[BIO39] Application of cucumber green mottle mosaic virus vector as peptide presentation system

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Introduction

Recently plant viruses have been exploited as an alternative production method for pharmaceutically important peptides [1-9]. Antigenic peptides that were produced through this approach have been shown to be immunogenic. Thus, chimeric plant virus vector could be used as vaccine production system. Immunological studies also showed that chimeric virus based vaccine can be administered both orally and through subcutaneous route [10, 11]. This makes chimeric virus vector a new, safe, versatile and cost effective method for vaccine production.

The "a" determinant of the hepatitis B surface antigen (HBsAg) is present in all serotypes of HBV; it has been used as the basis for almost all HBsAg diagnostic kit and vaccine development.

The "a" determinant of HBsAg is a conformational epitope. Although the structure of HBsAg has yet to be determined, physical properties of HBsAg have been very well characterized. The epitope is heavily cross-linked through disulfide bridges [12], and glycosylated at several sites [12-15]. *In vitro* production of HBsAg will have to maintain the epitope in correct conformation and proper post-translational modification is necessary to maintain its antigenicity and antibody binding capabilities [11, 15-18]. That is why the HBsAg cannot be produced in prokaryotic system. Plant based production system has been developed [17]. The HBsAg produced from the system was shown to be antigenic, and binds to specific HBsAg monoclonal antibody.

We have successfully constructed a novel Cucumber Green Mottle Mosaic Virus (CGMMV) vector, and base on this vector a chimeric CGMMV vector was built. Both constructs were used to produce infectious transcript that was later used to inoculate muskmelon plants. The coat protein of the chimeric virus vector produces chimeric CGMMV that randomly displayed HBsAg on the surface. It produces two types of coat proteins; the native coat protein and recombinant coat protein that have HBsAg "a" determinant fused to the C-terminal. The present of HBsAg on the virus particles was successfully detected using commercial HBsAg diagnostic kit.

Materials and methods.

The CGMMV vector and the chimeric CGMMV vector.

The map of the CGMMV vector that was constructed for use as vaccine production system (pCGT7X) is shown in (Figure 1). The pCGT7X was used to construct chimeric CGMMV vector. The map of the chimeric CGMMV vector (pCGHB310803) is shown in (Figure 2). The pCGHB310803 was constructed by ligating the HBsAg coding sequence into the *Hin*d III restriction site of the pCGT7X and amber stop codon of the CGMMV coat protein gene was modified to accommodate amber stop read through sequence.

Preparation of in vitro transcription template DNA.

Template for *in vitro* transcription was synthesized in a 50µl PCR reaction. The reaction contained 1x Thermopolymerase buffer (NEB) with $MgSO_4$ concentration adjusted to 3mM, 200µM of PCR nucleotide mix (Finzymes), 1µM of each forward and reverse primer (sequences are not shown due to patenting requirement), 15ng of pure pCGHB310803 or pCGT7X, 2U of DeepVent$_R$® DNA polymerase (NEB) and 1U of *Taq* DNA polymerase (Promega). The PCR cycles were as follows: 95°C initial denaturation for 3 minutes, followed by 32 cycles of 98°C denaturation for 10 second, and 65°C annealing and elongation for 7 minutes.

The PCR product was purified through two rounds of phenol-chloroform extractions followed by ethanol-Lithium precipitation. The precipitated DNA was then reconstituted in 10µl of ultra-pure water for each 50µl of starting PCR product.

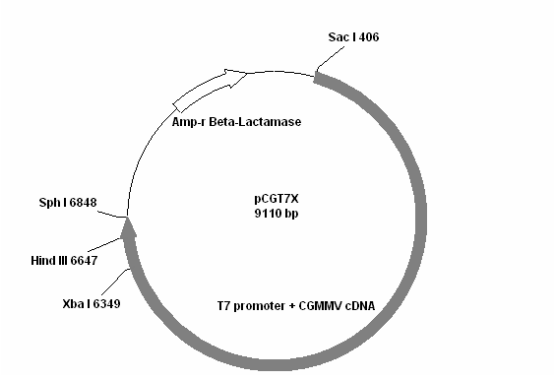

FIGURE 1 The schematic diagram of the pCGT7X The cDNA of the CGMMV and T7 promoter are clone into a *Hin*d III deletion variant of pUC19.

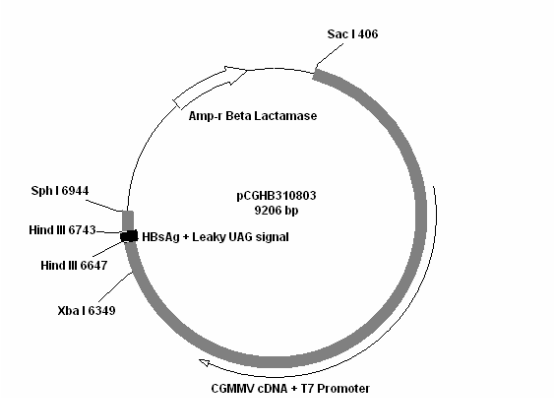

FIGURE 2 The schematic diagram of the pCGHB310803. The HBsAg "a" determinant coding sequence is cloned down stream of the CP gene. The expression of the HBsAg is controlled by leaky UAG sequence.

<u>In vitro</u> transcription to produce infectious RNA transcript.

The *in vitro* transcription reaction was carried out using Ambion® mMessage mMachine™ T7 kit, according to manufacturer protocol.

Double antigen sandwiched (DAS) ELISA with MONOLISA HBsAg plus Diagnostic kit (BioRad).

All reactions were carried out according to manufacturer protocol.

Inoculation of infectious transcript onto muskmelon plants.

Inoculation of infectious transcript onto muskmelon plants was carried out by mixing the transcription mix with inoculation buffer that contains (5% celite and 10% Diatomaceous Earth (Sigma), in 25mM Tris-glycine buffer pH 7.5). The mixture is then rubbed onto cotyledons of 7 day-old muskmelon seedlings.

RT-PCR to detect chimeric virus infection in muskmelon plants.

Total RNA was isolated and purified from muskmelon plant using QIAGEN RNeasy Plant mini kit. 5µg of the RNA were then mixed with 0.5µM of primer CGMMV3'UTR (5'AAA GCA TGC TGG GCC CCT ACC CGG GGA A-3'), and 100µM of PCR nucleotide mix (Finzymes) in 13µl reaction mix. This reaction mix was heated to 65^{o}C to denature the RNA followed by quick quench on ice. After that, 4µl of 5x First-strand buffer (InvitrogenTM™), 1µl of 0.1M DTT, 1µl RNaseOUT™ (Invitrogen™) and 200u of Superscripts™ III (Invitrogen™) reverse transcriptase

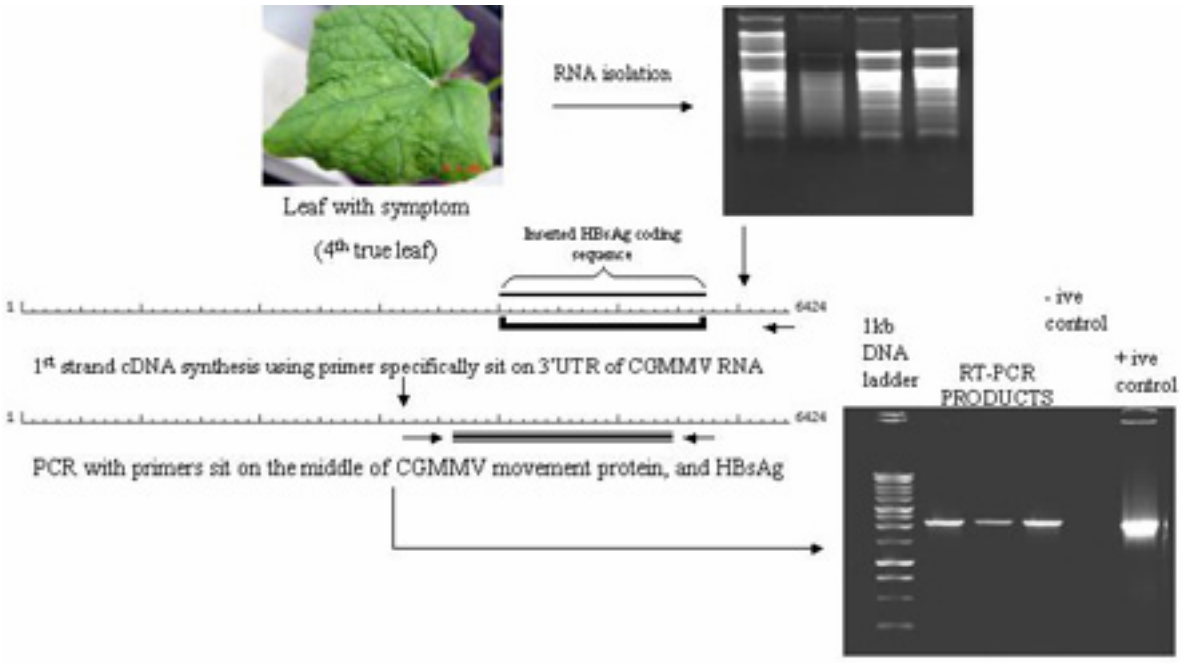

FIGURE 3 Diagram shows the detection of chimeric virus infection. Two steps RT-PCR used for this purpose is designed to be highly specific for detection of chimeric CGMMV infection. First strand cDNA synthesis was carried out at using primer that is specific for the 3' terminal of the viral RNA. Then PCR amplification of the HBsAg coding sequence was carried out using primers that are specific for the epitope coding sequence.

were added and the final reaction volume was 20µl. The reaction was carried out at 55°C for 1 hour to synthesis the first strand cDNA.

After the first strand synthesis, PCR was carried out with primer pair "PSTSENSE" (5'-TAG GAA AAA ACC AGA AGA TCT GCA GGA ATT TTT CTC-3'and "HindAmberHB" (sequence not disclose due to patent requirement) this pair of primers flanks a region of around 2100 base pairs and the HindAmberHB is specific for HBsAg sequence (Figure 3). The PCR reaction was carried out in a 50µl reaction mix containing, 1x Taq DNA polymerase buffer (Promega®), 1.5mM $MgCl_2$, 0.5µM of PCR nucleotide mix (Finzymes), 100µM of each primer, 2µl of first strand DNA, and 2U of *Taq* DNA polymerase (Promega). The PCR cycle condition was initial denaturation at 95°C for 3 minutes, and 30 cycles of denaturation at 98°C for 10 seconds, annealing at 60°C for 30 seconds and 72°C for 2 minutes.

Virus purification.

Virus isolation and purification was carried out according to the method reported by [19]. Virus infected plant materials were frozen overnight in –20°C. 1ml per-gram-plant material of phosphate buffer (0.1M, pH 7.5) containing 0.1% β-mercaptoethanol was added to frozen infected plant materials and then the plant material was homogenized with food processor. The homogenized plant material was then sieved through two layers of muslin cloth. After that, while stirring, equal volume of butanol:chloroform (1:1) was then added to the filtrate. The mixture was further stirred for another 30 minutes. The coagulated plant materials (chloroplast) and organic solvents were separated through centrifugation at 10,000g for 30 minutes. After that, 4g of Sodium Chloride and 4g of PEG 6000 were then added to each 100ml of supernatant. The mixture was stirred until all the NaCl and the PEG 6000 dissolved. The mixture was then stored overnight at 4°C to further precipitate the virus. On the second day, the precipitated virus particle was separated from the solution through centrifugation at 10,000g for 30 minutes. The virus pellet was then re-suspended in 20ml of ultra-pure water for each 100ml of initial plant extract. The virus

suspension was then cleared by centrifugation at 10,000g for 15 minutes. After this centrifugation step, 5ml of 0.2M EDTA was added to each 100ml of supernatant. The mixture was then subjected to ultra-centrifugation at 110,000g for 1 hour 30 minutes at 4^{o}C. After the ultra-centrifugation step, the pellet, which contained the virus, was then re-suspended in ultra-pure water again. Then, the unresolved materials in the suspension were separated by centrifugation at 10,000g for 15minutes. These cycles of high-speed and low-speed centrifugations were carried out until the pellet is clear. The final virus pellet was re-suspended in ultra-pure water.

Result and discussion

The CGMMV virus vector (pCGT7x) that was constructed has the first nucleotide of the viral genome located at the +2 position of the T7 promoter, where the +1 position was an extra-viral Guanidine nucleotide. Thus, in theory, the extra viral Guanidine nucleotide will be transcribed into the m^{7}GpppCap structure, therefore, the transcript would be almost identical to that of the native virus. However, after the vector was constructed, the shorten T7 promoter was found to be inefficient in generating transcript, because of that, not enough transcript was generated for inoculation purpose. Thus, the clone could not be used directly as transcription template. Since the +1 and +2 sequence after the T7 promoter are essential for transcription efficiency [20], a pair of primers "T7doubleG" and "CGMMV3'UTR" were designed to carry out PCR to generate template for *in vitro* transcription. PCR product that is produced with this pair of primers will have an extra-viral guanidine nucleotide at the 5' end of This new PCR generated template performed well in transcription reaction (Figure 4). The *in vitro* synthesized transcripts were also used to inoculate cotyledons of seven-day old muskmelon seedling. Two weeks after inoculation, all inoculated muskmelon seedlings showed typical mosaic symptom. Infectivity of the *in vitro* synthesized transcript was also found to be to the same degree to that of purified viral RNA (result not show).

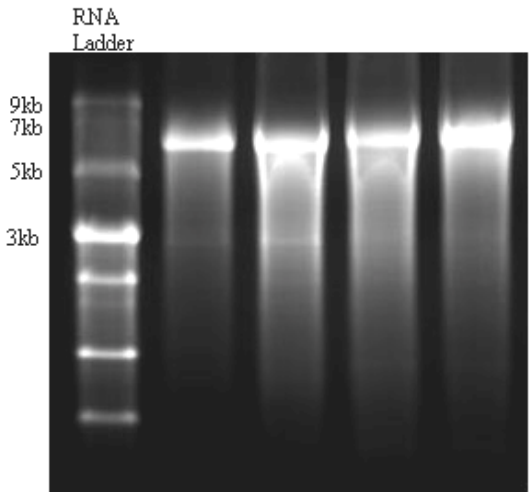

FIGURE 4 *In vitro* synthesized RNA analyzed using 1% native agarose gel electrophoresis. The RNA samples were pre-denatured in 7M urea before loaded into the gel. The Dominant ~6k bases band is the *in vitro* synthesized RNA.

The use of shorten T7 promoter to generate infectious viral transcript has been reported in several papers [21-25], however, there were also cases reported that the shorten T7 promoter failed to produce any transcript at all. While some papers reported that extra-viral nucleotide at the 5' end will render the transcript to become non-infectious. The pCGT7X that was constructed in this study showed that the shorten T7 RNA polymerase promoter failed to effectively initiate transcription reaction. This could be due to the multiple adenine nucleotide repeat at the 5' UTR region of the CGMMV genome. Multiple adenine nucleotides repeat directly after bacteriophages promoter have been found to cause premature abortion of transcription product[20, 26], as well as causing RNA polymerase to drop off the template. This is because the hydrogen bonds that hybridized adenine to Uracil is relatively weaker, and the interaction was suggested to stabilized ternary complex that formed among synthesized RNA, template DNA and the polymerase. Addition of an extra guanidine nucleotide to the +2 position of T7 RNA polymerase could have stabilized the ternary complex, thus resulting in transcription that is more efficient. The extra-viral guanidine nucleotide was also not causing any observable reduction in infectivity. Although direct comparison between transcript without extra-viral Guanidine nucleotide and the

transcript with extra-viral nucleotide was not able to carry out, preliminary infectivity study showed that there is no observable effect (result not show).

Once it was confirmed that the transcription CGMMV vector is working, construction of chimeric CGMMV (pCGHB310803) that expressed HBsAg on its surface was carried out. This chimeric virus vector was designed in such a way that the production of recombinant fusion protein is controlled by a leaky UAG signal. Introduction of the leaky UAG signal will cause occasional translation read-through, resulting in a new monopartide virus that produces two different coat proteins.

The chimeric virus vector was successfully constructed, and the *in vitro* transcription template was generated through the same PCR method that was developed for pCGT7x. The *in vitro* synthesized transcript was inoculated onto the cotyledons of seven-day old muskmelon seedling, and typical mosaic symptom was observed after two weeks post inoculation. Infected plants were further screened with RT-PCR to confirm that the symptom was indeed caused by the chimeric virus (Figure 5).

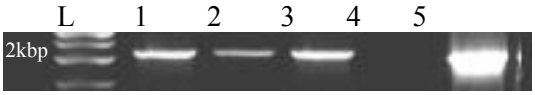

FIGURE 5 RT-PCR product analyzed on 0.8% native agarose gel. Lane L was NEB 1kbp DNA ladder, lane 1 to lane 3 were sample results lane 4 was negative control (native CGMMV infected leaves) while lane 5 was *in vitro* synthesized RNA.

The mosaic symptom appeared on new leaves two weeks after the inoculation. This signified that the virus had moved from the inoculated leave to young leaves, and caused systemic infection. Since the movement of virus requires the virus to be assembled into virus particle [27, 28]. Systemic infection caused by the virus also showed that the present of the recombinant fusion protein does not hamper the assembly of virus particles. Furthermore, virus isolation procedure was also able to purify the virus particles. Even though the virus was able to assemble into particles, it does not mean that the recombinant fusion protein has taken part in the formation of virus particles. To test this ELISA was carried out to find out if the virus particles were indeed displaying the HBsAg peptide. Double antigen sandwich ELISA with commercial clinical diagnostic kit (MONOLISA® HBsAg Plus BioRad™) showed the present of HBsAg in both infected plant materials and purified chimeric virus particles (Figure 6). The ability of the chimaric virus to bind monoclonal antibody specific to HBsAg shows that the presented peptide was in correct conformation.

The ability to produce the chimeric virus displaying HBsAg in the correct conformation shows that this system is suitable for use as a production system for commercially important peptide and even small proteins.

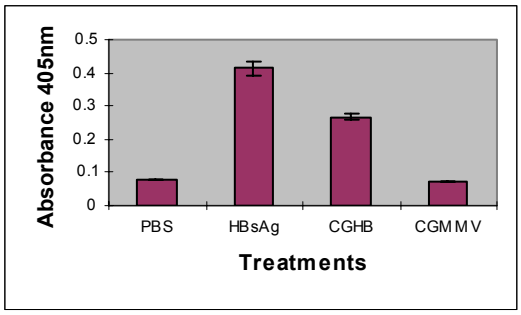

FIGURE 6 ELISA detection of the present of HBs antigen. PBS and native CGMMV were used as negative control while purified HBsAg was used as positive control. The CGHB stands for chimeric CGMMV.

While the mechanism of how the present the native coat protein help the production of viable virus is yet to be determined, a simple experiment where a mutant virus vector with the UAG at the leaky stop site replaced by CAA failed to produce viable virus (result not shown). This has further confirmed the important of the present of the native virus coat protein.

Our group has rediscovered the use of translation read-through signal in CGMMV chimeric virus system to be advantageous and it is suitable to be used as a versatile, high yielding epitope production system.

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