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Analysis of Symptom Determinant of *Cucumber mosaic virus* RNA3 *via* Pseudorecombinant Virus in Zucchini Squash

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Isolates of *Cucumber mosaic virus* (CMV) collected in Korea, were compared with their pathological features in tobacco and zucchini squash. Full-length cDNA clone of RNA3 was generated by using long-distance RT-PCR. Transcript RNA3 from the cDNA clone was inoculated onto host plants with transcripts RNA1 and RNA2 of Fny strain, generating RNA3-pseudorecombinant CMV. Timing and severity of systemic symptom was not significantly different among the pseudorecombinant CMVs in tobacco, compared with strains Fny-CMV and Pf-CMV. However, the pseudorecombinant CMVs induced two different systemic symptoms (mosaic vs. chlorotic spot) in zucchini squash. Based on symptom induction, the pseudorecombinant CMVs were categorized into two classes. The severity and timing of symptoms were correlated with viral RNA accumulations in systemic leaves of zucchini squash, suggesting that different kinetics of virus movement associated with CMV proteins are crucial for systemic infection and symptom development in zucchini squash. The analysis of movement proteins (MP) of CMV strains showed high sequence homology, but the differences of several amino acids were found in the C-terminal region between Class-I-CMV and Class-II-CMV. The analysis of coat proteins (CP) showed that the CMV isolates tested belonged to CMV subgroup I and the viruses shared overall 87-99% sequence identity in their genomes. Phylogenetic analysis of MP and CP suggested that biological properties of Korean CMV isolates have relationships associated with host species.

Keywords **:** *Cucumber mosaic virus*, infectious cDNA, pseudorecombinant, RT-PCR, zucchini squash

Plant viruses are responsible for severe diseases in many crops, ornamental plants, and various plants, resulting in major economical losses (Hull, 2002). Virus symptoms usually vary from local discoloration to severe perturbation of growth and development, or even death of the plant. In most relationships between virus and susceptible host, the induction of disease symptoms is likely to be the last step in colonization of plant virus in which the agent modifies the metabolism of its host at many different levels. In turn, host plant shows various responses to virus, depending upon genetic backgrounds, development stage and surrounding environment (Hull, 2002).

Cucumber mosaic virus (CMV) is a prevalent plant pathogen all over the world and has the widest host range of over 885 plant species in 65 families (Palukaitis et al., 1992). CMV, the type species of the genus *Cucumovirus*, has a tripartite genome of positive-sense single-stranded RNAs, designated as 1, 2 and 3 in order of decreasing size (Peden and Symons, 1973). RNA2 codes for the 2a protein, which is an RNA-dependent RNA polymerase of replication complex, whereas, RNA1 codes for the 1a protein, another subunit of CMV replicase complex (Hayes and Buck, 1990). RNA3 encodes for two proteins involved in viral movement and encapsidation (Canto et al., 1997; Kaplan et al., 1997).

In several genetic studies, the determinants of the various phenotypes of CMV have been mapped to one or more of genomic RNAs (Gal-On et al., 1994; Huppert et al., 2002; Rao and Francki, 1982; Palukaitis and Garcia-Arenal, 2003; Shintaku et al., 1992; Suzuki et al., 1995; Szilassy et al., 1999; Takeshita et al., 1998 and 2001). Strains of CMV usually show differences in biological properties as a manner of host-specific. For example, both Fny and M strains of CMV infect tobacco, but show differences in symptom development (mosaic versus chlorotic: Shintaku et al., 1992). In the case of the M strain of CMV, very slow movement in inoculated cotyledons of zucchini squash, due to change of two specific amino acids in the capsid protein, resulting in a lack of systemic infection (Wong et al., 1999). This observation was explained that a host-specific response that restricts to movement of M-CMV was neutralized by infection of a potyvirus, allowing systemic infection of M-

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CMV in zucchini squash (Choi et al., 2002; Rao and Francki, 1982). Similarly, there was no difference of symptom induction between the Fny and Pf strains of CMV in tobacco, although these strains showed a distinct difference of timing and intensity of symptom development in zucchini squash (Choi et al., 2005). Various CMV-encoded proteins or combinations of proteins have been implicated in local or systemic infection in host plants (Palukaitis and Garcia-Arenal, 2003; Saitoh et al., 1999; Takeshita et al., 2001). Therefore, to investigate further genetic characterization of the interactions between CMV strains and hosts leading to various symptom inductions, we analyzed the nature and genetic basis of the differences in pathogenicity associated with CMV isolates collected in Korea. For this purpose, their biological properties of the CMV isolates using infectious cDNA clones were characterized by comparisons with the better-characterized and faster-moving Fny-CMV or slower-moving Pf-CMV had been used in similar previous studies (Choi et al., 2005; Roossinck and Palukaitis, 1990).

Materials and Methods

Plants, virus source and plant maintenance. Tobacco plants (*Nicotiana tabacum* cv. Xanthi-nc) at the four-leaf stage were used for propagation of virus, as described previously (Canto et al., 1997). Korean CMV isolates, Fny-CMV (Rizzo and Palukaitis, 1990) and Pf-CMV were propagated in tobacco plants and further purified by the method of Peden and Symons (1973) for subsequent experiments (Table 1). Sap inoculum was prepared from CMV-infected plants and purified virus or in vitro transcripts generated from cDNA clones (Zhang et al., 1994). Cotyledons of zucchini squash (*Cucurbita pepo* cv. Black Beauty) were inoculated with sap from infected tobacco plants or mix of pseudorecombinant transcripts as described below. Inoculated plants were grown in controlled greenhouse conditions with the temperature at 20-26°C.

Construction of full-length cDNA clones of CMV isolates. Viral RNAs of CMV isolates were extracted from purified virus particles or infected leaf tissues using buffered SDS/phenol extraction (Choi et al., 1999; Gal-On et al., 1994). PCR for RNA3 was carried out in a 50 μl reaction volume contained 5 μl RT solution, 5 μl of 10X Expand Long Template PCR buffer (Roche Diagnostics) containing 2.5 mM $MgCl_2$, 10 ng forward primer, 10 ng reverse primer, 1 mM dNTPs, and 2.5 U Expand Long-Template Enzyme mixture (Roche) in a programmable thermal cycler (iCycler; Bio-Rad Laboratories), as previously described (Fig. 1A and Choi et al., 2003a). The synthesized full-length RT-PCR product of RNA 3 was directly digested by *Bam*HI and *Pst*I, and then the digested fragment was purified from an agarose gel using the QIAquick Gel Extraction kit (Qiagen), as the manufacturer's instructions. The purified cDNA of the CMV RNA3 was ligated into linearized pUC18 digested previously with *Pst*I and *Bam*HI. In some cases, the synthesized full-length cDNA of RNA3 was cloned directly into pGEM-T easy vector.

Infectivity test of Pseudorecombinant CMV. Each full-length cDNA of CMV RNA3 was digested with *Pst*I to produce a linear template DNA and blunted with Klenow enzyme to remove non-viral sequences at downstream of CMV 3' end. Transcripts *in vitro* were synthesized, as described previously (Choi et al., 2003a). Each of the synthesized RNA3 transcripts was mixed with transcripts of Fny-CMV RNAs 1 and 2, and then the mixed transcripts were inoculated to tobacco plants. Zucchini squash plants were mechanically inoculated with sap derived from tobacco plants infected with RNA3 pseudorecombinant virus or with the purified virions. The systemic symptom of zucchini squash was observed at different time points until 2 week post-inoculation. All experiments were performed at least three times.

Northern blot hybridization and sequence analysis. Total RNA was purified from the systemic leaf tissues of zucchini squash. The purified total RNA was subjected to analysis of Northern blot hybridization and RT-PCR amplification with *Cucumovirus*-specific primers, as previously described (Canto et al., 1997; Choi et al., 1999). Briefly, systemically infected leaves of zucchini squash were sampled by harvesting 6 leaf discs (ca. 50 mg). The sampled leaf discs were ground in 300 μl extraction buffer [50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% SDS, and 0.5% 2-mercaptoethanol]. The samples were further extracted with phenol/chloroform (25:24, v/v), and RNA was precipitated by ethanol, according to standard protocol (Sambrook et al., 1989). Total RNAs were fractioned by electrophoresis on 1.6% agarose-formaldehyde denaturing gel and transferred to positively charged nylon membrane (Roche) by capillary method. The membrane was cross-linked by UV and hybridized to DIG-labeled RNA probes complementary to the 3'-noncoding region of all Fny-CMV RNAs. Signals were detected using DIG Luminescent Detection Kit with CSPD (Roche). The full-length cDNA clone of CMV RNA3 was used for sequence determination by using proper subcloning procedures, according to a standard protocol (Sambrook et al., 1989). In some cases, RT-PCR product amplified from total RNA was directly cloned into pGEM-T easy vector (Promega). Subsequently, the cDNA of virus in the selected clones was sequenced by

using dye-termination method (Sanger et al., 1977). The deduced amino acid sequences were analyzed by using BLAST search and DNASTAR software.

Results

Analysis of pseudorecombinant CMVs. Since the proteins encoded by CMV RNA3 have been known as the crucial determinants of disease-symptom type in plants (Choi et al., 2005; Huppert et al., 2002; Palukaitis et al., 1992; Palukaitis and Garcia-Arenal, 2003; Ryu et al., 1998; Shintaku and Palukaitis, 1992; Suzuki et al., 1995; Takeshita et al., 2001), we first constructed full-length cDNA of RNA3 of CMV isolates collected from various plants in Korea (Table 1). Subsequently, the synthesized full-length cDNAs were cloned into pUC18 vector at the multi-cloning sites of *Pst*I and *Bam*HI, or directly into pGEM-T easy vector (Fig. 1A).

To examine the role of RNA3 in the determination of disease-symptom type, RNA3 transcript of each Korean CMV isolates was mixed with transcripts RNA1 and 2 of Fny strain to make RNA3-pseudorecombinant CMV (Fig. 1B). For nomenclature, each pseudorecombinant was simply symbolized by using the isolate name of CMV from which it was originated. For example, Lc indicates a pseudorecombinant CMV containing transcripts RNA1 and RNA2 of Fny strain plus transcript RNA3 of Lc strain. The reconstituted Fny-CMV and Pf-CMV were used as controls for comparing the severity and timing of symptom (Choi et al., 2005; Rizzo and Palukaitis, 1990).

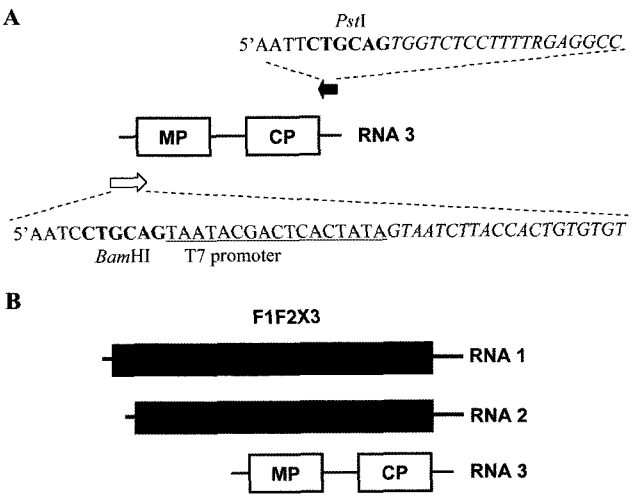

Fig. 1. Schematic diagram of construction of infectious cDNA clone of CMV RNA3 and the generation of pseudorecombinant CMV. (A) Long distance RT-PCR was used for synthesis of full-length cDNA of RNA3 with forward and reverse primers. (B) Transcripts RNA1 and RNA2 of Fny plus transcript RNA3 of each CMV isolate was mixed and inoculated to host plants. X means simple nomenclature named by using the CMV RNA3 from which it was originated.

Table 1. Symptom analysis of RNA3-pseudorecombinant of CMV in zucchini squash

	isolate[a]	Symptom[b]	Time[c]	reference
Class I	As	severe mosaic	5	Ryu et al., 1994
	Gs	severe mosaic	5	Choi et al., 1998
	Mf	severe mosaic	4	Choi et al., 1998
	Pa	severe mosaic	5	Choi et al., 1998
	Ph	severe mosaic	5	Choi et al., 1998
	Rs	severe mosaic	4	Choi et al., 1998
	Rsk1	severe mosaic	4	in this study
	Rsk2	severe mosaic	4	in this study
	Rsk3	severe mosaic	4	in this study
	Sa1	severe mosaic	5	Choi et al., 1998
	Fny	severe mosaic	3	Rizzo and Palukaitis, 1990
Class II	Ga	chlorotic spot	9	Choi et al., 1998
	Lc	chlorotic spot	9	Choi et al., 1998
	LK3	chlorotic spot	8	Choi et al., 2004
	LK4	chlorotic spot	8	Choi et al., 2003b
	Pf	chlorotic spot	9	Choi et al., 2005

[a]Pseudorecombinant CMV was generated by mix of transcripts RNA3 plus transcripts RNA1 and 2 of Fny strain.
[b]Symptom was evaluated by observations in systemic leaves of squash until 14 dpi.
[c]Timing of symptom development were determined in systemic leaves of zucchini squash by initial symptom observation.

All the pseudorecombinant CMVs induced indistinguishable green-mosaic symptoms in systemic leaves of tobacco plants (Fig. 2), so the symptom difference was not recognized among them (Fig. 2). Furthermore, the quantities of virus particles purified from the infected tobacco were similar (data not shown), suggesting that the strain classification was not possible in tobacco, according to systemic symptoms.

To differentiate symptom based on host specificity, zucchini squash was inoculated with pseudorecombinant-infected tobacco leaves. Zucchini squash has been previously demonstrated as a good differential host for CMV classification (Gal-On et al., 1994; Roossinck and Palukaitis, 1990; Palukaitis et al., 1992). Zucchini squash inoculated with pseudorecombinant CMVs developed two kinds of distinct responses. One group of the pseudorecombinant CMV isolates induced severe symptoms consisting of severe mosaic and stunt on systemic leaves initiating at 4-5 dpi, similar to Fny strains, which named Class I (Fig. 2). The other group of CMV isolates designated Class II induced mild symptom consisting of chlorotic spot on systemic leaves starting at 8-9 dpi, similar to Pf strain (Fig. 2 and Table 1). Any pseudorecombinant CMVs that could induce intermediated severity and timing were not observed

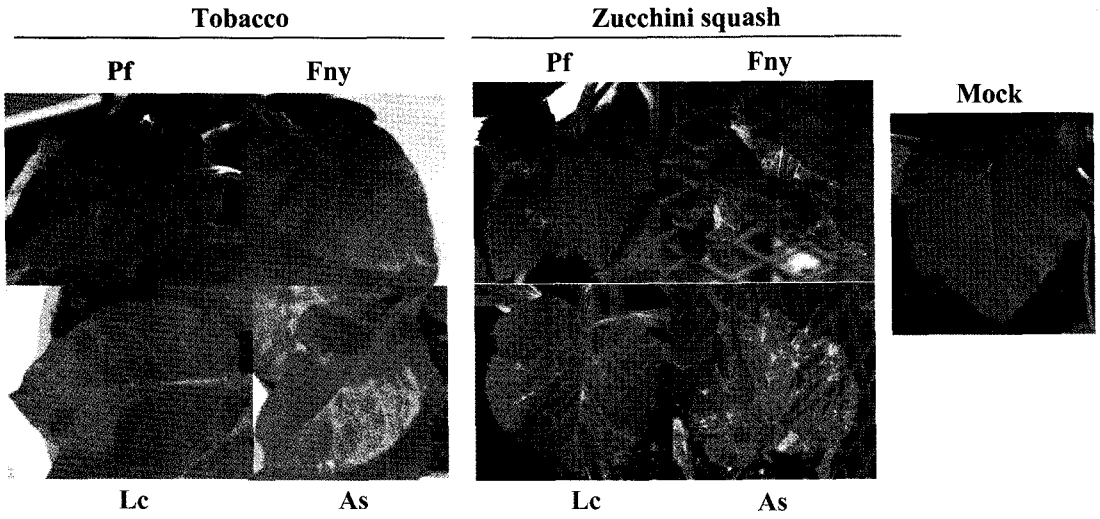

Fig. 2. Symptoms of host plants infected with Class-I-CMV (Fny and As) and Class-II-CMV (Pf and Lc).

in this study.

To confirm the maintenance of genetic information of RNA3 of pseudorecombinat CMV to zucchini squash, the nucleotide sequence of CP gene amplified by RT-PCR from the infected systemic leaves was analyzed. As a consequence, RNA3 was proved to be originated from the transcript of RNA3 in pseudorecombinant CMV and any nucleotide substitution of original CP gene was not founded (data not shown). These results suggested that two distinct types of systemic symptoms in zucchini squash are deeply related to the genetic information of RNA3, and that the determinants of disease symptom seem to be located in RNA3 genome.

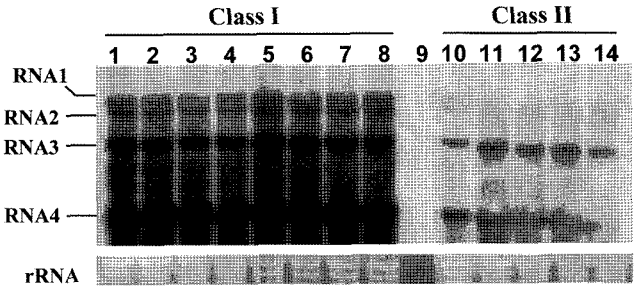

Fig. 3. Analysis of viral RNA accumulations in zucchini squash by Northern blot hybridization. Lane 1,As; lane 2,Gs; lane 3, Pa; lane 4, Ph; lane 5, Rs; lane 6, Rsk1; lane 7, Sa1, lane 8, Fny; lane 9, mock; lane 10, Ga; lane 11, Lc; lane 12, LK3; lane 13, LK4; lane 14, Pf.

Analysis of virus accumulation in zucchini squash. Disease severity has been correlated with accumulation of viral RNAs, which were also depending on the kinetics of virus movement in the host (Choi et al., 2005; Gal-On et al., 1994; Wong et al., 1999). Therefore, the accumulations of viral RNAs between two classes of pseudorecombinant CMVs were examined in the systemically infected zucchini squash. By the way, it was not possible of the direct comparison of viral RNA accumulation between Class-I and Class II, because squash plants infected with Class-I-CMV generally showed the poor growth of non-inoculated leaves or plant death at 9 dpi. Thus, the accumulation of viral RNAs in the systemic leaves of squash plants infected was ascertained at 7 dpi, a time at which plants infected with Class II-CMV isolates including Pf strain had not shown the distinct production of systemic symptoms.

Zucchini squash plants infected by Class I-CMV isolates displayed typical mosaic symptoms earlier or at 7 dpi. High accumulation levels of viral RNAs were observed in systemic leaf tissues (Fig. 3). In turn, it was clearly shown that viral RNA accumulations of Class-II isolates were lower than those of Class I at 7 dpi. Interestingly, the genomic RNAs of Class-II-CMV in systemic leaves were detected by Northern blot hybridization at 7 dpi, although the plants infected with Class II-CMVs displayed very mild symptoms or symptomless.

Conclusively, our results suggested that the accumulation of viral RNAs is correlated with the induction and timing of symptom in zucchini squash. Furthermore, our results were coincident with previous studies that had been analyzed the relationships between symptom severity and RNA accumulation in the same host plants (Choi et al., 2005; Gal-On et al., 1994; Roossinck and Palukaitis, 1990). Again, these results extended that 3a MP and/or CP encoded in RNA3 of CMV played an important role in the differential infection phenotypes of the tested CMVs, on the basis of comparisons with strains Fny and Pf (Taliansky and Garcia-Arenal, 1995).

Table 2. Sequence analysis of movement and capsid proteins between Korean CMV isolates and Fny-CMV[a]

Isolate	3a MP		Accession No.	CP		Accession No.
	nt	aa		nt	aa	
As	93.0[a]	93.5	AF013291	94.5	97.2	AF013291
Gs	–[b]	–	–	98.6	99.5	AB290915
Mf	97.5	99.3	AJ276481	98.5	99.5	AJ276481
Pa	89.9	91.4	AB290913	95.6	98.6	AB290152
Ph	–	–	–	98.6	99.5	AB290153
Rs	–	–	–	96.5	98.2	AB290154
Rsk1	–	–	–	96.3	98.2	AB290155
Rsk2	–	–	–	96.3	98.2	AB290156
Rsk3	–	–	–	96.3	98.2	AB290207
Sa1	96.1	97.8	AB290914	96.3	98.2	AB290208
Fny	100	100	NC_001440	100	100	NC_001440
Ga	96.9	98.2	AB290912	91.5	94.0	AB290210
Lc	95.7	98.2	AB290911	94.4	87.6	AB290209
LK3	95.1	98.6	AJ495841	97.0	98.6	AJ495841
LK4	95.0	98.6	AB354399	96.7	98.2	AB290151
Pf	98.1	99.3	AJ237850	94.2	99.1	AJ237850

[a]Sequence identity (percent) of 3a MP and CP genes between Fny and other strains was presented.
[b]–: not determined.

Analysis of sequences associated with systemic symptom in zucchini squash. Since pathogenic properties of strain Fny (Class-I) in zucchini squash were dramatically changed by transcripts RNA3 that was originated from strains Lc, Ga, LK3 and LK4, we focused on the analysis of genetic features in RNA3 of Class II isolates associated with timing and severity of symptoms. Previous studies showed that some amino acids in 3a MP were crucial for symptom development using tests with various chimeras of RNA3 or mutations on the RNA3 in squash plants (Choi et al., 2005; Gal-on et al., 1994). The MP sequences of Class-II-CMVs (Ga, Lc, LK3, and LK4) and Class-I-CMVs (Pa and Sa1) were determined using full-length cDNA clones of RNA3. The MP sequences of other 11 CMV strains were obtained from GenBank on NCBI website. All MP sequences were analyzed by using BLAST and DNA Star program.

Overall, the amino acids of MPs among 16 CMV isolates shared high sequence identity (approx. 93%, Table 2).

Comparative sequence analysis for MPs between Class I

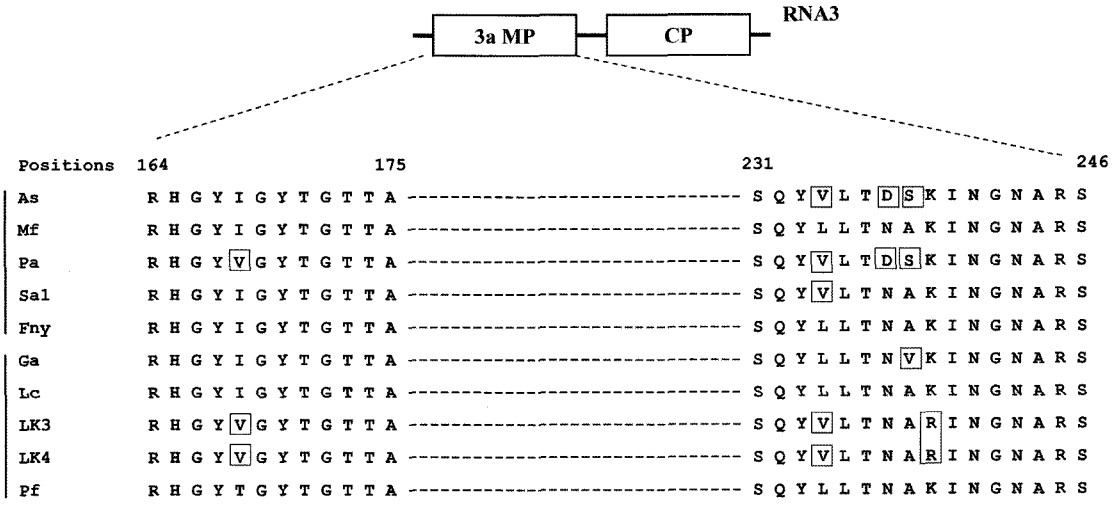

Fig. 4. Multiple alignments of MP sequences between two classes of CMVs. Positions of amino acids from the first methionine in MP was indicated on sequences. Rectangle indicates different amino acids from conserved context of aa sequence. Positions aa 175-231 conserved completely also indicated as dash-bars. Classification of CMV isolates in this study was presented on the left.

and Class II-CMVs isolates was shown that the specific amino acids responsible for the difference in disease symptom were not clearly identified. However, high frequency of amino acid substitutions between two CMV classes were observed specifically at the five positions of 168, 234, 237 238, and 240 in the C-terminal region (Fig. 4). It assumed that the substituted context of amino acids or the net charge of amino acids in C-terminal region of MP, rather than specific amino acids, may play an important role in the development and timing of symptom in two-class CMV. isolates. Currently, however, many factors have been reported to be involved in the development of virus symptoms in the host plants. Interaction of 3a MP with other viral proteins or unidentified host proteins can affect replication and movement of virus, and thereby various disease symptoms could be specialized in squash plants. To confirm the maintenance of genetic information of RNA3 of pseudorecombinat CMV to zucchini squash, the CP genes were amplified by RT-PCR and analyzed. As a consequence, RNA3 was proved to be originated from the transcript of RNA3 in pseudorecombinant CMV and any nucleotide substitution was not founded (data not shown). These results suggested that two distinct types of systemic symptoms in zucchini squash are deeply related to the genetic information of RNA3, and that the determinants of disease symptom seem to be located in RNA3 genome. It remains to be determined whether combinations or specific interaction of virus proteins is crucial.

Molecular classification of CMV isolates. To classify the Korean CMV isolates at molecular level, the sequences of CP genes were determined and analyzed with those of representative CMV strains as a distinct control. The nucleotide length of the CP genes from Korean isolates was 657-nt as same as the previously reported CP genes of various CMV strains. The overall sequence similarity of the CP ORFs was approximately ranged from 87-99%, compared with the Fny-CMV (Table 2). In general, the N-terminal region of CP ORF (approx. 95%) was revealed more conserved than the C-terminal region (approx. 89%). However, there were no distinct regions showing remarkable variability in the CP ORFs and the specific amino acids or regions were not found which might be controlling the pathogenecity difference in zucchini squash.

Phylogenetic analysis with CP genes showed that all the CMV strains belonged to subgroup I, and that there was no strain belonged to subgroup II (Fig. 5). Each CMV was subdivided into subgroup IA (S-IA) and IB (S-IB). Phylogenetic analysis of the CP sequences showed that all CMV isolates used for analysis were placed in subgroup IA (S-IA), except for strains As and K (S-IB). Close relationships were observed in lily-infecting CMV isolates (LK3, LK4 and Ly2) and crucifer-infecting CMV isolates (Rsk1, Rsk2, Rsk3, and Rs) in S-IA, which it was consistent with host species isolated originally (Fig. 5). Phylogenetic tree analysis of MPs was very similar to that of CPs, indicating the high relationship between CMV isolates and original hosts was observed, but the relationship from MP analysis was not be perfectly coincident. Our analysis also indicated that the classification based on phylogenetic analysis was no complete correlation with the pathological phenotypes of Class I and II. For instance, the Class-II-CMVs (Ga, Lc, and LK4) were grouped into the different trees, even though they showed similar phenotypes in zucchini squash. Another example was that the isolate Pa was closer to As on the basis of phylogenetic tree analysis of MP (data not

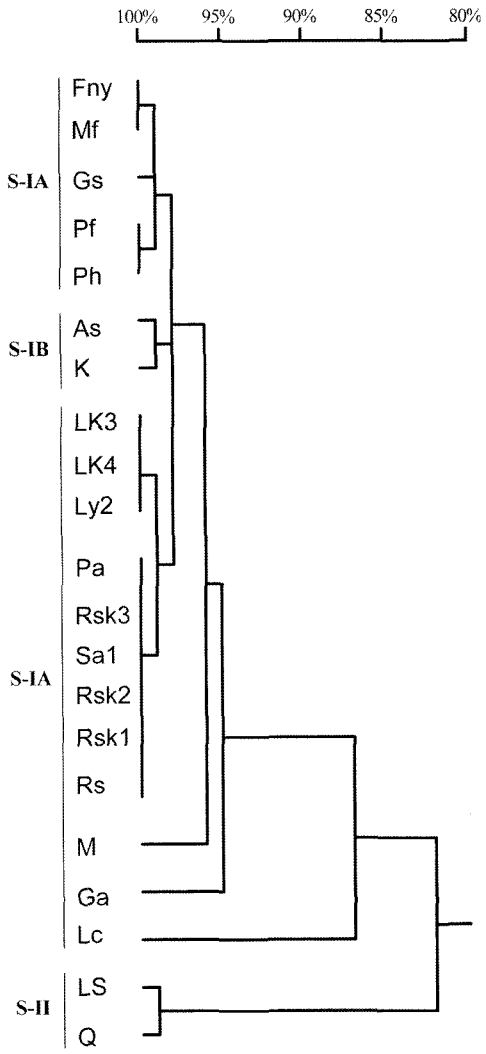

Fig. 5. Phylogenetic tree analysis of CP genes among CMV strains. Sequence homology was indicated on the top. Published CMV strains analyzed together were as followings: M-CMV (D10539), Ly2 (AJ296154), K (AF127977), Q (M21464), and LS (AF127976). The subgroups belonged to CMV isolates were indicated.

shown and Table 2). It was expected that the isolate Pa belongs to S-IB, but analysis of CP ORFs finally showed the inaccuracy of this assumption (Fig 5).

Discussion

This study was designed to figure out the determinant factor controlling the type of disease-symptom with the materials of Korean CMV isolates. To achieve this, the RNA3 of each CMV from infectious cDNA clone was mixed with RNA1 and RNA2 of Fny-CMV to investigate the role of RNA3 genome in disease symptom. Even though two types of symptoms in timing and severity on zucchini squash were obviously confirmed by infections of pseudorecombinant CMVs, very similar factors seem to be associated with MP and CP in tobacco, on the basis of the same mosaic symptoms (Fig. 2). In particular, the systemic symptom has been known to be dependent on the amplification and movement of virus in plant tissue. To amplify the viral RNAs, the 2a protein interacts with helicase domain of the 1a protein, resulting in the formation of an active replicase complex (Hayes and Buck, 1990; O'Reilly et al., 1998). And it was also revealed that the MP can directly bind 2a replicase protein (Hwang et al., 2005), even though the amino acids involved in the interaction between 2a protein and MP have not been determined yet. Besides, CP showed a possibility of cooperation with replicase complex (1a, 2a) and MP (Palukatis and Garcia-Arenal, 2003). As the CP has not displayed the important role in cell-to-cell movement of CMV in plant (Canto et al., 1997; Sanchez-Navarro et al., 2006), it demonstrates that MP can affect the viral RNAs replication in addition to virus movement. Although it is not completely exclusive of some subtle differences of the interactions among CMV proteins in virus movement, MP was shown to be more associated with determination of disease symptom than CP in this study. It is conceivable that the combination of replicase and MP associated with viral RNA and/or with host factor(s) affect indirectly collaboration for virus movement (Blackman et al., 1998; Scholthof, 2005; Waigmann et al., 2004).

Although it is not clear which amino acids of MP are unequivocally crucial for timing and severity of symptom, many differences in C-terminal amino acids in MP were observed from sequence analysis (Fig. 4). The C-terminal amino acids of MP have been regarded as an important region in viral movement. Amino acid positions 164-175 lied in putative zinc finger domain, putative nucleic acid binding domain and in vitro RNA binding domain (Li et al., 2001). Also, aa positions 231-246 lied in RNA binding domain containing essential sequences for CMV movement. It was shown that the C-terminal 33 amino acids of CMV MP affected virus movement, RNA binding and inhibition of infection and translation (Kim et al., 2004). The 33 aa-deleted MP also increased a greater RNA binding affinity than the wild-type MP. It suggested that it is the C-terminal 33 amino acids of CMV MP are involved in specificity for viral genome in plant (Canto et al., 1997; Nagano et al., 2001). A recent study with *Alfalfa mosaic virus* (AMV) showed that the added C-terminal AMV MP sequence allows the cell-to-cell movement of chimeric MP of CMV via interaction with the AMV CP (Sanchez-Navvaro et al., 2006). Furthermore, the mutation in amino acid of 168 position (Pf strain), 51 and 240 (Sny strain) changed the disease symptom in tobacco and zucchini squash, respectively (Choi et al., 2005; Gal-on et al., 1996). It seems that the symptom difference among pseudorecombinant CMVs was like due to difference in virus movement rather than virus replication. Furthermore, several studies with CMV showed that single amino acid or two more amino acids in the same RNA affected the kinetics of virus movement, resulting in different symptom inductions (Choi et al., 2005; Gal-On et al., 1996; Kim and Paluakaitis, 1997; Takeshita et al., 1998). The mutations of aa 631 and 641 of Fny 2a protein allowed host reactions from hypersensitive reaction to systemic mosaic symptom in cowpea (Kim and Palukaitis, 1997). Additionally, mutation of aa 20 and 21 of the 3a MP of CMV has been shown to affect movement between epidermal cells and affect local lesion production in *Chenopodium* spp. (Canto and Palukaitis, 1999), while mutation of aa 51 affects movement in bottle gourd (Takeshita et al., 2001).

It is another explainable reason why viral RNA accumulations of Class I-CMV isolates were consistently higher than those of Class II-isolates. We assumed that these differences of symptoms among pseudorecombinant CMVs were likely to be due to differences in virus movement, both cell-to-cell and long-distance movement in the inoculated tissue as well as the vasculature to upper leaves, rather than virus replication. As the view of viral proteins that significantly affected virus movement, it is conceivable that CMV 2b protein has affected to express their symptom phenotypes, because 2b protein derived from Fny RNA2 was commonly existed in all experiments. Although this seems unlikely, it cannot be ruled out at this moment. Therefore, the phenotypes of pseudorecombinant CMV described here might be resulted from subtle sequence differences in the coding proteins.

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. It remains the exquisite delimitation of genomic sequences responsible for pathogenicity determination associated with virus movement.

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