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Completion sequence and cloning of the infectious cDNA of a chb isolate of cucumber green mottle mosaic virus

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Summary. – Cucumber green mottle mosaic virus (CGMMV) is an important and widespread seed-borne virus that infects *Cucurbitaceous* plants. It is a member of the genus *Tobamovirus* in the family *Virgaviridae* with a monopartite (+) ssRNA genome. Here we report the complete genome sequence, construction and testing of the infectious clones of a chb isolate of CGMMV. Full-length CGMMV cDNA was cloned into the vector pUC19. The linearized vector containing full-length cDNA was used as template for *in vitro* transcription, and the synthesized capped transcript was highly infectious in *Chenopodium amaranticolor* and cucumber (*Cucumis sativus*). Inoculated plants showed symptoms typical of CGMMV infection. The infectivity was confirmed by mechanical transmission to new plants, RT-PCR and western blot. Progeny virus derived from infectious transcripts had the same biological and biochemical properties as wild-type virus. To our knowledge, this is the first detailed report of a biologically active transcript from CGMMV.

Keywords: cucumber green mottle mosaic virus; Tobamovirus; cucumber; infectious clone

Introduction

Cucumber green mottle mosaic virus (CGMMV) is an emerging disease pathogen in watermelons in China. In 2005, a disease outbreak occurred in watermelon fields in Liaoning province which was confirmed to be caused by CGMMV (Chen *et al.*, 2006). CGMMV is a seed-borne virus that infects *Cucurbitaceous* plants and causes severe mosaic symptoms, discoloration and deformation (Celix *et al.*, 1996; Ali *et al.*, 2004). CGMMV remains a serious threat to cucurbit plant production, because seed-borne viruses act as initial inoculum in their hosts. CGMMV infected seeds can cause significant loss of yield (Francki *et al.*, 1986; Kim and Lee, 2000). In December 2006, the Chinese Ministry of Agriculture imposed a quarantine measure to restrict movement of watermelon seeds and seedlings to China (Chen and Li, 2007). In 2009, we reported the distribution pattern of CGMMV in China during 2007 to 2008. The virus has spread in five provinces of China, including Liaoning, Hebei, Guangdong, Hubei and Shandong (Liu *et al.*, 2009). The complete genome sequence of the isolates from Liaoning and Shandong has been obtained, but to our knowledge, the genome information of the other isolates has not been described.

CGMMV belongs to the genus *Tobamovirus*, which consists of a positive-sense single-stranded RNA encoding at least four proteins. The 5'-proximal ORFs encode the short and the long viral replicase, the third ORF encodes the movement protein (MP). The 3'-proximal ORF encodes the coat protein (CP). CGMMV was first isolated in the United Kingdom in 1935 (Ainsworth, 1935). It has been reported in Europe, Israel, Saudi Arabia, India, Pakistan, Korea, Japan and China (Komuro *et al.*, 1968; Antignus *et*

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Abbreviations: CGMMV = cucumber green mottle mosaic virus; KGMMV = Kyuri green mottle mosaic virus; ZGMMV = zucchini green mottle mosaic virus

al., 1990; Lee *et al*., 1990; Celix *et al*., 1996; Lee, 1996; Ali *et al*., 2004; Chen and Li, 2007). To date, several isolates of CGMMV have been described and they are closely related to CV3 and CV4 isolates from the U.K (Ainsworth, 1935, Francki *et al*., 1986), including CGMMV-W (watermelon) and the highly similar CGMMV-SH from Japan (Ugaki *et al*., 1991); CGMMV-Sp from Spain (Celix *et al*., 1996); an Indian isolate C (Vani and Varma, 1993) and CGMMV-Is from Israel (Antignus *et al*., 1990). Two other viruses that were initially determined as the cucumber (CGMMV-C) and the Yodo (CGMMV-Yodo) isolates of CGMMV were subsequently considered to be isolates of a distinct virus, named Kyuri green mottle mosaic virus (KGMMV) and the Yodo isolate of KGMMV (KGMMV-Y), respectively, based on serological and sequencing studies (Tan *et al*., 2000). CGMMV-KOM, CGMMV-KW and CGMMV watermelon strains were reported in Korea (Kim *et al*., 2003). Three Chinese isolates, CGMMV-LN and CGMMV-Liaoning from Liaoning province, CGMMV-TANG from Shandong province, had been reported (Chen *et al*., 2006; Liu *et al*., 2009). Here we report the genome of CGMMV-chb isolate, which was isolated from Hebei province of China.

To date, 10 infectious cDNA clones for *Tobamovirus* have been obtained: tobacco mosaic virus (TMV) (Meshi *et al*., 1986), tomato mosaic virus (ToMV) (Weber *et al*., 1992), odontoglossum ringspot virus (ORSV) (Yu and Wong, 1998), Kyuri green mottle mosaic virus (KGMMV) (Yoon *et al*., 2001), zucchini green mottle mosaic virus (ZGMMV) (Yoon *et al*., 2002), obuda pepper virus (ObPV) (Junqueira *et al*., 2014), turnip vein-clearing virus (TVCV) (Zhang *et al*., 1999), pepper mild mottle virus (PMMoV) (Ichiki *et al*., 2009), ribgrass mosaic virus (RMV) (Ryu *et al*., 2012), maracuja mosaic virus (MarMV) (Song *et al.,* 2006), and Chinese rape mosaic virus (CRMV) (Aguilar *et al*., 1996). However, little information has been reported for CGMMV so far.

In this study, we determined the complete nucleotide sequence and analyzed the genome organization for the chb isolate of CGMMV (KJ658958). We studied the relationship of chb isolate to other isolates submitted in GenBank. We also described the construction of a full-length cDNA clone of CGMMV-chb, from which highly infectious transcripts could be synthesized. The biological activity of the *in vitro* transcripts of CGMMV-chb in local lesions of the host and systemic host plants were described. Such an *in vitro* transcription system could be very useful for manipulating the viral RNA to study genome functions or plant-virus interaction mechanism.

Materials and Methods

Virus and plant sources and viral RNA extraction. The isolate chb of CGMMV (CGMMV-chb) used in this study was originally isolated from diseased watermelon plants in Hebei province of China, which was isolated and purified from the local lesion of the host *Chenopodium amaranticolor* and then propagated on systemic host cucumber (*Cucumis sativus*) cv. Jin You No.1. Cucumber cv. Jin You No.1 and *Ch. amaranticolor* were grown for CGMMV *in vitro* transcripts infectivity test. Total RNA was extracted from sample tissues using Trizol Reagent as previously described by Liu *et al.* (2009). The total RNA was kept at -80°C and used as template for cDNA synthesis and as a positive control of transcript bioassay experiments.

Primer design, cDNA synthesis, molecular cloning and sequencing for the genome of CGMMV-chb. The nucleotide sequences of CGMMV-chb were obtained using a series of primers (Table 1), which were based on the conserved sequences of published genomes

Table 1. List of primers designed for determination of CGMMV-chb genome, construction of infectious cDNA clone and identification of CGMMV

Primer	Primer sequence in 5'-3' orientation	Position[a]	Product size	Purpose
pr5RACE1-R	CTCCATATCTTCAGTTACATCCA	1922-1944	1944 bp	5'-RACE
pr5RACE2-R	CATGTGTGTGTCTGCTCTGACCA	992-1014	1014 bp	5'-RACE
prCGchb2-F	CATGTGAAGATATGGATGTAACTGAAG	1910-1936	1563 bp	Determination of genome
prCGchb2-R	GCAAACATGGTCAAGATCGACTG	3451-3473		
prCGchb3-F	GATGCAGTTACAAGTATAATAGCGGATG	3409-3436	1617 bp	Determination of genome
prCGchb3-R	CGAGTTCTCGACTGACACCTTAC	5004-5026		
pr3RACE1-F	GAGTGATAAGCGCCTTTTCCGTAG	4959-4982	1465	3'-RACE
pr3RACE2-F	GTCTGTCGTCTCTTCCGATCAG	5542-5563	882 bp	3'-RACE
prCGQC-F	ATGGATCC*TAATACGACTCACTATA*GGGTTTTAATTTT TATAATTAAAC	1-22	6424 bp	Construction of infectious clone
prCGQC-R	CAGAGCTCTACGTATGGGCCCCTACCCGGGG	6408-6424		
prCGCP-F	CTTTGACAAAGTTCCTATTTCAGCG	5605-5629	721 bp	Identification of CGMMV
prCGCP-R	TGAGCAAACCGTTCGATTTA	6307-6326		

[a]The reference accession number (National Center for Biotechnology Information) for the determination of the primer positions is CGMMV-KW (AF417242). prCGQC-F: *Bam*HI is indicated by shading. T7 promoter is shown in italics. prCGQC-R: *Sac*I and *Sna*BI is indicated by shading.

of CGMMV. The amplification of the 5' part and 3' part of the genome was carried out using 5'-full RACE kit with TAP (tobacco acid pyrophosphatase) and 3'-full RACE core set with PrimeScriptTM RTase (TaKaRa, China) according to the manufacture's instructions. Two antisense-oligonucleotides were synthesized for rapid amplification of cDNA 5'-ends (5'-RACE) (pr5RACE1-R and pr5RACE2-R). Two sense oligonucleotides were also synthesized for 3'-RACE (pr3RACE1-F and pr3RACE2-F). Successful amplification of nucleotide segments of the expected size was confirmed by electrophoresis in 1% (w/v) agarose gel. The PCR products were then purified by the DNA gel extraction kit (TaKaRa, China) and cloned into the pMD18-T vector (TaKaRa) according to the manufacture's instructions. The recombinant vectors were transformed into *E. coli* strain JM109. For each segment at least three independently isolated clones were sequenced (Shanghai Sangon Biotech Company, China). The sequences were compared with the corresponding virus sequence retrieved from the GenBank database.

Sequence and phylogenetic analysis. Sequence segments were assembled by VECTOR NTI, version 10.0 software (Informax, USA). Other twelve CGMMV genome sequences were obtained from NCBI, including CGMMV_TANG (HM008919), CGMMV-LN (EU352259), CGMMV-KOM (AF417243), CGMMV-KW (AF417242), CGMMV-SH (D12505), CGMMV-W (AB015146), Liaoning isolate of CGMMV (EF611826), CGMMV strain Watermelon (AB369274), CGMMV isolate Ah (KF155232), CGMMV isolate Ec (KF155231), CGMMV isolate Rd (KF155230), CGMMV isolate TY (KF155229), KGMMV (NC_003610) and ZGMMV (NC_003878) were used as outgroup control. The multiple alignments were made using the Align in VECTOR NTI, version 10.0 software. Sequence comparison or phylogenetic tree construction was made using a neighborjoining of Bootstrap test in MEGA5.0 (version 5.0, Japan).

Construction of a full-length cDNA clone of CGMMV-chb. Full-length genome of CGMMV-chb was amplified with prCGQC-F and prCGQC-R by RT-PCR (Table 1 and Fig. 1). The prCGQC-F, contains *Bam*HI recognition site and T7 RNA promoter sequences and two additional G, which can improve the efficiency of transcription. The prCGQC-R contains *Snab*I and *Sac*I site for cloning and *in vitro* transcription as shown in Fig. 1. PCR was performed with first-strand cDNA from viral RNAs and LA Taq™ (Takara) to amplify long template DNA. The PCR program was performed in a standard thermocycler (Bioer, China), with the following conditions: denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 30 sec, and extension at 72°C for 7 min. The PCR products (5 µl) were analyzed by electrophoresis in 1.0% agarose gel containing 0.5 µg/ml ethidium bromide in 1×TAE buffer (40 mmol/l tris-acetate, 1 mmol/l EDTA) for 30 min at 120 V. Amplified products were digested with *Bam*HI and *Sac*I restriction endonucleases and cloned into pUC19 plasmid. The full-length cDNA clone was used as template DNA for *in vitro* transcription reaction.

In vitro transcription and infectivity assay. Full-length cDNA clone of the CGMMV-chb in pT7CGBS was linearized by *Sna*BI and used as template for *in vitro* transcription. The reaction was performed with T7 RNA polymerase in the presence of Ribo m7G cap analog (Takara) according to manufacture's instructions. Quantity and quality of RNA transcripts were assayed in 1.0% agarose gel. Carborundum-dusted cotyledons of cucumber and *Ch. amaranticolor* plants were inoculated by 2.0 µg to 3.0 µg of the transcripts. Appearance and development of virus symptoms on inoculated plants were observed and compared to those of wild virus RNA. RT-PCR and western blot analyses were performed to verify the infectivity.

Western blot analyses. Western blot was performed using commercial antibody against CGMMV (Agdia Inc., USA) according to the manufacture's instructions. Crude extracts of the inoculated and healthy tissues were separated by 15% SDS-PAGE and transferred onto a nitrocellulose (NC) membrane using electrotransfer. Hybridization was carried out in a hybridization incubator with homologous antibody for western blot analysis (Ryu *et al.*, 2012).

Results

Molecular characterization of CGMMV-chb

The complete genome of CGMMV-chb (GenBank Acc. No. KJ658958) is 6424 nt in length. Nucleotide sequence analysis of CGMMV-chb indicated the presence of four ORFs and two UTRs organized similarly to that of other CGMMV isolates. The first two ORFs encode the 128.7 kDa

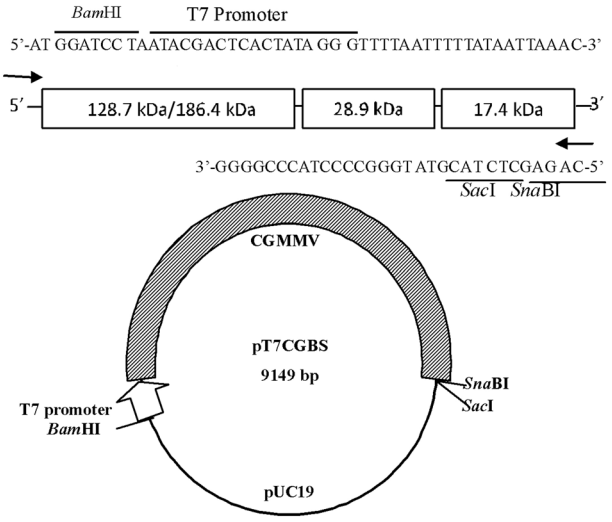

Fig. 1
Construction of a full-length cDNA clone (pT7CGBS) of cucumber green mottle mosaic virus (CGMMV)
The 5'-end and 3'-end primer sequences are shown in detail on the corresponding region. The sites of T7 RNA promoter and restriction endonuclease are marked in the map of the cDNA clone.

(nt 61–3492) pre-read through and 186.4 kDa read through (nt 61–5004) proteins. ORF 3 encodes the 28.9 kDa protein involved in cell to cell movement (MP, nt 4994–5785) and ORF 4 encodes the 17.4 kDa CP (nt 5763–6245). It also has approximately 60 nucleotides of 5'UTRs and 179 nucleotides of uncompleted 3'UTRs.

Phylogenetic analysis

A sequence comparison was made between CGMMV-chb and all other isolates of CGMMV as well as KGMMV and ZGMMV as outgroup from GenBank. The results showed that the full genome sequence of CGMMV-chb shared 94%~100% sequence identity with the other twelve isolates and the highest sequence identity (100%) with CGMMV-KW, and a relatively low sequence identity (94%) with CGMMV isolate Ec (Table 2). The sequence of each ORF, 3'UTRs, 5'UTRs was also compared between CGMMV-chb and other CGMMV isolates. The result showed that CGMMV-chb shared 94%~100% sequence identity with other isolates for 129 kDa and 186 kDa protein, 96%~100% for MP, 92%~100% for CP, 95%~100% for 5'UTR and 98%~100% for 3'UTR. 3'UTR is the most conserved part in the genome (Table 2). The sequence of four ORFs between CGMMV-chb and other isolates was also compared at the amino acid level, which showed that CGMMV-chb shared 100% identity with CGMMV-KOM, CGMMV-KW, CGMMV-LN (EU352259), CGMMV-SH (D12505) and CGMMV-TANG (Table 2), indicating that the six isolates may be the closest among all CGMMV isolates. Phylogenetic trees based on genome sequence alignments indicated that all the CGMMV isolates are closely related, and formed a distinctive cluster. However CGMMV-chb was closer to CGMMV-KW and Liaoning isolate of CGMMV than other isolates. KGMMV belongs to a separate clade and is in the same sub-cluster with ZGMMV (Fig. 2).

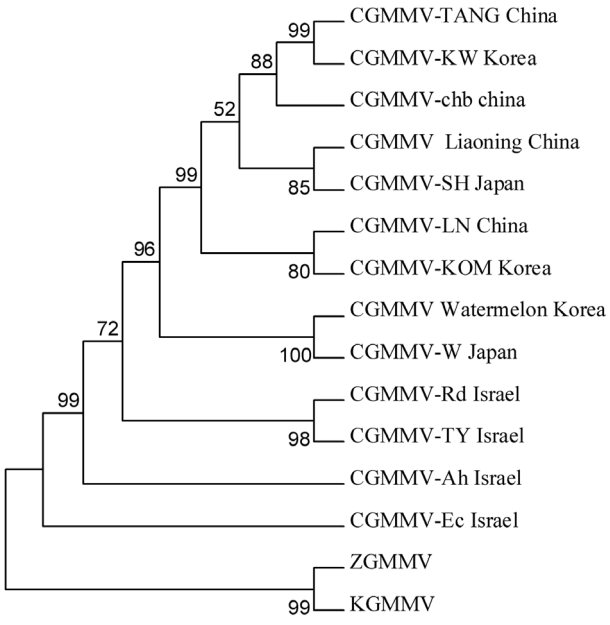

Fig. 2
Phylogenetic relationship of CGMMV-chb with the other twelve isolates

Infectivity of in vitro transcript of CGMMV-chb from cloned cDNA

The full-length cDNA of CGMMV-chb was amplified by RT-PCR with 5'-end upstream (prCGQC-F) and 3'-end downstream primers (prCGQC-R) (Table 1 and Fig. 1). High quality full-length cDNA product was obtained by RT-PCR, then digested and cloned to plasmid pUC19. The recombinant vector pT7CGBS was analyzed by single digestion (*Sna*BI, *Sac*I, *Eco*RI, *BamH*I) and double digestion (*BamH*I and *Sac*I). The result showed the construction of pT7CGBS

Table 2. Nucleotide and amino acid sequence identities (%) comparison between CGMMV-chb and other isolates

Source of virus	Genome (%)	129 K (%)		186 K (%)		MP (%)		CP (%)		5'UTR (%)	3'UTR (%)
	nt	nt	aa	nt	aa	nt	aa	nt	aa	nt	nt
CGMMV-Ah	98	97	99	97	99	99	100	97	99	98	98
CGMMV-Ec	94	94	99	94	99	96	98	92	98	98	98
CGMMV-Rd	98	98	99	98	99	99	99	97	99	95	98
CGMMV-TY	98	98	99	98	99	99	100	98	99	97	99
CGMMV-KOM	99	99	100	99	100	98	100	100	100	100	98
CGMMV-W	98	97	99	97	99	100	99	99	100	100	100
CGMMV-KW	100	99	100	99	100	100	100	100	100	98	100
CGMMV Liaoning	99	99	99	99	99	100	99	100	100	100	100
CGMMV Watermelon	99	99	99	99	99	100	99	100	100	100	100
CGMMV-LN	99	99	100	99	100	100	100	100	100	100	100
CGMMV-SH	99	99	100	99	100	100	100	100	100	100	100
CGMMV-TANG	99	99	100	99	100	100	100	99	100	98	99

Table 3. Assay of infectivity of *in vitro* transcript RNAs product of CGMMV-chb transcribed from cloned cDNA on two host plants[a]

	Cucumber	*Ch. amaranticolor*
Inoculated plant	9	10
Infected plant	5	10
Incident rate (%)	56	100

[a] Number of infected or inoculated plants.

is correct (Fig. 3 Lane 1–6). The full-length cDNA clone pT7CGBS was linearized with *Sna*BI and transcribed with T7 RNA polymerase. A 6424 bp long RNA transcript was produced (Fig. 3 Lane 7) and mechanically inoculated in to two plant species. The pT7CGBS transcripts were infectious in 5 of 9 (56%) cucumber plants and 10 of 10 (100%) *Ch. amaranticolor* plants, respectively (Table 3). The symptoms and the timing of appearance of the pT7CGBS transcripts were identical to those induced by the wild CGMMV RNA on cucumber (Fig. 4c) and *Ch. amaranticolor* (Fig. 4d). The presence of CGMMV in inoculated plants was determined by RT-PCR with CGMMV specific primers (prCGCP-F and prCGCP-R) or by western blot analyses. The results for cucumber are shown in Fig, 4a and Fig. 4b. The amounts of viral CP detected were at a similar level in plants inoculated with *in vitro* transcripts (Fig. 4b lane 2 and lane 3) and control plants inoculated with wild-type CGMMV RNA (Fig. 4b, lane 1). The pT7CGBS is the first CGMMV cDNA clone from which highly infectious transcript can be produced.

Virus from plants infected by *in vitro* transcript was inoculated to healthy plants, producing the same symptoms (data not shown). Progeny virus derived from infectious *in vitro* transcripts was efficiently transmitted by sap inoculation to its host plants (data not shown), showing the sTable replication competency of the virus derived from the clone. These results indicate that we have obtained a full-length infectious clone of a CGMMV-chb with high biological activity yielding the *in vitro* transcript which can yield symptoms indistinguishable from those of the native virus.

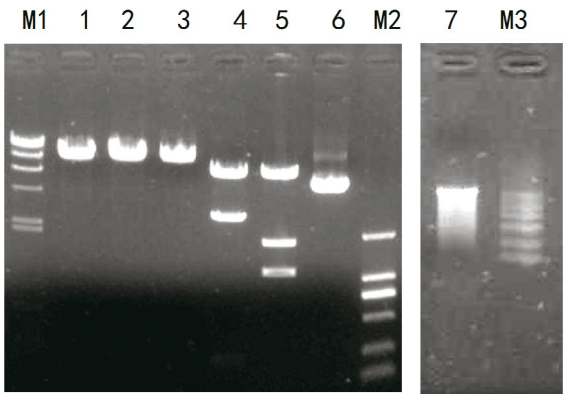

Fig. 3
The verification of pT7CGBS by restriction endonuclease digestion and *in vitro* transcription using linearized pT7CGBS as template
λ-*Hind*III digest DNA marker (M1); DL2,000 DNA Marker (M2); pT7CGBS digested by *Sna*BI (1); pT7CGBS digested by *Bam*HI (2); pT7CGBS digested by *Sac*I (3); pT7CGBS digested by *Bam*HI and *Sac*I (4); pT7CGBS digested by *Eco*RI (5); pT7CGBS plasmid control (6); *in vitro* transcripts using linearized pT7CGBS as template (7); RL6000 RNA Marker (M3)

Discussion

Cucurbitaceous plants, such as cucumber, melon, watermelon, zucchini squash, cantaloupe and other, are economically important vegetable crops in China. CGMMV

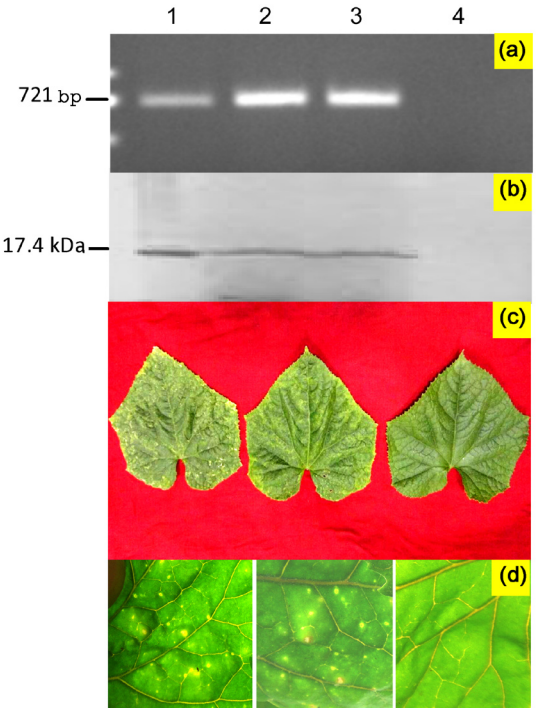

Fig. 4
The symptoms and detection of virus-specific gene or coat protein in plants inoculated with *in vitro* transcript RNAs from full-length cDNA clone (pT7CGBS) of chb isolate of cucumber green mottle mosaic virus (CGMMV-chb)
RT-PCR analysis for inoculated plant tissues with CGMMV (a); Western blot analysis of CGMMV coat protein hybridized with CGMMV antibody (b). Total nucleic acids or total protein samples were prepared from cucumber cotyledons or upper leaves. Wild CGMMV-chb total RNAs-inoculated upper leaf of cucumber (positive control, A1 and B1), transcripts-inoculated cotyledon of cucumber (A2 and B2), transcripts-inoculated upper leaf of cucumber (A3 and B3), phosphate buffer inoculated upper leaf of cucumber (negative control, A4 and B4). The symptoms of the upper leaf of cucumber and treated leaf of *Ch. amaranticolor* inoculated by wild CGMMV-chb total RNAs as positive control (C left and D left), upper leaf of cucumber and treated leaf of *Ch. amaranticolor* inoculated by transcripts (C middle and D middle), upper leaf of cucumber and treated leaf of *Ch. amaranticolor* inoculated by phosphate buffer as negative control (c right and d right).

is a new emerging *Tobamovirus* on *Cucurbitaceous* plants and represents a serious threat to the production for the country (Ainsworth, 1935). CGMMV-chb was originally isolated from naturally grown watermelon plants in Hebei province of China which exhibited green mottle and mosaic symptoms on leaves and severe distortion symptoms (our unpublished results). At present, only three whole genome sequences of CGMMV isolates were reported in China. The lack of sequence data hinders the classification and evolution studies of the virus. The complete genome sequence of CGMMV-chb obtained in this study will be utilized to reveal the CGMMV classification status and virus evolutionary patterns.

The similarity and phylogenetic relationships of CGMMV-chb with the other isolates had been analyzed. It showed that CGMMV-chb shared the highest identity with CGMMV-KW, which was found in Korea, not only for the complete genome sequence but also for each ORF. The two isolates also belong to the same sub-cluster in the phylogenetic tree, indicating that the two isolates may be evolutionarily closer than other isolates. The *Cucurbitaceous* plants import and export seed trade between Korea and China might be the reason for the introduction of the virus. The consequence is in accordance with Liu *et al.* (2009) and Tian *et al.* (2009). As the seed-borne virus, immediate attention should be paid to the use of certified seeds, investigation of effective seed treatment, and education of the farming community about the importance of phytosanitary measures in disease control. In addition, the four encoded proteins of the CGMMV-chb showed higher identities with the other CGMMV isolates in the nucleotide and amino acid levels. In particular, the amino acid sequence is very conservative. This probably indicates that some factors such as negative selection and bottleneck episodes can result in low genetic diversity and the small changes may play an important role for its evolution and prevalence (Kim *et al.*, 2010). The four CGMMV isolates from China were clustered to one sub-group. It showed that the variation of the virus may be correlated with geographical origin and the climate factors. The result was in accordance with Li's finding (2010). Focusing on such genetic variability of CGMMV, this study could be a helpful baseline for understanding genetic characterization of CGMMV isolates and developing control strategies for the breeding of virus resistant cultivars and plant quarantine (Kim *et al.*, 2010).

Using of infectious clones has become the most important tool to study the biology, pathogenesis, and virulence determinants of both positive and negative stranded RNA viruses (Bing *et al.*, 2014). They also provided further insight on functions of different viral proteins or the mechanisms of interaction between viruses and their host plant or vector (Klein *et al.*, 2014). The full-length clone of CGMMV was constructed for the first time by Ooi *et al.* (2006), for use as the vector for expression system for hepatitis B surface antigen. The infectivity of the full-length clone of CGMMV was not shown in detail. In our study, the full-length cDNA clone of CGMMV-chb was produced and evaluated for infectivity after T7 transcription *in vitro* (pT7CGBS). The symptoms were indistinguishable from those generated by inoculation of CGMMV wild strain RNA. The method was simplified compared with Ooi *et al.* (2006). Similar results for *in vitro* transcription with T7 promoter was reported for KGMMV (Mizumoto *et al.*, 2010), ZGMMV (Wu *et al.*, 2009) and CFMMV (Rhee *et al.*, 2014). Production of the infectious cDNA clone of CGMMV-chb will facilitate the evaluation of mutants to investigate CGMMV-chb gene function and interactions with host plants (Yoon *et al.*, 2001; Wu *et al.*, 2009; Mizumoto *et al.*, 2010).

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