농학박사학위논문

Glycine soja 에서 분리 동정된 *Cucumber mosaic virus* 의 분자 생물학적 특성 구명

Molecular and biological characterization of an isolate of *Cucumber mosaic virus* from *Glycine soja*

2014 년 8 월

서울대학교 대학원

농생명공학부 식물미생물전공

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Molecular and biological characterization of an isolate of *Cucumber mosaic virus* from *Glycine soja*

A dissertation submitted in partial
fulfillment of the requirement for
the degree of

DOCTOR OF PHILOSOPHY

to the Faculty of
Department of Agricultural Biotechnology

at

SEOUL NATIONAL UNIVERSITY

by

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August 2014

농학박사학위논문

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이 논문을 농학박사학위논문으로 제출함
2014년 5월

서울대학교 대학원
농생명공학부 식물미생물전공

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A THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Molecular and biological characterization of an isolate of *Cucumber mosaic virus* from *Glycine soja*

UNDER THE DIRECTION OF DR. KOOK-HYUNG KIM

SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF SEOUL NATIONAL UNIVERSITY

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AUGUST 2014

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THE DEGREE OF DOCTOR OF PHILOSOPHY
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Molecular and biological characterization of an isolate of *Cucumber mosaic virus* from *Glycine soja*

Mi Sa Vo Phan

ABSTRACT

Host adaption is one of the major factors for sequence convergence or divergence in viral evolution. In this study, a *Cucumber mosaic virus* (CMV) isolate from *Glycine soja* (wild soybean), named as CMV-209, was characterized molecular and biological properties by generating its infectious full-genome cDNA clones. Sequence analysis of full-length genome of CMV-209 showed CMV-209 belonged to subgroup I of CMV, but neither subgroup IA nor subgroup IB since the CMV-209 only shared overall 89-93% sequence identity in their genomes. Phylogenetic analysis of both each CMV-209 genomic

segment and each viral protein also exhibited that CMV-209 was located in a new branch separated from subgroups IA and IB. The investigation of CMV-209 host ranges suggested that CMV-209 is a host-selective strain. CMV did not infect most of the tested legume cultivars except causing local lesion on *Vigna sinensis* (cowpea) and systemic infection on *Pisum sativum* (pea) and *Glycine soja*. CMV-209 seems to have adapted itself to infect pea because other typical CMV strain such as CMV-Fny could not infect pea. In the other hand, the symptom expression of CMV-209 in *Nicotiana benthamiana* was further analyzed by comparing with that of CMV-Fny, which is a representative strain of CMV. CMV-209 induced latent symptom in *Nicotiana benthamiana* whereas CMV-Fny caused typical symptoms including mosaic, stunting, or distortion of leaves. Pseudorecombinant results demonstrated that RNA2 of CMV was responsible for symptom development, e.g. Fny-RNA2 was accountable for mosaic, stunting or leaf distortion and 209-RNA2 for latent symptom. Furthermore, the compatibility among CMV genomic RNA segments also played a role in symptom expression because pseudorecombination between distinct strains of a species can result in emergence of virus progenies carrying higher infectivity or causing severe damages. The pseudorecombinants containing 209-RNA1 and Fny-RNA2 induced more severe leaf

distortion and stunting than those consisting of Fny-RNA1 and Fny-RNA2. In summary, results of our study indicate that CMV-209 is a novel strain and belongs to subgroup I of CMV in the genus *Cucumovirus*.

Key words: *Cucumber mosaic virus*, sequence analysis, phylogenetic analyses, infectious clones, wild soybean, symptom expression, pseudorecombination

Student Number: 2009-31284

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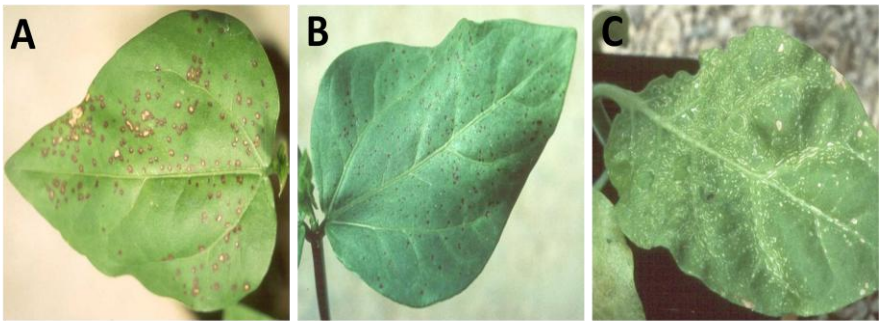
GENERAL INTRODUCTION

Cucumber mosaic virus (CMV) is the type species of the genus *Cucumovirus* in the family *Bromoviridae* (Roossinck et al., 2005). CMV has a high degree of diversity, including a numerous strains differing in both biological and molecular characteristics. In the first place, CMV strains were divided into two subgroups, designated subgroups I and II based on converging biological, serological, and physical criteria, whereby subgroup I contained strains were resistant to heat while strains of subgroup II were sensitive (Jacquemond, 2012). Moreover, they were also distinguished from the differences in symptom induction in cowpea (*Vigna unguiculata)* and tobacco plant (*Nicotiana tabacum cv*. Xanthi nc*).* Most strains of subgroup I induce large necrotic lesions (Fig. 1A) and no visible symptom, but most strains in subgroup II induce small necrotic local lesions (Fig. 1B) and etched rings (Fig. 1C) in inoculated leaves of cowpea and tobacco, respectively. Next, based on nucleotide variation in the 5' non-coding region of RNA3, subgroup I strains are further divided into IA and IB (Roossinck et al., 1999). However, recently the molecular biology of CMV has been extensively studied and analyzing of CMV genome structures has led to new in-sights of their genetic variability, evolution, and taxonomy, e.g. some new strains were reported to be in a new



Palukaitis, P. and García-Arenal, F. 2003

Fig. 1. Different symptom induction in d*iagnostic species* of CMV strains in subgroup I and subgroup II. CMV strains in subgroup I and II induced large and small necrotic local lesions, respectively on inoculated leaves of cowpea (*Vigna unguiculata* cv. Blackeye 5) (A, B, respectively). CMV strains in subgroup II caused etched rings on inoculated leaves of tobacco (*Nicotiana tabacum* cv. Xanthi nc) (C).

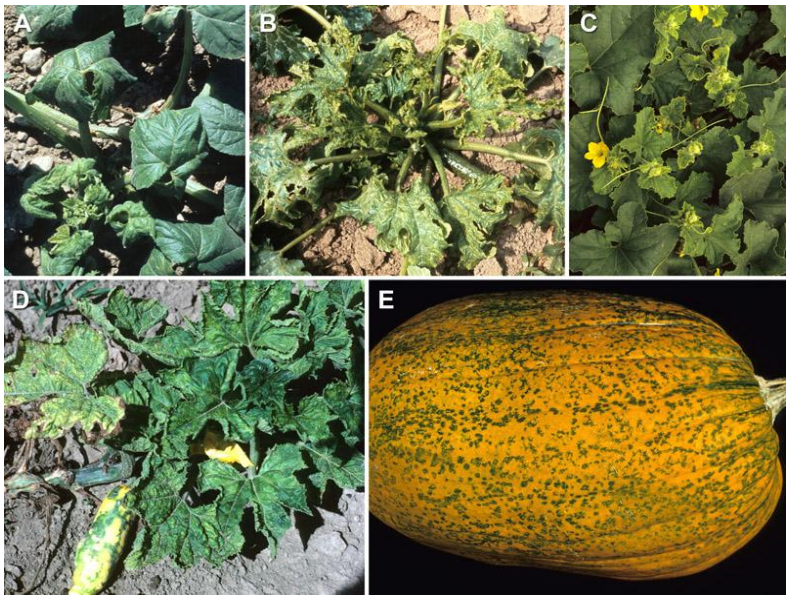
subgroup III (Liu et al., 2009).

CMV is found worldwide, very easily spreads and causes severely damage to the host. It has the broadest host range of any known virus, infecting more than 1200 species in over 100 plant families (Edwardson and Christie, 1991). Depending on the host genotype and the strain, more or less severe symptom will be developed (Jacquemond, 2012), from necrosis, severe stunting, leaf deformation to chlorosis, ringspot, mosaic, mottle, etc.… (Fig. 2, 3). Furthermore, the age of the crop when the infection occurs also affected on symptoms caused by CMV. For instance, in the case of tomato in the field, if plants are young at the time of infection, the infected ones will remain small, sometimes severely stunting, with a bushy appearance and severe deformation, upward cupping or rolling on leaves. In opposite, if the plants are more mature at the time of infection they will only show obvious mosaic symptoms, no severe leaf distortion, cupped or rolled (Zitter and Murphy, 2009). Almost all CMV viral proteins are involved in symptom development. For example, protein 1a of CMV-Ns induces necrotic lesions on several *Nicotiana* spp; protein 2a of CMV-Fny causes necrotic local lesions on cowpea; CP of CMV-M is responsible for chlorotic symptoms; etc. (Ding et al., 1995; Divéki et al., 2004; Jacquemond, 2012; Kim and Palukaitis, 1997; Shintaku et al., 1992;

Suzuki et al., 1995; Zhang et al., 1994). In fact, many biological processes of host plants such as photosynthesis, pigment metabolism and plant-pathogen interaction are influenced by CMV infection during the symptom development (Lu et al., 2012). Among of above processes, the pigment metabolism including "porphyrin and chlorophyll metabolism", carotenoid biosynthesis" and "anthocyanin biosynthesis" may be directly responsible for the disease development (Lu et al., 2012). Despite CMV has been extensively studied, the mechanisms underlying symptom development are still poorly understood (Jacquemond, 2012).

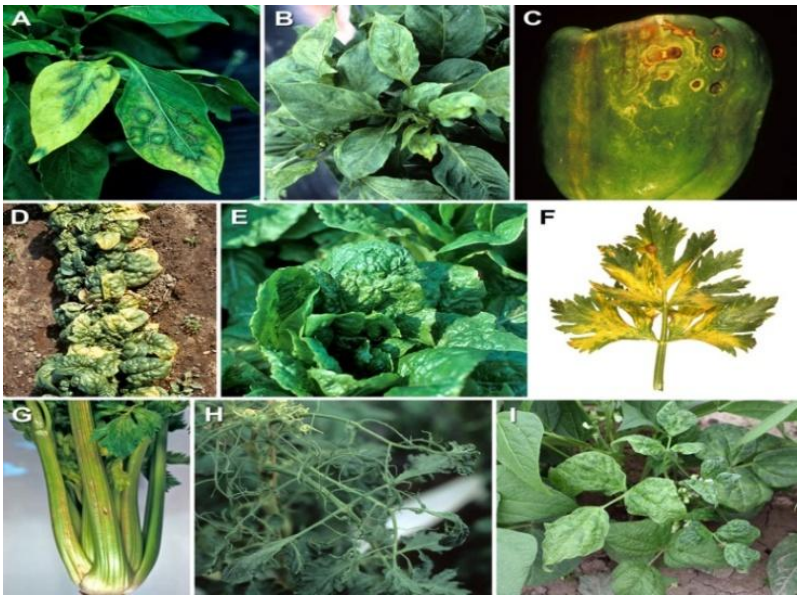
In the other hand, so far as is known, whether viruses are able to replicate and move through out plant largely depends on whether each viral protein precisely interacts with protein or other components of the plants (Whitham and Wang, 2004) as well as whether viruses can suppress or meddle in plant defense mechanisms (Brigneti et al., 1998; Kasschau and Carrington, 1998). CMV is an extremely successful multipartite virus, whose genome consists of three single-stranded positive-sense RNAs (Fig. 4A) and codes for five proteins 1a, 2a, 2b, 3a and CP (Fig. 4B, C, D) (Jacquemond, 2012). Proteins 1a and 2a are necessary for viral replication in association with host proteins. Protein 1a localizes to the vacuolar membrane (the tonoplast) (Cillo et al.,

2002), interacts with tonoplast host protein to be anchored (Huh et al., 2011; Kim et al., 2006) and then protein 2a is recruited to tonoplast to conduct the viral replication (Chen et al., 2001; Cillo et al., 2002). However, the exact roles of host proteins in addressing and anchoring protein 1a and 2a to the tonoplast and in the replication complex still need additional studies. In the same manner, protein 3a or movement protein (MP), essential for virus movement (Boccard and Baulcombe, 1993; Canto et al., 1997; Suzuki et al., 1991), also interacts with and severs F- actin filaments to increase the plasmodesmal size exclusion limit (SEL) (Su et al., 2010) for easily trafficking itself along with viral RNAs through plasmodesmata (Sasaki et al., 2006). The most interesting ability of CMV is that protein 2b acts as a silencing suppressor (Brigneti et al., 1998). It restrains the long-distance spread of the gene-silencing signal (Guo and Ding, 2002) and binds to small interfering RNA (siRNA) in the RNA silencing pathway (Goto et al., 2007). It also inhibits the cleavage activity of Argonaute1, a component of the antiviral RNA-induced silencing complex to counter plant defense 1 (Zhang et al., 2006) and supports for viral infection of shoot apical meristems (SAMs) and for efficient invasion of leaf primordia (Sunpapao et al., 2009) where are mostly considered to escape virus invasion (Retheesh and Bhat, 2010). In summary, most CMV strains



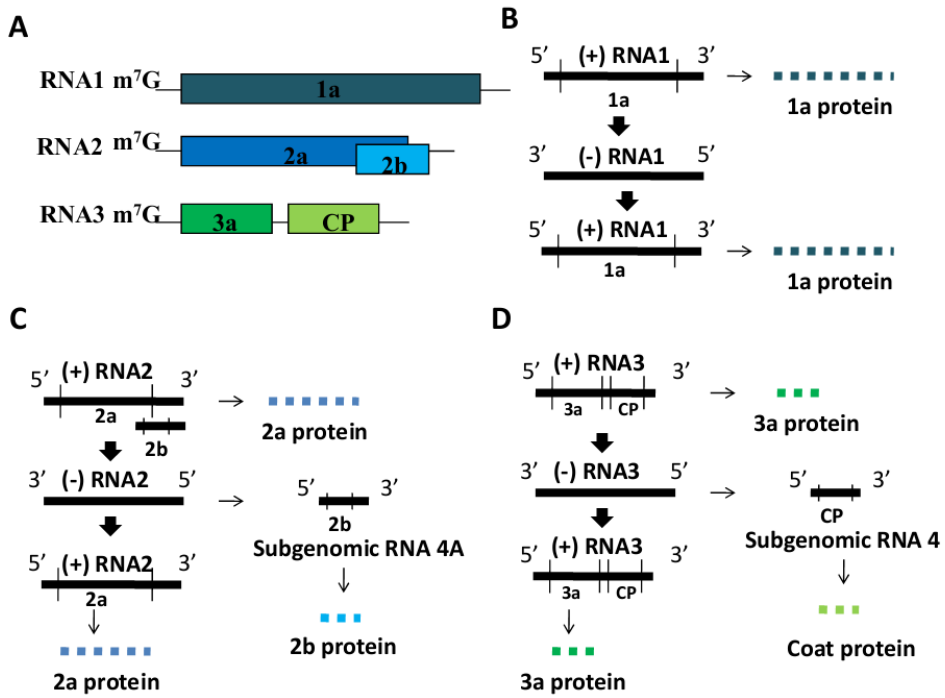
Zitter, T. A., and J. F. Murphy. 2009.

Fig. 2. Symptoms induced by CMV strains in cucurbits. CMVs induced severe epinasty, downward bending of the petiole and leaf surface along with leaf reduction in summer squash (A), severely stunted and malformed leaves in zucchini (B), severely stunted growing tips in muskmelon (C), color breaking on yellow squash fruit (D), and mosaic pattern on pumpkin fruit (E).



Zitter, T. A., and J. F. Murphy. 2009.

Fig. 3. Symptoms induced by CMV strains in non-cucurbit crops. CMVs caused chlorosis, chlorosis mosaic and ringspot on leaves and fruit in peper (A, B, C, respectively), chlorosis mottle, narrowing, wrinkling with vein distortion and inward leaf roll on leaves in spinach (D), severe roughness, mottling on leaves in lettuce (E), yellowing and veinal necrosis on leaves and sunken beige colored lesions on petioles in celery (F, G, respectively), filiformity leaves in tomato (H), and leaf curl, green mottle and blistering on leaves in bean (I).



Palukaitis, P. and García-Arenal, F. 2003

Fig. 4. CMV biology. The genome of CMV consists of three single-stranded positive-sense RNAs (A). Protein 1a, 2a, 3a are translated directly from genomic RNAs 1, 2, 3 respectively (B, C, D, respectively). Protein 2b and CP are expressed from sub genomic RNA4A and RNA4, respectively (C, D, respectively). ORFs are indicated by boxes and named according to the proteins they code for. CP, capsid protein; m7G, cap structure; (+)RNA, genomic positive-sense RNA; (-)RNA, complementary minus-sense RNA.

have efficient strategies to invade a variety of plant species. Nevertheless, in viral evolution, host adaptation of CMV seems somewhat related to narrowing host range. As an example, CMV strains adapting to Lily cannot infect tobacco, cucurbits and tomato (Lee et al., 2007; Masuta et al., 2002; Ryu and Choi, 2002). Similarly, isolates from soybean are also not to be able to infect cucumber (Hong et al., 2007). This raises an interesting question that if the narrow host range can be the advantage for virus in nature (Jacquemond, 2012).

In this study, a new strain of *Cucumber mosaic virus*, identified as CMV-209, was isolated from wild soybean (*Glycine soja*). We encountered a similar situation that CMV-209 is a host-selective strain because of its lacking of ability to infect most of the tested legume cultivars except *Pisum sativum*. Phylogenetic analyses and nucleotide comparison of this strain with the other CMV strains indicated that it clustered separately within neither subgroup IA nor IB. CMV-209 is a novel virus and further investigation of CMV-209 is paramount to give new insights into genetic variability, evolution, taxonomy and host adaptation of CMV.

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CHAPTER 1

Molecular and biological characterization of an isolate of *Cucumber mosaic virus* from *Glycine soja* by generating its infectious full-genome cDNA clones

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The Plant Pathology Journal (2014), 30(2): 159-167

ABSTRACT

Molecular and biological characteristics of an isolate of *Cucumber mosaic virus* (CMV) from *Glycine soja* (wild soybean), named as CMV-209, was examined in this study. Comparison of nucleotide sequences and phylogenetic analyses of CMV-209 with the other CMV strains revealed that CMV-209 belonged to CMV subgroup I. However, CMV-209 showed some genetic distance from the CMV strains assigned to subgroup IA or subgroup IB. Infectious full-genome cDNA clones of CMV-209 were generated under the control of the *Cauliflower mosaic virus* 35S promoter. Infectivity of the CMV-209 clones was evaluated in *Nicotiana benthamiana* and various legume species. Our assays revealed that CMV-209 could systemically infect *Glycine soja* (wild soybean) and *Pisum sativum* (pea) as well as *N. benthamiana*, but not the other legume species.

INTRODUCTION

Cucumber mosaic virus (CMV) is a type member of the genus *Cucumovirus* and has a very broad host range (Palukaitis and García-Arenal, 2003). Its genome comprises three single-stranded plus-sense RNAs, named RNA 1–3 in order of decreasing size, which encode five viral proteins: 1a, 2a, 2b, 3a (movement protein; MP), and capsid protein (CP). Initially, CMV strains were classified into two main subgroups, I and II, based on converging biological, serological, physical criteria(Hu et al., 1995; Ilardi et al., 1995; Wahyuni et al., 1992), nucleic acid hybridization (Owen and Palukaitis, 1988), andgene sequences (Owen et al., 1990; Sala and Bala, 1999). CMV strains were further divide into three subgroups including IA, IB, and II based upon phylogenetic analyses of a large number of the sequences of the CP gene as well as rearrangements in the 5′ non-translated region (NTR) of RNA 3 (Roossinck et al., 1999). However, additional analyses with full-genome sequences of CMV strains demonstrated that some strains were grouped in neither subgroup I nor subgroup II but in a new subgroup, named subgroup III (Jacquemond, 2012; Liu et al., 2009); while some strains were in between subgroups I and II (Chen et al., 2007; Jacquemond, 2012; Maoka et al., 2010), and the others were in

an intermediate position between subgroups IA and IB (Jacquemond, 2012; Lin et al., 2004; Masuta et al., 2002). Thus, analysis of at least one part of each genomic segment was necessary for confident genotyping of CMV strains (Jacquemond, 2012).

Ever since the first report of CMV strain isolated from soybean (Koshimizu and Izuka, 1958), several additional CMV strains have been isolated (Hong et al., 2003; Senda et al., 2004; Takahashi et al., 1980). The firstly isolated CMV soybean strain induced mild mosaic and stunting on infected soybean plants and was transmissible through seeds with a high efficiency by aphids (Koshimizu and Iizuka, 1963; Takahashi et al., 1980). Even though the biological characteristics of CMV soybean strain have been studied extensively, there was no available sequence information on CMV soybean strain genomes. A previous report analyzing the phylogenetic relationship of the nucleotide sequences of the 3a and CP genes of seven CMV soybean strains showed that these CMV soybean strains formed a distinct cluster separated from the other CMV strains(Hong et al., 2003).

Construction of infectious cDNA clones corresponding to the genomes of RNA viruses is necessary since viruscDNA clones are essential sources for maintaining their original RNA genomes and for engineering viral vectors (Boyer and Haenni, 1994; Nagyová and Subr,

2007). In addition, easy manipulation of virus cDNA clones allows mutagenesis (deletions, insertions, substitutions) of specific regions of virus genomes and subsequent introduction of the mutated or wild type genomes into host plants for studies of virus life cycle, functions of viral proteins and their interactions with various host factors involved in virus replication, cell to cell movement, resistance responses (Bendahmane et al., 2002; Kim and Palukaitis, 1997; Paalme et al., 2004; Prüfer et al., 1995).

In this study, we obtained a CMV isolate from *Glycine soja* (wild soybean) and named CMV-209. Using this isolate, we generated infectious full-genome cDNA clones of CMV-209. Using the cloned CMV-209, its molecular characteristics were examined by determining complete nucleotide sequences of each genomic RNA segment and comparing to those of the other representative CMV strains. The biological characteristics were investigated by inoculating the cloned CMV-209 in various legume species as well as *Nicotiana benthamiana*.

MATERIALS AND METHODS

I. Virus source and construction of the full-length cDNA clones of CMV-209

Wild soybean samples showing typical mosaic symptoms were collected in Suwon, Korea 2006. To find the samples infected with CMV, the collected samples were screened by RT-PCR using the CMV specific primers CMV R3 561 Fw and CMV R3 1473 Rv (Table 1). One (sample No. 209) of the wild soybean samples was found to be infected with CMV. Total RNA was extracted from the CMV-infected wild soybean (No. 209) using TRI Reagent method (MRC, USA)and subjected to RT-PCR to amplify full-length cDNAs corresponding to CMV RNA1, RNA2, and RNA3 using the specific primer pairs that contain *Bam*HI restriction site(Table 1). The amplicons were digested with *Bam*HI and ligated with the modified binary vector pSNU1 (Park and Kim, 2006) which was digested with *Bam*HI. The resulting clones carrying the full-length cDNA of RNA1, RNA2 or RNA3 of CMV were named as pCMV-209R1, pCMV-209R2 and pCMV-209R3, respectively (Fig. 1).

Table 1. Primers for constructing, detecting and sequencing infectious CMV clones

Name	Sequence (5′ → 3′)[a]
CMV R3 561 Fw	TTGGGAATCGTAAGCGGTGTTTTG
CMV R3 1473 Rv	TTACAACGTTCACTCCCCACAAAG
CMV 3 end *Bam*HI	GC**GGATCC**TGGTCTCCTTTTRGAGRCC
CMV R1R2 5 end *Bam*HI	GC**GGATCC**TTTATTTACAAGAGCGTACGG
CMV R3 5 end *Bam*HI	GC**GGATCC**GTAATCTTACCACTGTGTGTGTG
CMV detR2 F	CCCTGTTGGTGATCCGAGTAA
CMV detR2 R	CCGTAAGCTGGATGGACAAC

[a] The *Bam*HI sequence is shown in boldface

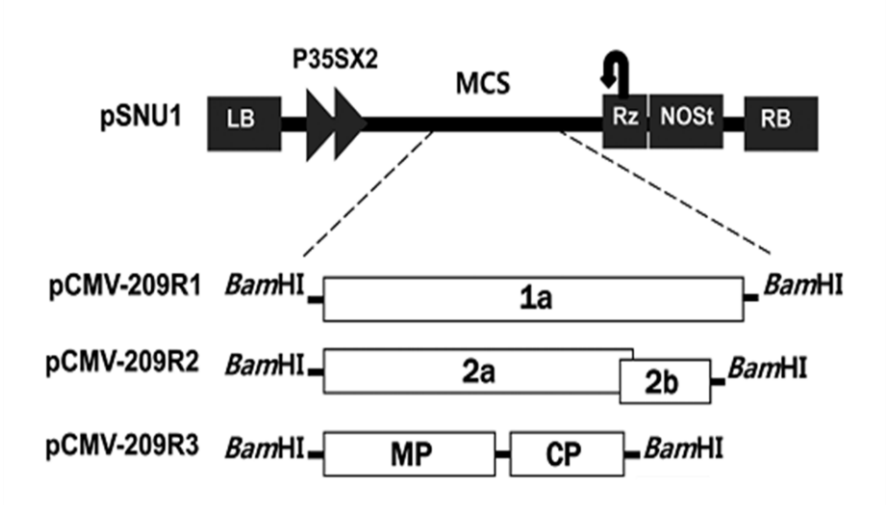

Fig. 1. Schematic representation of the T-DNA vector, pSNU1, and CMV-209 cDNA constructs. The pSNU1 vector contains, in sequential order, a left border of T-DNA (LB), a double 35S promoter (P35Sx2), multiple cloning site (MCS), a cis-cleaving ribozyme sequence (Rz), a NOS terminator (NOSt), and a right border of T-DNA (RB). The constructs pCMV-209R1, pCMV-209R2 and pCMV-209R3 express RNA1, RNA2 and RNA2 of CMV strain 209, respectively. The restriction enzyme cleavage sites used to make the constructs are shown.

II. Infectivity test and screening host range for CMV-209

All of the CMV constructs (i.e. pCMV-209R1, pCMV-209R2, and pCMV-209R3) were individually transformed into *Agrobacterium tumefaciens* strain GV2260 by electroporation and cultured as previously described (Park and Kim, 2006). The *Agrobacterium* transformants were grown at 30^{o}C until OD_{600} = 0.5 ± 0.05. A mixture of three *Agrobacterium* cultures harboring constructs pCMV-209R1, pCMV-209R2, and pCMV-209R3 in a 1:1:1ratio to yield a total OD_{600} of 0.17 ± 0.02 was used for co-agroinfiltration. This final Agrobacterium mixture was infiltrated into the abaxial surface of the N. benthaminana leaves (3 to 4 weeks old) by using a 1-ml syringe without a needle. pCMV-Fny clones (pCR1(+), pCR2(+), and pCR3(+); Seo at al., 2009) were also included in agroinfiltration as control. After 7 days, the upper non-inoculated *N. benthamiana* leaves were subjected to RT-PCR using the primer pairs CMV detR2 F and CMV detR2 R (Table 1) to examine whether the infiltrated plants are systemically infected with CMV-209. As shown in Table 2, the sap extracts from the *N. benthamiana* plants systemically infected with CMV-209 were used as viral inoculums for sap-inoculation of 14 legume cultivars including *Pisum sativum* (pea), *Vigna sinensis* (cowpea), *V. angularis* (cv. Chungbupat), *Phaseolus vulgaris* (Kidney bean), *Ph. vulgaris* (Pinto

bean), *Glycine max* (cv. Somyong), *G. max* (cv. Pureunkong), *G. max* (cv. Sowon), *G. max* (cv. Danbaek), *G. max* (cv. L29), *G. max* (cv. Daewon), G. *max* (cv. V94-5152), *G. max* (cv. Huanggumkong), *G. max* (cv. Lee74), and *G. soja* (wild soybean).

III. Sequencing, comparative, and phylogenetic analyses

The full-length cDNA sequences in the infectious clones of CMV-209 (pCMV-209R1, pCMV-209R2 and pCMV-209R3) were sequenced using the appropriate primers (the list of primers used for the sequencing is available on request). The nucleotide sequences and deduced amino acids sequences of CMV-209 were compared with those of other representative CMV strains retrieved from the GenBank database. Sequences of PSV-P, PSV-W and PSV-Mi as well as TAV-KC were used as out-groups (Table 3). Multiple sequence alignments of all the analyzed sequences were obtained using CLUSTALS (Thompson et al., 1997). The sequence similarity comparisons were performed using Lasergen Package. The phylogenetic analyses were carried out using the Neighbor-Joining method (Saitou and Nei, 1987). The confidence probability was estimated using the bootstrap test (1000 repetitions) (Dopazo, 1994; Felsenstein, 1985; Rzhetsky and Nei, 1992). The evolutionary distances were computed using the p-distance method

(Nei and Kumar, 2000) and were the units of the number of nucleotide or amino acid differences per site. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

Table 2. The infectivity of CMV-209 in various legume species[a]

Species	Symptoms[b] on the leaves	
	Inoculated	Upper
Pisum sativum (pea)	+/ns	+/st
Vigna sinensis (cowpea)	+/nll	-
V. angularis (cv. Chungbupat)	-	-
Phaseolus vulgaris (Kidney bean)	-	-
Ph. vulgaris (Pinto bean)	-	-
Glycine max (cv. Somyong)	-	-
G. max (cv. Pureunkong)	-	-
G. max (cv. Sowon)	-	-
G. max (cv. Danbaek)	-	-
G. max (cv. L29)	-	-
G. max (cv. Daewon)	-	-
G. max (cv. V94-5152)	-	-
G. max (cv. Huanggumkong)	-	-
G. max (cv. Lee74)	-	-
G. soja (wild soybean)	+/ns	+/M, st

[a]Inoculation with the plant sap infected with CMV-209.

[b] Symbols: +, virus infection confirmed by RT-PCR;-, no virus infection confirmed by RT-PCR; ns, no symptom or latent infection; nll, necrotic local lesion; st, stunt; M, mosaic.

Table 3. Sequences obtained from the GenBank database

Virus strains	Subgroup	Database accession number		
		RNA1	RNA2	RNA3
Fny	IA	D00356	D00355	D10538
Rs	IA	AJ511988	AJ517801	AJ517802
Y	IA	D12537	D12538	D12499
Legume	IA	D16403	D16406	D16405
Mf	IA	AJ276479	AJ276480	AJ276481
New Delhi	IB	GU111227	GU111228	GU111229
PI1	IB	AM183114	AM183115	AM183116
NT9	IB	D28778	D28779	D28780
Tfn	IB	Y16924	Y16925	Y16926
LS	II	AF416899	AF416900	AF127976
Trk7	II	AJ007933	AJ007934	L15336
LY	II	AF198101	AF198102	AF198103
Q	II	X02733	X00985	M21464
Bx	III	DQ399548	DQ399549	DQ399550
PHz	III	EU723568	EU723570	EU723569
PSV-P	I	EU570236	EU570237	EU570238
PSV-W	II	U33145	U33146	U31366
PSV-Mi	III	AY429431	AY429430	AY775057
TAV-KC	-	AJ320273	AJ320274	AJ237849

RESULTS AND DISCUSSIONS

I. Infectivity and host range of CMV-209

To verify the infectivity of the full-genome cDNA clone of CMV-209, we first infiltrated *N. benthamiana* plants with a mixture of agrobacterium cultures harboring pCMV-209R1, pC-MV-209R2, and pCMV-209R3. All the infiltrated plants showed very mild or no visible symptom (Fig. 2B) while showing typical symptoms including mosaic, stunting, or distortion of the leaves from CMV-Fny infiltrated plants (Fig. 2C). Although there was no obvious visible symptom from CMV-209 infiltrated plants, progeny viruses were detected from the upper systemic leaves by RT-PCR using CMV-specific primers (Fig. 2D) demonstrating that the full-genome cDNA clones of CMV-209 are fully infectious.

Next, since CMV-209 was isolated from wild soybean, the host range and infectivity of CMV-209 were examined in various legume species. To this end, twelve legume species were inoculated with the sap extracted from the *N. benthamiana* plants systemically infected by the CMV-209 infectious clone. Among the tested legume species, only *G. soja* (wild soybean) and *P. sativum* (pea) were systemically infected by CMV-209 (Table 2). The pea plants infected with CMV-209 showed severe stunting symptom (Table 2; Fig. 3A), rosetting of stem (Fig. 3B)

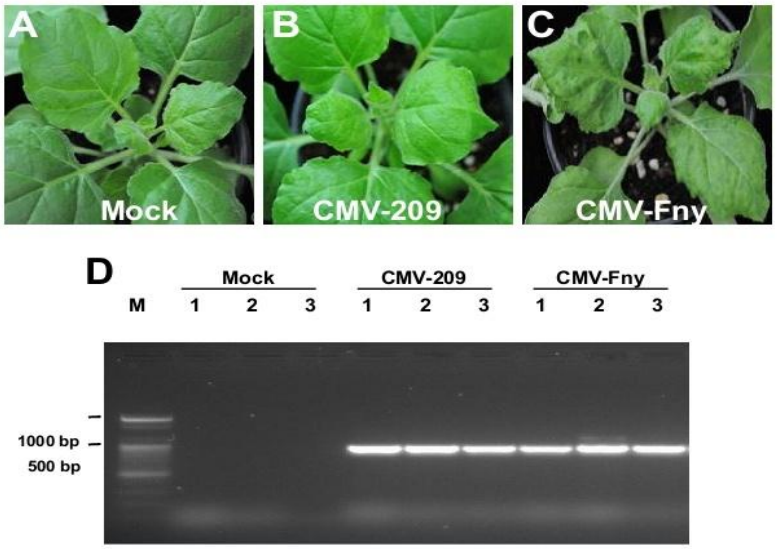

Fig. 2. Symptom of CMV-209 in *Nicotiana benthamiana*. pCMV-209 induced latent symptom in *Nicotiana benthamiana* (B) while pCMV-Fny caused typical symptoms includ-ing mosaic, stunting, or distortion of leaves (C). Mock represents healthy plant (A). Photographs were taken at 13 days post-inoculation. (D) Detection of progeny viruses in *N. benthamiana* plants inoculated with CMV strains. Total RNAs, isolated from upper uninoculated leaves of each *N. benthaminana* plant harvested at 7 days post-agroinfiltration, were analyzed by RT-PCR. M indicates a 100 bp DNA ladder (Bioneer). Lanes 1 to 3 represent results from three RNA samples extracted from three *N. benthaminana* plants.

and their roots were significantly shorter and fewer than those of healthy and CMV-Fny infected pea plants (Fig. 3A). CMV-209 inoculated *Vigna sinensis* (cowpea) showed necrotic spots on inoculated leaves (Fig. 3C).

Host adaptation is one of the main factors for sequence convergence or divergence of viruses. CMV-209 could systemically infect pea (*P. sativum*) and *N. benthamiana*, but not the other host plants tested in this study, suggesting that CMV-209 is host-selective strain (Table 2). CMV-209 seems to have adapted itself to infect pea because other typical CMV strain such as CMV-Fny could not infect pea (Fig. 4A). In fact, Hong et al. (2003) also reported that CMV soybean strains might have adapted to soybeans in their evolutionary history and that they had evolved faster than CMV subgroup IA.

Interestingly, CMV-209 also induced necrotic spots in inoculated leaves of *V. sinensis* as other CMV strains did (Fig. 4C). Although two amino acid residues of the 2a protein at positions 631 and 641 being Phe and Ala, respectively, were also conserved in CMV-209, which have been reported to be crucial for causing a local hypersensitive response in cowpea (Kim and Palukaitis, 1997), it remains to be determined whether these two conserved amino acid residues are actually responsible for causing necrotic local lesions. The

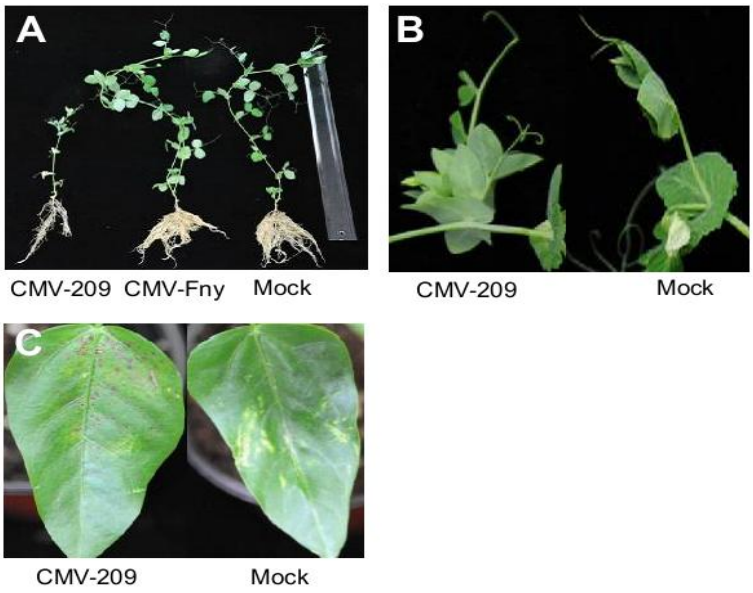

Fig. 3. Symptom of CMV-209 in legume cultivars. CMV-209 induced severe stunting (A), rosetting (B) in *Pisum sativum*, and necrosis local lesions in inoculated *Vigna sinensis* leaves (C). CMV-Fny did not infect *Pisum sativum* (A). Mock represents healthy plant. CMV-209 and CMV-Fny represents plants infected by CMV-209 and CMV-Fny, respectively. Photographs were taken at 18 days (A, B) and 9 days (C) post-inoculation.

difference of host range and/or infectivity between the CMV-209 and other reported CMV strains in tested host plants observed in this study might be due to sequence changes in CMV-209 RNA genome segments. In this regard, it is worth mentioning that the RNA3 segment including 3a and CP proteins has been reported as a host range determinant and thus might be more responsible as host-interactive constraints than the other viral gene products (Hong et al., 2007; Hong et al., 2003; Roossinck, 2002; Ryu et al., 1998). We are currently conducting experiments with reasserted RNA segment using several CMV strains including Fny and 209 to further determine whether RNA3 segment is responsible for determining host range and/or infectivity.

II. Molecular characteristics of CMV-209

The complete nucleotide (nt) sequence of CMV-209 was determined and deposited in the GeneBank database (Accession Nos: KJ400002, KJ400003, KJ400004). The sizes of the genomic RNAs varied for the different strains and species. The sizes of RNAs 1, 2, and 3 of the strains in CMV subgroup I were in range of 3357-3365 nt, 3036-3060, and 2213-2220 nt, while in CMV subgroup II were 3389-3391, 3038-3053, 2197-2209 nt, respectively (Palukaitis and García-Arenal, 2003). The length of RNA1, RNA2, and RNA3 of CMV-209

were 3,360 nt, 3,057 nt, and 2,243 nt, respectively, indicating that CMV-209 belongs to CMV subgroup I.

The coding part of the genome consists of five ORFs, i.e. 1a, 2a, 2b, MP, and CP. The first ORF encodes the largest protein (111.65 kDa; 993 aa) which possesses a putative methyltransferase domain and a helicase motif. The second ORF codes the RNA-dependent RNA polymerase (RdRp) protein (97.043 kDa; 858 aa). The third ORF encodes the smallest protein (12.846 kDa; 110 aa), which has function as a silencing suppressor. The fourth is a putative MP (30.595kDa; 279 aa) and the fifth is the 24.136kDa CP consisting of 218 aa.

The nucleotide positions of the five ORFs (1a, 2a, 2b, 3a, and CP) for CMV-209 and length of which were presented in Table 4. The initiation condon for the 1a, 2a, 2b, 3b, and CP genes of CMV-209 were positioned at position 95, 78, 2413, 110, and 1280 compared with 95-98, 85-87, 2417-2419, 120-121, and 1257-1260 of subgroup IA and 95-96, 79-80, 2414-2415, 122, and 1255-1261 of subgroup IB, respectively. The size (2577 nt) of CMV-209 ORF2a was slightly different from subgroups IA and IB, which was 2574 nt in length. The length of CMV-209 ORF 3a (840 nt) were same as subgroup IA (840 nt) but slightly differ from that of subgroup IB (852 nt). ORFs 1a, 2b, and CP of CMV-209 were 2982 nt, 333 nt, 657 nt, respectively, in length as were in

subgroups IA and IB (data not shown). Further information of the other components of RNAs was indicated in detail in Table 4.

Table 4. Molecular characteristics of CMV-209

RNAs	Components	Position	Nucleotide (base)	Protein (aa/kDa)
RNA1	5′UTR	1-94	94	-
	ORF1a	95-3076	2982	993/111.365
	3′UTR	3077-3360	284	-
RNA2	5′UTR	1-77	77	-
	ORF2a	78-2654	2577	858/97.043
	ORF2b	2413-2745	333	110/12.846
	3′UTR	2746-3057	312	-
RNA3	5′UTR	1-109	109	-
	ORF3a	110-949	840	279/30.595
	IR	950-1279	330	-
	ORFCP	1280-1936	657	218/24.136
	3′UTR	1937-2243	307	-

III. Comparative analysis of the CMV-209 genome sequence

According to the International Committee on Virus taxonomy (ICTV) criteria for discriminating species from the genus *Cucumovirus*, subgroups ordinarily have at least 65% sequence identicalness. The nt sequence similarity for the complete genomic RNAs among the CMV-209 and the representativeCMV strains was ranged from 71.8 % to 93.4% while the similarity among CMV-209 and other cucumoviruses such as PSV and TAV was in the range of 56.1-68.7% (Table 5). The genome sequence of CMV-209 had highest similarity with that of CMV-Fny (Table 5).

In CMV, within-subgroup nt homologies of subgroup II and III were very high (more than 98% and 94%, respectively). The nt sequence of CMV-209 had the sequence identity to subgroup II and III only around 76% and 89.5%, respectively (data not shown). In the case of subgroup I, the sequence identities of RNA1, 2, and 3 within subgroup IA were more than 92.7, 95.8, and 97.1%, respectively, and were more than 97.8, 91.2 and 97.4%, respectively, within subgroup IB. The sequence identities of RNA1, 2, and 3 between sister group, subgroup IA and IB, were in the range of 89.8-92.2, 89.1-91.5, and 91.8-93.9%, respectively (data not shown).

Genomic segment comparison of CMV-209 with the strains in

subgroup I, including subgroup IA and IB, showed their identity levels of 89.6-91.2, 90.0-92.4, and 91.7-93.4% in RNA1, 2, and 3 sequences, respectively (Table 5). The 3′UTR of CMV has information required for minus strand synthesis of the viral RNA during replication and is highly conserved within subgroup. However, the 3′UTR nt sequences of CMV-209RNA1, RNA2, and RNA3 showed the similarities of 87.5-91.1, 86.0-88.7, and 92.0-93.7%, respectively, when compared to those of subgroup IA and IB (Table 5). Taken together, our analyses suggest that CMV-209 could belong to a distinct sister group from subgroup IA and IB.

Table 5. Sequence identity between the RNAs, ORFs, and encoded proteins of the CMV-209 strain and those of other strains of CMV, PSV, and TAV

Virus strains	RNA1			
	Complete	5′UTR	ORF1a (nt/aa)	3′UTR
Fny (IA)	92.1	94.6	92.4/96.8	88.8
Rs (IA)	91.9	94.6	92.1/96.5	88.6
Y (IA)	91.2	91.4	91.5/95.1	87.5
Legume (IA)	92.1	94.6	92.4/96.4	88.9
Mf (IA)	91.6	93.5	91.6/94.7	90.3
New Delhi (IB)	89.6	91.4	89.4/94.0	91.1
PI1 (IB)	90.5	91.4	90.5/96.3	89.7
NT9 (IB)	90.6	91.4	90.6/96.2	90.4
Tfn (IB)	90.5	91.4	90.5/96.2	90.5
LS (II)	76.4	84.3	77.1/85.1	68.4
Trk7 (II)	76.4	84.3	77.1/84.6	68.8
LY (II)	76.3	83.1	77.0/84.4	68.5
Q (II)	76.4	84.6	77.1/84.4	67.6
Bx (III)	89.5	92.9	89.7/94.4	85.9
PHz (III)	89.5	96.8	89.7/94.5	84.8
PSV-P (I)	67.5	65.5	67.7/73.6	65.1
PSV-W (II)	67.6	62.7	68.2/73.5	62.9
PSV-Mi (III)	68.7	67.5	68.6/72.1	64.1
TAV-KC	68.7	72.8	68.5/73.5	69.9

Table 5. Sequence identity between the RNAs, ORFs, and encoded proteins of the CMV-209 strain and those of other strains of CMV, PSV, and TAV (*Continued*)

Virus strains	RNA2				
	Complete	5′UT	ORF2a(nt/aa)	ORF2b (nt/aa)	3′UT
Fny (IA)	92.4	87.0	92.9/95.2	87.1/80.0	87.8
Rs (IA)	92.3	84.4	92.8/95.1	87.7/80.0	88.2
Y (IA)	92.8	84.4	93.4/95.6	87.7/80.0	87.8
Legume (IA)	91.5	85.5	92.3/94.6	84.0/66.4	86.0
Mf (IA)	92.3	87.0	92.9/94.9	87.4/78.2	86.8
New Delhi (IB)	90.0	83.1	90.7/91.4	81.8/72.5	88.7
PI1 (IB)	90.8	96.1	91.4/93.9	84.8/73.4	86.3
NT9 (IB)	90.9	96.1	91.4/93.9	85.2/74.3	87.0
Tfn (IB)	91.1	96.1	91.6/93.8	85.2/74.3	86.7
LS (II)	71.8	76.6	72.8/75.5	64.7/52.0	66.9
Trk7 (II)	71.8	76.6	72.8/75.3	63.4/52.0	66.3
LY (II)	71.8	76.6	72.9/75.1	65.0/52.0	65.5
Q (II)	72.0	76.6	73.0/74.3	-/-	65.6
Bx (III)	88.4	84.7	89.1/87.0	78.4/70.0	86.1
PHz (III)	88.3	84.7	89.0/89.7	78.7/70.9	87.1
PSV-P (I)	58.5	62.5	58.5/53.8	50.7/35.8	59.0
PSV-W (II)	58.4	64.8	58.9/53.9	-/-	-
PSV-Mi (III)	59.9	61.1	59.2/55.1	52.2/40.2	66.3
TAV-KC	61.5	68.8	60.7/58.8	43.9/28.7	68.8

Table 5. Sequence identity between the RNAs, ORFs, and encoded proteins of the CMV-209 strain and those of other strains of CMV, PSV, and TAV (*continued)*

Virus strains	RNA3					
	Complete	5′UTR	ORF3a (nt/aa)	IR	ORF3b (nt/aa)	3′UTR
Fny (IA)	93.0	95.4	91.9/92.8	89.8	94.4/95.9	93.0
Rs (IA)	93.1	95.4	92.0/92.8	90.1	94.2/95.9	93.7
Y (IA)	92.4	89.7	91.5/93.2	90.1	93.6/94.0	93.0
Legume (IA)	92.8	92.5	92.1/93.2	90.1	93.9/95.0	92.3
Mf (IA)	93.0	93.5	92.6/92.8	89.1	94.1/95.4	93.0
New Delhi (IB)	91.7	91.6	89.1/83.2	87.8	94.2/96.3	92.3
PI1 (IB)	92.7	91.6	92.5/92.8	86.3	93.8/95.9	92.3
NT9 (IB)	93.4	93.5	92.9/93.2	88.5	94.4/96.3	92.3
Tfn (IB)	93.2	93.5	93.0/93.2	86.3	94.7/96.8	92.0
LS (II)	75.4	46.4	78.7/81.4	70.9	75.0/81.6	70.7
Trk7 (II)	74.9	46.4	77.3/82.1	69.7	74.3/80.2	71.0
LY (II)	75.2	45.2	78.9/81.7	70.9	75.0/82.0	70.7
Q (II)	75.2	47.0	78.5/81.4	70.9	75.5/82.5	70.7
Bx (III)	89.4	90.6	90.0/91.4	82.4	91.6/94.0	88.0
PHz (III)	89.6	89.4	90.6/91.4	83.9	91.2/94.0	86.9
PSV-P (I)	57.8	40.0	65.7/66.7	48.8	26.5/47.7	62.1
PSV-W (II)	56.1	37.2	45.8/61.5	48.8	52.2/39.7	62.6
PSV-Mi (III)	57.0	39.4	65.0/64.5	45.8	51.7/48.4	63.4
TAV-KC	58.0	40.0	67.2/67.5	58.2	53.3/43.1	67.7

IV. Phylogenetic analysis

First, phylogenetic trees for each CMV-209 genomic segment were constructed, in which PSV and TAV were used as out groups. The trees revealed obviously that CMV-209 was clustered in subgroup I. For all three RNAs, a first branching distinguished from clear clustering of group II strains, a second one separated into subgroup III, and the next branching of the trees precisely divided subgroups IA, IB, and CMV-209. In the case of RNAs 1 and 2, CMV-209 shared same branching points to subgroup IA (Fig. 4A, B), suggesting that these two RNAs of CMV-209 had closer relationship to subgroup IA than subgroup IB. Although RNA3 of CMV-209 had the same progenitor with subgroup IA and IB, it formed a distinct branch before separation of branches of subgroup IA and IB (Fig. 4C).

Next, phylogenetic analyses of 1a, 2a, 2b, 3a, and CP genes based on amino acid sequences were established. The 2a and 3a protein trees had identical patterns with the full length RNA 2 and 3 nt sequence trees, respectively (Fig 5B, D). The 1a and 2b protein trees presented a complete segregation of CMV-209 out of subgroups IA and IB (Fig. 5A, C), whereas 1a and 2b ORF nt sequence trees (data not shown) and genomic RNA 1 and 2 trees showed CMV-209 close to subgroup IA. Both CP aa sequence tree and CP nt sequence tree (data

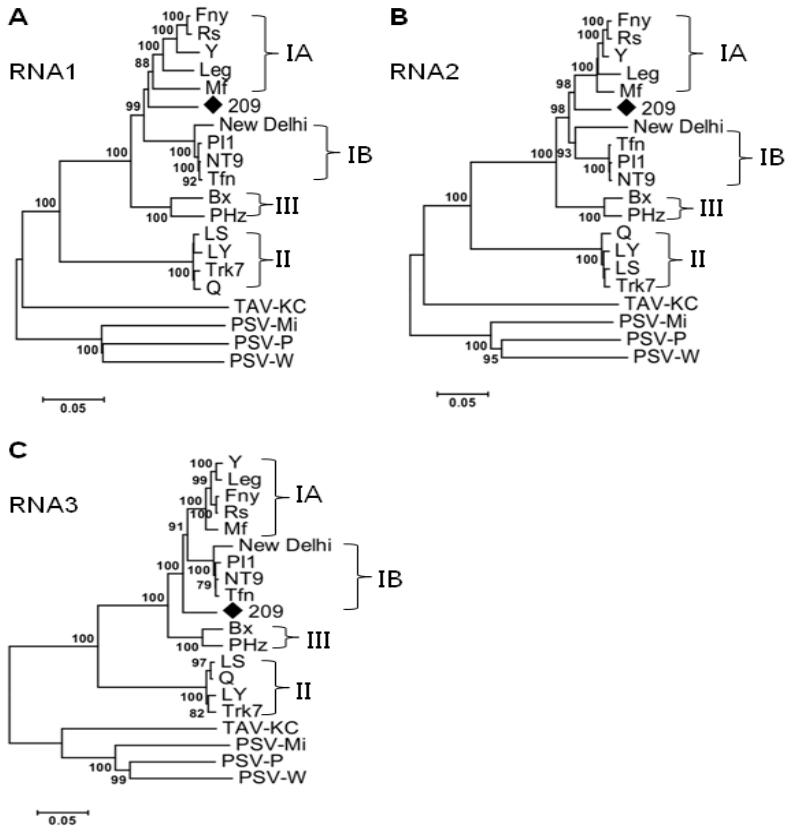

Fig. 4. Phylogenic trees of cucumoviruses obtained from analysis in MEGA 6 for each genomic segment (panels A, B, and C for RNA1, 2, and 3, respectively). Numbers at nodes indicate the percent occurrence of nodes in 1000 bootstrap resampling. Bootstrap values lower than 70 are not indicated. Roman numerals indicate respective CMV subgroubs.

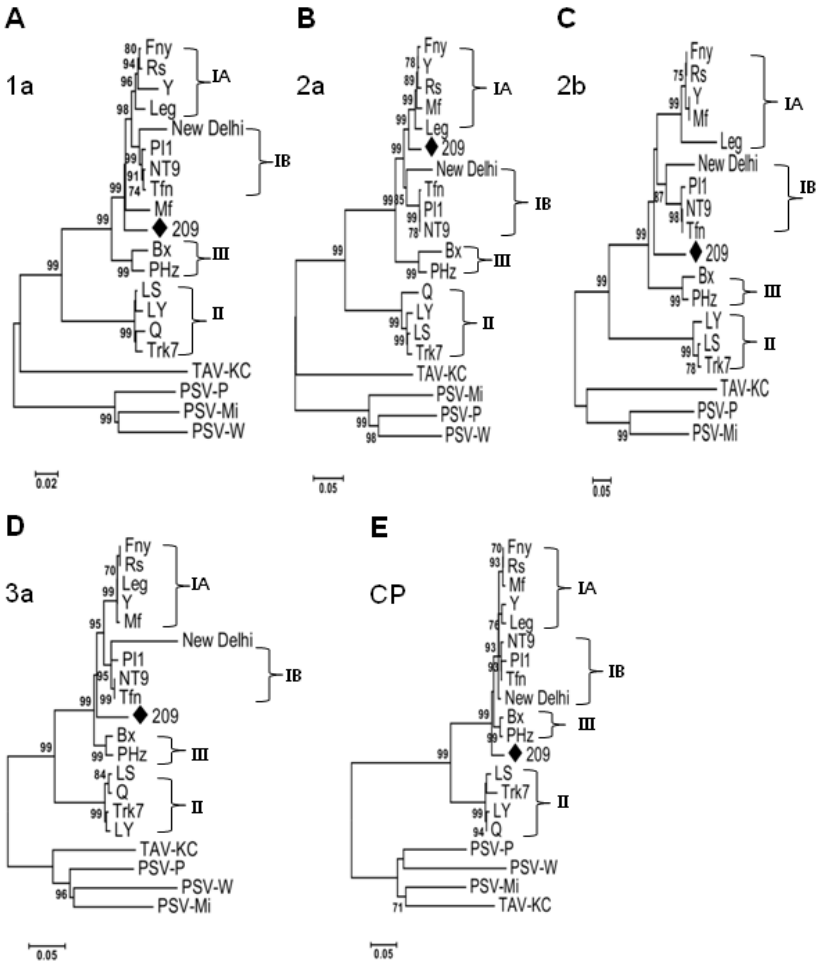

Fig. 5. Phylogenic trees of cucumoviruses obtained from analysis in MEGA 6 for each ORF amino acid sequences. Numbers at nodes indicate the percent occurrence of nodes in 1000 bootstrap resampling. Bootstrap values lower than 70 are not indicated. Roman numerals indicate respective CMV subgroubs.

not shown) exhibited that CMV-209 was located in a new branch separated from subgroups IA and IB; however, CP protein tree gave a clearer separation. This may suggest that the difference of the biological characteristics among CMV-209 and subgroups IA and IB appeared at amino acid level. In general, amino acid phylogenetic analyses of CMV-209 and the CMV strains had unquestionably placed CMV-209 in another subgroup of subgroup I.

In summary, our analyses suggest molecular evidences that CMV-209 may belongs to a distinct subgroup within CMV subgroup I and that the adaptation of CMV-209 in pea (*P. sativum*) could be due to aa changes of RNA 3 segment based upon sequence analyses. Further study will test whether RNA3 is indeed responsible for adapting CMV-209 in pea by inoculating swapped RNA segments with the other CMV strains and mutant clones which contain site-directed mutations.

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CHAPTER 2

Pseudorecombination between two distinct strains of *Cucumber mosaic virus* results in enhancement of symptom severity

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The Plant Pathology Journal (2014), http://dx.doi.

org/10.5423/PPJ.NT.04.2014.0031

ABSTRACT

Recently, a *Cucumber mosaic virus* (CMV) strain, named as CMV-209, was isolated from *Glycine soja*. In this study, symptom expression of CMV-209 was analyzed in detail in *Nicotiana benthamiana* by comparing with that of CMV-Fny, which is a representative strain of CMV. Using infectious cDNA clones of CMV strains 209 and Fny, symptom expression of various pseudorecombinants between these two strains were examined in the early and late infection stages. In the early infection stage, the pseudorecombinants containing Fny-RNA2 induced stunting and leaf distortion on the newly emerged leaves whereas the pseudorecombinants containing 209-RNA2 caused no obvious symptoms. In the late infection stage, the pseudorecombinants containing 209-RNA1 and Fny-RNA2 induced severe leaf distortion and stunting, while CMV-209 induced mild symptom and CMV-Fny caused typical mosaic, general stunting and leaf distortion symptoms, indicating that RNA 2 encodes a symptom determinant(s) of CMV, which is capable of enhancing symptoms. Furthermore, our results support the possibility that natural recombination between compatible viruses can result in emergence of novel viruses causing severe damages in crop fields.

INTRODUCTION

Cucumber mosaic virus (CMV) is an economically important plant pathogen all over the world and has a broad host range of more than 1,200 plant species (Palukaitis and García-Arenal, 2003). The CMV genome comprises of three single-stranded plus-sense RNAs (Palukaitis et al., 1992). RNA1 encodes one large 1a protein which contains a putative methyltransferase domain (Mi et al., 1989; Mi and Stollar, 1991; Rozanov et al., 1992) and helicase motif (Gorbalenya et al., 1989; Habili and Symons, 1989; Hodgman, 1988). RNA2 encodes the 2a protein containing the GDD motif typical for RNA-dependent RNA polymerase (RdRp) (Hayes and Buck, 1990) and one small 2b protein which has a function of a silencing suppressor (Brigneti et al., 1998; Lucy et al., 2000). RNA3 encodes the viral movement protein (3a protein) and the capsid protein (CP) (Davies and Symons, 1988; Ding et al., 1995a; Kaplan et al., 1995). The 2b protein and CP are translated from the subgenomic RNA4A (Ding et al., 1994) and RNA4 (Schwinghamer and Symons, 1975), respectively.

The specific strains of CMV have been characterized to induce various symptoms on different hosts (Palukaitis et al., 1992). In tobacco, most strains of CMV induced a light-green or dark-green mosaic

(Takanami, 1981). However, some strains induced distinct symptoms such as leaf distortion and stunting (Szilassy et al., 1999), bright yellow chlorosis (Fulton, 1950; Takanami, 1981) or necrotic lesions (Divéki et al., 2004; Troutman and Fulton, 1958; Zhang et al., 1994). To date, symptom determinants of CMV have been analyzed extensively and almost the location of these symptom determinants were either on RNA 3 (Shintaku, 1991; Shintaku et al., 1992; Suzuki et al., 1995; Szilassy et al., 1999; Zhang et al., 1994) or RNA 2 (Ding et al., 1995b; Ding et al., 1996; Du et al., 2007; Du et al., 2008; Shi et al., 2002; Soards et al., 2002; Ziebell et al., 2007). In a few cases, the determinants of expression symptom has been mapped to RNA 1 (Divéki et al., 2004) or both RNA 1 and RNA 2 (Zhang et al., 1994).

CMV-209 is a CMV strain, which was recently isolated from *Glycine soja*. In this study, symptom expression of CMV-209 was analyzed in *Nicotiana benthamiana* based on pseudorecombination of genomic RNAs of CMV-209 with those of CMV-Fny, which is a typical strain of CMV. Our results demonstrated that, in the early infection stage, the pseudorecombinants harboring Fny-RNA2 caused stunting and leaf distortion on the newly emerged leaves although the pseudo-recombinants holding 209-RNA2 exhibited no obvious symptoms. In the late infection stage, the pseudorecombinants consisting of 209-

RNA1 and Fny-RNA2 induced severe leaf distortion and stunting, while CMV-209 induced mild symptom and CMV-Fny caused typical mosaic, general stunting and leaf distortion symptoms, indicating that the symptom determinant(s) of CMV is included in RNA 2 and is capable of enhancing symptom severity. Additionally, our results support the possibility that novel viruses causing severe damages in crop fields can be emerged by natural recombination between compatible viruses.

MATERIALS AND METHODS

I. Virus source and construction of the full-length cDNA clones of CMV-209 and CMV-Fny

The full-length cDNAs of genomic RNA of CMV-209 and -Fny were amplified using the cDNA clones of CMV 209 (i.e. pCMV-209R1, pCMV-209R2 and pCMV-209R3) (Vo Phan et al., 2014) and Fny [i.e. pCR1 (+), pCR2 (+) and pCR3 (+)] (Seo et al., 2009) as templates. The primers, CMV-R1R2-5-end-T7, CMV-R3-5-end-T7, and CMV-3-end-*SmaI* (Table 1), were used for PCR, thereby the resulting products contained a T7 promoter and *SmaI* site at the 5' and 3' ends, respectively. The resulting PCR products were cloned directly into pGEM®-T Easy vector (Promega, USA). The final cDNA clones of CMV-209 and -Fny constructed for *in vitro* transcription were named pT7-CMV-209R1, pT7-CMV-209R2, pT7-CMV-209R3, pT7-CMV-FnyR1, pT7-CMV-FnyR2, and pT7-CMV-FnyR3, respectively (Fig. 1).

Table 1. Primers used for CMV-detection and construction of the CMV cDNA clones

Name	Sequence (5′ → 3′)[a]
CMV-3-end-*Sma*I	G**CCCGGG**TGGTCTCCTTTTRGAG RCC
CMV-R1R2-5-end-*T7*	*taatacgactcactata*GTTTATTTACAAG AGCGTACGG
CMV-R3-5-end-*T7*	*taatacgactcactata*GTAATCTTACCACT GTGTGTGTG
CMV-detR1-F	TTTTTGAACCGTCCACTGACATGA
CMV-detR1-R	AGTGAAGCCTTATCGGCTTGGG

[a] The *Sma*I sequence is shown in boldface and the sequence of T7 promoter is written in italic.

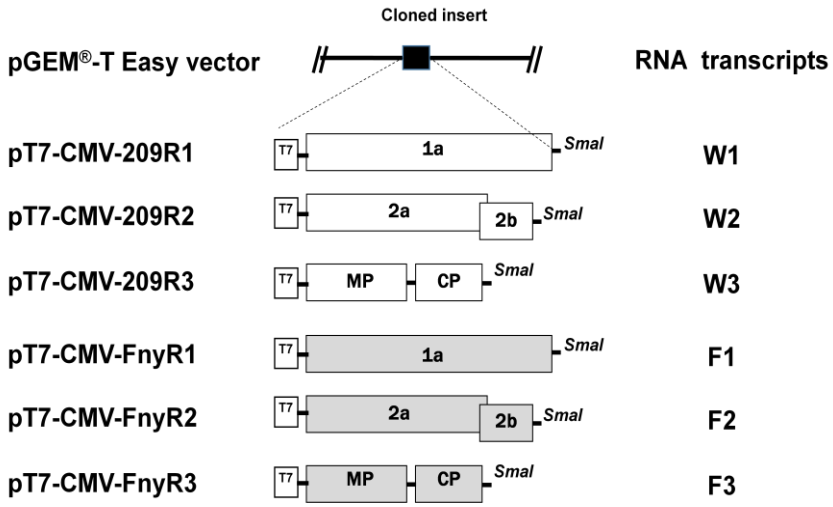

Fig. 1. Schematic diagram of construction of infectious cDNA clones of CMV-209 and CMV-Fny strains and names of their RNA transcripts. The constructs pT7-CMV-209R1, pT7-CMV-209R2, pT7-CMV-209R3 and pT7-CMV-FnyR1, pT7-CMV-FnyR2, pT7-CMV-FnyR3 express RNA1, RNA2 and RNA2 of CMV strain 209 and Fny, respectively. W1, W2, W3 and F1, F2, F3 are RNA transcripts synthesized from templates pT7-CMV-209R1, pT7-CMV-209R2, pT7-CMV-209R3 and pT7-CMV-FnyR1, pT7-CMV-FnyR2, pT7-CMV-FnyR3 , respectively by *in vitro* transcription.

II. Construction and characterization of pseudorecombinants of CMV-209 and CMV-Fny

The CMV-209 and -Fny clones were linearized by *SmaI*. The cDNA-derived infectious RNAs of CMV-209 and –Fny were generated by *in vitro* transcription with T7 RNA polymerase (Takara, Japan) in the presence of 7-methylguanosine "cap" structure (Promega, USA) according to the manufacturer's protocol. The *in vitro* transcripts from the full-length cDNA clones of CMV-209 and -Fny RNAs 1, 2 and 3 were named W1, W2, W3, F1, F2, and F3, respectively (Fig. 1) and mixed to mimic the original CMV RNAs (i.e. CMV-209, W1W2W3; CMV-Fny, F1F2F3). Six different pseudorecombinants, W1W2F3, W1F2W3, F1W2W3, F1F2W3, F1W2F3, and W1F2F3, were also generated by mixing the *in vitro* transcripts. All of the parental and pseudorecombinant RNAs were diluted in the inoculation buffer (0.05 M potassium phosphate, pH 7.5) and total 1.5 μg of RNA transcripts (500 ng of each RNA transcript 1, 2 and 3) was inoculated per plant. All the *N. benthamiana* plants inoculated with transcripts were lightly dusted with carborundum before inoculation. Mock-treated plants were inoculated with the inoculation buffer. The inoculated plants were maintained in an insect-free green house at 25^{o}C under a 14 h photoperiod. The disease symptoms were observed at two stages, the

early infection stage [7 days post inoculation (dpi)] and the late infection stage (12 dpi). CMV infection of the inoculated plants was confirmed by RT-PCR at 7 dpi using primers CMV-detR1-F and CMV-detR1-R (Table 1).

RESULTS

I. CMV RNA 2 contains a symptom determinant (s) causing stunting and leaf distortion

The *N. benthamiana* plants infected with CMV-Fny (F1F2F3) showed general stunting, vein clearing and leaf distortion on the infected systemic leaves in the early infection stage (Fig. 2C). In contrast, the plant inoculated with CMV-209 (W1W2W3) developed no obvious symptoms and indistinguishable from healthy plants although they were systemically infected with CMV-209 as confirmed by a specific RT-PCR (Fig. 2B and 3). Thus, we sought to examine what differences between two strains of CMV affect symptom expression. To this end, we performed inoculation assays using pseudorecombinants of CMV-Fny and-209. Six pseudorecombinants (F1W2W3, W1F2W3, W1W2F3, W1F2F3, F1W2F3 and F1F2W3) were inoculated in *N. benthamiana*. At 7 dpi, the symptoms induced in the inoculated *N. benthamiana* plants were observed in detail (Fig. 2 and Table 2). Viral infection of the inoculated plants was confirmed by RT-PCR using total RNA extracted from upper un-inoculated leaves (Fig. 3). Interestingly, pseudorecombination resulted in induction of different symptoms according to combinations of viral genomic RNAs. The pseudorecombinant of F1W2W3 induced vein clearing but no leaf

distortion on the upper leaves of infected plants (Fig. 2D). The pseudorecombinants containing F2 (i.e. F1F2W3, W1F2F3, and W1F2W3) produced leaf distortion and stunting symptoms (Fig. 2E, G and I). However, two pseudorecombinants of W1W2F3 and F1W2F3 induced no obvious symptoms (Fig. 2F and H). In summary, the pseudorecombinants containing F2 induced stunting and leaf distortion on the newly emerged leaves, while those containing W2 (except for F1W2W3, which induced vein clearing) caused no obvious symptoms in the early infection stage. Therefore our results suggested that CMV RNA 2 contains a symptom determinant(s) causing stunting and leaf distortion in the early infection stage.

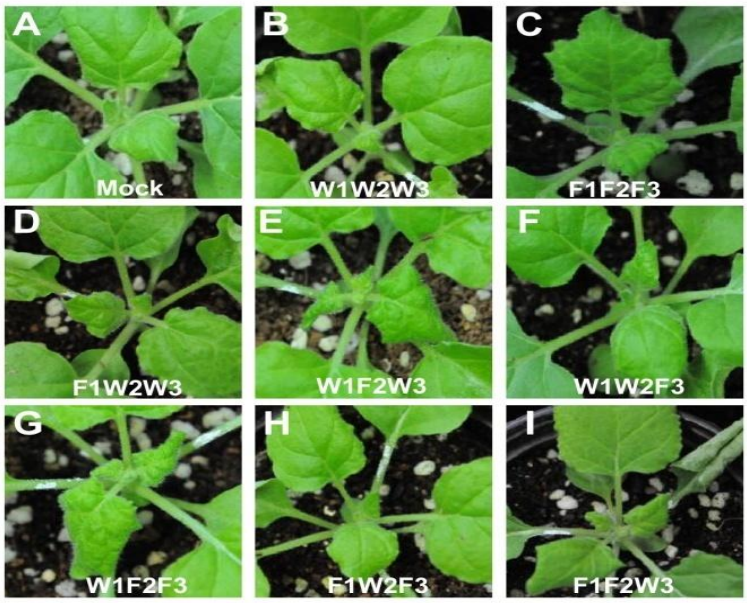

Fig. 2. Symptoms induced in *Nicotiana benthamiana* by CMV-209, CMV-Fny and their pseudorecombinants constructed *in vitro* from the three genomic segments of the two virus strains at 7 dpi. W1W2W3 (CMV-209), W1W2F3 and F1W2F3 caused mind symptom (B, F, H, respectively). F1F2F3 (CMV-Fny) and F1F2W3 caused general stunting, vein clearing and leaf distortion (C, I, respectively). F1W2W3 induced vein clearing symptom (D). W1F2W3 and W1F2F3 induced severe stunting and leaf distortion (E, G, respectively). Mock represents healthy plant. Photographs were taken at 7 days post inoculation.

Table 2. Characteristics of symptoms induced by CMV-209, CMV-Fny and their pseudorecombinants on *Nicotiana benthamiana* plants

Inoculum[a]	Disease symptom[b]/severity of systemic symptom[c]	
	7 dpi	12 dpi
W1W2W3	ns/-	ns/-
W1W2F3	ns/-	ns/-
W1F2W3	ld, gst/++	sld, sst/++++
F1W2W3	vc/+	vc, mld/+
F1F2F3	vc, ld, gst/++	vc. mld, smo /+++
F1F2W3	vc, ld, gst/++	vc, mmo/++
F1W2F3	ns/-	ns/-
W1F2F3	ld, gst/++	sld, sst/++++

[a]Inoculums of CMV-209, CMV-Fny and their pseudorecombinants were generated by mixing of *in vitro* RNA transcripts.

[b] Symbols: ns, no obvious symptom; ld, leaf distortion; sld, severe leaf distortion; mld, mild leaf distortion; gst: general stunting; sst, severe stunting; vc, vein clearing; smo, severe mosaic; mmo; mild mosaic.

[c] -, no obvious symptom, +, mild symptom; ++, intermediate symptom; +++, severe symptom; ++++, very severe symptom.

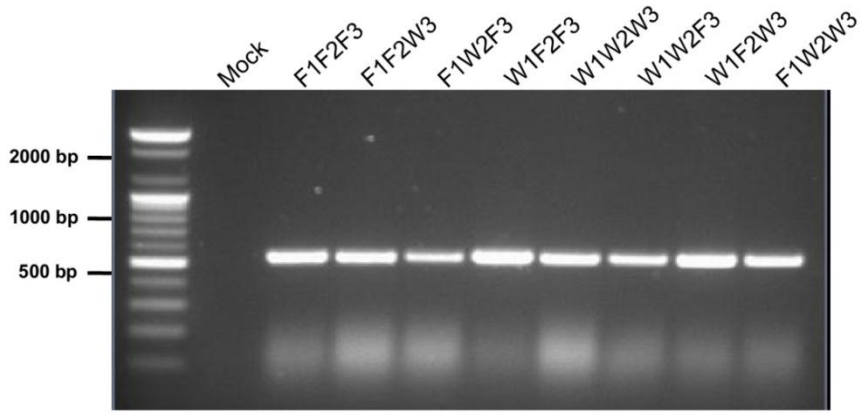

Fig. 3. Detection of CMV in *N. benthamiana* plants infected by CMV-209, CMV-Fny and their pseudorecombinants. Total nucleic acids were extracted from un-inoculated upper leaves of infected plants at 7 days post inoculation, and subjected to RT-PCR analysis using primer CMV-detR1-F and CMV-detR1-R.

II. The compatibility among CMV genomic RNA segments plays a role in symptom expression

We further observed the infected plants to investigate whether the symptoms induced by the pseudorecombinants could be intensified in the late infection stage. To this end, the *N. benthamiana* plants infected with each pseudorecombinant were observed at 12 dpi. Similar to the observation in the early infection stage, the plant inoculated with CMV-209 (W1W2W3) exhibited no obvious symptoms and indistinguishable from healthy plants (Table.2, Fig. 4B), whereas CMV-Fny (F1F2F3) induced vein clearing, general leaf distortion and severe mosaic symptoms on the infected systemic leaves at 12 dpi (Table.2, Fig. 4C). The pseudorecombinants, W1W2F3 and F1W2W3, also did not cause any obvious symptoms as similar to CMV-209 (Table.2, Fig. 4F and H). However, the pseudorecombinants, W1F2W3 and W1F2F3 containing CMV-Fny RNA 2 induced produced severe leaf distortion and stunting symptoms (Table.2, Fig. 4E and G). Exceptionally, the plant infected with F1F2W3, showed vein clearing and mild mosaic symptoms (Table.2, Fig. 4I), while those infected with F1W2W3 developed vein clearing and mild leaf distortion in the late infection stage (Table.2, Fig. 4D). Our results implied that the compatibility among CMV genomic RNA segments plays a role in symptom

expression and, as observed in the cases of W1F2W3 and W1F2F3, pseudorecombination between distinct strains of a virus species can result in emergence of virus progenies carrying higher infectivity or causing severe damages.

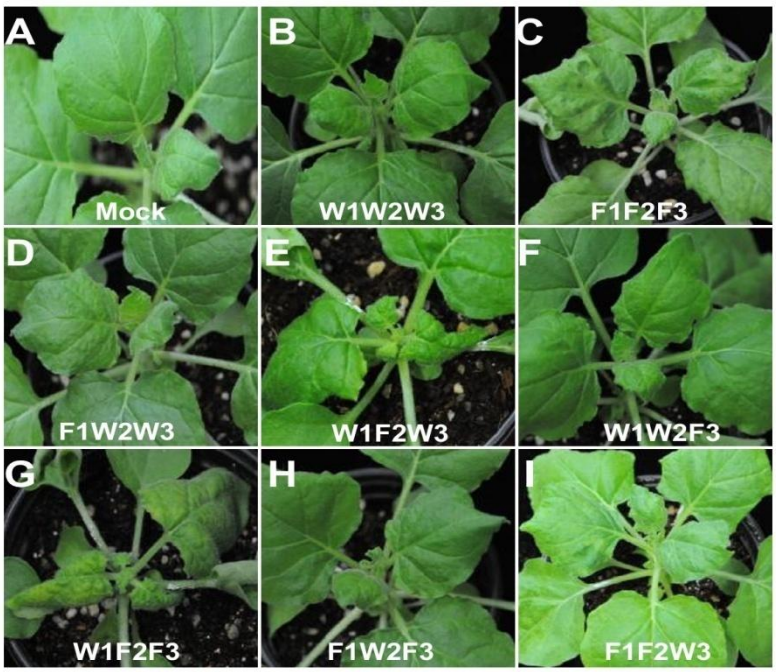

Fig. 4. Symptoms induced in *Nicotiana benthamiana* by CMV-209, CMV-Fny and their pseudorecombinants constructed *in vitro* from the three genomic segments of the two virus strains at 12 dpi. W1W2W3 (CMV-209), F1W2F3, W1W2F3 and F1W2F3 caused mind symptom (B, D, F, H, respectively). F1F2F3 (CMV-Fny) and F1F2W3 caused general stunting, mosaic and leaf distortion (C, I, respectively). W1F2W3 and W1F2F3 induced severe stunting and leaf distortion (E, G, respectively). Mock represents healthy plant. Photographs were taken at 12 days post inoculation.

DISCUSSION

In general, disease symptom development of *N. benthamiana* plant induced by CMV-Fny could be divided into two visually distinct stages. Under our plant growth condition, the newly emerged leaves after inoculation of CMV-Fny initially showed vein clearing and general stunting and leaf distortion at 7 dpi (Early infection stage, Fig. 2C) and then the next leaves emerged at 12 dpi showed green mosaic (Late infection stage, Fig. 4C). CMV-Fny RNA 1 might play a role in vein clearing production in the early infection stage, because it was evident that the pseudorecombinants, F1W2W3 and F1F2W3 induced vein clearing while the other recombinants containing W1 did not (Fig. 2). Zhang et al. (1994) also suggested that both RNAs 1 and 2 of CMV-Fny were involved in determining the severity of systemic symptom on tobacco working with CMV-Fny and CMV-LS and (Zhang et al., 1994). However, Rao and Franki (1982) handled with eighteen pseudorecombinants constructed *in vitro* from three strains of CMV (CMV-U, CMV-M and CMV-K) showed that RNA 1 had little effect on symptom induction (Rao and Francki, 1982). The fact that involvement of CMV RNA 1 in symptom determination was reported rather rare, except for mapping of the genetic determinant for necrosis

of CMV-Ns to the protein 1a (Divéki et al., 2004).

Our results clearly show that CMV-Fny RNA 2 played a crucial role in symptom induction in both the early and late infection stage. F1W2F3 caused no obvious symptom (Fig. 2H, 4H) while W1F2W3 induced stunting and leaf distortion (Fig. 2E, 4E) all the time during the viral infection. According to the previous reports that the mutant CMV-Fny-Δ2b, in which the 2b ORF was deleted, could move systemically in tobacco and *N. benthamiana* but could not induce symptom (Soards et al., 2002; Ziebell et al., 2007) and transgenic plants expressing the CMV-Fny 2b protein exhibited strong symptom-like phenotypes such as leaf distortion, general stunting (Lewsey et al., 2007), CMV-Fny 2b protein seemed to have ability to control symptom expression (Ding et al., 1995b; Ding et al., 1996; Du et al., 2007; Shi et al., 2002; Soards et al., 2002). Recently, the importance of specific domains within the 2b protein for symptom induction has been investigated carefully (Ding et al., 1996; Goto et al., 2007; Lewsey et al., 2009). Lewsey et al. (2009) demonstrated that two nuclear localization signals (NLS1 and NLS2) and the N-terminal domain (5T) of CMV-Fny 2b protein were required and essential for symptom induction, respectively.

The C-terminal domain (3T) and two serine residues within a putative

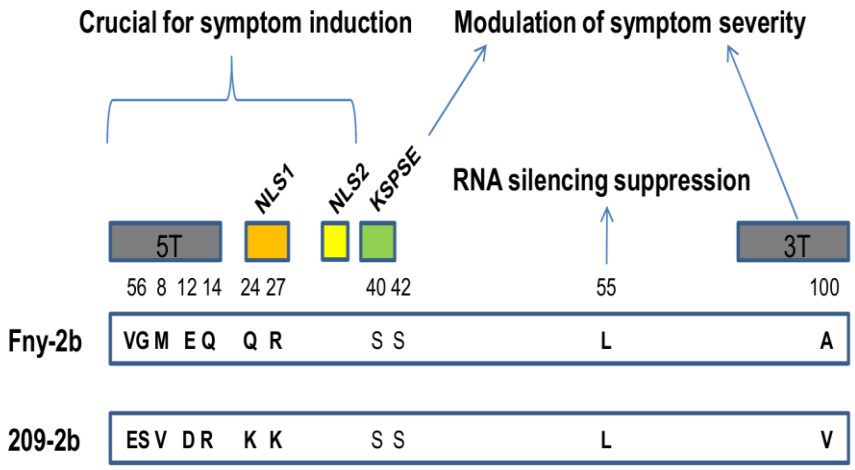

Fig. 5. Schematic map describes locations and roles of amino acids in the 2b protein of CMV-Fny and -209. 5T, the first 17 amino acids of 2b protein; NLS, arginine-rich nuclear localization sequence; KSPSE, the putative phosphorylation sequence; 3T, the 16 C-terminal residues of the 2b protein. Numbers indicate amino acid residue position.

phosphorylation domain (KSPSE) of CMV-Fny 2b modulated symptom severity (Fig. 5). The comparison of amino acids sequence of 2b protein of CMV-Fny and CMV-209 revealed that most of various amino acids were located at the N-terminal domain, two of them at NLS1 domain and one at C-terminal domain (Fig. 5). It is worth to determine which amino acid residues are responsible for symptom induction. Interestingly, although CMV-209 caused latent symptom on *N. benthamiana* (Fig. 2B, 4B), the amino acid residues of the 2b protein at position 55 being Leu was conserved in CMV-209 (Fig. 5), which has been reported to be crucial for the RNA silencing suppression and the induction of viral symptoms (Xu et al., 2013). It remains to be examined whether this conserved amino acid residue is actually responsible for suppressing silencing.

Reassortment of genomic segments is one of the mechanisms for genetic variation and new strain generation of multipartite RNA viruses (Morse, 1994). There is evidence that reassortment occurs in natural populations of plant virus (Chen et al., 2007; Fraile et al., 1997; Lin et al., 2004; Maoka et al., 2010). In spite of some studies demonstrated that the reassortment between CMV subgroups was a rare event (Bonnet et al., 2005; Fraile et al., 1997; Jacquemond, 2012), it did not mean that reassortment was not important in CMV evolution (Lin et

al., 2004; Roossinck, 2002; White et al., 1995). For example, CMV-Tsh was a natural reassortant between CMV subgroup IA and II strains, in which RNAs 1 and 3 of CMV-Tsh was derived from one or two subgroup II strain(s), while RNA2 was derived from a subgroup I strain, revealed an evolution than its parents. It was probably that RNA 2 of subgroup II was replaced by RNA 2 of subgroup IA due to its low efficiency in suppression of host defense mechanism during the mixed infection of CMV-Tsh parental viruses (Chen et al., 2007). In the present work, we found that the reassortment between CMV-209 and CMV-Fny caused more severe symptom than wild-type strains (e.g. W1F2W3 and W1F2F3 induced severe leaf distortion and stunting (Fig. 4E, G) whereas W1W2W3 showed latent symptom (Fig. 4B) and F1F2F3 exhibited mosaic, leaf distortion and general stunting (Fig. 4C)). In addition, we also observed that a pseudorecombinant comprising of RNA 1 of CMV-209 and RNAs 2 and 3 of CMV-12, an isolate form Azuki bean, showed more severe symptom than its parents (data not shown). These results indicated that natural reassortment between compatible viruses can result in emergence of novel viruses causing severe damages in crop fields.

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Glycine soja 에서 분리 동정된 *Cucumber mosaic virus* 의 분자 생물학적 특성 구명

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초록

기주적응은 바이러스의 진화에 있어 바이러스 게놈서열의 수렴과 변이에 주요한 요인 중 하나이다. 이 연구에서 우리는 *Glycine soja* (wild soybean) 로부터 CMV-209라 명명한 CMV strain을 분리하였으며 전체 게놈의 감염 가능한cDNA clone을 제작함으로써 분자적, 생물학적 특징을 규명하였다. CMV-209의 전체 게놈 서열을 이용한 계통유연학적 분석은 CMV-209 strain이 CMV의 subgroup I에 속한다는 것을 보여주었으나, 기존 subgroup IA와 subgroup IB에 속하는 CMV strain들과는 서열상 89-93%의 상동성을 보이는 것으로 보아 IA 또는 IB에도 속하지 않은 것으로 나타났다. 또한 CMV-209 게놈의 각각의 segment와 각각의 바이러스 단백질에 대한 계통학적 분석은 CMV-209가 subgroup IA와 IB와는 다른 계통에 위치한다는 것을 나타냈으며 CMV-209의 기주범위에 대한 조사는 CMV-209가 기주 선택적 strain이라는 것도 나타냈다.

CMV는 *Vigna sinensis*에서는국부 병반을야기하고 *Pisum sativum*과 *Glycine soja*에서는전신감염하였으나 실험에 사용된 여타

콩과 다른 품종들 대부분은 감염하지 못했다. CMV-209는 전형적인 CMVstrain인 CMV-Fny가 콩과 식물을 감염시키지 못한다는 것을 고려했을 때 콩과 식물을 감염시킬 수 있도록 적응해 온것으로 보여진다.한편 본 연구에서는 CMV-Fny와의 비교를 통하여 CMV-209의 *Nicotiana benthamiana*에서의 병징에 대한 분석을 수행하였으며 CMV-Fny가 *Nicotiana benthamiana*에감염하여 모자이크, 왜화, 잎의 기형화 등의 전형적인 병징을 보이는데 반해 CMV-209는 눈에 뛰는 병징을 보이지 않으면서 감염하는 잠복감염의 특성을 보인다는 것을 알게 되었다. CMV-Fny와 CMV-209의 각 RNA 절편들의 pseudorecombinant들을 이용한 감염실험 결과는 CMV의 RNA2가 병징의 발생에 기여한다는 것을 증명하였다. 예를 들면 Fny-RNA2는 모자이크, 왜화또는 잎의 기형화를 나타내는데 반해 209-RNA2는 잠복 감염의 특성을 보인다.또한 우리는 구별된 strain간의 pseudorecombination이 더욱 강한 감염성과 심각한 피해를 야기할 수 있다는 점을 통해 strain간의 RNA 절편들이 서로 호환 가능하다는 것을 관찰하였다. 209-RNA1과 Fny-RNA2를 지닌 pseudorecombinant는 Fny-RNA1과 Fny-RNA2의 그것에 비해 더욱 심한 잎의 기형화 및 왜화 증상을 나타내었다.요약하면 본 연구를 통하여 CMV-209는 *Cucumovirus*속 CMVsubgroup I에 보다 가까운 새로운 strain이라는 것을 보여준 것이다.