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Development of a cucumber green mottle mosaic virus-based expression vector for the production in cucumber of neutralizing epitopes against a devastating animal virus

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ABSTRACT

Virus-based expression systems have been widely exploited for the production of recombinant proteins in plants during the last thirty years. Advances in technology have boosted scale-up manufacturing of plant-made pharmaceuticals to high levels, via the complementation of transient expression and viral vectors. This combination allows proteins of interest to be produced in plants within a matter of days and thus, is well suited for the development of plant-made vaccines or therapeutics against emerging infectious diseases and potential bioterrorism agents. Several plant-based products are currently in varying stages of clinical development. To investigate the viability of virus-based expression systems for plant-made vaccines against porcine reproductive and respiratory syndrome virus (PRRSV), the most devastating threat to the pork industry in Canada, we cloned the full-length genome of a cucumber green mottle mosaic virus (CGMMV) isolate and developed a CGMMV-based expression vector. We further employed this vector to express the neutralizing epitope (NE) of PRRSV glycoprotein 5 (GP5) in cucumber leaves via agroinfiltration. The coding region of the GP5 NE was inserted downstream of the open reading frame for coat protein (CP) and expressed by a readthrough mechanism. The chimeric virus particles were stable and the expression levels reached as high as 35.84 mg/kg of cucumber leaf fresh weight. This study offers a promising solution to the production of a low cost, versatile and robust vaccine for oral administration against PRRSV through a chimeric virus particle display system.

1. Introduction

Cucumber green mottle mosaic virus (CGMMV) is an economically important seed-borne virus with cucurbitaceous crops as its major natural hosts. The virus was first identified in England (Ainsworth, 1935), and has subsequently been detected in many countries in Europe, Asia and the Middle East (Al-Shahwan and Abdalla, 1992; Fletcher et al., 1969; Liu et al., 2009; Ugaki et al., 1991; Yoon et al., 2008). In recent years, several reports have noted the occurrence of this virus in Australia and North America, including the United States and Canada (Kehoe et al., 2017; Li et al., 2015; Tesoriero et al., 2016; Tian et al., 2014). The virus has a single-stranded, positive-sense RNA of about 6400 nucleotides (nt) as its genome and belongs to subgroup II of the genus *Tobamovirus* in the family *Virgaviridae*. CGMMV particles are rod-shaped and approximately 300 nm x 18 nm in size. The genomic RNA encodes a 126-kDa protein (small replicase subunit), a 186-kDa readthrough protein known as RNA-dependent RNA polymerase (RdRp), a 30-kDa movement protein (MP) and a 17-kDa coat protein (CP) (Ooi et al., 2006; Teoh et al., 2009; Zheng et al., 2015). In infected leaves, CGMMV can yield a large amount of viral particles in a short period. The simple monopartite genome of CGMMV makes genetic engineering relatively easy for expression of exogenous proteins. Therefore, CGMMV has potential for the development of a virus-based expression system (Ooi et al., 2006; Zheng et al., 2015). Moreover, CGMMV particles produce naturally symmetrical nanostructures, and thus have a proficiency to carry and display small peptides, e.g. an antigen, attached to the outer part of their capsids (Brennan et al., 1999; Sugiyama et al., 1995). These chimeric virus particles could act as efficient carriers in the development of plant-based vaccines against animal diseases (Dalsgaard et al., 1997). In this study, we determined

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the full-length cDNA sequence of CGMMV isolated from cucumber plants in Ontario, Canada and developed a full-length cDNA clone. We further modified this vector for the production of chimeric virus particles bearing the neutralizing epitope (NE) of GP5 of porcine reproductive and respiratory syndrome virus (PRRSV), in cucumber plants.

2. Material and methods

2.1. RNA extraction, cDNA synthesis, small RNA deep sequencing and determination of the full-length genomic sequence of CGMMV

Greenhouse cucumber leaves (cultivar Picowell RZ F1, Rijk Zwaan) showing mottle and mosaic symptoms were used for extraction of total RNA. Purification of total RNA and small RNA, construction and sequencing of small RNA libraries, and next generation sequencing data analysis were performed essentially as described previously (Chen et al., 2015).

The near full-length genome sequence of CGMMV was confirmed by RT-PCR and subsequent DNA sequencing essentially as described (Cui et al., 2013). The 3′ and 5′ ends of the genome of the virus were determined using the random amplification of cDNA fragments (RACE) kits essentially following the supplier's instructions (Invitrogen, Burlington, Ontario, Canada).

2.2. Construction of infectious CGMMV clone

The binary vector pCB-Rz was obtained by modification of pCB 301 (Xiang et al., 1999) to contain a double *35S* promoter and a ribozyme sequence at the 3′ end, upstream of the terminator (*Rz-T*). The double *35S* promoter and *Rz-T* fragments were amplified from pCass4-Rz (Annamalai and Rao, 2005). The full-length cDNA sequence of the CGMMV genome was cloned into pCB-Rz via several steps. The resulting clone pCB-CG was confirmed by PCR and DNA sequencing. Primers used in this study are listed in Supplementary Table S1.

2.3. Construction of chimeric CGMMV presenting the NE of GP5

To test if the CGMMV vector can be used to express the NE (a nine-amino-acid peptide: Ser^{37}-Gln-Leu-Gln-Ser-Ile-Tyr-Asn-Leu^{45} which induces the production of neutralizing antibodies against the disease in pigs) of PRRSV GP5 (Roques et al., 2013) as a CP-NE fusion protein, pCG-NE was generated (Supplementary Figure S1). In brief, the NE coding sequence was inserted in frame right before the stop codon of CP of the *Nhe*I and *Bam*HI fragment by overlapping PCR. Then the modified cDNA fragment was inserted into the corresponding sites of pCB-CG to generate pCG-NE.

To modify the CGMMV vector for readthrough translation, the CGMMV clone pCB-CG was engineered to include the readthrough (RT) sequence, either "TCT-*AAA-TAG-CAA-TTA*" (RT1) or "TCC-*AAA-TAG-CAA-TTA*" (RT2) (Ooi et al., 2006; Teoh et al., 2009) by replacing the wild type CP stop codon. The resulting vector was further modified by insertion of the coding sequence for GP5 NE to generate the expression vector pCG-RT1NE, or a short cDNA fragment encoding a C-terminal cmyc-tagged NE to construct the chimeric CGMMV vector pCG-RT2NEcmyc. The sequences of all the modified CGMMV vectors were verified by Sanger sequencing.

2.4. Agroinfiltration

The CGMMV based constructs were agroinfiltrated into plants using *Agrobacterium tumefaciens* (strain GV3101). Preparation of agrobacterial culture and agroinfiltration were as described previously (Cui et al., 2013; Kapila et al., 1997). Essentially, the agrobacterial suspension was infiltrated into either the abaxial leaf epidermis of 3-week-old *Nicotiana* species, or the cotyledons of 10-day-old cucumbers (*Cucumis sativus* var. Straight 8), watermelons (*Citrullus lanatus* var. Greybelle) and cantaloupes (*Cucumis melo* var. Hami), with a 1 ml syringe. For each plant species, five seedlings (two leaves or cotyledons each) were used. The sequence of the modified CGMMV in inoculated plants was verified by sequencing of RT-PCR products.

2.5. Protein analysis

For detection of CGMMV, double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was performed using a CGMMV ELISA kit (catalog number SRA 45701; Agdia, Elkhart, IN, USA) and following the manufacturer's protocol. To study the protein production of chimeric CGMMV in cucumber, western blot analysis was performed using systemic leaf tissue samples (Cui et al., 2013). Immunoblots were visualized by Immobilon Western (Millipore Corporation, Billerica, MA, USA). The protein marker was taken as a blight-field image and then overlapped with the immunoblot images to determine the protein sizes.

For transmission electron microscopy (TEM), CGMMV particles were extracted following the procedure as described (Seo et al., 2013). The extracted virus particles were added to the anti-cmyc agarose conjugate (Sigma-Aldrich) and incubated for 2 h on an orbital shaker at 4 °C. After incubation, the virus-bead complexes were then precipitated by centrifugation and washed six times. The beads were resuspended in 100 µl of washing buffer in the final step. CGMMV wild type-infected leaves were processed in the same procedure as a negative control. The resulting suspension was subjected to negative staining procedure with 2% uranyl acetate (pH 4.5) and visualization using TEM.

3. Results

3.1. Cloning and sequencing of the full-length genome sequence of CGMMV

Next-generation sequencing of the small RNA libraries derived from cucumber leaves generated a total of 9177495 raw reads and 8254371 (89.94%) clean reads (> 17 nt) after trimming. These clean reads were assembled into large contigs, and those longer than 100 nt were used to search against the NCBI database using BLASTN. The Blast results with 59 contigs identified only one virus, CGMMV. This isolate was named CGMMV-Ca. RT-PCR and 5′ and 3′ RACE were employed to determine the complete genome sequence of CGMMV-Ca. The genome sequence of the CGMMV-Ca isolate was submitted to the NCBI database (GenBank accession number MH426842). CGMMV-Ca is 6423 bp in length. BLASTN searches showed that CGMMV-Ca shares > 98% sequence identity at both nt and amino acid (aa) levels with CGMMV-W (GenBank accession # AB015146), an isolate from watermelon in Japan and > 99% with the ABCA13-01 isolate (accession # KP772568) from cucumber in Alberta, Canada. The 5′ and 3′ UTRs of CGMMV consist of 58 and 174 nt, respectively. The first ORF (including readthrough) (nt 59 to 5005) encodes a 186 kDa protein and a 129 kDa non-readthrough protein (nt 59 to 3493). These two proteins contain the domains including methyltransferase, helicase domain and RNA-dependent RNA polymerase (only in the 186 kDa readthrough protein), which are essential for viral replication. The other two ORFs downstream of ORF1 encode MP (264 aa) and CP (161 aa) (Fig. 1B).

3.2. Construction of a CGMMV-Ca-based infectious clone for agroinfiltration

The full-length cDNA of CGMMV was successfully cloned into plasmid pCB-Rz (Fig. 1A) between the double *35S* promoter and the ribozyme sequence (*Rz*), to generate pCB-CG (Fig. 1B). Since the 5′ UTR sequence is important for efficient replication and biological activities of the positive strand RNA virus (Boyer and Haenni, 1994; Janda et al., 1987), the transcription start site was designed precisely at the nucleotide "G" of the CGMMV genome to produce the authentic viral 5′ end. Transcription of the full-length CGMMV cDNA, followed by

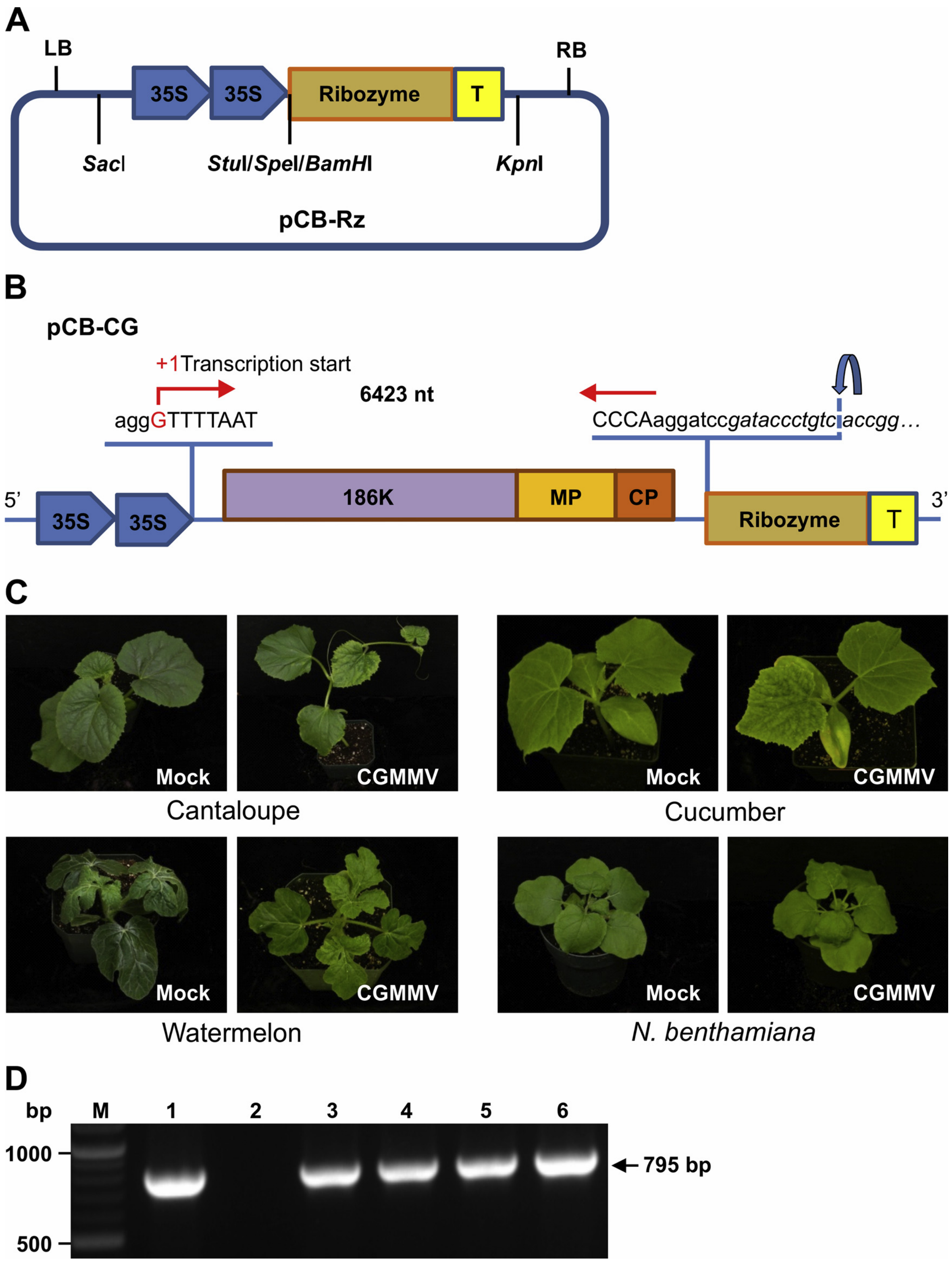


Fig. 1. Construction of the CGMMV infectious clone. **A**. Schematic representation of T-DNA vector, pCB-Rz, which was used to clone the cDNA of CGMMV genomic RNA between the left border (LB) and right border (RB). The vector contains a double *CaMV 35S* promoter (*35S*), a ribozyme sequence (*Rz*) and a *CaMV 35S* terminator (*T*) derived from pCass4-Rz. **B.** Insertion of CGMMV cDNA into pCB-Rz to create pCB-CG. Single lines and open box represent noncoding and coding regions, respectively. The self-cleavage site of ribozyme is indicated by a curved arrow. The uppercase letters present the authentic viral sequence, whereas, the lower case ones show the additional sequence on backbone vector. The transcription start site was designed precisely at the nucleotide (nt) "G" of the CGMMV genome to produce the authentic viral 5′ end. Transcription of the full-length CGMMV cDNA, followed by ribozyme self-processing would produce the entire viral genome with an additional 18-nt extension at the 3′ UTR. **C.** Symptoms of plants inoculated with the CGMMV infectious clone, pCB-CG, at 15 dpi. Cantaloupe (*Cucumis melo* var. Hami): mosaic and crinkled leaves; Watermelon (*Citrullus lanatus* var. Greybelle): mosaic and clearing vein symptoms; Cucumber (*Cucumis sativus* var. Straight Eight): mosaic and crinkled leaves; and *N. benthamiana*: mild symptoms with crinkle and mosaics at leaf edges. **D.** RT-PCR analysis of infiltrated plants at 15 dpi. The coding sequence of MP was amplified as 795 bp in size. Lane M: 100 bp DNA marker; 1: Positive control (CGMMV infected cucumber); 2: Negative control (buffer-infiltrated cucumber); lanes 3 through 6: Cucumber, watermelon, cantaloupe and *N. benthamiana* infiltrated with pCB-CG, respectively.

ribozyme self-processing would produce the entire viral genome with an additional 18-nt extension at the 3′ UTR (Fig. 1B).

A pathogenicity test of the CGMMV derived clone, pCB-CG, was conducted using its natural hosts including cucumbers, cantaloupes and watermelons, as well as experimental hosts, *N. benthamiana* and *N. tabacum.* The inoculated cucurbit plants showed obvious mosaic, chlorosis, necrosis and crinkle symptoms on newly emerging leaves at 10 dpi, similar to the symptoms of wild-type CGMMV infected plants (Fig. 1C). Mild mottling and distortion appeared on the upper leaves of *N. benthamiana* at 15 dpi (Fig. 1C). No typical systemic symptoms were displayed in *N. tabacum*. No mock-treated plants infiltrated with buffer exhibited any of these symptoms (Fig. 1C). To further confirm the infectivity, samples of systemic leaves were collected and subjected to DAS-ELISA and RT-PCR. Both DAS-ELISA and RT-PCR detected CGMMV in cucumber, watermelon, cantaloupe and *N. benthamiana* plants infiltrated with the pCB-CG clone (Fig. 1D), but not in *N. tabacum* plants. These data suggest that the full-length cDNA clone of CGMMV was infectious in cucumbers, watermelons, cantaloupes and *N. benthamiana,* but not in *N. tabacum*. This is consistent with the known host range of CGMMV.

3.3. pCG-RT1NE can infect both N. benthamiana and cucumber but does not produce chimeric CGMMV

To use the CGMMV infectious clone as a vector for the expression of a potential vaccine against PRRSV, we first constructed a CGMMV modified clone pCG-NE to express the NE as a CP-NE fusion. Unfortunately, this direct fusion compromised viral infectivity (Supplemental Figure S1), suggesting CGMMV cannot tolerate with addition of the NE. We thus tested if the NE can be expressed via a readthrough mechanisms.

pCG-RT1NE was generated using the readthrough sequence (TCT-*AAA-TAG-CAA-TTA*) named RT1 (Fig. 2A). This readthrough sequence was operational in muskmelon (Ooi et al., 2006). pCG-RT1NE was agroinfiltrated into cucumber and *N. benthamiana*. The pCG-RT1NE clone could infect both cucumber and *N. benthamiana*. The infected *N. benthamiana* showed very mild symptoms, with clearing veins and blistering on leaf edges (Fig. 2B bottom), whereas more severe symptoms such as wrinkled and mosaic leaves were observed in the infected cucumber seedlings (Fig. 2B top). RT-PCR results confirmed the presence of chimeric CGMMV in all infected cucumber and *N. benthamiana* plants infiltrated with pCG-RT1NE (Fig. 2C). We further extracted total proteins from the plants infected by the chimeric virus and the protein extracts were subjected to immunoblotting analysis, using anti-CP antibody, to detect the chimeric CP containing the NE of GP5. Unexpectedly, only one single protein, of approximately 17 kDa in size, corresponding to the predicted size of the CP from the wild-type clone of CGMMV was detected (Fig. 2D). No proteins with the predicted size for the CP fusion protein (CP-NE) was detected. This result indicated that the RT1 sequence may not be an optimum sequence for the expression of protein of interest.

3.4. pCG-RT2NEcmyc can infect only cucumber and produce chimeric CGMMV

To overcome the non-readthrough translation of pCG-RT1NE, pCG-RT2NEcmyc was constructed using the readthrough sequence (TCC-*AAA-TAG-CAA-TTA*) designated RT2 which was previously reported to be operational in muskmelon as well (Teoh et al., 2009) (Fig. 3 A). As shown, both RT1 and RT2 contain the minimal readthrough context "*AAA-TAG-CAA-TTA*", however, they are different at the third nucleotide of the 5′ three additional nucleotides, "TCT" in RT1 and "TCC" in RT2. TCC and TCT encode the same amino acid, serine. In order to facilitate quantification and detection of NE via western blot and immunoprecipitation, a cmyc tag was fused to the C-terminus of the NE (adding 10 amino acids Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu to the NE). In contrast to pCG-RT1NE, pCG-RT2NEcmyc could only infect cucumber. The infected cucumber displayed blistering and cupped leaves that were different from the symptoms induced by pCG-RT1NE (Fig. 3B). RT-PCR confirmed the presence of the chimeric CGMMV in infiltrated cucumber (Fig. 3C). Further immunoblotting analysis detected both the chimeric CP containing NEcmyc (CP-NEcmyc) with a predicted size of approximately 20 kDa, and wild-type of CP (17 kDa) in cucumber infiltrated with pCG-RT2NEcmyc using the anti-CGMMV CP antibody (Fig. 3D) or the CP-NEcmyc using the anti-cmyc antibody (Fig. 3E). The CP-NEcmyc fusion protein in cucumber was quantified (Supplementary Figure S2). The expression level of CP-NEcmyc was estimated to be 35.84 μg/g fresh weight (0.067% TSP) at 21 dpi, and decreased to 16.60 μg/g fresh weight (0.038% TSP) at 50 dpi (Fig. 3F).

3.5. The NE is present on the surface of the chimeric CGMMV CP that is stable for at least 45 dpi

To determine whether the NE of PRRSV GP5 was displayed on the surface of CGMMV capsids, the chimeric virions extracted from pCG-RT2NEcmyc-infiltrated leaves immunoprecipitated using anti-cmyc beads and were imaged by TEM, as shown in Fig. 4A. The amount of chimeric CGMMV bound to anti-cmyc beads was substantially more abundant when compared to wild type CGMMV, as a negative control, as observed at 19 000X magnification (Fig. 4A top). Moreover, there were more beads unbound by wild type CGMMV when compared to chimeric CGMMV in the same field. Each virion shape was an intact rod about 300 nm in length and 18 nm in width, which is typical for CGMMV (Fig. 4A bottom). The results demonstrated that the affinity for chimeric virions of anti-cmyc beads was much higher than that for the wild type CGMMV virions, due to the immuno-attraction between cmyc antigen and the anti-cmyc beads. These data affirmed that NE, as well as cmyc, was successfully displayed on the surface of chimeric CGMMV capsids.

To investigate the stability of pCG-RT2NEcmyc in cucumber, RT-PCR and subsequent sequencing of the amplified DNA fragment were conducted using RNAs isolated from newly emerging young leaves of the infiltrated plants at 45 dpi. The results from RT-PCR confirmed that the amplicon (738 bp in length) of the coding sequence for the chimeric CP and 3′UTR was preserved in the plants infiltrated with pCG-RT2NEcmyc, which is larger than the amplified fragment (663 bp in length) from the RNA sample isolated from the plants infiltrated with pCB-CG (Fig. 4B). The NEcmyc sequence present in the chimeric virus was also confirmed by sequencing of the RT-PCR product (Fig. 4C).

4. Discussion

CGMMV is an important viral pathogen that impedes Cucurbita crop production in the world. CGMMV belongs to the tobamovirus group. As *Tobacco mosaic virus* (TMV), a member of tobamoviruses, can direct expression of large amounts of foreign proteins in plants (Donson et al., 1991), we examined the possibility to develop a CGMMV-based expression vector. At first, we determined the genomic sequence of CGMMV-Ca, a CGMMV isolate in Ontario, Canada, and developed a full-length cDNA infectious clone derived from this isolate. This clone could be introduced into plants directly through agroinfiltration. We further used this vector as a viral expression system for peptide display on the surface of CGMMV capsids. A few previous studies have reported the decoration of CGMMV capsids with different peptides such as *Hepatitis B virus* surface antigen (HBsAg) (Ooi et al., 2006) and a truncated dengue virus envelope protein (Teoh et al., 2009) via readthrough translation. However, the infectious clones used in these studies had to be transcribed via in vitro transcription to obtain the viral RNA transcripts, which were then mechanically inoculated onto muskmelon. Recently, Zheng et al. (2015) have reported the development of a viral vector derived from a CGMMV isolate from bottle gourd in China. Infection with this vector can be established via agroinoculation. *GFP*

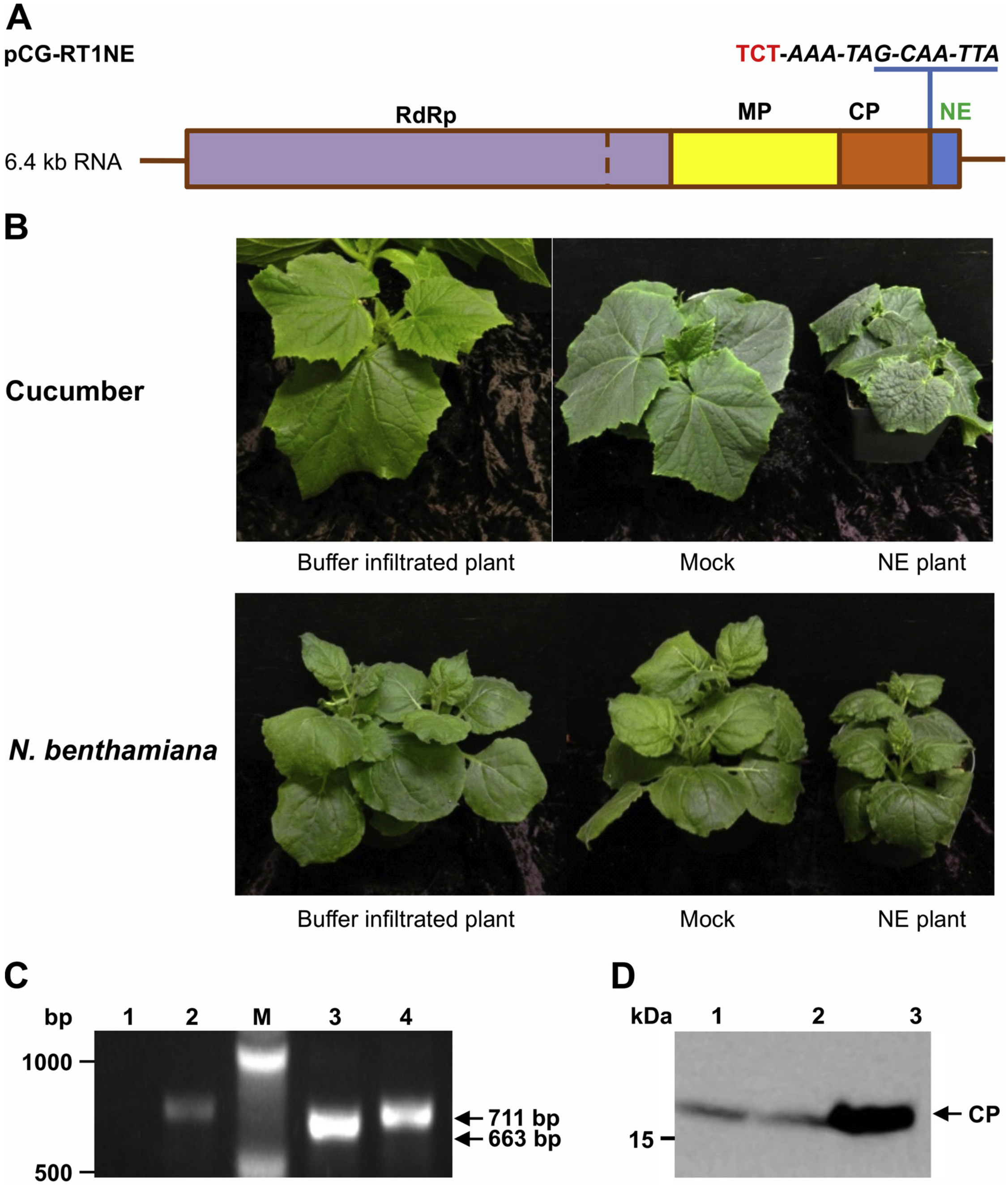


Fig. 2. Construction of pCG-RT1NE. **A.** Schematic representation of pCG-RT1NE. **B.** Symptoms in cucumbers and *N. benthamiana* infiltrated with pCG-RT1NE at 21 dpi. **C.** RT-RCR results of pCG-RT1NE infiltrated plants at 21 dpi. The coding sequence of CGMMV CP fused with NE plus 3′ UTR was amplified (711 bp). Lane 1: Negative control (buffer-infiltrated cucumber); 2: Infiltrated *N. benthamiana*; M: 100 bp DNA ladder; 3: pCB-CG infiltrated cucumber as a positive control (663 bp); 4: Infiltrated cucumber. **D.** Western blot analysis of pCG-RT1NE-infected leaves using anti-CP antibody. The protein size label is derived from protein molecular weight markers. Lane 1: Chimeric virus-infected cucumber; 2: Chimeric virus-infected *N. benthamiana*; 3: Wild-type virus- infected *N. benthamiana*.

encoding green fluorescent protein (GFP) was placed between MP and CP sequences and transcribed by an additional native subgenomic promoter of CP. Transcription of wild type CP was driven by the native subgenomic promoter. GFP was initially detected as a free protein in new leaves of bottle gourd, and along with infection, deletion of *GFP* occurred due to the recombination of repeating promoters. Jailani et al. (2017) also found that the inserted foreign gene was not stable in a recombinant CGMMV. In this case, most of the CP sequence was deleted and GFP was fused in frame downstream to the N-terminal region of CP. Although GFP expression was significantly enhanced in inoculated *N. benthamiana* plants at 5 dpi, the *GFP* gene was lost at 9 dpi. Despite a huge success with the TMV-derived vector (Donson et al., 1991), CGMMV seems not suitable for expression of long polypeptides. Therefore, we modified the full length cDNA of CGMMV-Ca to express the NE of PRRSV GP5 as a fusion to the CP C-terminus in cucumber. In our study, the infectivity rate of cucumber plants via agroinfiltration was 100%. Moreover, vacuum infiltration could also be applied to highly efficiently introduce the vector into a large quantity of plants.

Systemic movement in infected plants is one of the key processes of viral pathogenesis and results in a large amount of viral proteins produced throughout the plant. Taking advantage of this property, a chimeric CGMMV pCG-NE, expressing the NE of PRRSV GP5 was designed to preserve the ability of wild type CGMMV to spread systemically in cucumber (Supplementary Figure S1). The direct fusion of the nine-

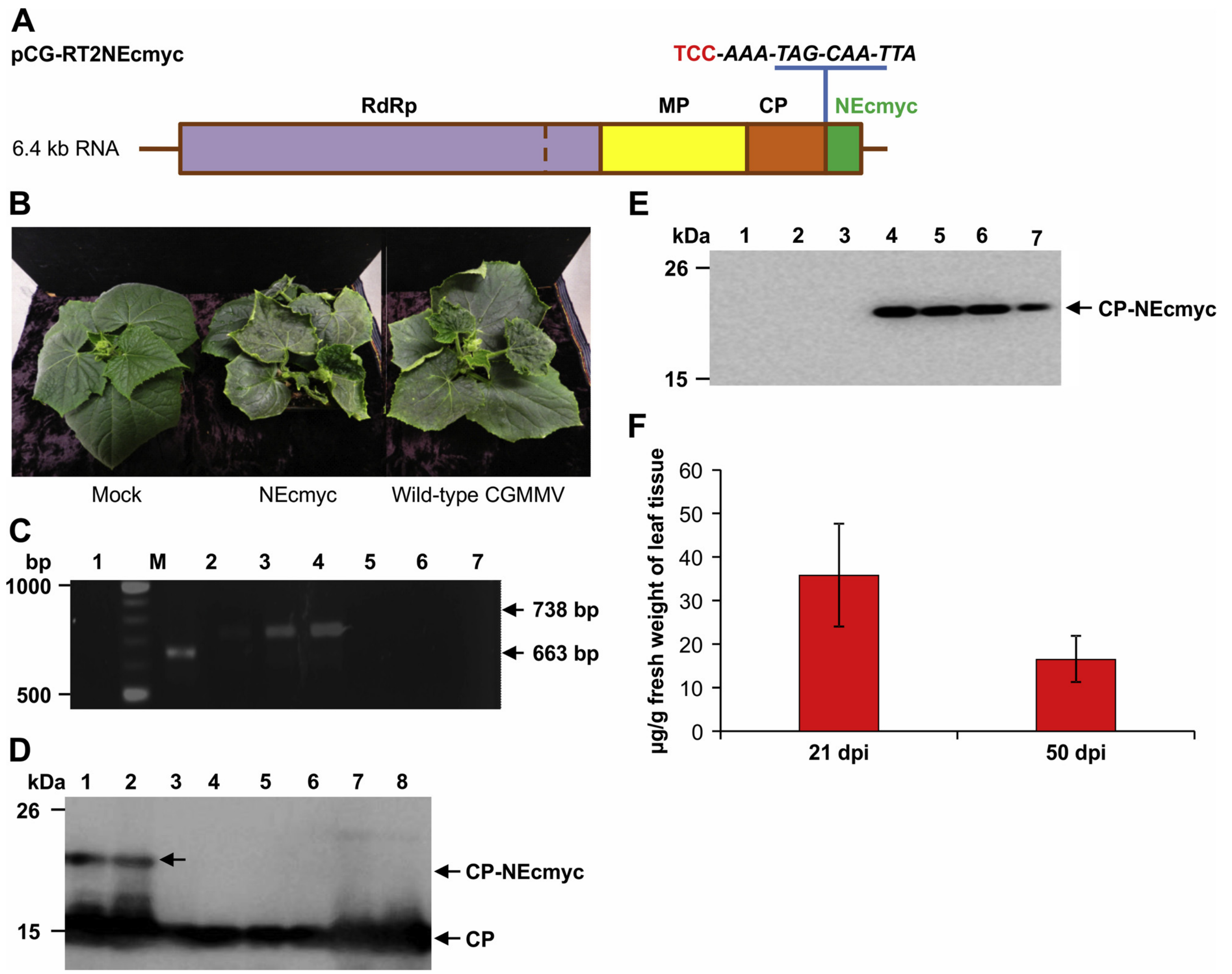
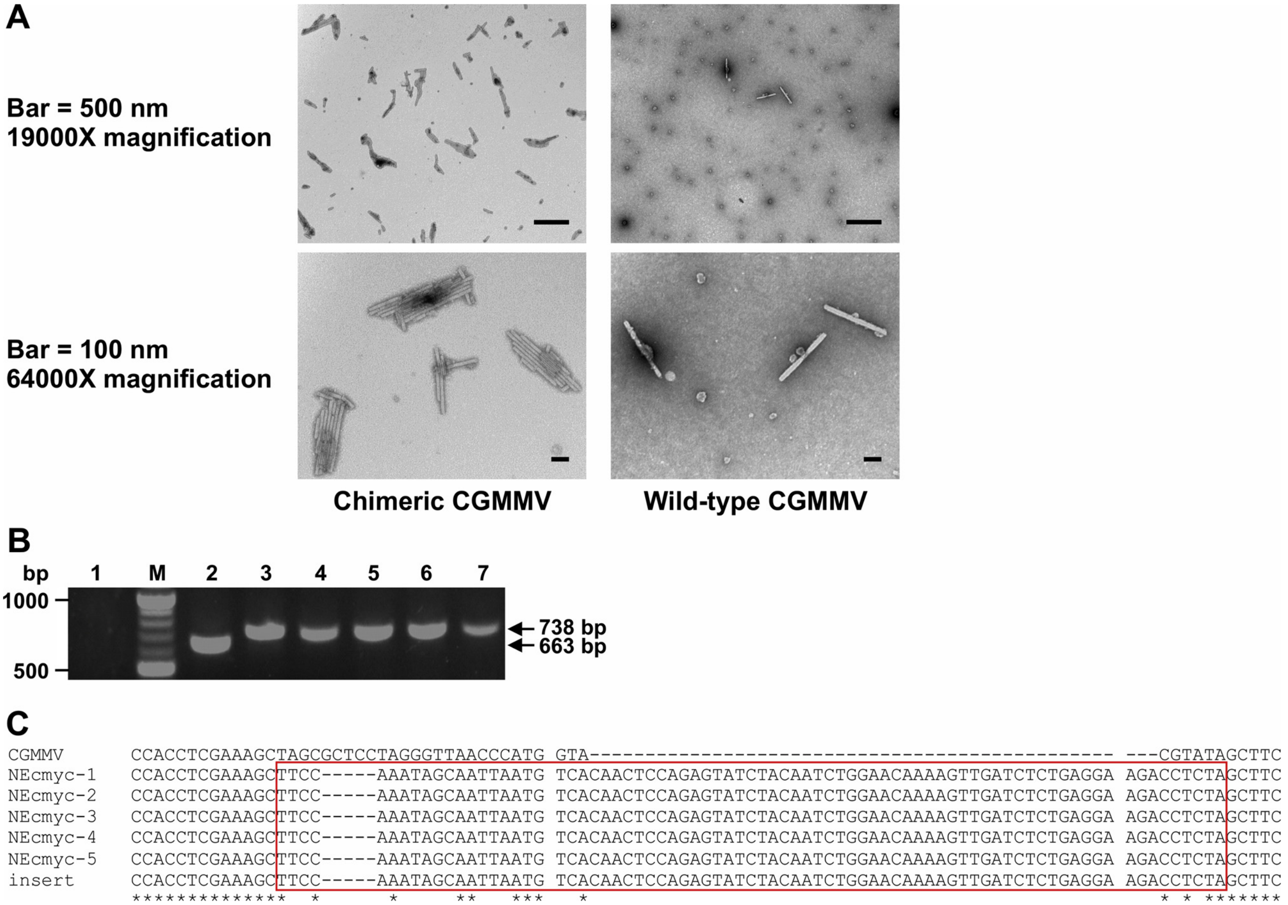


Fig. 3. pCG-RT2NEcmyc including CP extension with the readthrough translational context RT2. **A.** Schematic representation of pCG-RT2NEcmyc. **B.** Symptoms in cucumber infiltrated with pCG-RT2NEcmyc at 21 dpi. **C.** RT-PCR analysis of plants infiltrated with pCG-RT2NEcmyc at 21 dpi by amplification of the extended CP sequence (738 bp). Lane 1: Negative control (buffer-infiltrated cucumber); M: 100 bp DNA ladder; 2: Wild-type CGMMV-infected plant (663 bp); 3 to 5: pCG-RT2NEcmyc infiltrated cucumber; 6 to 8: pCG-RT2NEcmyc-infiltrated *N. benthamiana.* **D.** Western blot analysis of pCG-RT2NE-infected leaves using anti-CP antibody. The protein size labels are derived from protein molecular wieght markers. Lanes 1 and 2: Chimeric virus-infected cucumber. Lanes 3 to 6: Wild-type virus-infected *N. benthamiana*. Lanes 7 and 8: Wild-type virus-infected cucumber. **E.** Western blot analysis of pCG-RT2NE-infected plants with anti-cmyc antibody. CP fusions containing NEcmyc were detected (˜20 kDa). The protein size labels are derived from protein molecular wieght markers. Lane 1: Non-infiltrated plant; 2: Buffer-infiltrated plant; 3: pCB-CG infiltrated plant; 4 to 7: pCG-RT2NEcmyc infiltrated cucumber. **F.** Chimeric CP expression levels in infected leaves (µg per g fresh weight). Error bars represent the standard deviation of the mean (n = 3).

amino-acid NE to CP resulted in chimeric CGMMV which demonstrated compromised infectivity in the infiltrated plants, probably due to the incompetence of virus assembly (Dawson et al., 1989; Saito et al., 1990; Takamatsu et al., 1990). Thus, we exploited ribosomal readthrough translation to maintain systemic virus movement. Several families of plant viruses including tobamoviruses, luteoviruses, benyviruses and tombusviruses have been reported to use the stop codon readthrough as a gene expression strategy (Harrell et al., 2002). In this study, two sequences named RT1 and RT2, were used to express NE and NEcmyc, respectively. Both sequences met the minimal context required for CGMMV readthrough translation and only differed in one nucleotide (upstream of the minimal context) which did not alter the amino acid sequence. Interestingly, RT1, "TCT-*AAA-TAG-CAA-TTA*", failed to direct readthrough translation and pCG-RT1NE could efficiently infect both the natural host cucumber and the experimental host *N. benthamiana* (Fig. 2). In contrast RT2, "TCC-*AAA-TAG-CAA-TTA*" successfully led to expression of the readthrough protein CP-NEcmyc and pCG-RT2NEcmyc could only infect cucumber but not *N. benthamiana* (Fig. 3). Taken together these data suggest that expression of the NE either as a CP fusion or via translational readthrough apparently negatively affects vial infectivity. Nevertheless, the CP-NEcmyc expression vector pCG-RT2NEcmyc seemed very stable as no viral deletion mutants were found in cucumber after 45 dpi (Figs. 3F; 4B and C), which offers great potential for optimization and application of this vector.

Theoretically, CGMMV is a highly promising platform for antigen display. CGMMV possesses a symmetrical helical structure composed of about 2100 molecules of CP along its rod shape. The topography of all viruses in this family, including CGMMV, shares similar CP structures, with a four-a helix CP core and both the N- and C-termini exposed on the surface of virus particles (Lobert et al., 1987; Namba and Stubbs, 1986). Our immunoprecipitation and TEM results supported that NEcmyc protruded outward from the CGMMV virion, together with the C-terminal portion of CP and was able to bind to anti-cmyc beads, leading to the formation of virus-bead complexes (Fig. 4A). The rate of translational readthrough for tobamoviruses is estimated to be 5–10%, which seems low. However, as each virion could potentially carry approximately 105–210 copies of PRRSV-NE on its surface and the



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CGMMV        CCACCTCGAAAGCTAGCGCTCCTAGGGTTAACCCATG GTA----------------------------------------------- ---CGTATAGCTTC
NEcmyc-1     CCACCTCGAAAGCTTCC-----AAATAGCAATTAATG TCACAACTCCAGAGTATCTACAATCTGGAACAAAAGTTGATCTCTGAGGA AGACCTCTAGCTTC
NEcmyc-2     CCACCTCGAAAGCTTCC-----AAATAGCAATTAATG TCACAACTCCAGAGTATCTACAATCTGGAACAAAAGTTGATCTCTGAGGA AGACCTCTAGCTTC
NEcmyc-3     CCACCTCGAAAGCTTCC-----AAATAGCAATTAATG TCACAACTCCAGAGTATCTACAATCTGGAACAAAAGTTGATCTCTGAGGA AGACCTCTAGCTTC
NEcmyc-4     CCACCTCGAAAGCTTCC-----AAATAGCAATTAATG TCACAACTCCAGAGTATCTACAATCTGGAACAAAAGTTGATCTCTGAGGA AGACCTCTAGCTTC
NEcmyc-5     CCACCTCGAAAGCTTCC-----AAATAGCAATTAATG TCACAACTCCAGAGTATCTACAATCTGGAACAAAAGTTGATCTCTGAGGA AGACCTCTAGCTTC
insert       CCACCTCGAAAGCTTCC-----AAATAGCAATTAATG TCACAACTCCAGAGTATCTACAATCTGGAACAAAAGTTGATCTCTGAGGA AGACCTCTAGCTTC
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Fig. 4. The presentation of NEcmyc on the surface of chimeric CGMMV. **A.** TEM results of chimeric CGMMV under different magnification, 19 000X and 64 000 × . The number of chimeric virus particles bound to anti-cmyc beads was substantially larger when compared to the wild-type viruses. At 64 000X magnification, these virus particles displayed the rod shapes which are characteristics for CGMMV. **B.** RT-PCR analysis of infiltrated plants at 45 dpi. Lane 1: Negative control (buffer-infiltrated plant); M: 100 bp ladder; 2: pCB-CG infiltrated cucumber (663 bp); lanes 3 to 7: pCG-RT2NEcmyc infiltrated cucumber (738 bp). **C.** Sequence alignment of chimeric virus. The insert sequence of NEcmyc was detected in all infiltrated cucumber plants at 45 dpi.

particle display maximizes the capacity of the NE as an antigen, the data from this study warrant a feeding test to examine the ability of the chimeric CGMMV-NEcmyc as an effective vaccine against PRRSV.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.04.006.

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