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BRIEF REPORT

Development of an agroinoculation system for full-length and GFP-tagged cDNA clones of cucumber green mottle mosaic virus

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Abstract The complete 6243-nucleotide sequence of a cucumber green mottle mosaic virus (CGMMV) isolate from bottle gourd in Zhejiang province, China, was determined. A full-length cDNA clone of this isolate was constructed by inserting the cDNA between the 35S promoter and the ribozyme in the binary plasmid pCB301-CH. A suspension of an *Agrobacterium tumefaciens* EHA105 clone carrying this construct was highly infectious in *Nicotiana benthamiana* and bottle gourd. Another infectious clone containing the green fluorescence protein (GFP) reporter gene was also successfully constructed. This study is the first report of the efficient use of agroinoculation for generating CGMMV infections.

Cucumber green mottle mosaic virus (CGMMV; genus *Tobamovirus*, family *Virgaviridae*) causes a serious disease of cucurbit crops with significant economic losses in several countries [2, 10, 20, 24]. In China, it was first discovered in 2003 [4] and has since been reported in Guangdong, Liaoning, Hebei, Hubei, Shangdong and many other provinces [15]. It has now assumed epidemic proportions.

Infectious cDNA clones are powerful tools for investigating many viruses. A T7-promoter-driven CGMMV infectious clone has been successfully constructed, but although it has been useful for experimental studies [17, 22], the need for *in vitro* transcription makes it inconvenient and expensive to use. Agroinoculation has proved to be an effective approach for introducing binary plasmids containing the full-length cDNA of virus genome components into their hosts and to establish infection [1, 3, 6, 14, 25, 27]. Some such clones and their modified constructs are now widely used as virus-based vectors, including those for tobacco mosaic virus (TMV), potato virus X (PVX) and tobacco rattle virus (TRV). These have provided great benefit to researchers, not only for studying the viruses but also as powerful tools for understanding the functions of host genes [5, 9, 18, 23]. We now report the construction of a full-length infectious clone of CGMMV driven by the cauliflower mosaic virus (CaMV) 35S promoter that can be used in agroinoculation and its use for expressing green fluorescent protein (GFP), providing a tool for studying the interaction between CGMMV and its host plant.

The CGMMV isolate (CGMMV-ZJ) was isolated originally from a bottle gourd plant in Zhejiang province and was usually propagated on bottle gourd (*Lagenaria siceraria*, Hangzhou chang gua). To construct the infectious cDNA clone of CGMMV, the genomic sequence of CGMMV-ZJ was first determined. Total RNA was extracted with TRIzol (Ambion), and the first-strand cDNA was synthesized with primer M4-T using ReverTra Ace (Toyobo) [7]. All primers (p1-p9, Supplementary Table 1)

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were designed based on the published sequence of a CGMMV isolate (GenBank: NC_001801) (Supplementary Table 1), and CGMMV fragments were amplified by PCR using KOD Fx Neo (Toyobo) according to the manufacturer's instructions. The sequences of the 5' and 3' ends of CGMMV-ZJ were determined by 5' and 3' rapid amplification of cDNA ends (RACE), using a SUPERSWITCH^{TM} RACE cDNA Synthesis Kit (Hangzhou Sonice Biotechnology Co., Ltd) according to the manufacturer's instructions. Sequence comparisons were done with CLUSTALW [11]. Phylogenetic analysis was done by the maximum-likelihood method using MEGA 6.06 [21].

The complete nucleotide sequence of CGMMV-ZJ (GenBank: KP244682) was 6423 nucleotides (nt) long and had the typical genome organization for a member of the genus *Tobamovirus* (Fig 1A). There was a 14-nt overlap between the open reading frames (ORFs) of the RNA-dependent RNA polymerase (RDRP) and the movement protein (MP), and a 26-nt overlap between the ORFs of the MP and coat protein (CP) (Fig. 1). The 5' and 3' nontranslated regions (NTRs) consisted of 60 and 176 nucleotides, respectively. Based on a multiple sequence alignment, the complete nucleotide sequence of CGMMV-ZJ was estimated to be 90-99 % identical to the other sequenced isolates of CGMMV (Fig. 1B). A phylogenetic tree based on the complete nucleotide sequence suggested that CGMMV-ZJ belongs to a distinct subset of Chinese (KC852073, KC851866, KC852074, KC852072, HM008919) and Korean (AF417242) isolates of CGMMV (Fig. 1B).

To construct the full-length infectious CGMMV cDNA, two overlapping segments covering the full genome of CGMMV were amplified from the cDNA of CGMMV-ZJ with primer pairs p10/p11 and p12/p13 (Supplementary Table 1). The plasmid pCB301-CH (a modified version of pCB-301 [26]), which was used as the backbone, was digested overnight with *Stu* I and *Sma* I. Then, the digested vector and the two fragments of CGMMV were ligated together using a ClonExpress^{TM} MultiS One Step Cloning Kit (Vazyme) according to the manufacturer's protocol. In

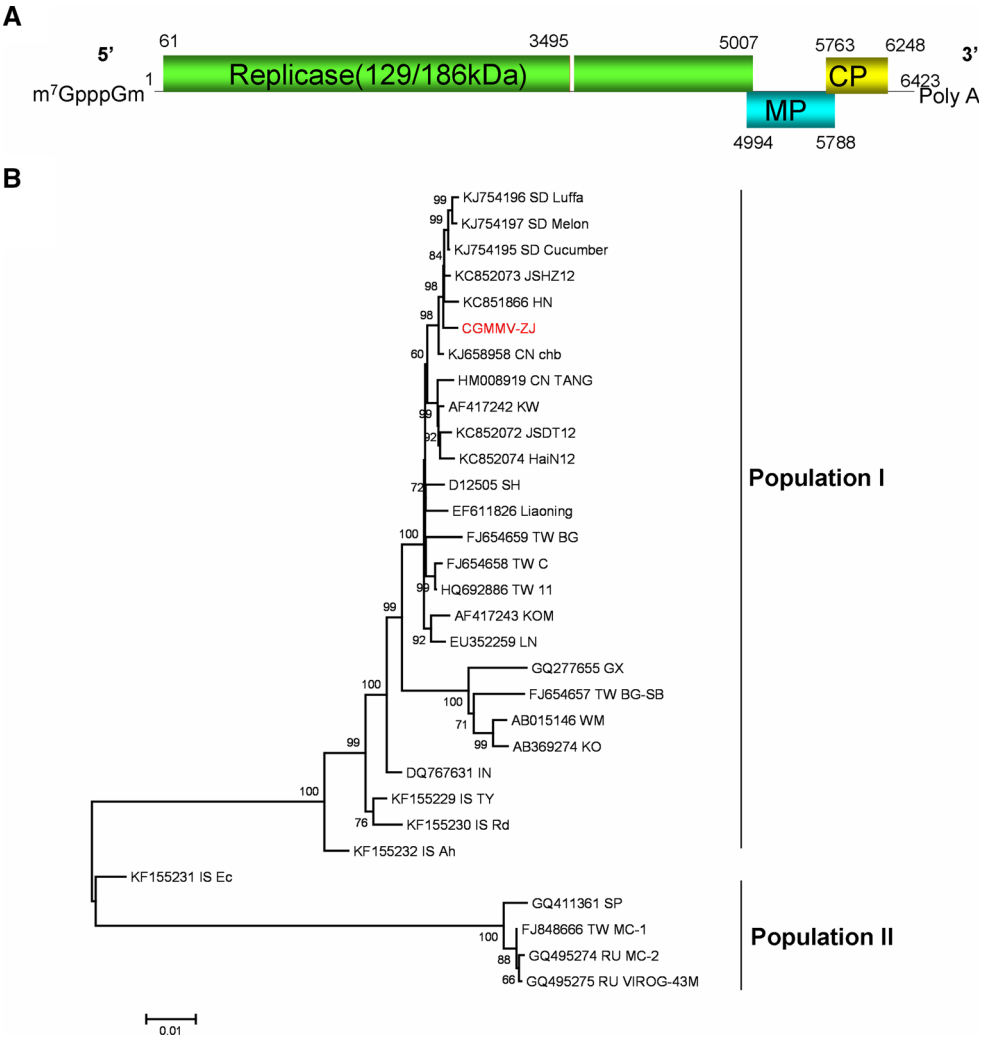

Fig. 1 Genome organization of the CGMMV Zhejiang isolate (A) and maximum-likelihood phylogenetic tree of the complete nucleotide sequences of all fully-sequenced CGMMV isolates (B). Figures at the branches show percent bootstrap support from 1000 replicates (where >60 %)

this way, the full-length CGMMV cDNA was inserted between an upstream 35S promoter and a downstream hepatitis delta virus ribozyme and 35S terminator in the binary vector pCB-301-CH to construct pCB-CGMMV (Fig. 2A). To investigate infectivity, symptoms and evidence of viral replication, *A. tumefaciens* strain EHA105 containing pCB-CGMMV was infiltrated into leaves of *N. benthamiana*, bottle gourd (*Lagenaria siceraria*, Hangzhou chang gua), watermelon (*Citrullus lanatus*, Zaojia 84-24) and cucumber (*Cucumis sativus Linn*, No. 9 Yuan-fengyuan) plants. Symptoms of viral infection appeared on all hosts (Fig. 2C). In bottle gourd and watermelon,

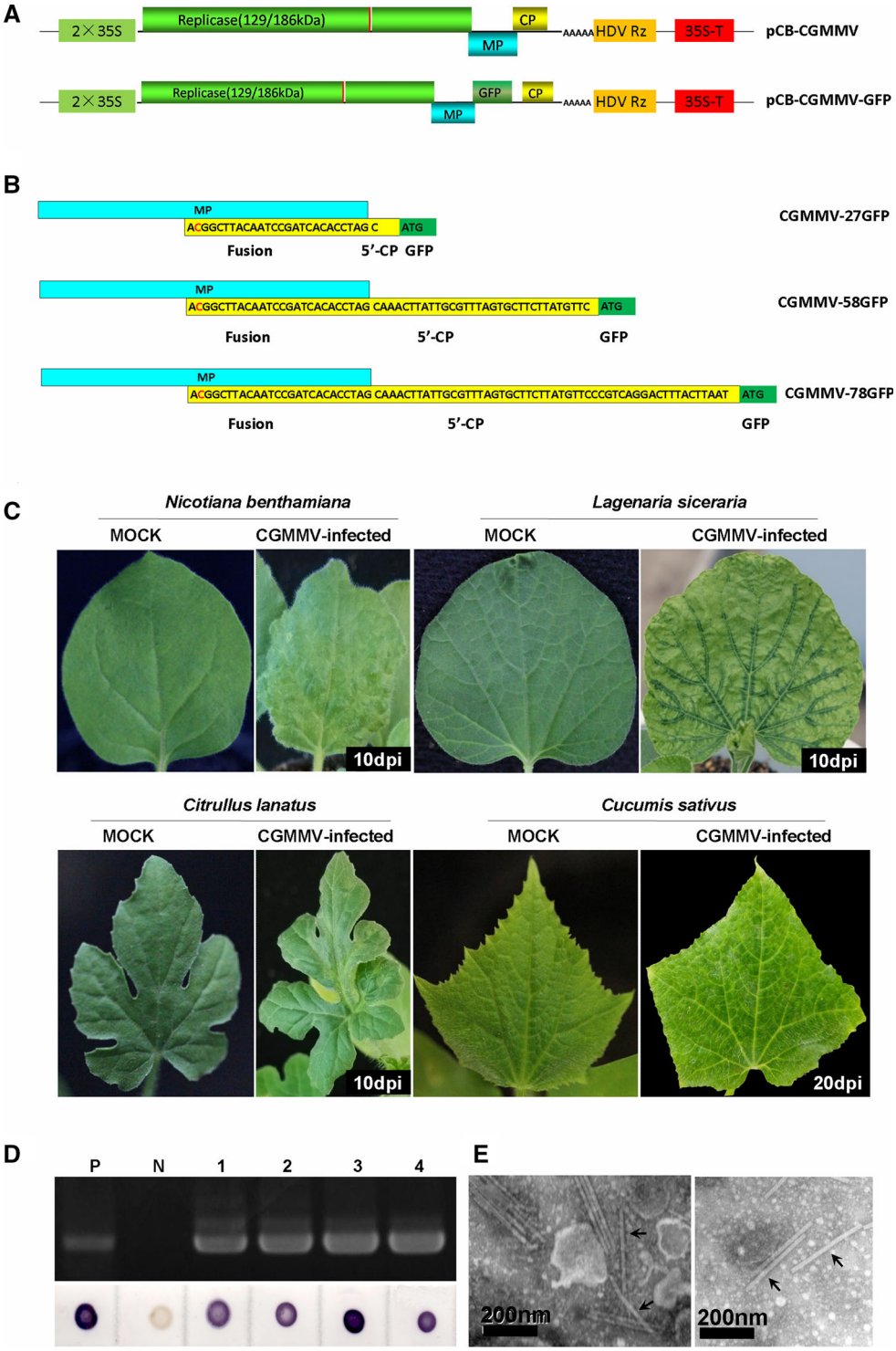

Fig. 2 Construction of pCB-CGMMV and pCB-CGMMV-GFP vectors and inoculation of pCB-CGMMV to *N. benthamiana*, bottle gourd, watermelon and cucumber plants. (A) pCB-CGMMV was produced by inserting the CGMMV-ZJ sequence into the pCB301-CH plant binary expression vector. pCB-CGMMV-GFP was the reconstructed version of CGMMV with different lengths of CP promoter (27, 58 and 78 nucleotides) and the GFP ORF added between the MP and CP. (B) CGMMV-27GFP, CGMMV-58GFP and CGMMV-78GFP are pCB-CGMMV CP subgenomic promoter mutants. In pCB-CGMMV-GFP, the CP subgenomic promoter drives GFP transcription. In order to capture the entire subgenomic promoter, which may extend into the CP ORF, varying lengths of the 5'-CP ORF were retained, as indicated by the construct name (e.g., 27 nucleotides retained in CGMMV-27GFP). Note that the 5'-CP ORF naturally overlaps the MP ORF and the CP start AUGs were mutated to ACGs. (C) Symptoms on hosts inoculated with pCB-CGMMV and the healthy controls photographed at 10 or 20 dpi. (D) RT-PCR (top) and dot-ELISA (below) detection of pCB-CGMMV after inoculation to different hosts. P, positive control; N, negative control; 1, *N. benthamiana*; 2, *L. siceraria*; 3, *C. lanatus*; 4, *C. sativus*. (E) Rod-shaped virions (arrows) in sap of pCB-CGMMV-inoculated bottle gourd (left panel) and *N. benthamiana* (right panel) observed by transmission electron microscopy

systemic mosaic occurred at about 10 days post-inoculation (dpi), followed quickly by typical green mottle mosaic symptoms. Mosaic and malformation appeared on newly expanded leaves of inoculated *N. benthamiana*, while only very slight mosaic appeared on some cucumber plants, even after one month. CGMMV infection was confirmed by RT-PCR and Dot-ELISA at 20 dpi (Fig. 2D). The presence of rod-shaped virions typical of a tobamovirus was confirmed by transmission electron microscopy in bottle gourd and *N. benthamiana* (Fig. 2E).

It was reported previously that a linearized blunt-ended CGMMV clone pCGHB310803 driven by the T7 promoter could not be transcribed *in vitro* unless an extra guanidine residue was added by LD-PCR [17]. In our study, we produced two vectors – with and without an extra guanidine nucleotide residue before the viral genome. Both vectors were infectious and caused typical symptoms on hosts, suggesting that the extra guanidine may not be necessary for transcription by the 35S promoter. A polyA tail was also added or omitted in our constructs, but there was no obvious difference in their infectivity, although recent research has shown that CGMMV possesses a small fraction of gRNA with polyadenylated tails [12].

In order to construct a cDNA clone of CGMMV tagged with green fluorescent protein (GFP), the coding region for GFP was placed in frame between the sequences coding for the MP and CP, generating the recombinant plasmid pCB-CGMMV-GFP. Until now, the CP promoter of CGMMV has not been identified. The coding strategy of CGMMV differs from other well-studied members of the genus *Tobamovirus*, such as TMV and pepper mild mottle virus (PMMoV). The MP overlaps only the RDRP in TMV, and no genes overlap in PMMoV, while in CGMMV, the MP overlaps both the RDRP and CP. In order to use the viral promoter to drive heterologous ORF expression with CGMMV vectors, the 5'-NTR of the CGMMV CP subgenomic sequence was determined. The primer pair p14/p3 (Supplementary Table 1) was used to amplify the subgenomic RNA of CGMMV by 5'-RACE, which demonstrated that most of the clones (12/13) had only 13 nucleotides (GTTTCTTTTGAAGATG) before the CP start codon, suggesting that the 5'-UTR of the CP subgenomic RNA is rather short. Based on our RACE result and the sequences of CP subgenomic RNA promoters of TMV, we chose to use 234 extra nucleotides before the CP initiation codon as the CP 5' subgenomic promoter.

It has also been suggested that RNA sequences within the CP ORF may form part of the sgRNA promoter [8], and when TMV-based transient expression vectors included 5-proximal CP ORF sequences upstream of the foreign gene, sgRNA levels increased [19]. We therefore prepared three constructs including differing lengths of the 5'-proximal CP ORF to test their efficiency for foreign protein expression. These pCB-CGMMV-GFP constructs were named according to the number of CP ORF bases used: CGMMV-27GFP, CGMMV-58GFP and CGMMV-78GFP, respectively (Fig. 2B). The AUG start codon at the beginning of the CP ORF was mutated so that only the AUG of the inserted GFP gene would be recognized in translation, at the same time maintaining the Asp amino acid encoded in the MP at the appropriate position. Using p35S-30B:GFP as template, primer pair p15/p16 (Supplementary Table 1) was used to amplify the turboGFP fragment. Using pCB-CGMMV as template, primer pairs p17/p18 and p19/p20 (Supplementary Table 1) were used to amplify the MP-GFP and GFP-CP fragment, respectively. The MP::GFP::CP fragment of CGMMV-27GFP was then constructed by overlap PCR. Similarly, primer pairs p21/p22 and p23/p24 were used to construct CGMMV-58GFP, and p21/p22, p24/p25 and p24/p26 (Supplementary Table 1) were used to construct CGMMV-78GFP. Then, the corresponding PCR fragments were overlapped by PCR, and the fusion PCR products were ligated into pGEM-T Easy Vector (Promega) and used to transform *E. coli*. The junctions and the inserted sequences were confirmed by sequencing. The MP::GFP::CP fragment was digested with *Nru* I-*Apa*LI and ligated into similarly digested pCB-CGMMV, creating the recombinant plasmid pCB-CGMMV-GFP. The cloned CGMMV-27GFP, CGMMV-58GFP and CGMMV-78GFP constructs were transferred to *A. tumefaciens* EHA105, and three transformants per binary vector construct were tested by agroinoculation of plant leaves.

Within two weeks of agroinoculation, green fluorescence could be seen on the new leaves of bottle gourd systemically infected by all three CP subgenomic promoter mutants, and two to three days later, the typical mosaic symptoms were obvious on the leaves (Fig. 3A). The presence of GFP and viral sequences in the symptomatic tissues was confirmed by RT-PCR (using p27/p28 for GFP and p29/p30 for CP detection) and western blot (Fig. 3B and C). When primer pair p31/p32 (Supplementary Table 1) from the MP and CP was used to amplify sequences from the leaves derived from pCB-CGMMV-GFP, an extra band of similar size to that detected by the same primer pair in the wild type could be seen, which suggested that the GFP gene had been deleted from the viral genome. Further sequencing of the deletion products of the three constructs suggested that all three constructs had reverted perfectly to wild type by deleting the GFP gene and the repeat sequence used as the CP promoter.

When we inoculated *N. benthamiana* directly with the three GFP constructs, no GFP fluorescence could be detected. When bottle gourd sap infected with CGMMV-GFP was used to inoculate *N. benthamiana,* only weak GFP fluorescence could be detected as a spot after 2 weeks,

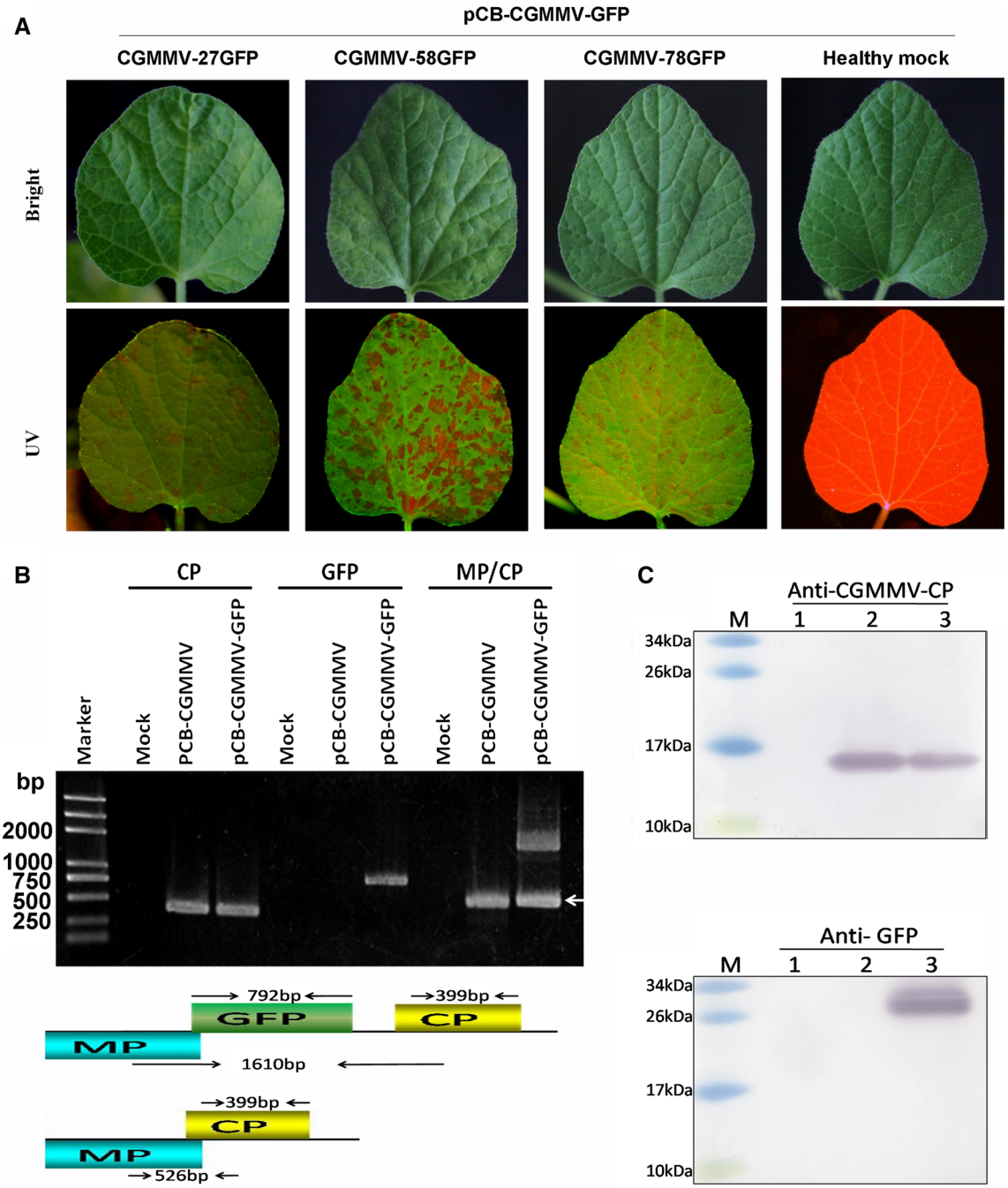

Fig. 3 Inoculation of pCB-CGMMV-GFP to bottle gourd. (A) Photographs under white and UV light of bottle gourd inoculated with pCB-CGMMV-GFP 19 dpi. (B) RT-PCR analysis of mRNA from CGMMV-infected or CGMMV-78GFP-infected bottle gourd. The primers for detecting CP, GFP and MP/CP and the corresponding lengths of the expected fragments are indicated by arrows with numbers in the construct maps at the bottom of the panel. The white arrow indicates an extra band similar in size to that amplified by the MP/CP primer pair from the wild type, suggesting that the GFP gene had been deleted from the construct genome. (C) Western blot analysis of CGMMV-78GFP coat protein and GFP in systemically infected bottle gourd. Lane M, protein marker; lane 1, healthy control plant; lane 2, CGMMV-infected plant; lane 3, CGMMV-78GFP-infected plant

which then disappeared several days later (data not shown), suggesting that the construct could infect *N. benthamiana* but that the GFP is not stable in this host. This may be because *N. benthamiana* is not the most suitable host for virus propagation, but a more important reason might be that insertion of heterologous ORFs in place of the CP ORF may markedly reduce transcriptional activity relative to wild-type levels [19].

It has been reported that the TRBO expression vector, a 35S-promoter-driven TMV replicon that lacks the TMV coat protein gene sequence, can express very high levels of foreign proteins in plants, even at levels of 3 to 5 mg/g fresh weight of plant tissue [13]. A similar vector based on sunn hemp mosaic virus (SHMV) yielded 600 μg GFP/g fw (25 % TSP) in *N. benthamiana* when co-expressed with the p19 silencing suppressor of tomato bushy stunt virus, while in the absence of p19, SHEC/GFP expression was nearly eliminated [16]. We also constructed three CGMMV/GFP expression vectors without the CP gene (CGMMV-27GFP-EV, CGMMV-58GFP-EV and CGMMV-78GFP-EV), but only a few fluorescent green spots occurred on inoculated leaves of *N. benthamiana* whether they were inoculated alone or co-inoculated with the p19 suppressor (data not shown).

The full-length infectious clone of CGMMV we have established clearly has biological activity and will assist future work on gene function and pathogenesis of the virus.

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