농학석사학위논문

Construction of infectious full-length clones of *Cucumber green mottle mosaic virus* and *Zucchini yellow mosaic virus* isolated from cucurbitaceous crops in Korea and their application as viral vectors

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2014년 2월

서울대학교 대학원

농생명공학부 식물미생물학 전공

강민지

A THESIS FOR DEGREE OF MASTER SCIENCE

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BY
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February 2014

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2014년 2월

위 원 장 _____ (인)

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A THESIS FOR DEGREE OF MASTER OF SCIENCE

Construction of infectious full-length clones of *Cucumber green mottle mosaic virus* and *Zucchini yellow mosaic virus* isolated from cucurbitaceous crops in Korea and their application as viral vectors

UNDER THE DIRECTION OF
DR. KOOK-HYUNG KIM

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

BY MINJI KANG

MAJOR IN PLANT MICROBIOLOGY
DEPARTMENT OF AGRICUTURAL BIOTECHNOLOGY

FEBRURY 2014

APPROVED AS A QUALIFIED THESIS OF MINJI KANG
FOR THE DEGREE OF MASTER OF SCIENCE
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ABSTRACT

Construction of infectious full-length clones of *Cucumber green mottle mosaic virus* and *Zucchini yellow mosaic virus* isolated from cucurbitaceous crops in Korea and their application as viral vectors

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Major in Plant Microbiology

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Cucumber green mottle mosaic virus (CGMMV) and *Zucchini yellow mosaic virus* (ZYMV) infect many cucurbitaceous crops causing significant economic damages in many fields. The CGMMV, which belongs to the genus *Tobamovirus*, is a rod-shaped virus with approximately 300 nm in length and contains a plus-sense single strand RNA (ssRNA) genome of 6.4 kb. The ZYMV, which belongs to the genus *Potyvirus*, is a flexuous rod-shaped virus of about 750 nm in length and contains a plus-sense ssRNA of 9.5 kb. In this study, two CGMMV isolates called CGMMV-KW and -KOM, which are

isolated from watermelon and oriental melon, respectively and one ZYMV isolate called ZYMV-PA, which is obtained from pumpkins in Andong, were used to construct full-length infectious clones. RT-PCR products were cloned into binary vector under the control of the *Cauliflower mosaic virus* 35S promoter and NOS terminator and were designated as pCGMMV-KW, pCGMMV-KOM, and pZYMV-PA, respectively. For infectivity test of full-length clones, the pCGMMV-KW and pCGMMV-KOM, which were transformed into *Agrobacterium tumefaciens*, were infiltrated into *Nicotiana benthamiana* by agroinfiltration methods. The two pCGMMVs caused systemic mosaic symptom on *N. benthamiana*. Interestingly, the two pCGMMVs caused local chlorotic spot symptom on *Chenopodium amaranticolor* when saps of *N. benethamiana* infected with these two pCGMMVs were used as inoculums. Since it has been reported that CGMMV-KOM isolate do not infect *C. amaranticolor*, I sequenced the two pCGMMV isolates and compared them with previously reported the CGMMV genome sequences. The difference in sequences between the pCGMMV-KOM and the reported CGMMV-KOM had no relationship with the symptom development on *C. amaranticolor*. Further work is required to identify the factors that are related to the symptom development. When pZYMV-PA was inoculated onto *Cucurbita pepo* L. var. *zucchini* by mechanical methods, same symptoms were observed as that in the previous report. Compared with the previously reported ZYMV-PA genome sequences, the sequence of pZYMV-PA revealed difference of eight amino acids. The pZYMV-PA was engineered as a viral vector, because it shows systemic symptom in cucurbitaceous crops. The ZYMV-based viral vector enables the systemic expression of foreign proteins in cucurbitaceous crops.

This ZYMV-based viral vector was useful in expressing two reporter genes, *gfp* and *bar*, in cucurbitaceous crops indicating that pZYMV-based viral vector can be applied for expression of specific foreign genes in cucurbitaceous plants.

Key words : *Cucumber green mottle mosaic virus*, *Zucchini yellow mosaic virus*, infectious clone, expression viral vector, GFP, herbicide resistance gene and Cucurbitaceous crops

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INTRODUCTION

Cucurbitaceous crops belong to the family *Cucurbitaceae* with about 825 species in 118 genus. In Korea, cucurbitaceous crops including watermelon, oriental melon, cucumber, and squash, are mostly cultivated. According to the reported research from the Rural Development Administration, the cucurbitaceous crops are one of the most important crops and virus diseases of these cucurbitaceous crops are the most serious concern for growers (Kim et al., 2012). Of the viruses that can infect cucurbitaceous crops, *Cucumber green mottle mosaic virus* (CGMMV) and *Zucchini yellow mosaic virus* (ZYMV) infect many cucurbitaceous crops causing significant economic damages in the fields and thus are the economically important viruses infecting Korean cucurbitaceous crops.

CGMMV was first isolated in Korea in 1989 (Lee et al., 1990) and caused widespread ‘blood flesh’ in watermelons and considerable economic damage in 1995 (Lee, 1996). Since then, viral disease of cucurbit plants has been reported occasionally (Park et al., 2001). The disease symptoms caused by CGMMV are diverse which include mottling and systemic mosaic symptoms on leaves and deterioration on fruit in cucurbitaceous crops including watermelon, oriental melon, cucumber, and zucchini (Lee et al., 1991). CGMMV, a member of the genus *Tobamovirus*, is a rod-shaped virus with approximately 300 nm in length and contains a plus-sense single strand RNA (ssRNA) genome of 6.4 kb (Ugaki et al., 1991). CGMMV genome contains 60 and 176 nucleotides of 5′ and 3′ untranslated regions (UTRs), respectively, and four open reading frames (ORF1-4). ORFs 1 and 2 encode proteins of 129 and 186 kDa, which have replicase activity while ORFs 3

and 4 endcode 29kDa and 17.4kDa proteins, which assists virus movement and forms virion, respectively.

ZYMV has been reported on all continents and has become a major pathogen in cucurbitaceous crops worldwide (Antignus et al., 1989) within a decade since the first report (Lisa et al., 1981). ZYMV causes severe stunting and yellowing symptoms on leaves, stream and fruit deformation in Cucurbitaceous crops (Kwon et al., 2005). ZYMV, which belongs to the genus *Potyvirus*, is a flexuous rod-shaped virus of about 750 nm in length and contains a plus-sense ssRNA of 9.5 kb. The ZYMV possesses a covalently linked 5′-terminal viral protein (VPg) and a 3′-terminal poly (A) tail (reviewed in Riechmann et al., 1992). The genome of ZYMV encodes a large ORF and a putative pretty interesting potyviral protein at N terminus of P3 (P3N-PIPO) ORF. The large ORF is cleaved by self-encoded proteases into functional proteins including the coat protein (CP); the helper component protease (HC-Pro); the cylindrical inclusion protein (CI); the combined VPg-protease protein; and RNA replicase. The putative P3N-PIPO ORF of potyviruses was recently described as small putative protein and was deduced within the P3 protein gene (Chung et al., 2008).

Plant RNA viruses are easily able to generate quasispecies in host plants and are thus unstable in maintaining their genome intact. Constructing infectious clones is, therefore, needed to keep biological characteristics and for studying molecular aspects for each viral isolate. Many kinds of plant viral infectious clones under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter or T7 RNA promoter have been constructed. For example, *Potato virus X* (PVX) infectious clone, which belongs to the genus *Potexvirus*,

is able to infect many solanaceous plants (Ruiz et al., 1998) and several tobamovirus infectious clones including *Tobacco mosaic virus* (TMV) infect tomatoes and many other plants (Dawson et al., 1989). In addition, *Soybean mosaic virus* (SMV) infectious clone, which belongs to the genus *Potyvirus*, causing typical SMV symptoms on soybean plants (Seo et al., 2009) was constructed. Plant viral infectious clones can also be applied for the over-expression of special foreign genes in plants.

In this study, to construct the CGMMV and ZYMV infectious clones for further molecular study of the CGMMV and ZYMV Korean isolates (Cho, 1998; Cho, 2002) and to engineer them as expression vectors in cucurbitaceous crops, two CGMMV isolates named CGMMV-KW and -KOM, which are isolated from watermelon and oriental melon, respectively, and one ZYMV isolate called ZYMV-PA, which is obtained from pumpkins in Andong, were used. I constructed two CGMMV and one ZYMV full-length clones and confirmed that these full-length clones are infectious on *Nicotiana benthamiana* and on *Cucurbita pepo* L. var. *zucchini*, respectively. ZYMV-based viral vector was also constructed and tested as vector for expression of foreign gene products on *C. pepo* L. var. *zucchini*.

MATERIAL AND METHODS

1. Source of virus and viral RNA extraction

Two CGMMV isolates (CGMMV-KW and -KOM; Kim et al., 2003) and ZYMV isolate obtained from pumpkins at Andong (ZYMV-PA; Kwon et al., 2005) were propagated in *Cucumis melo* L. (oriental melon) in a greenhouse. To determine the symptom development and the infectivity by these two CGMMV (KW and KOM) isolates and ZYMV-PA isolate, I inoculated three isolates in *N. benthamiana* and *C. pepo* L. var. *zucchini* by mechanical sap inoculation, respectively. Virus-inoculated plants were placed in growth chamber at 25℃ under 16/8 hours photoperiod. The total RNA including the viral RNA was extracted from the virus-inoculated *N. benthamiana* leaves using Total RNA Isolation TRI_{ZOL} Reagent (MRC, USA) following the protocols provided by the manufacturer. The concentrations and qualities of viral RNAs were checked by NanoPhotometer (Implen, Germany).

2. Construction of the CGMMV-KW, CGMMV-KOM and ZYMV-PA infectious full-length clone

Specific primer-pairs were designed based on previously reported CGMMV-KW, CGMMV-KOM, and ZYMV-PA sequences in GenBank (accession numbers: AF417243, AF417242, and AY278998, respectively) to synthesize full-length cDNA for two

CGMMV isolates (KW and KOM) and ZYMV-PA. The full-length cDNA of two CGMMV isolates synthesized using SuperScript™ III reverse transcriptase (Invitrogen, USA) with CGMMV-R (Table 1) was used for PCR amplification of dsDNA corresponding to viral genome using Pfu Ultra II DNA polymerase (Agilent Technologies, USA) with CGMMV-*Sac*I-F and CGMMV-R primer pair (Table 1). Full-length dsDNA of CGMMV-KW and -KOM were then digested with *Sac*I and inserted between the *Sac*I and *Sma*I sites in pSNUI, respectively (Park et al., 2006), which is a modified binary vector including CaMV 35S promoter (Fig. 1A, pCGMMV-KW and CGMMV-KOM). In case of ZYMV, the three partial cDNA fragments of ZYMV-PA were synthesized using SuperScript™ III reverse transcriptase with ZYPA-1807-*BamH*I-R, ZYPA-5938-R and ZYPA-dT-*Bam*HI-R, respectively (Table 1). First, a ZYMV DNA segment from the 5′end of ZYMV to the *Kpn*I site in HC-Pro region was amplified by Phusion® High-Fidelity DNA Polymerase (NEB, England) using ZYPA-F and ZYPA-1807-*Bam*HI-R (Table 1) and digested with *Bam*HI. The digested PCR product was inserted between *Stu*I and *Bam*HI of pCass-RZ, which is a binary vector including CaMV 35S promoter. The resulting subclone was named pZYMV-F1. Secondly, a ZYMV DNA segment from *Kpn*I site in HC-Pro to *Bam*HI site in VPg region was amplified by Phusion® High-Fidelity DNA Polymerase using ZYPA-1788-F and ZYPA-5938-R (Table 1). This PCR product was digested with *Kpn*I and *Bam*HI and was inserted between *Kpn*I and *Bam*HI sites of ZYMV-F1 subclone. This subclone was named pZYMV-F2. Finally, the last ZYMV DNA segment was amplified by Phusion® High-Fidelity DNA Polymerase using ZYPA-5911-F and ZYPA-*Bam*HI-R (Table 1) and digested with *Bam*HI. The amplified PCR product

was cloned into pZYMV-F2, which was digested with *Bam*HI (Fig. 1 B). The final clone was designated as pZYMV-PA.

Table 1. CGMMV and ZYMV specific primers used for construction of full-length clones

Primers	Sequences (5′-3′)
CGMMV-*Sac*I-F	C GAGCTCGTTTTAATTTTTATAATTAA ACAAACAACAACAACAAC
CGMMV-R	TGGGCCCCTACCCGGGGAAA
ZYPA-F	AAAATTGAAACAAATCACAAAGACTATAAG
ZYPA-1807-*Bam*HI-R	AC GGATCC AACTTACG**GGTACC**ATTT
ZYPA-1788-F	CAAAT**GGTACC**CGTAAGTTGG
ZYPA-5938-R	CCAGTGAGA**GGATCC**ACAA
ZYPA-5911-F	CATCAGATTTGT**GGATCC**TCTC
ZYPA-*Bam*HI-R	GCTG GGATCC GTGGTAAA
ZYPA-dT-*Bam*HI-RT	*GCTG GGATCC* *GTGGTAA*ATTTTTTTTTTTTTTTTTTTTTT

F, sense primer; R, antisense primer; RT, antisense primer for Reverse transcription; Restriction sites inserted for cloning are underlined; The bold type faces show the restriction sites that the virus itself contains; The tagging enzyme sites are italicized to hybridize to the ZYMV poly(A) tail.

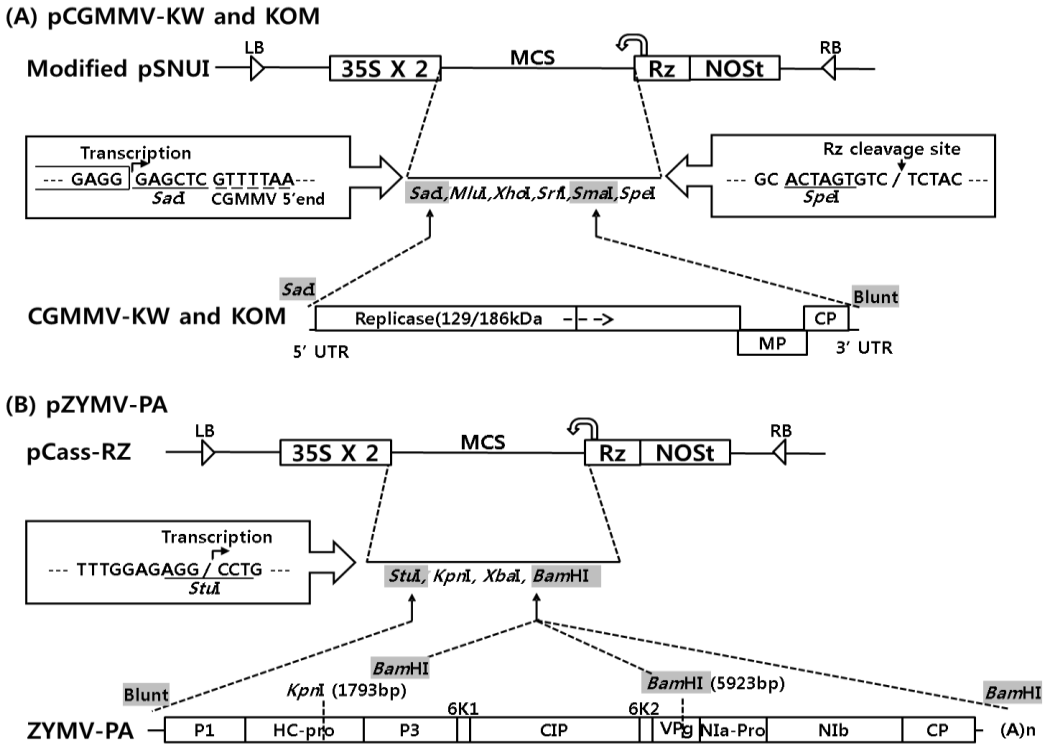

Fig. 1. Schematic representation of the construction of CGMMV and ZYMV full-length clones. The modified pSNUI and pCass-RZ vectors contain, in sequential order, a left border of T-DNA (LB), a double CaMV 35S promoter (35S), a cis-cleaving ribozyme sequence (RZ), a NOS terminator (NOSt), and a right border of T-DNA (RB). The restriction enzyme cleavage sites used to make the constructs are shown in gray boxes. (A) The full-length PCR products amplified from cDNAs of two CGMMV isolates (KW and KOM) are inserted in the modified pSNUI binary vector. (B) The three cDNAs synthesized from RNAs of ZYMV-PA are amplified to three segments using three primer pairs, respectively. These segments were inserted in pCass binary vector in order using the restriction enzyme site that the virus itself has or was inserted into the primer.

3. Construction of CGMMV-based viral vector with duplicated subgenomic RNA promoter

I constructed CGMMV vector, which harbor duplicated CP subgenomic promoter (SGP) for MCS and MCS (*Xho*I and *Hpa*I) for foreign gene insertion in the *Nru*I site. The fragment from *Nru*I to movement protein (MP) 3′ end with MCS (*Xho*I and *Hpa*I) tagged (Fig. 2A, box a′) not only plays the role of the new back portion of the MP but also functions as the CP SGP of the MCS. The original portion (Fig. 2A, box a) will function as the SGP of CP. To solve the problem of overlapped sequences (26 nucleotides) between MP and CP at CP SGP of MCS, one nucleotide (cytosine) was inserted into the end of MP. By inserting one nucleotide in front of MCS, the subgenomic RNA (sgRNA) of MCS was translated *in frame* from start codon of CP to stop codon of foreign gene. PCR products based on pCGMMV-KW were amplified by PCR using phosphorylated primer pair (CG-*Nru*I-B-F and CG(MCS)InC-B-R, Table 2) and were inserted into *Nru*I-digested pCGMMV-KW. The resulting clone was named as pCGMMV-Insertion-C (pCGMMV-InC) (Fig. 2A). Moreover, this clone created the three sgRNAs by individual promoter. The coding region of green fluorescent protein (GFP, 735 bp), which contains 6 histidine tag in the upstream of 5′ end without the stop codon, was amplified by Ex-Taq polymerase (TAKARA BIO, Japan) from pPZP-sGFP (Park et al., 2009) using phosphorylated primer pair (GFP-B-F and GFP-B-R, Table 2). The amplified fragments were inserted in pCGMMV-InC, which was opened with *Hpa*I to make the pCGMMV-inc-GFP (pCGMMV-iGFP) (Fig. 2B).

Table 2. Specific primers used for the construction of CGMMV-based viral vector with duplicated sgRNA promoter

Primers	Sequences (5′-3′)
CG-*Nru*I-B-F	CGATCCTTGGTCTTTATTTGTGAG
CG(MCS)InC-B-R	CTCGAG GTTAAC CTAGGTGTGATCGGATTGTAAG
GFP-B-F	ATGCATCATCATCATCATCATGTG
GFP-B-R	TTACTTGTACAGCTCGTCCATGC

F, sense primer; R, antisense primer; MCS (*Xho*I and *Hpa*I) for insertion of foreign genes are underlined

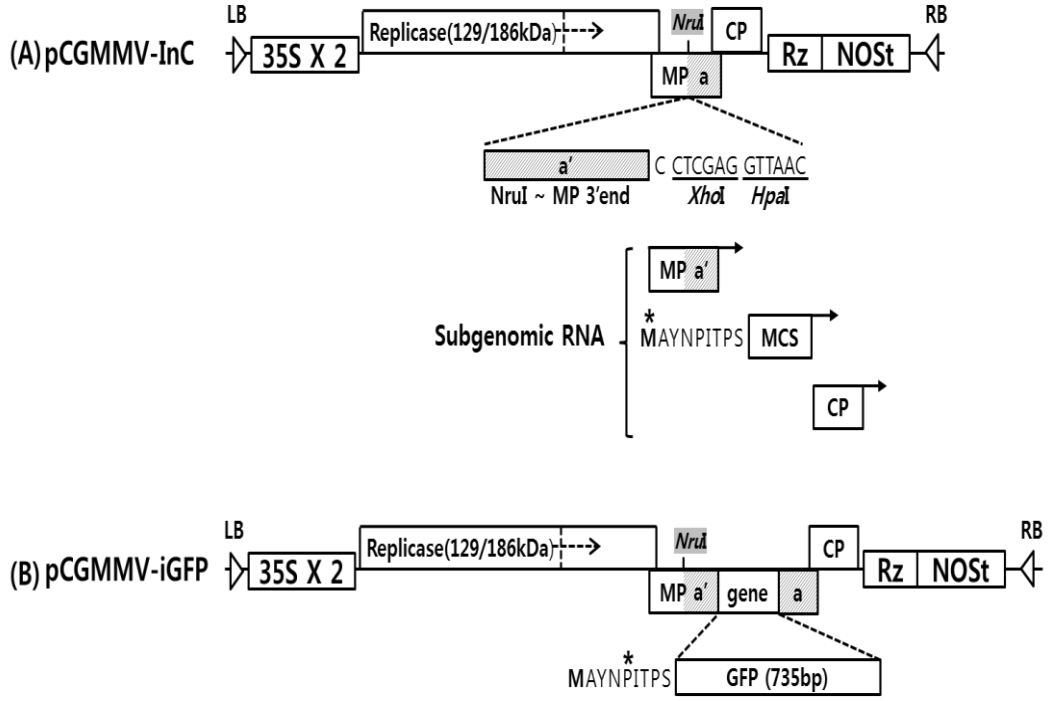

Fig. 2. Schematic representation of the construction of pCGMMV-InC and pCGMMV-iGFP. (A) CGMMV genome organization and construction of an available gene insertion cassette with duplicated sgRNA promoter (a′). The sgRNA of MCS among three sgRNAs created by individual promoter was translated *in frame* from start codon of CP (the marked asterisk) to stop codon of foreign gene. (B) Diagrammatic representation of insertion of the GFP into the pCGMMV-InC. The pCGMMV-iGFP were cloned by inserting GFP fragment into the the pCGMMV-InC.

4. Construction of ZYMV-based viral vector

In order to systemically express a protein in a plant using a plant virus, the gene should be inserted to a particular portion of the clone to prevent stopping or shifting when the virus RNA is transcribed. The multi cloning sites (MCS; *Apa*I, *Spe*I, and *Sal*I) and NIa-Pro cleavage sites were inserted between NIb and CP region of pZYMV-PA using two *Sac*I enzyme sites which exists in NIb region of pZYMV-PA and pCass vector respectively. The PCR product amplified using primer pair (ZYPA-NIb-F and ZYPA-NIb(NIa_MCS)-R; Table 3) was cloned into pGEM-T Easy Vector (Promega, USA). This intermediate clone was named pPA-MCS1-T. Secondly, PCR fragments were amplified using primer pair (ZYPA-(MCS_NIa)CP-F and pCass-*Sal*I-R, Table 3) and digested with *Sal*I. These fragments were inserted in pPA-MCS1-T vector that was opened with *Sal*I. The resulting subclone was named pPA-MCS2-T and was digested with *Sac*I. The fragments, which contain NIb/CP region harboring the *Apa*I, *Spe*I, and *Sal*I sites and the NIa-Pro cleavage sites (S and DTVMLQ), were selected from the digested pPA-MCS2-T and were inserted in pZYMV-PA, which was digested with *Sac*I. This clone was designated pZYMV-MCS (Fig. 3A) and used for a systemic expression of foreign proteins in cucurbitaceous crops. The GFP was amplified by Ex-Taq polymerase from pPZP-sGFP (Park et al., 2009) using a primer pair that has *Spe*I sites attached at the ends (GFP-*Spe*I-F and GFP-*Spe*I-R, Table 3). The amplified fragments were digested with *Spe*I and inserted into pZYMV-MCS, which was digested with *Spe*I. The clone that GFP was inserted into pZYMV-MCS was named as pZYMV-GFP (Fig. 3B). The *bar* gene, which is a herbicide resistance gene encoding phosphinothricin acetyltransferase (549 bp), was

amplified by Ex-Taq polymerase from pBPMV-Bar (Zhang et al., 2010) using a primer pair that has *Spe*I sites attached at the ends (Bar-*Spe*I-F and Bar-*Spe*I-R; Table 3). The strategy to construct the Bar clone was same with that of pZYMV-GFP. The clone containing *bar* gene between NIb and CP region of pZYMV-MCS was designated as pZYMV-Bar (Fig. 3B).

5. Nucleotide sequencing

The sequences analysis of all clones obtained from the previous sectionincluding two pCGMMVs (KW and KOM), pCGMMV-InC, pCGMMV-iGFP, pZYMV-PA, pZYMV-MCS, pZYMV-GFP, and pZYMV-Bar were confirmed through the dideoxy nucleotide termination method and an ABI PRISM 3700 XL DNA Analyzer (Applied Biosystem, Foster City, CA, USA) located at the National Instrumentation Center for Environmental Management (NICEM, Seoul National University).

Table 3. Specific primers used for the construction of ZYMV-based viral vector

Primers	Sequences (5′-3′)
ZYPA-NIb-F	CCTGTGGAATGGATCTTTAAAGGCTGAGCTCAGAC
ZYPA-NIb(NIa_MCS)-R	GACT GTCGACACTAGTGGGCCC *CGA* CTGGAGCATCACAGTGTCTCCTTGTTCAAAGAAAATG
ZYPA-(MCS_NIa)CP-F	AGTC GTCGAC *ACTGTGATGCTCCAG* TCGGGCACTCAGCAAACT GTGG
pCass-*Sal*I-R	GACT GTCGAC TGATTTCAGCGTACCGAATTCGAGCTC
GFP-*Spe*I-F	G **ACTAGT** ATGCATCATCATCATCATCATGTG
GFP-*Spe*I-R	G **ACTAGT** CTTGTACAGCTCGTCCATGC
Bar-*Spe*I-F	G **ACTAGT** ATGAGCCCAGAACGACGC
Bar-*Spe*I-R	G **ACTAGT** AATCTCGGTGACGGGCAG

F, sense primer; R, antisense primer; The multi cloning enzyme sites are underlined (*Apa*I, *Spe*I, and *Sal*I); The highlighted letters show the restriction sites that the virus itself contains and use for cloning; The NIa-Pro cleavage sites is italicized; The restriction enzyme sites for cloning were bolded.

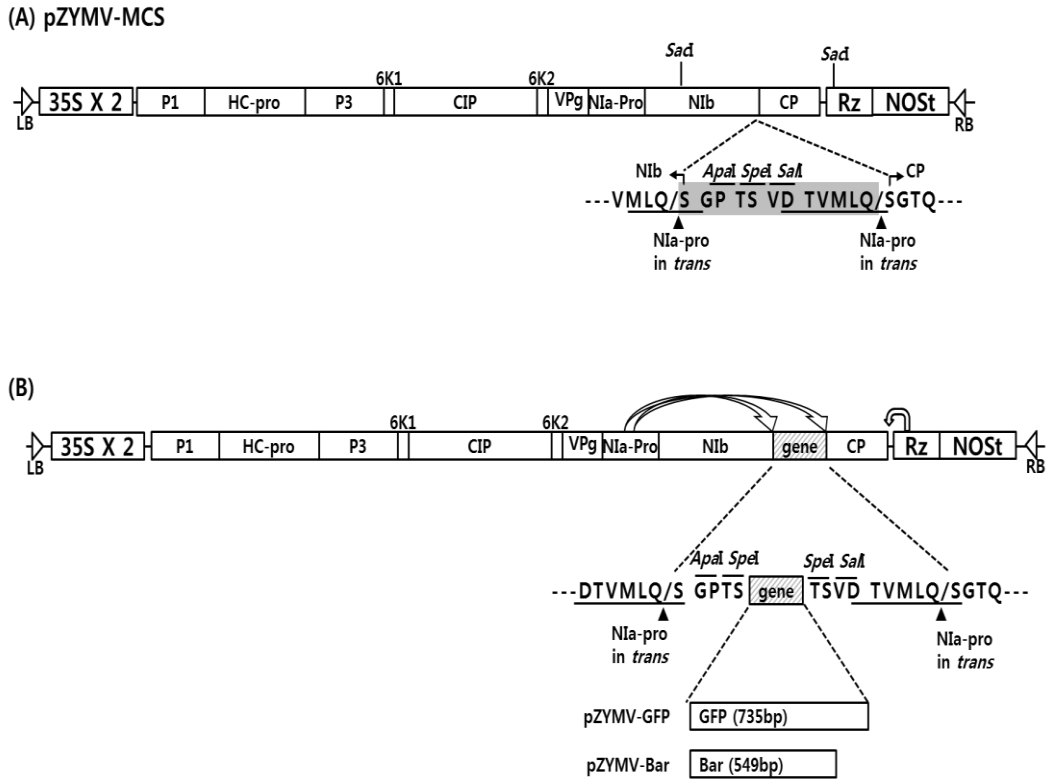

Fig. 3. Schematic representation to construct pZYMV-MCS and viral expression clones. (A) ZYMV genome organization and construction of an available gene insertion cassette between NIb-Pro and CP region. The shaded box indicates that the multi cloning site sites (MCS, *Apa*I, *Spe*I, and *Sal*I) and the NIa-Pro cleavage sites (S and DTVMLQ) were inserted into the polyprotein ORF between the NIb-Pro and CP region. (B) Diagrammatic representation of insertion of the *gfp* and *bar* genes into the ZYMV-based vector. The amplified expression genes inserted into the MCS site are shown in the hatched boxes.

6. Plant growths and inoculations

N. benthamiana, Chenopodium amaranticolor, and commercial cultivars of squash (*C. pepo* L. var. *zucchini*) plants were grown in a growth chamber at 25℃ with a 16/8-hours photoperiod. In case of the two pCGMMVs (KW and KOM) and the CGMMV viral vectors, agro-delivery system was utilized to insert theses viral genes into the genome of *N. benthamiana* plants. The four CGMMV clones (pCGMMV-KW, pCGMMV-KOM, pCGMMV-InC, and pCGMMV-iGFP) were transformed into *Agrobacterium tumefaciens* (GV 2260 strain) by heat-shock method. The *Agrobacterium* transformants were selected on YEP medium plates containing 100mg/L of Kanamycin and 50mg/L of rifampicin. After screening by colony PCR, the *Agrobacterium* transformants including four CGMMV clones were cultured for overnight at 28℃ with shaking in YEP liquid medium containing 100mg/L of Kanamycin and 50mg/L of rifampicin. The *Agrobacterium* cells were centrifuged at 4,000× g for 10 minutes and resuspended in infiltration buffer (10mM MES, 200μM acetosyringone, and 10mM $MgCl_2$) to a final OD_{600} of ~0.5. The resuspended cells were incubated with shaking for 4 hours at 28℃ to activate the *Agrobacterium* Vir genes. The cells containing the four CGMMV clones were infiltrated into *N. benthamiana* by agroinfiltration methods, respectively. The saps of *N. benthamiana* infected with the two CGMMV clones (pCGMMV-KW and pCGMMV-KOM) were used as inoculums for inoculation into *C. amaranticolor*. Four ZYMV clones (pZYMV-PA, pZYMV-MCS, pZYMV-GFP, and pZYMV-Bar) were inoculated into two cotyledons per plant by rub-inoculation with carborundum when the cotyledons of *C.*

pepo L. var. *zucchini* were fully expanded. All plasmid ZYMV clones were prepared using the plasmid Maxi Kit (QIAGEN, USA) as rub-inoculums on plants. Approximately 10 μg of plasmids were used for each inoculation. Wild-type (WT) viruses of two CGMMV isolates (KW-WT and KOM-WT) were inoculated by mechanical inoculation using sap extracts infected viruses with carborundum onto *N. benthamiana* and *C. amaranticolor* and the inoculation of ZYMV-PA WT was performed by mechanical inoculation using sap extracts infected virus with carborundum onto *C. pepo L. var. zucchini*.

7. RNA extraction and RT-PCR

Total RNAs are extracted from control plants or virus infected plants using the TRI_{zol} Reagent method. 1 μg of the extracted total RNAs were denatured at 70℃ for 10 minutes with 25 μM of reverse primers (CG-detection-R for detection of pCGMMVs (KW and KOM) and CGMMV WTs (KW and KOM); CG-InC-R for confirmation of pCGMMV-InC and pCGMMV-iGFP; ZYMV CP-R for detection of pZYMV-PA and ZYMV WT; PA MCS+386-R for confirmation of pZYMV-MCS, pZYMV-GFP, and pZYMV-Bar; Table 4). Reverse transcriptase (RTase) reaction mixtures were incubated at 42℃ for 1 hour with 160 U of GoScript™ RTase (Promega, Madison, WI, USA) and then were inactivated at 70℃ for 5 minutes. dsDNA was amplified by 25 cycles of PCR using Ex-Taq polymerase. PCR for CGMMV detection was carried out using a primer pairs (CG-detection-F and CG-detection-R for the two pCGMMVs and the two CGMMV

WTs; CG-InC-F and CG-InC-R for pCGMMV-InC and pCGMMV-iGFP whether MCS and foreign genes are inserted in CGMMV viral genomes; Table 4). ZYMV was detected by PCR using 10 μM of primer pairs (ZYMV CP-F and ZYMV CP-R for pZYMV-PA and ZYMV WT; PA MCS-364-F and PA MCS+386-R to confirm whether MCS and foreign genes are inserted into ZYMV viral genome, Table 4). After initial denaturation for 2 minutes at 94℃, each cycle for PCR consisted of 20 seconds at 94℃, 30 seconds at 55℃, 50 seconds at 72℃. Amplified PCR products were checked by electrophoresis on a 1% agarose gel containing ethidium bromide and were visualized under UV light.

8. Detection of GFP fluorescence

Tobacco plants agroinfiltrated with GFP fusion pCGMMV-iGFP and squash plants rub-inoculated with pZYMV-GFP were observed by visualizing under illumination with a hand-held UV-light source to confirm whether GFP was systemically expressed on plants (Dark Reader Hand Lamp HL28T; Clare Chemical Research, Dolores, CO, USA). Image data were photographed using a camera with a Dark Reader camera filter.

9. Herbicide treatment

Fully expanded cotyledons of squash plants were rub-inoculated with pZYMV-Bar. After 7 days, the Bar expressing squash plants were sprayed with non-selective herbicide that was diluted to 0.3% with distilled water. This herbicide contains glufosinate-ammonium

as the active ingredient with a concentration of 0.54% glufosinate-ammonium (w/v) in deionized water. Herbicide-treated squash plants were photographed at 7 days post treatment.

Table 4. CGMMV and ZYMV specific primers to confirm the infectivity of two pCGMMVs (KW and KOM) and variant pZYMVs

Primers	Sequences (5′-3′)
CG-detection-F	AGTTACAAGTATAATAGCGGATGT
CG-detection-R	TCAAATACTTGAAAACCGG
CG-InC-F	CGCGAATTCTCTGTTAGGTTCATAC
CG-InC-R	CCTGACGGGAACATAAGAAGCAC
ZYMV CP-F	CTTTGAACAAGGAGACACTG
ZYMV CP-R	GCGGTAAATATTAGAATTAC
PA MCS-364-F	CCATCAAGCAATGTTGGTTGAT
PA MCS+386-R	CGACTCCCATCTGTTGATCATT

F, sense primer; R, antisense primer.

RESULTS

1. Construction of two full-length CGMMV clones (KW and KOM) and their infectivity test in plants

Full-length dsDNAs corresponding to the genome regions of two CGMMV isolates (KW and KOM) were cloned into a modified binary vector, pSNUI, which contains the CaMV 35S promoter and NOS terminator (Fig. 1A; Park, et al., 2006). The final clones were designated as pCGMMV-KW and pCGMMV-KOM, respectively. I carried out *in vivo* infectivity test to confirm whether these full-length clones are infectious on *N. benthamiana* and *C. amaranticolor*. The pCGMMV-KW and pCGMMV-KOM, which were transformed into *A. tumefaciens*, were infiltrated into *N. benthamiana* by agroinfiltration methods. At 12 days post inoculation (dpi), the two pCGMMVs caused systemic mosaic symptom on *N. benthamiana* similar to those caused by two CGMMV WTs (Fig. 4A). It has been reported that CGMMV-KOM isolate does not replicate in *C. amaranticolor* plants (Kim et al., 2003). However, both two pCGMMV-KW and -KOM caused local chlorotic spot symptom on *C. amaranticolor* when saps of *N. benthamiana* rub-inoculated with these two pCGMMVs were used as inoculums (Fig. 4B and 4C). The viral transcripts derived from infectious clones were confirmed by RT-PCR analysis after extracting total RNAs from inoculated plants (Fig.4E and 4F). These results revealed that viral RNA transcirpts produced from pCGMMV-KW and pCGMMV-KOM have infectivity on *N. benthamiana* and *C. amaranticolor* plants.

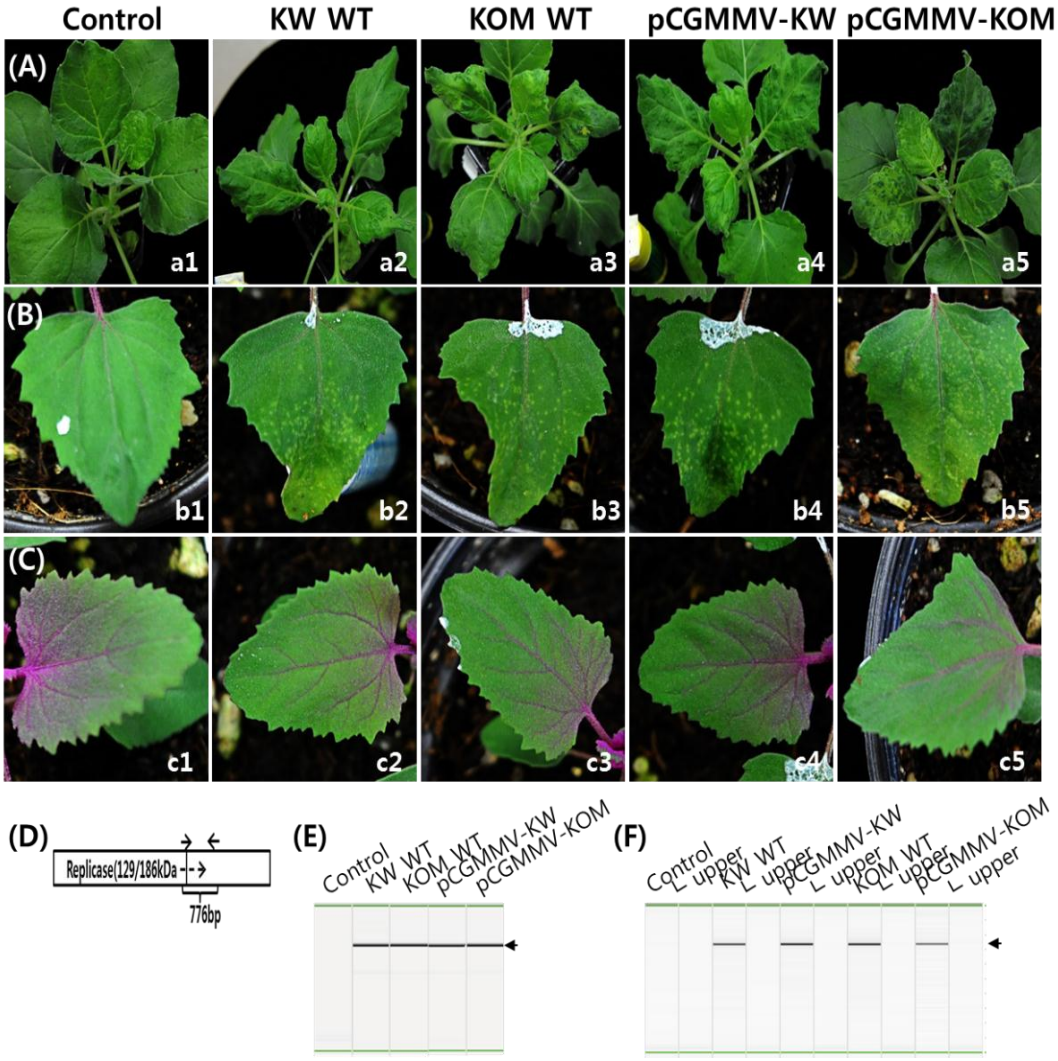

Fig. 4. CGMMV symptoms caused by two pCGMMVs (KW and KOM) and two CGMMV WTs (KW-WT and KOM-WT) in *N. benthamiana* and *C. amaranticolor* plants. Control means healthy plants. (A) pCGMMV-KW and pCGMMV-KOM (a4 and a5) displayed systemic mosaic symptom in *N. benthamiana* like two CGMMV WTs (a2 and a3). (B) Local chlorotic spot symptoms of CGMMV on the sap-inoculated leaves of *C.*

amaranticolor. All pCGMMVs (b2-b5) caused local chlorotic spot symptom on the sap-inoculated leaves of *C. amaranticolor*. (C) The upper leaves in the sap-inoculated *C. amaranticolor* plants. All pCGMMVs (c2-c5) did not show symptoms on the upper leaves of rub-inoculated *C. amaranticolor* plants. (D) CGMMV specific primer pair (the marked arrows), which was designed with sequences located at replicase-coding gene of CGMMV was used. (E and F) CGMMV viral RNAs were detected by RT-PCR onto *N. benthamiana* (E) and *C. amaranticolor* (F).

2. Comparative analysis of amino acid sequences in two CGMMV clones (KW and KOM)

I sequenced the two pCGMMVs and compared them with the previously reported CGMMV genome sequences (accession no.: AF417243 and AF417242, respectively). When comparing the sequences between the KW and KOM isolates of the previous work, 6 amino acids were different from the portion of both 129K and 186K replicase genes. After analyzing the nucleotide sequences of the pCGMMV-KW and comparing with the previously reported nucleotide sequences of CGMMV-KW strain, I confirmed that 5 nucleotides in 129K replicase gene, one nucleotide in 186K replicase gene, and one nucleotide in CP gene have changed (data not shown). However, among the 7 nucleotides, missense mutations have occurred in the 7^{th} amino acid of 129K replicase gene and the 1572^{th} amino acid of 186K replicase gene (Table 5). As the previous report, the pCGMMV-KW and the CGMMV-KW WT caused local chlorotic spots on *C. amaranticolor* leaves. When the sequences of the pCGMMV-KOM was analyzed and compared with the reported sequences, 3 nucleotides from 129K replicase gene has changed, and missense mutation has occurred in 228^{th} amino acid among the 3 nucleotides (Table 5). According to the previous report, the CGMMV-KOM WT does not cause symptom on *C. amaranticolor*. However, in this study, both pCGMMV-KOM and KOM WT caused local chlorotic spots on *C. amaranticolor* leaves.

Table 5. Comparison of amino acid sequences on two pCGMMVs (KW and KOM) and reported sequences

Region	Amino acid position	KW		KOM	
		NCBI[a] LCS[b]	Clone LCS	NCBI -[c]	Clone LCS
	7	Lys	Gln[*]	Gln	Gln
129K	228	Ala	Ala	Ala	Thr[*]
	699	Ile	Ile	Leu	Leu
	1212	Asn	Asn	Lys	Lys
186K	1238	Arg	Arg	Lys	Lys
	1572	Cys	Ser[*]	Ser	Ser

[a]NCBI, National Center for Biotechnology Information; [b]LCS, local chlorotic spot; [c]-, no infection; [*], The amino acid sequence that has changed from the reported sequence.

3. Construction of a full-length ZYMV-PA clone and their infectivity test in plants

The full-length cDNA of ZYMV-PA isolate was cloned into a binary vector, pCass-RZ, having the CaMV 35S promoter and NOS terminator (Fig. 1B). I carried out *in vivo* infectivity test to confirm infectivity of ZYMV full-length clone (pZYMV-PA) in *C. pepo* L. var. *zucchini*. When 10 μg of pZYMV-PA was inoculated on *C. pepo* L. var. *zucchini* by rub-inoculation method, pZYMV-PA caused yellowing, deformation, mosaic symptoms similar to those caused by ZYMV WT at 12 dpi (Fig. 5A). Total RNAs were prepared from healthy plants, wild-type ZYMV-PA (ZYMV-PA WT)-inoculated plants, and pZYMV-PA-inoculated plants, respectively, and RT-PCR was performed using primer pair that was detected CP region of ZYMV genome (Fig. 5B and 5C). A band of the predicted size (approximately 837 bp) was observed on 1% agarose gel. These results demonstrated that ZYMV transcripts produced from pZYMV-PA have infectivity on *C. pepo* L. var. *zucchini* plants.

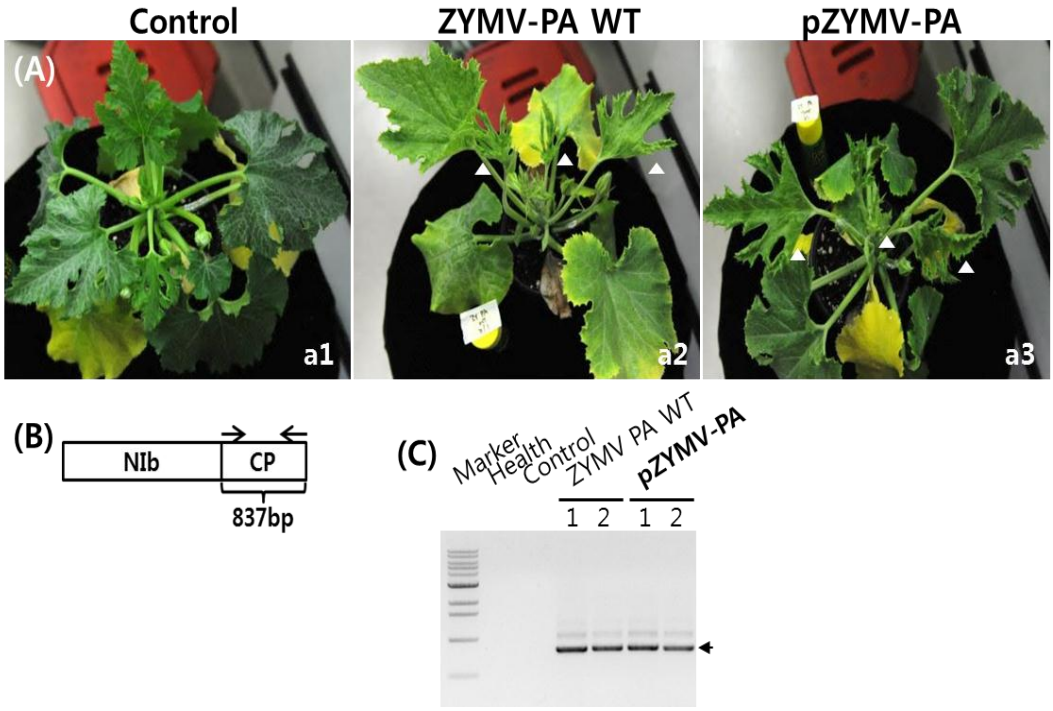

Fig. 5. ZYMV symptoms in *C. pepo* L. var. *zucchini* caused by pZYMV-PA and ZYMV-PA WT. (A) At 12 dpi, ZYMV-PA WT and pZYMV-PA (a2 and a3) caused yellowing, deformation of leaves (arrowheads) and mosaic symptoms. (B) The primer pair (the marked arrows) located at CP portion of ZYMV was used. (C) Replication of pZYMV-PA RNA was confirmed by RT-PCR used primer pair onto *C. pepo* L. var. *zucchini*. Arrow indicates partial ZYMV CP gene.

4. Amino acids sequences analysis of a ZYMV-PA clone

To determine the sequence similarity between pZYMV-PA and the previously reported sequence of ZYMV-PA (accession no.: AY278998), the full-length sequence of pZYMV-PA was analyzed and compared with the reported sequence (Table 6). The analysis of amino acid sequences showed that the pZYMV-PA differs by 8 amino acids from the reported sequences of ZYMV-PA. In addition, it has same sequence compare to that in the FRNK motif (Gal-On, 2000) that influences the symptom development and the DAG triplet (Gal-on et al., 1992) that is involved in aphid-transmission of ZYMV infection.

Table 6. Comparison of amino acid sequences on pZYMV-PA and reported sequences

Region	Amino acid position	NCBI[a]	Clone
		ZYMV-PA	ZYMV-PA
P1	42	Pro	Gln
	209	Glu	Lys
P3	965	Ala	Thr
CI	1328	Arg	Gln
	1711	Val	Ile
	1786	Pro	Leu
VPg	1870	Ile	Val
CP	2874	Lys	Asn

[a]NCBI, National Center for Biotechnology Information

5. Engineering CGMMV as viral expression vector with duplicated sgRNA promoter and its efficiency test

To construct pCGMMV-KW as viral vector for systemic expression of foreign genes in *N. benthamiana*, I designed pCGMMV-InC vectors, carrying a duplicated CP SGP for MCS (Fig. 2A). The resulting vectors, pCGMMV-InC, caused systemic mosaic symptom such as pCGMMV-KW at 12 dpi (Fig. 6A). I inoculated these clones into *N. benthamiana* by agroinfiltration and checked whether the virus genomes that contain the duplicated SGP and MCS were replicated in plants. Total RNAs were extracted from pCGMMV-KW- and pCGMMV-InC-inoculated plants and performed RT-PCR using the primers (Fig. 6B and 6C). After the desired size of portion containing MCS was detected from pCGMMV-InC, the GFP gene as a reporter gene was inserted into pCGMMV-InC (pCGMMV-iGFP) using *Hpa*I site of MCS to confirm the expression efficiency of pCGMMV-InC (Fig. 2B). However, no GPF signals were observed in pCGMMV-iGFP inoculated plants. Also, no viral bands were detected by RT-PCR in same plants (data not shown).

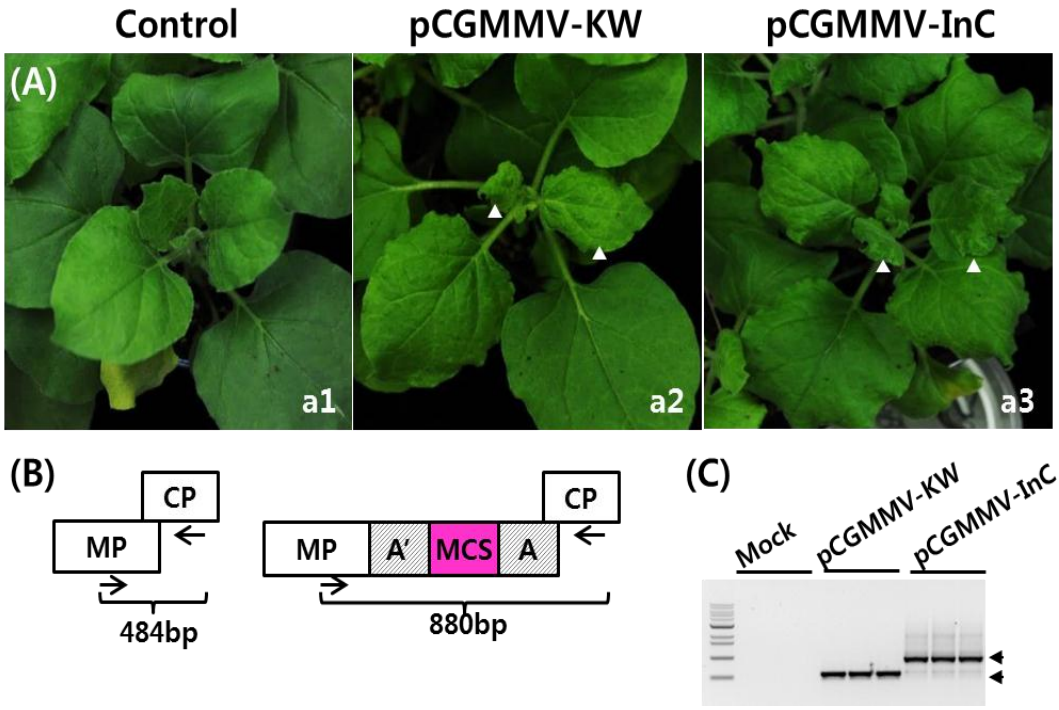

Fig. 6. Symptoms in *N. benthamiana* caused by pCGMMV-KW and pCGMMV-InC. (A) Unlike the mock-inoculated plant (a1), systemical mosaic symptom (marked arrowheads) was observed from pCGMMV-KW-inoculated and pCGMMV-InC-inoculated plants (a2 and a3, respectively). (B) The arrows show the primers that make it able to detect whether the artificially inserted MCS portion is preserved in the virus genome. (C) As a result of performing RT-PCR using the primers on total RNA that was extracted from infected plants, the desired sizes (pCGMMV-KW, 484 bp; pCGMMV-InC, 880 bp) were detected.

6. Engineering ZYMV as viral expression vector

To develop pZYMV-PA as viral vector for systemic expression of foreign genes in *C. pepo* L. var. *zucchini*, multiple cloning sites (MCS) including *Apa*I, *Spe*I and *Sal*I and additional NIa-Pro cleavage sites (S attached 5′end and DTVMLQ attached 3′end) were inserted into the polyprotein ORF between NIb-Pro and CP region (Fig. 3A). The resultant plasmid vectors, pZYMV-MCS, showed infectivity that caused yellowing, malformation of leaves and mosaic symptoms on *C. pepo* L. var. *zucchini* like pZYMV-PA at 12 dpi (Fig. 7A). I checked whether the ZYMV RNA transcripts induced from pZYMV-MCS include MCS. Total RNAs were prepared from healthy, pZYMV-PA-inoculated and pZYMV-MCS-inoculated plants and analyzed by RT-PCR using primer pair spanning the NIb-Pro and CP region (Fig. 7B and 7C). A unique band with the predicted size (approximately 728 bp for detecting pZYMV-PA and 764 bp for detecting pZYMV-MCS) was observed for ZYMV RNA transcripts including MCS from squash plants inoculated with pZYMV-MCS. As a result, ZYMV RNA transcripts derived from pZYMV-MCS were replicated on *C. pepo* L. var. *zucchini*.

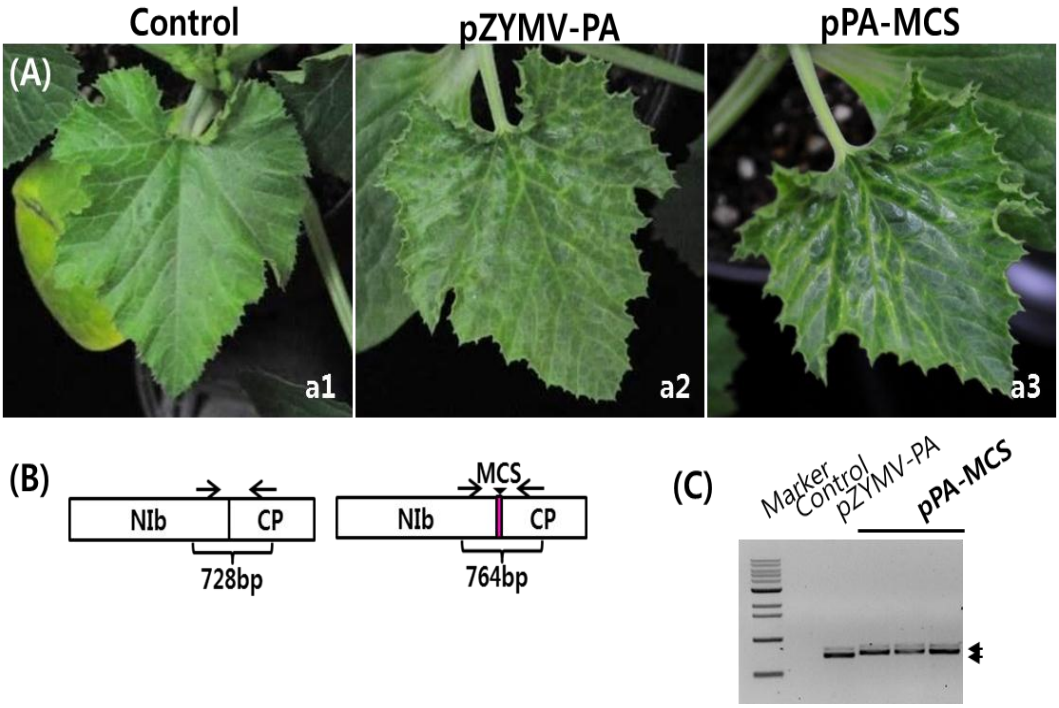

Fig. 7. Symptoms in *C. pepo* L. var. *zucchini* inoculated with ZYMV-based viral vector including MCS and NIa-Pro cleavage sites for expression of foreign genes. (A) pZYMV-PA and pZYMV-MCS (a2 and a3) caused yellowing, deformation of leaves and mosaic symptoms on *C. pepo* L. var. *zucchini*. Using primer pair containing MCS (B), Total RNA extracted from pZYMV-PA-inoculated and pZYMV-MCS-inoculated leaves were detected by RT-PCR using primer pair that contains MCS part to check *in vivo* infectivity of clones. (C) The upper arrows to the expected size of the PCR products containing the MCS portion.

7. The efficiency test of ZYMV-based viral vector

The pZYMV-PA was engineered as a viral vector, because it showed systemic symptoms in cucurbitaceous crops. The ZYMV-based viral vector was checked if it enables the systemic expression of foreign proteins in cucurbitaceous crops. I used reporter genes (*gfp* and *bar*) to evaluate the expression of foreign genes from the ZYMV vector in squash plants. PCR products of GFP and Bar were cloned into pZYMV-MCS, utilizing the *Spe*I site resulting in the construct designated as pZYMV-GFP and pZYMV-Bar (Fig. 3B). 10 μg of pZYMV-GFP and -Bar were inoculated onto the cotyledons of squash plants (var. *zucchini*) by rub-inoculation. pZYMV-GFP and -Bar caused typical yellowing and mosaic symptoms that are similar to the symtpoms by pZYMV-PA or -MCS. Systemic expression of GFP protein was also detected in upper leaves based on green fluorescence emitted by GFP under UV light (Fig. 8). The herbicide was treated on healthy, pZYMV-PA-, and pZYMV-Bar-inoculated plants at 7 dpi, respectively. After 7 days after herbicide treatment, the healthy and the pZYMV-PA-inoculated plants were damaged and died by the herbicide. However, in the pZYMV-Bar-inoculated plants, both inoculated and upper leaves of the plants did not die and grew properly because herbicide resistance was induced on the plants by systemic expression of Bar protein (Fig. 9).

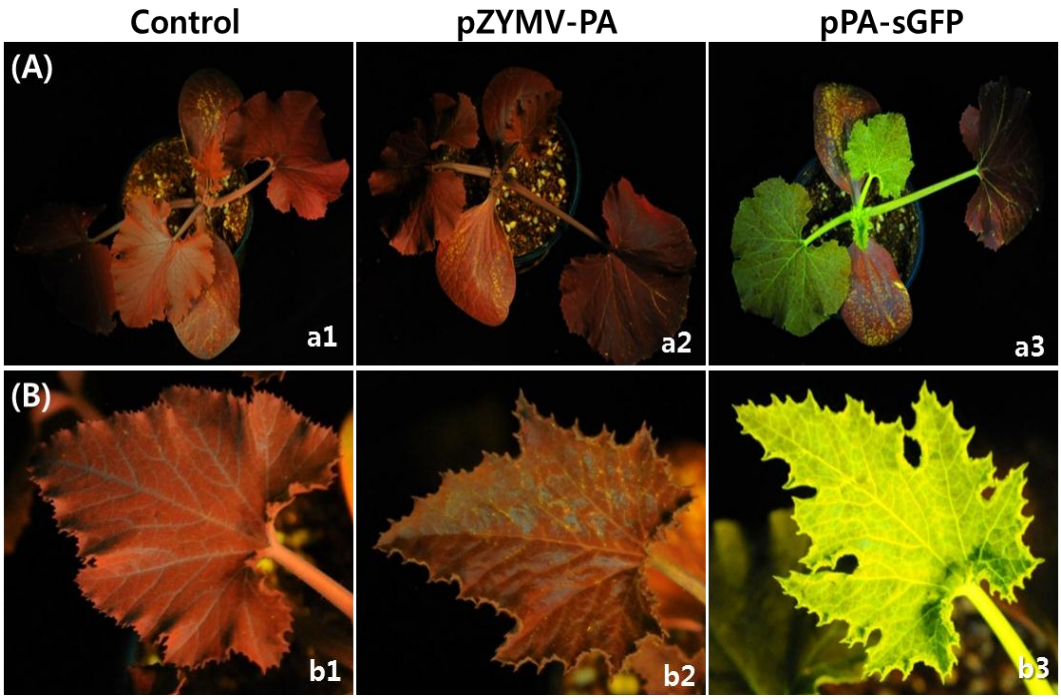

Fig. 8. Fluorescence emitted by GFP under UV light on leaves of squash plants inoculated with pZYMV-GFP. (A) pZYMV-PA and pZYMV-GFP-inoculated cotyledons of *C. pepo* L. var. *zucchini*. At 7 dpi, leaves inoculated with pZYMV-GFP (a3) showed intense fluorescence. No fluorescence was detected on leaves of healthy and pZYMV-PA-inoculated plants (a1 and a2). (B) Upper leaves were photographed under UV light at 12dpi. pZYMV-GFP caused systemic expression of GFP on *C. pepo* L. var. *zucchini*.

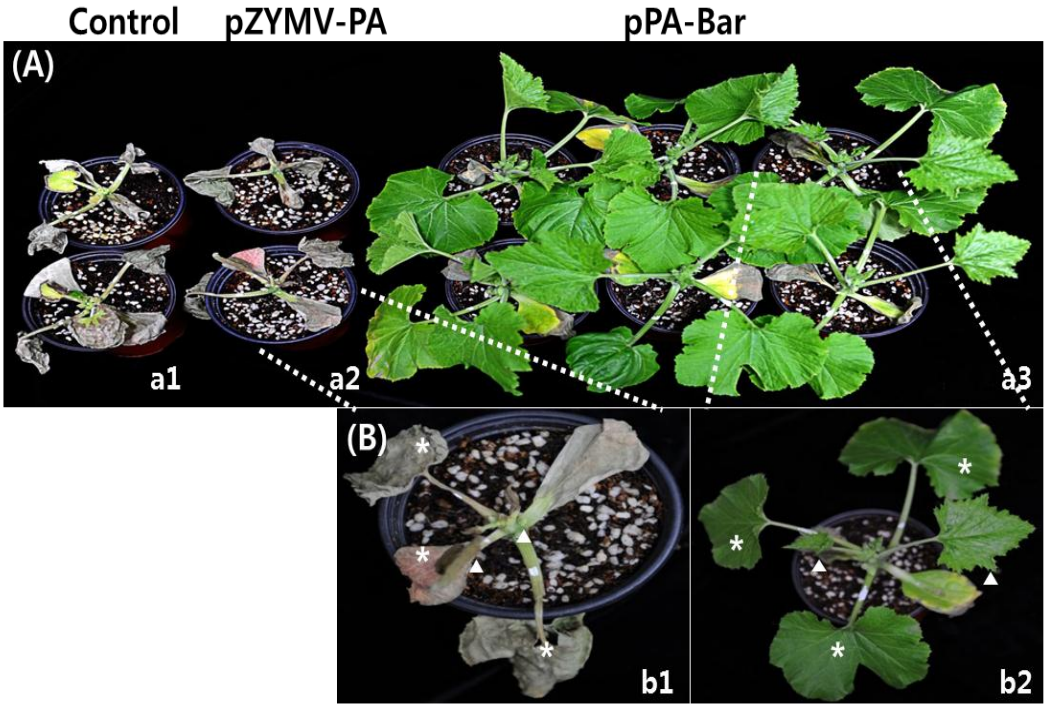

Fig. 9. Symptom appearance in squash plants inoculated with recombinant ZYMV carrying herbicide resistance gene after herbicide treatment (0.3% BASTA). (A) The representative photographs of squash plants inoculated with pZYMV-PA and pZYMV-Bar (a2 and a3) after herbicide treatment. Photographs were taken at 7 days after herbicide treatment. (B) Magnified images of pZYMV-PA and pZYMV-Bar (b1 and b2). Asterisks mean leaves treated with herbicide and arrowheads indicate upper leaves that grow up after herbicide treatment.

8. The stability of foreign gene expression

To evaluate the expression stability of the foreign genes (*gfp* and *bar*), pZYMV-GFP and -Bar were passaged three times from plant to plant by mechanical sap-inoculation. Total RNAs were prepared from plants infected with pZYMV-GFP and -Bar, respectively. The extracted total RNAs were analyzed if the expression genes were inserted stably in the viral genome by RT-PCR using primer pair. The primer pair was designed to detect the NIb-Pro and CP region containing MCS that inserted GFP and Bar genes (Fig. 10A). The predicted size of PCR products (approximately 1.5 kbp and 1.3 kbp for pZYMV-GFP and -Bar, respectively) was detected in pZYMV-GFP and -Bar-inoculated and its viral progeny-inoculated squash plants (Fig. 10B and 10C, respectively). GFP fluorescence was instantly detected in all squash plants infected with pZYMV-GFP or its viral progeny through serial passages (Table 7). In addition, pZYMV-Bar and its progeny virus induced herbicide resistance on *C. pepo* L. var. *zucchini* through serial passages (Table 7).

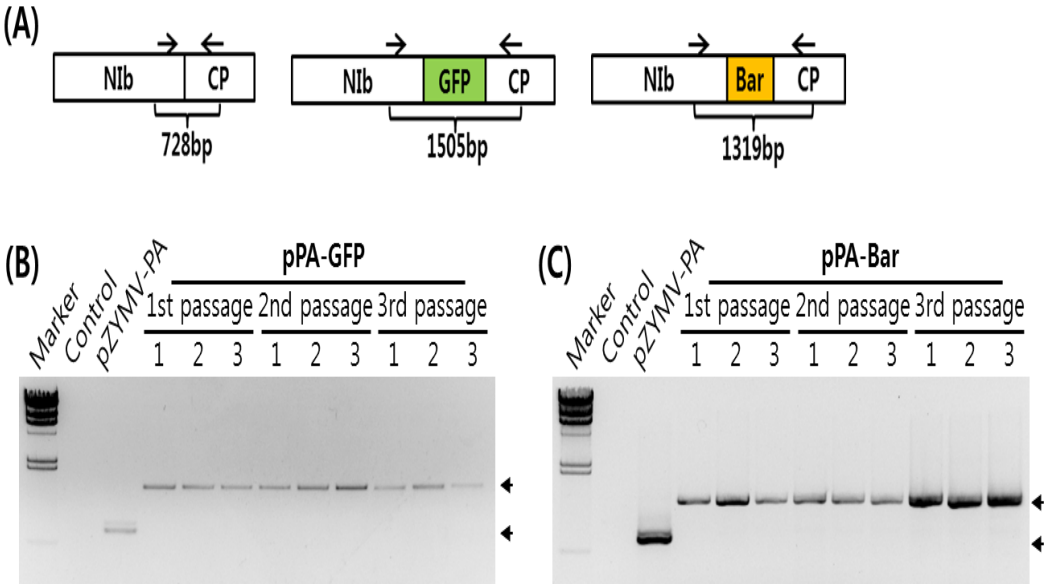

Fig. 10. Analysis of the stability of reporter genes in the viral expression vector by RT-PCR. (A) Schematic map of the NIb/CP, NIb/GFP/CP or NIb/Bar/CP region. Arrows indicate the primer pair for the region of detection by RT-PCR. (B and C) Total RNAs were isolated from healthy, pZYMV-PA-infected, pZYMV-GFP-infected, and pZYMV-Bar-infected leaves of each squash plant and were used for RT-PCR (B, pZYMV-GFP-infected; C, pZYMV-Bar-infected). Progeny viruses in plant sap inoculated with pZYMV-GFP or pZYMV-Bar were passaged three times to new plants. The numbered lanes in B and C correspond to each infected plants. The upper arrows indicate the PCR products containing the *gfp* or *bar* gene, respectively. The lower arrows shows the PCR products amplified from pZYMV-PA-infected leaves without reporter genes.

Table 7. The stability of pZYMV-PA and ZYMV-based expression clones by rub-inoculation in squash plants

Clones	Infectivity		
	Passage 1	Passage 2	Passage 3
pZYMV-PA	9/9[a]	9/9	9/9
pZYMV-GFP	9/9	9/9	9/9
pZYMV-Bar	9/9	9/9	9/9

[a] Number of the appearance of infectivity in plants / number of plants inoculated with clones respectively

DISCUSSION

Construction of infectious full-length clones of RNA viruses is useful for studying the molecular biology of these RNA viruses such as the characterization of the viral functions and manipulation of viral genome by mutagenesis or recombination for the studies of genome expression, replication, and pathogenesis. In addition, various viral expression vector systems are powerful tool for the expression and gene-silencing of the interesting genes in various plants.

In this study, I constructed the infectious full-length clones of CGMMV-KW, CGMMV-KOM, and ZYMV-PA, which are economically important in Korean cucurbitaceous crops. Both CGMMV and ZYMV clones were transcribed *in vivo* by CaMV 35S promoter, so the infectious transcripts can be produced *in vivo* by bypassing the difficulties of *in vitro* RNA transcription. The CGMMV clones were inoculated by *Agrobacterium*-mediated transformation to manipulate genes in *N. benthamiana* and caused similar symptoms like those of CGMMV WTs while the CGMMV clones were inoculated into *C. amaranticolor* by the saps of *N. benethamiana* infected with CGMMV clones as inoculums. In particular, the ZYMV clones were inoculated by rub-inoculation method on squash plants which caused yellowing and mosaic symptoms like ZYMV-PA WT. This DNA plasmid-mediated rub-inoculation method has an advantage of not requiring expensive and complicated steps such as agroinfiltration to deliver viral infectious clone into the genome of host plants.

Since it has been reported that there is a difference in symptom development between the CGMMV-KW and -KOM isolates in *C. amaranticolor* (Kim et al., 2003), I intended to execute the sequence analysis of the pCGMMV-KW and -KOM, which cause symptom development on *C. amaranticolor*, to find the symptom determinant(s) between the pCGMMV-KW and -KOM. Next, I compared them with previously reported CGMMV genome sequences, but did not identify any difference. The difference in sequences between the pCGMMV-KOM and the reported CGMMV-KOM had no relationship with the symptom development on *C. amaranticolor*. Therefore, it will be required to identify the factors that might be related to the symptom development in future study.

Moreover, when two 'unrelated' virus genera, *Tobamovirus* and *Potyvirus*, were simultaneously mechanically inoculated on their hosts, they verified the occurrence of synergistic interactions by observing symptom expression and cytopathology (Cho, 1998; Cho, 2002), but it remained to be elucidated on understanding the mechanism(s) for the synergistic interaction between genus *Tobamovirus* and *Potyvirus*. These infectious full-length clones constructed in this study may help us find the relationship between CGMMV and ZYMV. Utilizing these viral vectors has several advantages. Taking into account the helicoidal morphology of viral particles, no packaging limitations would be expected for rather large genome insertions (Scholthof et al., 1996). In this study, I used the CGMMV and ZYMV viruses isolated from cucurbitaceous crops in Korea and applied them as expression vectors. When I inserted GFP gene into CGMMV-InC vector, GFP was not expressed on *N. benthamiana*. This result suggests that the GFP gene might be excised from the viral vector by recombination of CP duplicated sgRNA promoter

during the replication in host plants. Later, in order to avoid the phenomenon, a design is needed for an alternative expression strategy based on a CP fusion protein that included the FMDV 2A catalytic peptide sequence and a promoter region including the longer 5′ end of CP. The FMDV-2A peptide sequences are used widely for this purpose since multiple proteins can be co-expressed by linking ORFs to form a single cistron (Sempere et al., 2011). In the previous report, the boundaries of the fully active promoter were between -157 and +54 although the minimal CP sgRNA promoter was mapped between -69 and +12 (Grdzelishvili et al., 2000). In this study, I tried to use +27 of CP 5′ region for CP sgRNA promoter of MCS. However, foreign gene was not expressed in plants. Construction of the CP sgRNA promoter region with +54 of CP 5′ region may be needed to enhance expression efficiency for further use of this clone as expression vector. It also remains to be determined whether this CGMMV vector can be used as silencing vector for virus-induced gene silencing (VIGS). It will provide a reverse genetics technique, which can be used to characterize gene functions of plants (Liu et al., 2002a; Burch-Smith et al., 2004; Wege et al., 2007).

In case of ZYMV, I found that the mechanical method for virus-induced gene expression system could be used to produce specific protein within cucurbitaceous crops rather than relying on the bombardment methods (Arazi et al., 2001). However, this ZYMV vector caused severe symptom and damage on *C. pepo* L. var. *zucchini* like ZYMV-PA WT. Therefore, this ZYMV vector can be appropriate meterial if it is developed as a vector with mild infectivity using site-directed mutagenesis on FRNK motif (Gal-On, 2000) and DAG triplet (Gal-on et al., 1992). The efficiency of the CGMMV and the ZYMV vectors

was confirmed by reporter gene (CGMMV viral vector using *gfp* gene; ZYMV viral vector using *gfp* and *bar* genes). Through the stability test of ZYMV viral vector, I demonstrated that the insertion of foreign gene in the ZYMV viral genome was maintained in host plants even after three serial passages.

In summary, I constructed the infectious full-length CGMMV and ZYMV clones and these clones were engineered as CGMMV-based and ZYMV-based viral vector system, but CGMMV-based viral vector did not work on plants in this study. The ZYMV-based viral vector will be successfully utilized for introduction, regulation, and production of expression of foreign gene in cucurbitaceous crops. It is reported that ZYMV latently infects onto inoculated leaves of *N. benthamiana*, but systemically not onto *N. benthamiana* (Kwon et al., 2005). I demonstrated that ZYMV is detected by RT-PCR on upper leaves of *N. benthamiana* when ZYMV and CGMMV are mixed infected (data not shown), and not detected by RT-PCR on upper leaves of *N. benthamiana* when ZYMV only is infected. In the future, ZYMV GFP expression clone derived from ZYMV-based vector can be used on determining whether certain ORF portions of CGMMV can affect systemic movement ability of ZYMV on *N. benthamiana*, by tracing emission of fluorescence.

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한국 박과작물에서 분리 동정된 *Cucumber green mottle mosaic virus*와 *Zucchini yellow mosaic virus*의 감염성 full-length 클론들의 제작 및 viral 벡터로의 응용

강민지

초록

박과작물은 *Cucurbitaceae*에 속하고 전 세계적으로 118속 825종이 분포하고 있으며, 국내에서는 주로 수박, 참외, 오이, 호박 등이 재배되고 있다. 박과작물은 현재 국내에서 주요 작물 중 하나로, 현재 농업현장에서 박과작물에 감염되는 바이러스는 경제적으로 심각한 문제가 되고 있는 것으로 보여진다. 박과작물에 피해를 입히는 바이러스 중 *Cucumber green mottle mosaic virus*(CGMMV)와 *Zucchini yellow mosaic virus*(ZYMV)를 이용하여 실험을 진행하였다. *Tobamovirus*속에 속하는 CGMMV는 약 300nm의 간상형 구조이며 6.4 kb의 게놈을 가지고 있다. *Potyvirus*속에 속하는 ZYMV는 약 750nm의 사상형 구조로 9.5 kb의 게놈을 가지며, 두 바이러스는 양성 외가닥 RNA로 이루어져 있다. 본 연구에서는 우리나라의 기주와 각 지역에 따라 분리 동정된 분리주를

이용하였다. 먼저 2003년도 선행 연구에 따르면 CGMMV는 기주가 따라, 우리나라의 watermelon(KW)과 oriental melon(KOM)에서 분리동정 되었다. ZYMV는 2005년도 선행 연구에 따르면 우리나라의 지역에 따라 Pumpkin-Andong(PA), -Euiryoung(PE) 그리고 -Suwon(PS)에서 분리동정 되었다. CGMMV의 두 개의 분리주(KW와 KOM)들과 ZYMV의 세 개의 분리주 중 PA을 이용하여 실험을 진행하였다. CGMMV와 ZYMV 같이 RNA 바이러스는 염기서열의 변이가 심하기 때문에 유전적 다양성이 존재할 수 있으므로 RNA보다 안정적인 DNA로 합성하여 클론으로 유지해야 한다. 따라서 CGMMV의 분리주들 (KW와 KOM)와 ZYMV PA 분리주는 감염성 클론(pCGMMV-KW, pCGMMV-KOM와 pZYMV-PA)을 제작하였고 식물체 내에서 병원성을 확인하였다. CGMMV의 분리주(KW와 KOM)들은 붉은 명아주에서 KW 분리주만이 국부 황백화 병징을 일으킨다고 보고 되었으나, 현재 실험에서 KW 분리주뿐만 아니라 KOM 분리주도 국부 황백화 반점을 일으키는 것으로 확인되었다. 클론의 염기서열을 분석하여 기존 보고된 염기서열과 비교해보았으나 붉은 명아주에서 병징 변화를 일으킬만한 아미노산을 확인할 수 없었다. CGMMV와 ZYMV 감염성 클론은 박과작물에 사용 가능한 바이러스 벡터로서 개발하였다. 먼저 CGMMV의 경우, *Agrobacterium*을 이용한 접종방법으로 *N. benthamiana*에서 확인할 수 있었다. ZYMV-PA 감염성 클론은 기계적인 접종에 의해 쉽게 박과작물에 접종 가능한 장점을 가지고 있기 때문에 박과작물에서 전반적으로 외래 유전자 발현을 시킬 수 있는 바이러스 벡터로 개발하였다. 먼저 바이러스의 특정 부위에 특정

유전자가 삽입될 수 있도록 멀티 클로닝 사이트 (MCS)를 삽입하였고, 바이러스 자체의 protease 역할을 하는 단백질에 의해 절단되어 외래유전자가 발현되도록 제작하였다(pZYMV-MCS). pZYMV-MCS에 삽입되는 단백질이 식물체 내에서 잘 발현되는지 확인하기 위해 리포터 유전자로 녹색 형광 단백질 유전자(*gfp*)와 제초제 저항성 유전자(*bar*)를 이용하였다. 이 유전자들은 MCS의 엔자임 사이트를 이용하여 pZYMV-MCS에 삽입되었다(pZYMV-GFP와 pZYMV-Bar). pZYMV-GFP을 접종한 쥬키니 호박에서 GFP 단백질이 전반적으로 발현되는 것을 확인하였다. 제초제를 처리했을 때, 아무것도 감염되지 않는 대조 식물이나 *bar* 유전자가 삽입되지 않은 pZYMV-PA에 감염된 식물은 고사되었지만 pZYMV-Bar을 접종한 식물은 제초제가 처리된 잎뿐만 아니라 상엽이 고사되지 않고 자라는 것을 확인하였다. ZYMV 기반 바이러스 벡터의 효율성은 상엽에서 RT-PCR을 이용하여 *gfp*나 *bar* 유전자를 포함하는 부분을 검출함으로써 확인하였다. 세대진전실험을 통해 ZYMV 기반 바이러스 벡터는 삽입된 유용 유전자들을 안정적으로 발현할 수 있다는 것이 확인할 수 있었다.

주요어 : *Cucumber green mottle mosaic virus*, *Zucchini yellow mosaic virus*, 감염성 클론, 식물 바이러스 발현용 벡터, 녹색 형광 단백질, 제초제 저항성 유전자, 박과작물

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