infected by lily isolates of *Cucumber mosaic virus*

Indicator plant	LK-CMV	LK4-CMV	LK5-CMV
Nicotiana benthamiana	–/M[a]	–/M	–/M
N. clevelandii	–/mM	–/CS	–/CS
N. tabacum cv. Samsun	N/–	N/–	N/–
Samsun NN	N/–	N/–	N/–
Xanthi-nc	N/–	N/–	N/–
N. glutinosa	–/–	–/–	–/–
Cucumis sativus cv. Baekdadaki	–/–	–/–	–/–
Cucumis melo	–/–	–/–	–/–
Cucurbita pepo cv. BlackBeauty	+/–	+/–	+/–
Sacheol	+/–	+/–	+/–
Vigna unguiculata	L/–	L/–	L/–

[a]Inoculated leaf/systemic leaf. CS = chlorotic spot; L = local legion; mM = mild mosaic; M = mosaic; N = necrotic ringspot; – = no symptom.

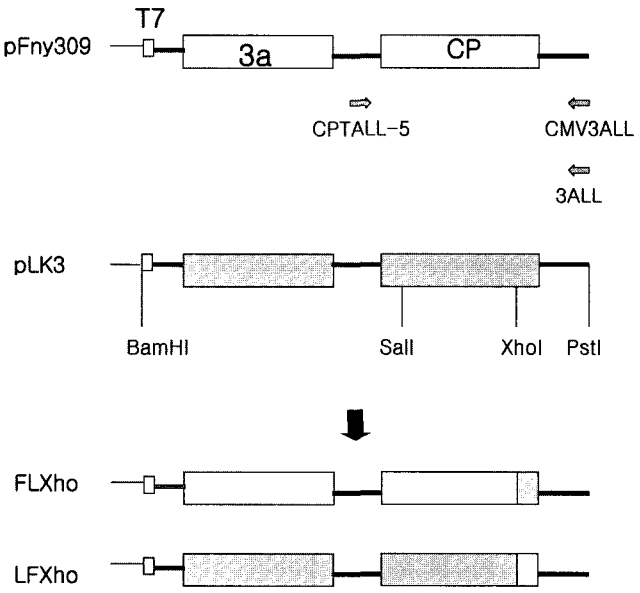

Fig. 1. Schematic representation of chimeric 3'NCR cDNA between LK-CMV and Fny-CMV. Fny-RNA3 and LK-RNA3 sequences are represented by open and closed rectangles. The conserved restriction enzyme sites are indicated by the line.

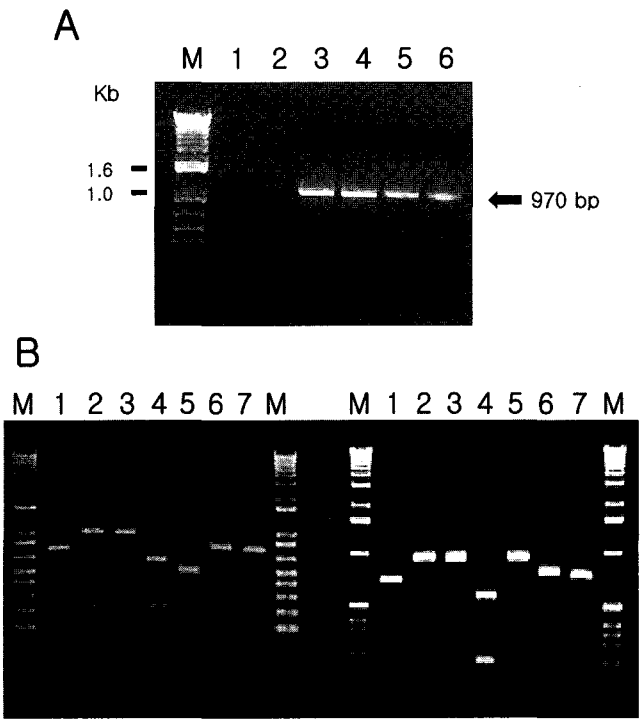

Fig. 2. Detection of *Cucumber mosaic virus* from lily and analysis of RT-PCR. (**A**) gel electrophoresis of RT-PCR product by *Cucumovirus*-genus specific primers. Lane M, 1 Kb plus DNA ladder (BRL); 1, LSV; 2, *Tomato aspermy virus* (KC strain); 3. *Lilium oriental hybrid* cv. Marcopolo; 4, *Lilium oriental hybrid* cv. Casa Blanca; 5, *Lilium lancitoium*; 6. Fny-CMV; (**B**) Analysis of synthesized RT-PCR from Fny-CMV (left) and LK-CMV (right) using restriction enzymes. Lane M, DNA size marker (BRL); 1, *Ava*I; 2, *Eco*RI; 3, *Eco*RV; 4, *Hinc*II; 5, *Hind* III; 6, *Sal*I; 7, *Xho*I.

The synthesized RT-PCR product of LK RNA3 was digested by *Bam*HI-*Pst*I enzymes and then purified by QUIAquick kit (QUIAGEN) prior to be cloned into pUC18 digested with the same restriction endnucleases, resulting in pLK3. Nucleotide sequences (nts) for 5'NCR, 3a gene and intercistronic region of LK-CMV RNA3 were determined using pLK3 clone by standard protocol (Sambrook et al., 1989) and compiled with the nts of CP gene and 3'NCR determined previously.

To construct chimeric cDNA of 3' NCR between LK-CMV and Fny-CMV, the first pair of reciprocal chimeras was constructed using *Pst*I-enzyme site located at downstream primer and the *Xho*I site that is in 3' terminal region of CP gene between pLK3 and pFny309. The fragments digested by *Xho*I-*Pst*I restriction endonucleases were purified from agarose gel using QIAquick gel extraction kit (Quiagen) following the manufacturer's instruction. The second pair of reciprocal chimeras was constructed using the common *Bam*HI site located at upstream of the T7 promoter and the *Sal*I site which is in CP gene. The third pair of chimeras was constructed by exchanging a fragment using the above *Bam*HI site and *Nhe*I site that is in the 3a gene (Fig. 2). The fourth pair of chimera was synthesized by using *Nhe*I site at the 3a gene and *Sal*I site at the CP gene. *In vitro* transcripts were generated from these RNA3 cDNA constructs; FLXho and LFXho (the *Xho*I reciprocals), FLSal and LFSal (the *Sal*I reciprocals), FLNhe, and LFNhe (the *Nhe*I reciprocals) and FLNhe/Sal, LFNhe/Sal (*Nhe*I-*Sal*I reciprocals).

These chimeric clones generated from pLK3 and pFny309 were linearized at the 3' end of the inserted sequence using *Pst*I, blunt-ended, and was used as template. Full-length capped transcripts of chimeric RNA3 from each chimeric clone were obtained by standard *in vitro* transcription system with T7 RNA polymerase (Promega) in the presence of cap analog m^7GpppG (New England Biolabs), and mixed with transcripts of RNA1 and 2 of Fny-CMV in equal volume to prepare inoculum at a concentration of 200-500 ng/μL. Young tobacco plants were inoculated with 15 μL per leaf dusted with Carborundum powder as described by Canto and Palukaitis [3] prior to inoculation on the cotyledons of zucchini squash (*Cucurbita pepo* cv. Black Beauty) plants.

Results and Discussions

Identification of causal viruses and host reactions. To identify the CMV isolates from three diseased lily plants, RT-PCR was performed using *Cucumovirus*-genus specific primers. Approximately 970 base-pair (bp) fragments were synthesized from the total RNAs of the lily samples (Fig. 2A). To determine their subgroups, RT-PCR products containing CP gene of CMV were assayed by restriction fragment length polymorphism (RFLP) using seven restriction enzymes. Lily isolates seemed to belong to subgroup IA (S-IA) by PCR-RFLP analysis (Fig. 2B), showing characteristics similar to that of Fny-CMV, a representative strain of S-IA. RFLP analysis of each isolate showed identical pattern, indicating that nucleotide sequences of their genomic RNAs were highly conserved, in spite of different isolation hosts.

For the host range test, all lily isolates were mechanically inoculated to indicator plants from systemic infected *N. benthamiana*. Systemic infections were observed in *N. benthamiana* and *N. clevelandii*, while local lesion was found in *Vigna unguiculata*. There were no infections in tobacco plants (*N. tabacum* cv. Samsun, *N. tabacum* cv. Samsun NN, and *N. tabacum* cv. Xanti-nc), *N. glutinosa*, *Cucumis sativus* cv. Baekdadaki, *Cucumis melo*, *Cucurbita pepo* cv. Black Beauty, and *Cucurbita pepo* cv. Sacheol (Table 1). Host reactions to the three isolates of CMV were identical, and all the isolates have highly similar biological properties (Table 1). Interestingly, results of their biological analysis were similar to Ly2-CMV and Li-CMV (Jung et al., 2000; Ryu et al., 2002). However, the pathology of LK-CMV is different from those of two CMV lily isolates, that is, LK-CMV can not infect tobacco plant (cv. Xanthi-nc) but Li-CMV is able to infect this host plant systemically (Ryu et al., 2002). Similarly, Ly2 strain is not even detected on inoculated cotyledons as well as systemic leaves in

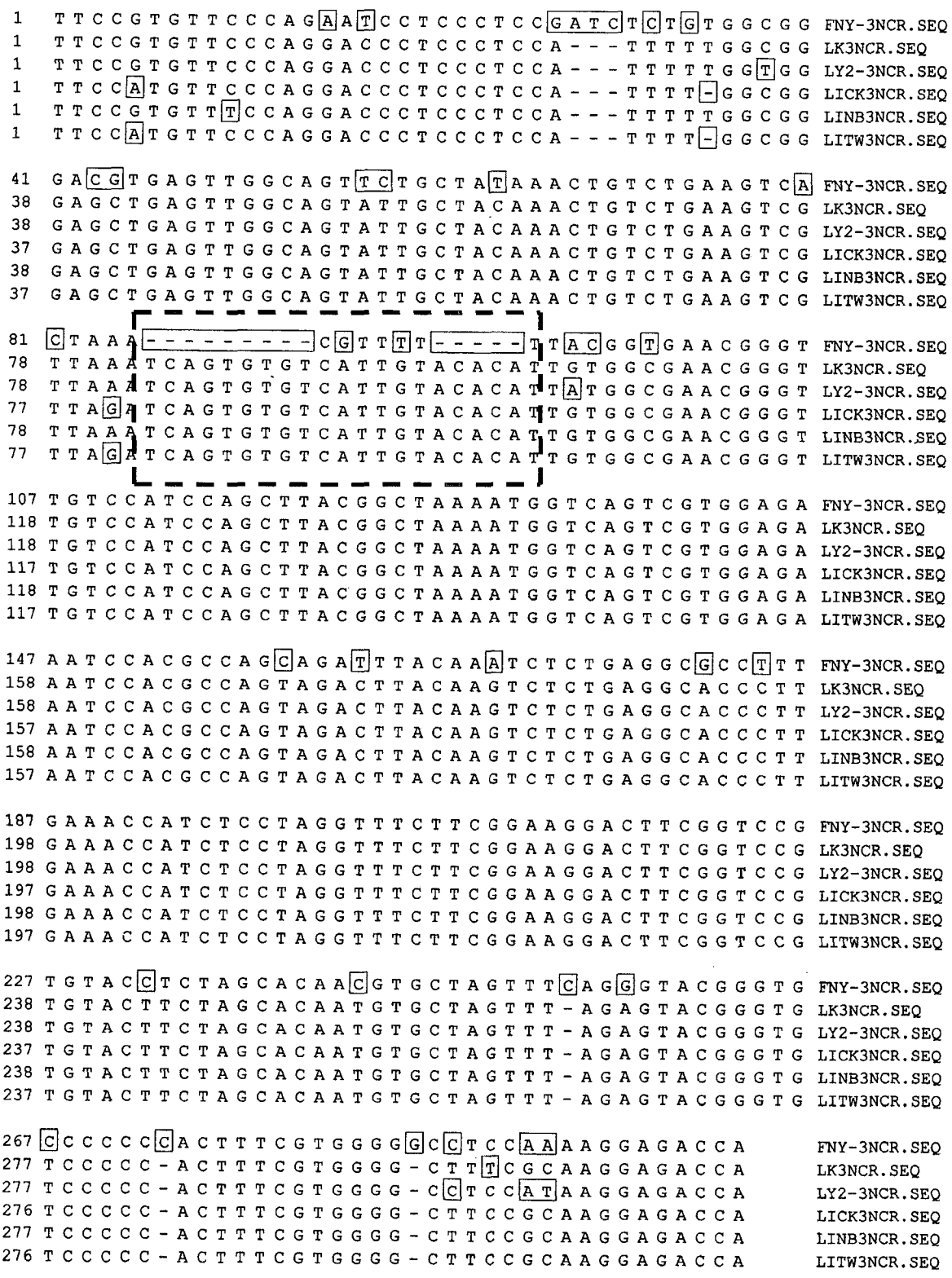

Fig. 3. Sequence alignment of 3'NCR of RNA3 between lily isolates and Fny strain of CMV. A box represents the additional 20 nts forming a loop-stem structure only found in the lily isolates of CMV. Bolded line indicates the conserved sequences in lily isolates, which be predicted a stem-loop structure by *mfold* program (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi). The RNAs 3 of CMV strains used in the study are as follow: Fny [28, D10538], LK [in this study, AJ495841), Ly2 [19, AJ296154], LiCK [6, AJ131616], LiNB [6, AJ131618] and LiTW [6, AJ131619].

squash plant (Jung et al., 2000). However, LK-CMV is able to replicate on the inoculated and move to adjacent cells (called cell-to-cell movement), although inhibition of systemic movement for LK-CMV causes blockage of systemic infection in squash plant. Additionally, HL-CMV from Japanese lily plant has been partially characterized, and its pathology resembles with our lily isolates (Masuta et al., 2002). It appeared that the highly conserved sequences of genomic RNA for lily isolates of CMV containing very similar biological properties were due to vegetative propagation of lily bulbs or host specificity (Carrere et al., 1999; Chen et al., 2001; Wong et al., 1999). Hence, it can be noted that lily isolates of CMV could not infect some common systemic hosts, such as tobacco and squash, unlike most subgroup-IA strains.

Sequence analysis of CP and 3'NCR of lily isolates of CMV. To characterize the lily isolates in the molecular level, RT-PCR covering CP gene and 3'NCR was carried out in lily isolates of CMV from purified RNA, using CPTALL-5 and 3ALL primer (Fig. 1; Materials and Methods). The expected RT-PCR product was synthesized (data not presented) and directly cloned into pGEM-T easy vector (Promega). The selected clones of each virus were sequenced. The sequence variations of CP and 3'NCR were demonstrated with other representative strain of each subgroup. Based on the analysis of sequence alignment, there was 90-99% homology of amino acid in CP region and 74-96% similarity of nucleotide in 3'NCR. The CP of each isolate was composed of 218 amino acids like other strains, while 3'NCRs of the isolates consisted of 311 nucleotides, somewhat longer than those of other strains. The sequence analysis of CP and 3'NCR indicated that the variations in coding or non-coding region among CMV isolates might have caused the diversity within the CMV subgroups (Schneider and Roossinck, 2001). However, it can be noted that most lily isolates in Korea are highly homologous to other lily isolates of distinct geographical origin, as well as, those isolated from ornamental varieties (Fig. 3). Interestingly, multiple alignments of 3'NCR showed that all the isolates from lily contained about 20 short nucleotides conserved highly, compared to that of Fny-CMV (Fig. 3).

Since 3'NCR of CMV consisted of RNA-RNA hybrid-formation resulting to stem-loop structure similar to cellular tRNA can be formed tRNA-like structure, which is necessary for efficient replication, minus-strand initiation and synthesis by the CMV replicase (Boccard and Baulcombe, 1993; Canto and Palukaitis, 1998; Fraile *et al.*, 1997), the secondary structure of 3'NCR of Fny RNA3 and LK-CMV is estimated by computer-aid RNA analysis. A unique region consisting of 20 nucleotides found in 3'NCR of LK-CMV was and able to form an additional stem-loop structure and that the residual secondary RNA structure of 3'NCR for LK-CMV was identical with that of Fny-CMV (data not shown). Therefore, to know whether the stem-loop structure formed by the additional 20 nts of 3'NCR of LK-CMV affected biological properties, the reciprocal chimeras of 3'NCR between Fny-CMV and LK-CMV were generated from cDNA clones, transcribed and mixed with transcripts RNA1 and RNA2 derived from cDNA clones of pFny109 and pFny209, resulting in FLXho-CMV and LFXho-CMV, respectively. Then, these chimeric viruses were inoculated to tobacco plant before inoculation to cotyledons of squash plants. The systemic symptom in zucchini squash induced by FLXho-CMV was similar severity of symptom to wild-type Fny-CMV (data not shown). Moreover, viral RNAs of FLXho-CMV were accumulated in the similar amount as that of Fny-CMV in both inoculated cotyledons and systemic leaves (data not shown). This sequence was still maintained in 3'NCR of chimeric construct during infection by direct-sequencing of RT-PCR product. These results indicated that the inability of lily isolates to cause symptoms in tobacco and some cucurbitaceous plants may be responsible for the encoded viral proteins rather than the RNA structure or sequence itself within tRNA-like structure found in 3'NCR of LK-CMV. However, it is interesting to note that this additional sequence of 20 nt inserted in the 3'NCR of RNA3 may be significant in host adaptation by recombination event leading to mutant viruses. Recently, Masuta et al. (2002) reported the natural recombinants of lily CMVs between S-IA and S-IB based on the sequence analysis of RNA3 of geographically isolated origins. They also suggested the lily isolates of CMV have evolved from common progenitor. In addition, RNA recombination is common for CMV, as suggested by Canto et al. (2001). They reported that generated recombinant CMV RNAs in transgenic tobacco expressed full-length RNA1 of CMV. The recombination events of CMV RNAs were observed in restricted 3'NCR regions by the combination of RNA1 and 3, or RNA1 and RNA2, but not RNA2 and RNA3. Nonetheless, recombinant viruses seldom occur frequently in nature, mainly on reduced fitness (Fraile et al., 1997). Based on the sequence analysis of 3'NCR on both S-I and S-II strains, only the lily isolates contained this additional sequence in 3'NCR. In alstroemeria, various sizes of nucleotide sequences that originated from RNA2 and RNA3 were inserted into 3'NCR of CMV by recombination (Chen et al., 2002). In tobacco plants, only non-recombinant sequences were generated, but the outcome was opposite in alstromeria, which suggested that these recombined RNA2 and 3 segments increased the biological fitness of CMV in alstromeria. Thus, the 20 nts segment found in 3'NCR of CMV lily isolates may be an adaptation product generated to escape the defense responses in the host plants, such as

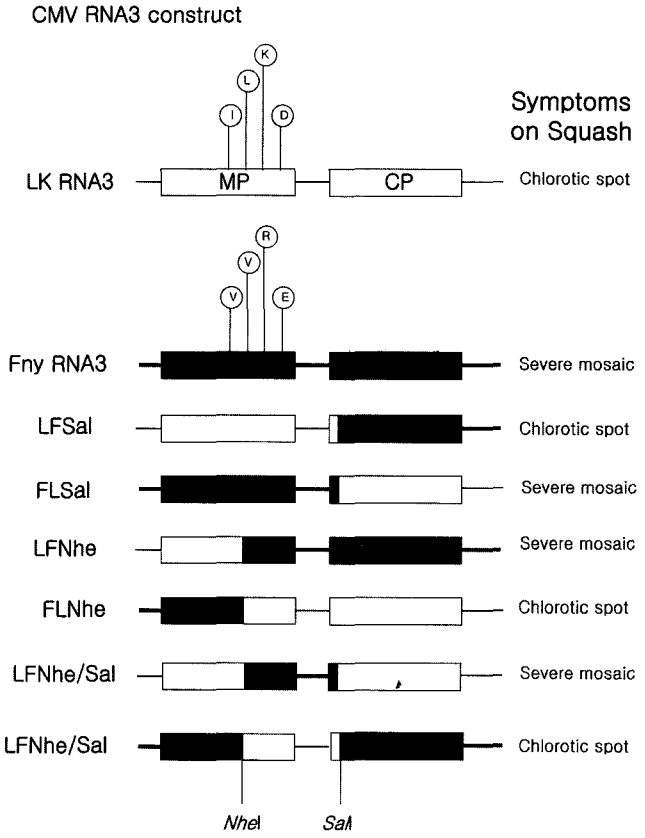

Fig. 4. Localization of sequences in CMV RNA3 specifying the phenotypes of CMV strains on zucchini squash. The transcripts derived from cDNA clones of RNA3 clones containing various RNA3 chimeras were mixed with RNA1 and RNA2 transcripts of Fny-CMV and were inoculated to tobacco plants. Virus from infected tobacco was inoculated to squash cotyledons. The systemic symptoms were checked 7 dpi. RNA3 transcripts were derived from the following plasmids to generate the corresponding viruses (in parentheses): pLK-CMV3 (LK-CMV); pFny309 (Fny-CMV); pLFSal (LFSal-CMV); pFL3Sal (FLSal-CMV); pLFNhe (LFNhe-CMV); pFLNhe (FLNhe-CMV); pLFNhe/Sal (LFNhe/Sal-CMV); pFLNhe/Sal (FLNhe/Sal-CMV). LK-CMV RNA3 sequences are indicated by open rectangles and Fny-CMV RNA3 sequences are indicated by filled rectangles. The amino acids at 3a protein positions 168, 234, 239 and 250 are indicated.

RNA silencing. Interestingly, the other chimeric LFXho-CMV is able to infect squash plants systemically and induce different symptom on upper leaves from Fny-CMV. Thus, we analyzed for this phenomenon using various RNA3 chimeras in detail.

Pathogenicity test of chimeric RNA3. Some reports suggest that viral proteins encoded by RNA3 of CMV are severity and determinant symptom in various hosts (Li et al., 2001; Palukaitis et al., 1992; Zhang et al., 1994). To reveal viral determinant for systemic symptom of squash plants generated by LFXho-CMV, we once synthesized RNA3 transcript of LK-CMV with RNA1 and 2 transcripts

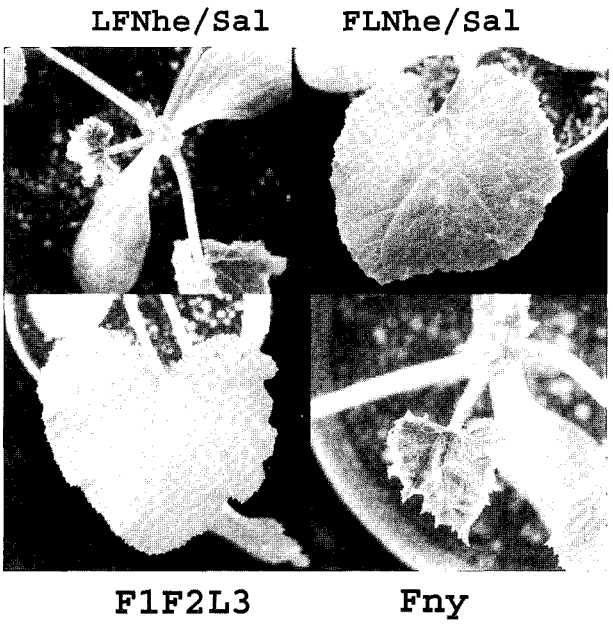

Fig. 5. Systemic symptoms on zucchini squash inoculated with CMVs containing RNA3 chimeras between LK-CMV and Fny-CMV. The used virus strains are indicated the lower or the upper hand of each segment.

of Fny-CMV using each full-length cDNA clones, resulting in F1F2L3-CMV, which inoculated to zucchini squash. Interestingly, a pseudorecombinant F1F2L3-CMV could infect on squash plants systemically, similar to LFXho-CMV. But wild-type LK-CMV was unable to infect squash plant systemically (Fig. 5). Moreover, the severity of systemic symptoms induced by F1F2L3-CMV in squash plants were drastically changed severe mosaic into chlorotic spot, compared to symptom produced by Fny-CMV (Fig. 5). These results indicate that pathogenicity determinant(s) of LK-CMV is not located on RNA3, but on RNA1/2 or on both RNAs in squash. However, we want to delimit which sequences within RNA3 of LK-CMV determine the induction of a chlorotic spot symptom on systemic leaves of zucchini squash.

We created a series of reciprocal chimeras between the cDNA clones of LK-CMV RNA3 and Fny-CMV RNA3 (Fig. 4). A pair of reciprocal chimeras was constructed using the common *Sal*I site, 1297 nucleotides from 5' end of LK-CMV RNA3, and 1293 nucleotides from 5' end of Fny-CMV RNA3. In vitro transcripts of these chimeras (LFSal and FLSal) were combined with the transcripts of Fny RNA1 and RNA2, and then inoculated onto zucchini squash. All of them induced a severe mosaic and stunt symptom on the systemic leaves like Fny-CMV. It indicated that the symptom determinant was likely to locate in MP. Thus, we constructed a second pair of chimeras by using the common *Nhe*I site in 3a gene, 641 nucleotides from 5'

end of LK-CMV RNA3, and 641 nucleotides from 5' end of Fny-CMV RNA3, in order to determine whether the 5'NCR or the 3a gene contained the sequences eliciting the chlorotic spot symptom. In vitro transcripts were generated from these reciprocal RNA3 cDNA chimeras, LFNhe and FLNhe, separately mixed together with transcripts of Fny RNA1 and 2, and then inoculated onto zucchini squash. The systemic symptoms of zucchini squash infected by either of these chimeras were indistinguishable from those induced by Fny-CMV, indicating that the sequenced of symptom determinant were located between *Nhe*I site and *Sal*I site of RNA3. Therefore, FLNhe/Sal and LFNhe/Sal, a third pairs of chimeras, were constructed by exchanging fragments of between *Nhe*I site of 3a gene and *Sal*I site of CP (there is no sequence variations in the CP gene upstream of the *Sal*I site.) Squash plants inoculated with viruses containing RNA3 derived from between the *Nhe*I and *Sal*I sites of pLK3 and pFny309 were showed that the zucchini squash plants infected by virus containing chimeric RNA3 of FLNhe/Sal-CMV were induced mild chlorotic symptom that was indistinguishable from those induced by F1F2L3-CMV (Fig. 5). Similarly, LFNhe/Sal-CMV, chimeric virus that contained a cDNA fragment of Fny RNA3 induced severe symptoms on zucchini squash that were indistinguishable from those induced by Fny strain. RT-PCR products representing chimeric RNA3 cDNA clones were synthesized from total RNA extracted from the infected plants and the genotype of each of chimeras was confirmed by sequencing. There were only three amino acids differences of amino acids encoded between the *Nhe*I-*Sal*I sites of LK- and Fny-CMV, at position 234, 239, and 250 (Fig. 4). Therefore, the amino acids at position 234, 239, and 250 in the 3a protein of LK-CMV RNA3 are important for determining symptoms of F1F2L3- and F1F2F3-CMV on zucchini squash.

Mutations at one or more of four positions within MP gene of *Brome mosaic virus* resulted in modification of symptom and compatibility with a new host (De Jong et al., 1995; Fujita et al., 1996). In zucchini squash, it has been demonstrated previously that the 3a sequences of Sny-CMV engendered reduced virus movement in squash cotyledons (Kaplan et al., 1997), a restriction of systemic infection and in some cases, a hypersensitive response. In addition, these sequences encoding at position amino acid 51 and amino acid 240 in 3a gene induced the high accumulation of the 3a protein and enhanced distribution of the virus in tobacco (Gal-On et al., 1996). These results suggested the sequences encoding the two amino acid determinants at position 51 and 240 of 3a gene for Sny strain affected indirectly or directly play a role in virus-host interaction(s) implicated in host range. Recently, Choi et al. (2003a) reported that RNA2 was involved for systemic infection of lily isolate of CMV, and this was responsible for the poor efficiency of virus movement.

The sequences containing the amino acid 234 in MP are necessary for CMV movement consisting of RNA binding domain (positions 209-236) and the sequences containing the amino acid 239 and 250 in MP are dispensable for CMV movement (positions 237-279) (Kaplan et al., 1995; Li et al., 2001; Zhang et al., 1994). Therefore, the sequences encoding amino acid 234 may be responsible for the alteration of symptom in zucchini squash, via modification of MP function(s) to promote the ability of RNA binding or entry into vasculature for systemic infection (Itaya et al., 2002; Li et al., 2001). A model has been proposed for the movement of CMV RNA into sieve elements (Blackman et al., 1998). This model suggested that the association with RNA-MP was critical for cell-to-cell movement with the interaction of CP. Therefore, the alteration of sequences in MP, such as nucleic acid biding domains, seems to affect the cell-to-cell movement, which may produce the slow and mild symptom expression in zucchini squash.

It is of interest that how virus movement is involved in pathogenicity connected with 3a MP to facilitate CMV systemic symptom.

Acknowledgments

This study was supported in part by the grant of Korea-Japan Joint Project (F01-2002-000-20001-0) from the Korea Science & Engineering Foundation in Korea to JKC, in part by the grant from the Agricultural R & D Promotion Center, the Ministry of Agriculture & Forestry in 2003 to KHR, and in part by the grant of BioGreen21 project from the Rural Development of Administration in Korea to KHR.

References

Blackman, L.M., Boevink, P., Santa Cruz, S., Palukaitis, P. and Oparka, K.J. 1998. The movement protein of cucumber mosaic virus traffics into sieve elements in minor veins of *Nicotiana clevelandii*. *Plant Cell* 10:525-533.

Boccard, F. and Baulcombe, D. 1993. Mutational analysis of cis-acting sequences and gene function in RNA3 of cucumber mosaic virus. *Virology* 93:563-578.

Canto, T., Choi S.K. and Palukaitis, P. 2001. A subpopulation of cucumber mosaic virus RNA1 contains 3' termini originating from RNAs 2 or 3. *J. Gen. Virol.* 82:941-945.

Canto, T. and Palukaitis, P. 1998. Transgenically expressed cucumber mosaic virus RNA1 simultaneously components replication of cucumber mosaic viruses RNA2 and 3 and confers resistance to systemic infection. *Virology* 250:325-336.

Carrere, I., Tepfer, M. and Jacquemond, M. 1999. Recombinants of cucumber mosaic virus (CMV): determinants of host range

and symptomatology. *Arch. Virol.* 144:365-379.

Chen, Y.K., Derks, A.F.L.M., Langeveld, S., Goldbach, R. and Prins, M. 2001. High sequence conservation among cucumber mosaic virus isolates from lily. *Arch. Virol.* 146:1631-1636.

Chen, Y.K., Goldbach, R. and Prins, M. 2002. Inter- and Intramolecular recombinations in the cucumber mosaic virus genome related to adaptation to alstroemeria. *J. Virol.* 76:4119-4124.

Choi, S.K., Choi, J.K., Park, W.M. and Ryu, K.H. 1999. RT-PCR detection and identification of three species of cucumoviruses with a single pair of primers. *J. Virol. Methods* 83:67-73.

Choi, S.K., Choi, J.K. and Ryu, K.H. 2003a. Involvement of RNA2 for systemic infection of *Cucumber mosaic virus* isolated from lily on zucchini squash. *Virus Res.* 97:1-6.

Choi, S.K., Choi, S.H., Yoon, J.Y., Choi, J.K. and Ryu, K.H. 2003b. A subpopulation of RNA3 of *Cucumber mosaic virus* quasispecies. Plant Pathol. J. 19:210-216.

Choi, S.K., Yoon, J.Y., Ryu, K.H., Choi, J.K. and Park, W.M. 2002a. First report of zucchini yellow mosaic virus on hollyhock (*Althaea rosea*). *Plant Pathol. J.* 18:121-125.

Choi, S.K., Choi, J.K., Ryu, K.H. and Park, W.M. 2002b. Generation of infectious cDNA clones of a Korean strain of tomato aspermy virus. *Mol. Cells* 13:52-60.

De Jong, W., Chou, A. and Alquist, P. 1995. Coding changes in the 3a cell-to-cell movement gene can extend the host range of brome mosaic virus systemic infection. *Virology* 214:464-474.

Ding, S.W., Anderson, B.J., Haase, H.R. and Symons, R.H. 1994. New overlapping gene encoded by the cucumber mosaic virus genome. *Virology* 198:593-601.

Ding, S.W., Li, W.X. and Symons, R.H. 1995. A noble naturally occurring hybrid gene encoded by a plant RNA virus facilitates long distance virus movement. *EMBO J.* 14:5762-5772.

Duggal, R., Rao, A.L. and Hall, T.C. 1992. Unique nucleotide differences in the conserved 3' termini of brome mosaic virus RNAs are maintained through their optimization of genome replication. *Virology* 187:261-270.

Fraile, A., Alonso-Prados, J.L., Aranda, M.A., Bernal, J.J., Malpica, J.M. and Garcia-Arenal, F. 1997. Genetic exchange by recombination or reassortment is infrequent in natural population of a tripartite RNA plant virus. *J. Virol.* 71:934-940.

Fujita, Y., Mise, K., Okuno, T., Ahlquist, P. and Furusawa, I. 1996. A single codon change in a conserved motif of a bromovirus movement protein gene confers compatibility with a new host. *Virology* 223:283-291.

Gal-On, A., Kaplan, I.B. and Palukaitis, P. 1996. Characterization of cucumber mosaic virus II. Identification of movement protein sequences that influence its accumulation and systemic infection in tobacco. *Virology* 226:354-361.

Itaya, A., Ma, F., Qi, Y., Matsuda, Y., Zhu, Y., Kiang, G. and Ding, B. 2002. Plasmodesmata-mediated selective protein traffic between "symplasmically isolated" cells probed by a viral movement protein. *Plant Cell* 14:2071-2083.

Jung, H.J., Ueda, S., Ryu, K.H., Lee, S.Y. and Choi, J.K. 2000. A novel strain of cucumber mosaic virus isolated from Lilium longiflorum. *Plant Pathol. J.* 16:306-311.

Kaplan, I.B., Shintaku, M.H.., Li, Q., Zhang, L., Marsh, L.E. and Palukaitis, P. 1995. Complementation of virus movement in transgenic tobacco expressing the cucumber mosaic virus 3a gene. *Virology* 209:188-199.

Kaplan, I.B., Gal-On, A. and Palukaitis, P. 1997. Characterization of cucumber mosaic virus: III. Localization of sequence in the movement protein controlling systemic infection in cucurbits. *Virology* 230:343-349.

Li, H., Li, W.X. and Ding, S.W. 2002. Induction and suppression of RNA silencing by an animal virus. *Science* 296:1319-1321.

Li, Q., Ryu, K.H. and Palukaitis, P. 2001. Cucumber mosaic virus-plant interactions: Identification of 3a protein sequences affecting infectivity, cell-to-cell movement, and long-distance movement. *Mol. Plant-Microbe Interact.* 14:378-385.

Loebenstein, G., Lawson, R.H. and Brunt, A.A. 1995. Virus and virus-like diseases of bulb and flower crops. John Wiley & Sons, United Kingdom.

Masuta, C., Seshimo, Y., Mukohara, M., Jung, H.J., Ueda, S., Ryu, K.H. and Choi, J.K. 2002. Evolutionary characterization of two lily isolates of Cucumber mosaic virus isolated in Japan and Korea. *J. Gen. Plant Pathol.* 68:163-168.

Palukaitis, P., Roossinck, M.J., Dietzgen, R.G.. and Francki, R.I.B. 1992. Cucumber mosaic virus. *Adv. Virus Res.* 41:281-348.

Peden, K.W.C. and Symons, R.H. 1973. Cucumber mosaic virus contains a functionally divided genome. *Virology* 53:487-492.

Rizzo, T.M. and Palukaitis, P. 1990. Construction of full-length cDNA clones of cucumber mosaic virus RNAs 1, 2, and 3: generation of infectious RNA transcripts. *Mol. Gen. Genet.* 222:249-256.

Ryu, J.H., Park, H.W., Park, W.M., Lee, S.Y. and Ryu, K.H. 2000. Molecular analysis of the 3-terminal region of Lily latent carlavirus from *Lilium lancitoium*. *Plant Pathol. J.* 16:231-235.

Ryu, K.H., Park, H.W. and Choi, J.K. 2002. Characterization and sequence analysis of a lily isolate of cucumber mosaic virus from *Lilium tsingtauense*. *Plant Pathol. J.* 18:85-92.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, New York.

Sanger, F., Nicklen, S. and Coulson, A.R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.

Schneider, W.L. and Roossinck, M.J. 2001. Genetic diversity in RNA virus quasispecies is controlled by host-virus interactions. *J. Virol.* 75:6566-6571.

Tomlinson, J.A. 1987. Epidemiology and control of virus disease of vegetables. *Ann. Appl. Biol.* 110:661-681.

Wong, S.M., Thio, S.S.C., Shintaku, M.H. and Palukaitis, P. 1999. The rate of cell-to-cell movement in squash of cucumber mosaic virus is affected by sequences of the capsid protein. *Mol. Plant-Microbe Interat.* 12:628-632.

Yoon, J.Y., Min, B.E., Choi, J.K. and Ryu, K.H. 2002. Genome structure and production of biologically active in vitro transcripts of cucurbits-infecting Zucchini green mottle mosaic virus. *Phytopathology* 92:156-163.

Zhang, L., Handa, K. and Palukaitis, P. 1994. Mapping local and systemic symptom determinants of cucumber mosaic cucumovirus in tobacco. *J. Gen. Virol.* 75:3185-3195.