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## Developing a plant virus-based expression system for the expression of vaccines against Porcine Reproductive and Respiratory Syndrome Virus

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Biology

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## Abstract

Virus-based expression systems have been widely exploited for the production of recombinant proteins in plants during the last three decades. 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 subunit vaccines against *Porcine reproductive and respiratory syndrome virus* (PRRSV), I have developed several viral vectors which express partial PRRSV glycoprotein 5 (GP5).

In this thesis, I demonstrated that viral vectors can increase the expression of proteins for vaccine development in a variety of host plants. Green fluorescent protein (GFP) and the C-terminus of GP5 fused with GFP (GP5C-GFP) were expressed at up to 37.29 mg/kg and 4.36 mg/kg fresh weight of leaf tissue, respectively, in soybean plants via biolistic bombardment, using viral vectors derived from *Bean pod mottle virus* (BPMV). A *Cucumber green mottle mosaic virus* (CGMMV)-based vector was employed to display chimeric virus particles, presenting the neutralizing epitope (NE) of GP5, at levels as high as 35.84 mg/kg of cucumber leaf fresh weight, via agroinfiltration. In addition, GFP and the ectodomain of GP5 fused with GFP (GP5e-GFP) were successfully produced at up to 2.03 g/kg and 36.53 mg/kg fresh weight of leaf tissue, respectively, in *Nicotiana benthamiana* using transient expression of *Pepino mosaic virus* (PepMV)-based vectors. Co-infiltration of viral vectors with p19, a silencing suppressor, can help to mitigate the toxicity of GP5e to plant tissue and enhance the accumulation of this protein. These plant-made products may be tested as oral subunit vaccines against PRRSV in pigs during future experimental trials. Overall, the results demonstrate that viral systems can produce low-cost, versatile and robust vaccines that have a great impact in the fight against viral diseases, especially in developing countries.

## **Keywords**

Plant-made vaccine, *Porcine reproductive and respiratory syndrome virus* (PRRSV), glycoprotein 5 (GP5), plant virus-based expression vectors, *Bean pod mottle virus* (BPMV), *Cucumber green mottle mosaic virus* (CGMMV), *Pepino mosaic virus* (PepMV), soybean, cucumber, *Nicotiana benthamiana*.

## **Co-Authorship Statement**

The following thesis contains materials from manuscripts in preparation which are co-authored by Hong Hanh Tran (HT), Dr. Hui Chen (HC), Bin Chen (BC), Dr. Xiaoyun Wu (XW), Dr. Norman P. A. Hüner (NH), and Dr. Aiming Wang (AW).

My supervisor Dr. Aiming Wang and co-supervisor Dr. Norman P. A. Hüner provided insight and strategic direction for this project and assisted in the preparation of this manuscript (Chapter 2-4).

### **Chapter 2. Author's contributions**

HT designed and performed the experiments and drafted the manuscript. HC generated pCass4-Nos (Chapter 2, Figure 2.3A). HC also assisted HT in the design and construction of pBPR1, pBPR2 and pBPR2(GFP). AW and NH provided advice and strategic direction. AW and NH edited the manuscript.

### **Chapter 3. Author's contributions**

HT designed and performed the experiments and drafted the manuscript. BC determined the CGMMV genome sequence and provided the full-length cDNA. AW conceived the study and provided strategic direction. AW and NH edited the manuscript.

### **Chapter 4. Author's contributions**

HT designed and performed the experiments and drafted the manuscript. XW determined the full-length cDNA sequence of PepMV and constructed the infectious clone. AW provided strategic direction. AW and NH edited the manuscript.

## **Dedication**

To my beloved family, Dad, Mom and my younger sister  
for their endless love, devotion, support and encouragement.

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## List of Abbreviations

35S	<i>Cauliflower mosaic virus</i> promoter
aa	Amino acid
BaMV	<i>Bamboo mosaic virus</i>
bp	Base pair
BPMV	<i>Bean pod mottle virus</i>
BSA	Bovine serum albumin
BTV	<i>Bluetongue virus</i>
CaCl <sub>2</sub>	Calcium dichloride
CaMV	<i>Cauliflower mosaic virus</i>
CBD	Cellulose-binding domain
CD	Cluster of Differentiation
CGMMV	<i>Cucumber green mottle mosaic virus</i>
CMV	<i>Cucumber mosaic virus</i>
CO <sub>2</sub>	Carbon dioxide
Co-pro	Proteinase cofactor
CP	Coat protein
CPMV	<i>Cowpea mosaic virus</i>
CR	Replication cofactor
CSFV	<i>Classic swine fever virus</i>
CTB	Cholera toxin B
CVP	Chimeric virus particle
CymRSV	<i>Cymbidium ringspot tombusvirus</i>
DAS-ELISA	Double-antibody sandwich enzyme-linked immunosorbent assay

dpi	Day post inoculation
EAV	<i>Equine arteritis virus</i>
EDTA	Ethylenediaminetetraacetic acid
ELP	Elastin-like polypeptides
ER	Endoplasmic reticulum
EU type	Western Europe type
F2A	2A-cleavage sequence of <i>Foot-and-mouth disease virus</i>
FDA	The United States Food and Drug Administration
FMDV	<i>Foot-and-mouth disease virus</i>
FoMV	<i>Foxtail mosaic virus</i>
g	Gram
GFP	Green fluorescent protein
GM1	Monosialotetrahexosylganglioside
GOI	Gene of interest
GP5	Glycoprotein 5
GUS	$\beta$ -glucuronidase
HFBI	Hydrophobin I
HBV	<i>Hepatitis B virus</i>
HCl	Hydrogen chloride
HC-Pro	Helper component protease
Hel	Helicase
HER2	Human epidermal growth factor 2
HIV-1	<i>Human immunodeficiency virus type 1</i>
HP-PRRSV	Highly pathogenic <i>Porcine reproductive and respiratory syndrome virus</i>
HRP	Horseradish peroxidase

HRV-14	<i>Human rhinovirus 14</i>
HT	Hyper-translatable
IgG	Immunoglobulin G
kb	Kilobase
kDa	Kilodalton
kg	Kilogram
kV	Kilovolt
l	Litre
LB	Luria-Bertani
L-CP	Large coat protein
LDV	<i>Lactate dehydrogenase-elevating virus</i>
LV	<i>Lelystad virus</i>
mAb	Monoclonal antibody
MES	2-(N- Morpholino)ethanesulfonic acid
MEV	<i>Mink enteritis virus</i>
mg	Milligram
MgCl <sub>2</sub>	Magnesium dichloride
ml	Millilitre
MLV	Modified-live virus
MP	Movement protein
NA type	North America type
NaCl	Sodium chloride
NDV	<i>Newcastle disease virus</i>
NE	Neutralizing epitope
ng	Nanogram



<i>Nos</i>	Nopaline synthase terminator
nsp	Non-structural protein
nt	Nucleotide
OD	Optical density
ORF	Open reading frame
ORSV	<i>Odontoglossum ringspot virus</i>
PCR	Polymerase chain reaction
PVDF	Polyvinylidene difluoride
PepMV	<i>Pepino mosaic virus</i>
pFc	Stabilizing domain- "Fragment of crystallizable chain of IgG"
pI	Isoelectric point
pp	Polyprotein
Pro	Proteinase
PRRSV	<i>Porcine reproductive and respiratory syndrome virus</i>
PTA	Potassium phosphotungstate
PTGS	Post-transcriptional gene silencing
PVX	<i>Potato virus X</i>
RdRp	RNA-dependent RNA polymerase
RMV	<i>Ribgrass mosaic virus</i>
rpm	Round per minute
RT	Readthrough
RT-PCR	Reverse transcription-polymerase chain reaction
S-CP	Small coat protein
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sg mRNA	Subgenomic messenger RNA

SGP	Subgenomic promoter
SHFV	<i>Simian hemorrhagic fever virus</i>
SHMV	<i>Sunn-hemp mosaic virus</i>
SOC	Super Optimal broth with Catabolite repression
T	<i>CaMV 35S Terminator</i>
TBSV	<i>Tomato bushy stunt virus</i>
<i>tCUP</i>	Tobacco cryptic upstream promoter
TEM	Transmission electron microscopy
TEV	<i>Tobacco etch virus</i>
TGB	Triple gene block
TMGMV	<i>Tobacco mild green mosaic virus</i>
TMV	<i>Tobacco mosaic virus</i>
TSP	Total soluble protein
USDA	The United States Department of Agriculture
UTR	Untranslational/untranslated region
UV	Ultraviolet
v/v	Volume/volume percent
VIGS	Virus-induced gene silencing
VLP	Virus-like particle
VP6	Viral protein 6
Vpg	Viral genome-linked protein
w/v	Weight/volume percent
μg	Microgram
μl	Microlitre

## **Chapter 1: General Introduction**

### ***1.1 Porcine reproductive and respiratory syndrome virus (PRRSV)***

#### **1.1.1 PRRSV and economic impacts**

Worldwide, pork is the most popular meat. Based on information from the USDA Foreign Agriculture Service in 2008, pork accounts for 40% of meat consumption, and is followed by poultry and beef (US National Pork Board 2012). China was the leader of the top ten pork-producing countries from 2012-2016, while Canada stood in seventh place (USDA 2016). Canada was also one of the largest pork exporters, having exported \$2.9 billion worth of pork to over 80 countries in 2011 (Brisson 2014). The three main Canadian pork producing provinces are Quebec, Manitoba, and Ontario (Farm Credit Canada 2012). Ontario contributed 24.7% of national pork production in 2015 (Honey 2016). These statistics emphasize that the swine industry plays a major role in contributing to national and regional profits. Biosecurity and disease control are key issues in maintaining the health of livestock and ensuring a profitable on-going business. Some diseases, especially those caused by viruses, usually emerge quickly and can lead to outbreaks, which kill pigs at all ages and decrease breeding efficiency. Such outbreaks require increased information, resources and procedures in place in order to act in a short time and avoid catastrophic losses.

Porcine reproductive and respiratory syndrome (PRRS) is the most devastating threat to this industry and has drawn concern of pork producers across the world for over three decades. The first outbreaks were recorded in the late 1980s in the United States, with the antibody to PRRS virus (PRRSV) having been collected in Iowa and Minnesota (Keffaber 1989; Loula 1991). The symptoms were incremental, with reproductive and respiratory failure that led to herd mortality. Almost simultaneously, PRRS was also observed in Europe and Asia (Wensvoort et al. 1991). In Canada, samples showing seropositivity to PRRSV confirmed that Ontario herds were first infected between 1979 and 1982 by the first Canadian isolate of the virus (Carman et al. 1995). To date, the virus is endemic in almost all pork producing countries except Australia, New Zealand, Norway, Switzerland, Finland, and Sweden (Niederwerder and Rowland 2017). Clinical signs can include anorexia, fever, dyspnea and

cyanosis, which will turn pig's ears blue and, hence, the disease is also known as "blue-ear pig disease."

As mentioned, the causal agent of the disease is PRRSV - an enveloped, positive-strand RNA virus belonging to the genus *Arterivirus* in the family *Arteriviridae*. Besides PRRSV, the genus includes *Equine arteritis virus* (EAV), *Lactate dehydrogenase-elevating virus* (LDV) and *Simian hemorrhagic fever virus* (SHFV) (Benfield et al. 1992; Cavanagh 1997). The predicted spherical or oval-shaped envelope of this virus is approximately 50-65 nm in diameter. The 15-kb genome is capped at the 5' terminus and polyadenylated at the 3' end, encoding 11 known open reading frames (ORFs). ORFs 1a and 1b encode two large replicase polyproteins (pp), i.e., pp1a and pp1ab, respectively (Allende et al. 1999). Subsequently, pp1a and pp1ab are proteolytically cleaved into 14 non-structural proteins (nsps), the first of which produces ten nsps: nsp1a, nsp1b, nsp2-nsp6, nsp7 $\alpha$ , nsp7 $\beta$  and nsp8, while the latter creates nsp9-12. Interestingly, the proteolytic cleavage is processed by nsp1a, nsp1b, nsp2 and nsp4. The nsp9-12 are believed to be involved in virus replication (Snijder 1998; Snijder and Meulenberg 1998). Recently, a short transframe (TF) ORF was discovered. It overlaps the nsp2-coding sequence in ORF 1a and expresses nsp2TF and nsp2N via -1/-2 programmed ribosomal frameshifting (Fang et al. 2012; Li et al. 2014). These two new proteins contain domains of papain-like cysteine proteinase. The 3' terminus of the PRRSV genome consists of a compact area of gene coding for five minor and three major structural proteins expressed by subgenomic mRNAs (sg mRNAs). The minor structural proteins are glycoprotein (GP) 2a, GP3, GP4, and un-glycosylated envelope proteins (ORF5a and E), all of which associate with the membrane. Three major structural proteins are GP5, an un-glycosylated membrane protein (M), and a nucleocapsid protein (N).

PRRSV strains discovered in the late 1980s - early 1990s in two distant continents, (Western Europe and North America) were classified into EU-genotype 1 and NA-genotype 2, respectively (Keffaber 1989; Wensvoort et al. 1991). *Lelystad virus* (LV) and ATCC VR-2332 are prototypes of genotypes 1 and 2, respectively. These virus strains are remarkably distinct in their nucleotide sequences, sharing only 55-70% sequence identity (Allende et al. 1999; Nelsen et al. 1999). PRRSV has evolved quickly, with many strains and varying

degrees of virulence. A highly pathogenic PRRSV (HP-PRRSV) strain has been detected in China and Southeast Asia since 2006 and is characterized by fast spreading outbreaks. This strain is associated with high fever and severe damage to pig's lungs, leading to high mortality in pigs (Tian et al. 2007). A new PRRSV subtype 3, designated as Lena strain, has also been isolated in Eastern Europe (Belarus), with both reproductive and respiratory failure and severe pulmonary lesions (Karniychuk et al. 2010). In general, there are two main genotypes, type 1 (EU) and type 2 (NA). Type 1 is divided into three subtypes, a pan-European subtype 1 (LV) and East European subtypes 2 and 3, to which Lena strain belongs (Stadejek et al. 2008). Type 2 contains NA (ATTC VR-2332) and Asian HP-PRRSV (Wernike et al. 2012).

Clinical cases have shown that PRRSV is the most common virus associated with the porcine respiratory disease complex (PRDC) (Brockmeier et al. 2002). PRRSV is able to suppress the host immune system, which consequently allows for secondary pathogens to attack the host, resulting in more severe and chronic diseases. In many cases, PRRSV can establish a mixed infection with *Mycoplasma hyopneumoniae*, *Bordetella bronchiseptica*, *Porcine circovirus*, *Porcine respiratory coronavirus*, *Swine influenza virus*, and *Haemophilus parasuis*, causing a complex of swine respiratory diseases. Due to this complexity, it's difficult to control these infections and recover the host. PRRSV infection can be classified into at least three prominent stages: acute infection, persistence, and extinction. In the final stage, the virus can be maintained in a host up to 250 days after infection. Thus, PRRSV can establish a “life-long” infection in swine production (Wills et al. 2003). Moreover, the disease is highly contagious, as PRRSV can quickly infect its host through mucosal and percutaneous surfaces, via respiratory and oral routes. Routes of transmission include either direct or indirect contact with fomites, e.g. aerial exposure, coitus/insemination, ingestion, inoculation, ear notching, tail docking, teeth clipping, and injection of drugs or vaccines (Baker et al. 2012; Hermann et al. 2009; Nathues et al. 2016; Otake et al. 2002). Moreover, fighting between pigs during sickness can also give rise to infection through bites, cuts, scrapes, and abrasions. Sick pigs may show aggressive behaviour by fighting healthy ones, resulting in virus transmission (Bierk et al. 2001).

PRRS is one of the most economically costly swine diseases around the world. Based on data from 2005-2010, PRRS caused an annual loss of \$664 million dollars in pork production (Holtkamp et al. 2013). Breeding herds accounted for 45% of this loss, which increased 33% in comparison to the previous report in 2005 (Holtkamp et al. 2013; Neumann et al. 2005). The high economic impact of this disease was also described for nine sow herds during and after outbreaks in the Netherlands (Nieuwenhuis et al. 2012). The average loss per sow was €126 during this 18-week outbreak, whereas, the cost was variable, from €3-160 per sow, after the epidemic. As these nine herds included between 250-1200 sows each, the total cost incurred by PRRS could potentially cause a massive negative impact on the national pork industry. In China, a fatal outbreak associated with a highly pathogenic PRRSV (HP-PRRSV) strain, emerged in 2006 and quickly spread out to 10 provinces (Li et al. 2007; Tian et al. 2007). This outbreak infected over 2,000,000 pigs and caused death in 400,000 cases. Moreover, the long-term persistence of this highly virulent and contagious disease drastically disrupted pork production in infected areas, causing significant economic loss to farmers. HP-PRRSV also disseminated from China into Southeast Asia, including Vietnam, Laos, Cambodia, Myanmar, The Philippines, Thailand, and Singapore. South Korea and Russia were also affected by this epidemic (An et al. 2010; Zhang et al. 2014). Disease outbreaks recurred several times during 2006-2010, with high infection and mortality rates in Vietnam, one of the major pork producing countries in Asia. Following these outbreaks, the market price for pork declined by 29% in 2008. Since pork production accounted for 58% of total agricultural gross domestic product (GDP) and 80% of total meat production, PRRS not only devastated pig farming, but also created a tremendous economic loss in Vietnam (Zhang et al. 2012). In Canada, it was estimated in 2010 that PRRS cost the national pork industry \$130 million dollars per year (The Pig Site 2010), and approximately \$37-\$75 million dollars per annum in Ontario, as reported recently (Mussell et al. 2011; Rosendal et al. 2014). These data show that PRRS is a devastating epidemic worldwide and causes a massive impact on the pork industry, as well as national economies. Thus, a solution is desperately needed to eradicate this disease. However, due to the complexity of this disease, it remains a challenging puzzle for scientists and veterinarians. Control of PRRSV outbreaks relies mainly on four important aspects: early diagnosis and monitoring, biocontainment, herd

management and vaccination (Pileri and Mateu 2016). Therefore, intense research has been conducted to develop effective vaccines against PRRSV.

### 1.1.2 Vaccines against PRRSV

Currently, commercial PRRSV vaccines are divided into two categories: killed-virus vaccines (inactivated vaccines) and attenuated or modified-live virus (MLV) vaccines. Inactivated vaccines are reported as being ineffective, or having limited efficacy (Kim et al. 2011; Meng 2000; Zuckermann et al. 2007). MLV vaccines are considered as an efficient method to control outbreaks, as they can establish protection against homologous re-infection and reduce viral shedding (Cano et al. 2007b; Linhares et al. 2012; Zuckermann et al. 2007). However, MLV possess some drawbacks, such as incomplete cross-protection (Cano et al. 2007a; Martelli et al. 2009; Murtaugh et al. 2002; Okuda et al. 2008), reversion to virulence under farm conditions (Bøtner et al. 1997; Storgaard et al. 1999), and immunosuppression in pigs (Key et al. 2003; Opriessnig et al. 2002). Therefore, a new generation of vaccines which are safe and efficient in the inhibition of virus infection, transmission, immunosuppression, and disease onsets, are definitely in need. Subunit vaccines are composed only of specific components (proteins or peptides) from a pathogen and are able to elicit a protective immune response when delivered into a host. These components are called antigens. Unlike inactivated or attenuated vaccines, subunit vaccines do not contain live components of the pathogen and, thus, reduce the risk of side effects, but still maintain the selectivity, specificity, safety and ability to activate immune protection (Rappuoli 2007). GP5 was specifically chosen as a candidate to develop PRRSV subunit vaccines, due to several advantages. As mentioned above, GP5 is a major glycoprotein, which is exposed on the surface of PRRSV and epitopes involved in virus neutralization are found on GP5 (Gonin et al. 1999; Pirzadeh and Dea 1997; Pirzadeh and Dea 1998; Wissink et al. 2003). Many experiments have shown that GP5 is able to induce neutralizing antibodies against PRRSV in pigs, and that vaccinated pigs were partially protected from the development of viremia and lung lesions, following PRRSV challenge (Jiang et al. 2006; Pirzadeh and Dea 1998; Qiu et al. 2005). It was also proposed that the ectodomain of GP5 may interact with the macrophage receptor, Cluster of Differentiation (CD) 169, which mediates the entry of the virus into host

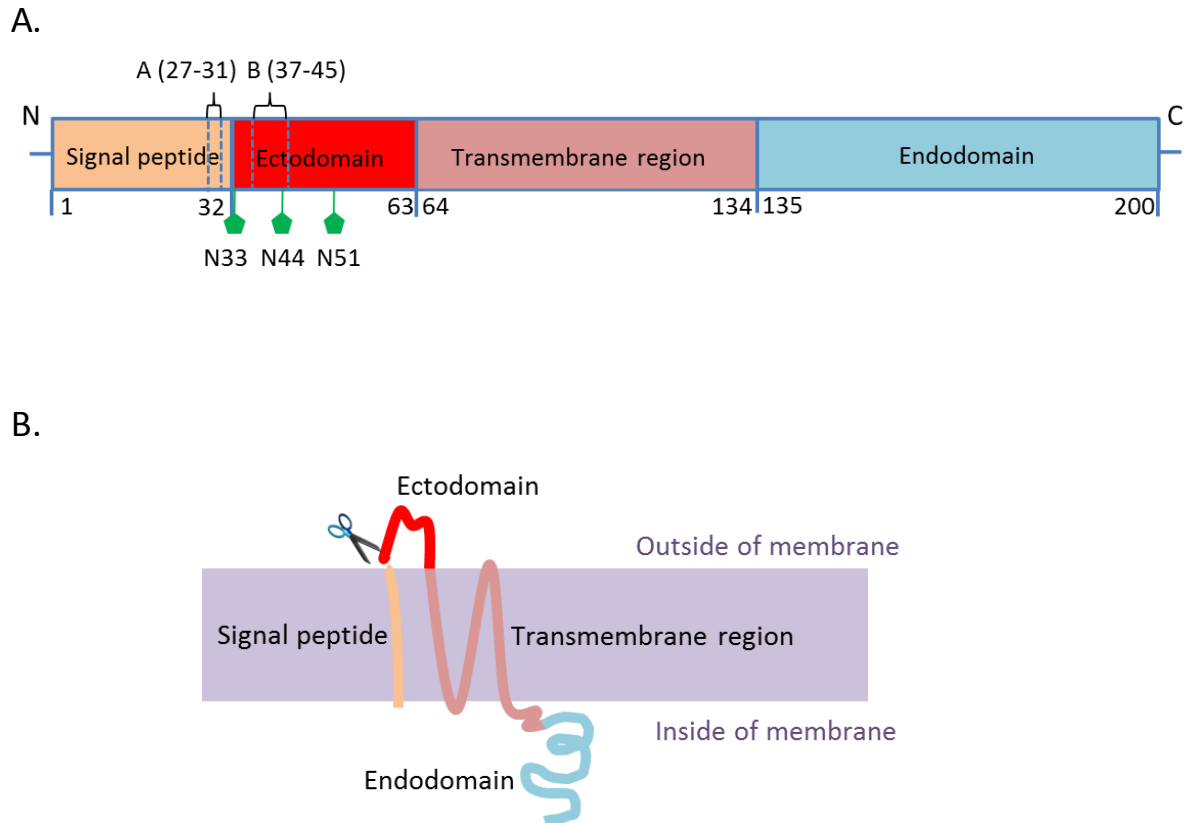
cells (Delputte and Nauwynck 2004; Van Breedam et al. 2010). Taken together, GP5, an antigen of PRRSV, is a promising trigger for immune system protection, as a component of a PRRSV subunit vaccine.

GP5 of the NA type 2 (VR-2332) virus is composed of 200 amino acids (aa), approximately 25 kDa in size, and divided into variable regions: (1) a N-terminal signal sequence (residues 1-32), which can direct the protein to the rough endoplasmic reticulum (ER) (Mardassi et al. 1995; Meng et al. 1995; Murtaugh et al. 1995); (2) an ectodomain (residues 33-63) containing N-glycosylation sites (N33, N44 and N51), in which N44 and N51 are highly conserved among different strains; (3) an extended hydrophobic region (residues 64-134) known as the transmembrane region; (4) a hydrophilic C-terminal section (residues 135-200), known as the endodomain, which is located in the matrix of the virus (Dea et al. 2000; Stadejek et al. 2002; Thaa et al. 2013) (Figure 1.1). The N-terminal part of GP5 contains two epitopes, epitope A (residues 27-31) and epitope B (residues 37-45) (Figure 1.1A). Epitope A is believed to work as a “decoy epitope”, one that is non-neutralizing, hypervariable and immunodominant, while epitope B is neutralizing and conservative among PRRSV isolates, but less immunodominant. Evidence has demonstrated that epitope A induces a strong non-neutralizing antibody response in the early stages of infection, whereas, epitope B elicits neutralizing antibodies against PRRSV much later, at more than four weeks post infection (Ostrowski et al. 2002; Yoon et al. 1994). Somehow, the decoy epitope A decreases or delays the induction of neutralizing antibodies against the nearby neutralizing epitope B, which is a common virus strategy to defeat host immune systems (Cleveland et al. 2000; Lopez and Osorio 2004; Ostrowski et al. 2002). Further elucidating the underlying mechanism would help to design a better subunit vaccine based on GP5.

## **1.2 Using plants as a biofactory to produce a subunit vaccine based on GP5**

GP5-based subunit vaccines have been produced in different host systems, such as bacteria (Bastos et al. 2002; Hu et al. 2016; Hu et al. 2012; Park et al. 2016; Prieto et al. 2011), yeast (Zhao et al. 2014), insect cells (Binjawadagi et al. 2016; Duran et al. 1997; Nam et al. 2013; Wang et al. 2012), mammalian cells (Eck et al. 2016; Gao et al. 2014; Jung et al. 2002; Matanin et al. 2008) and plants (Table 1.1).





**Figure 1.1** Characterization of PRRSV GP5 (adapted from Thaa et al. 2013). **A.** Schematic representation of GP5. The numbers under the bar indicate the position of amino acids in this protein, relative to the coding regions; signal peptide (in orange), ectodomain (in red), transmembrane domain (in brown) and endodomain (in blue). “N” and “C” are abbreviated for N-terminus and C-terminus of GP5. The N-glycosylation sites (N33, N44 and N51) are shown in green. The “decoy epitope” A includes residues 27-31, whereas the neutralizing epitope B is composed of residues 37-45. **B.** Putative topology of unprocessed GP5 on the virus membrane. Virus membrane is shown in purple. The signal peptide is cleaved, thus separated from the ectodomain, which presents on the outer membrane. The transmembrane region is hydrophobic and embedded in the membrane, while the endodomain is assumed to locate to the virus interior.

Since the late 1980s, the use of plants for the synthesis of recombinant proteins has been proposed as an alternative way to reduce the costs, while increasing biosafety and stability of products (Barta et al. 1986; Gutiérrez and Menassa 2014; Ma et al. 2003; Twyman et al. 2003). The word “molecular farming or pharming” describes the production of biopharmaceuticals, e.g. drugs, vaccines, etc. in plants using molecular biological techniques (Twyman et al. 2003). Plant platforms for the production of vaccines offer numerous advantages over the other systems mentioned above (Lomonossoff and D'Aoust 2016). Firstly, the production of plant-made vaccines is cost-effective, at as much as 1000 times lower than the cost of mammalian cell systems, and up to 50 times lower than *Escherichia coli* bioreactors, based on relative protein yields (Giddings et al. 2000; Mett et al. 2008). Plants have the ability to use natural resources, such as sunlight, CO<sub>2</sub>, water and nutrients to synthesize materials and energy, via photosynthesis, which reduces the cost and enhances the versatility to scale-up production, by increasing the cultivation area. Secondly, plants do not carry animal pathogens, e.g. microbes or prions, as opposed to mammalian cell systems and are, therefore, safer for human and animal use (Mason et al. 2002). Thirdly, plant systems can perform eukaryotic post-translational modifications (e.g. glycosylation or disulfide bond formation) which retain the desired structural properties of vaccines. Bacterial systems do not glycosylate peptides, while yeast and insect glycosylation are inconsistent, with high mannose glycoforms that can affect the immunogenicity of antigens (Chen and Lai 2013; Ma et al. 2003). Fourthly, plant platforms possess the flexibility for scaling-up production and, therefore, minimize the timeline of manufacturing. This can play a critical role when combatting infectious diseases or controlling outbreaks (Lomonossoff and D'Aoust 2016; Marsian and Lomonossoff 2016). Last, but not least, plant-made vaccines can also be produced in various edible parts of the plant, e.g. fruits, leaves, seeds, tubers, vegetables, etc. and are termed “edible vaccines”. Such vaccines have the potential for oral administration in hosts, without the use of needles and cold chains regularly required in vaccine delivery. Consequently, this makes edible vaccines more efficiently delivered, affordable, and accessible worldwide, particularly in developing countries (Mason et al. 2002; Rybicki 2014). Therefore, plants have become an attractive system for vaccine production. Stable

transformation and transient expression are often used for vaccine production in plants. With stable transformation, the gene of interest is incorporated into the plant genome and inherited by subsequent transgenic generations. Hence, all stable transgenic lines contain the gene and will be able to express the same protein of interest as their parents. However, stable transformation is labour-intensive and time-consuming for generating transgenic plants, and this is inappropriate for the required quick and large-scale production of vaccines against epidemics or bioterrorism attacks. Furthermore, recombinant proteins may be produced at inefficient yields in these transgenic plants, especially if they hinder plant development. GP5 has been expressed in various transgenic plants, including tobacco, potato, banana and Arabidopsis (Table 1.1). Aside from potato and Arabidopsis, the other plants gave low yields of protein expression at approximately 110-257 ng/g of leaf tissue fresh weight (Chan et al. 2013; Chia et al. 2010; Chia et al. 2011). Interestingly, this leaf tissue was able to induce specific mucosal, systemic humoral and cellular responses, when fed to PRRSV-challenged pigs. The viral load also decreased after inoculation. To circumvent the drawbacks of stable transformation, transient expression, in combination with viral vectors, was used to optimize protein expression levels, as well as reduce time and labour investments in this study. Transient expression is a conventional method for delivering a gene of interest into the somatic cells of leaf tissue, where the gene will be transcribed and translated into a protein of interest. In contrast to transgenic plants, the heterologous gene does not become a part of the plant genome and, thus, will not be inherited by the next generation. Proteins of interest can be produced within a week, when using this method. The desired gene may be introduced into plant cells by either agroinfiltration or biolistic bombardment. Agroinfiltration involves the pressurized saturation of leaves with an *Agrobacterium tumefaciens* culture harboring the gene of interest, whereas, biolistic bombardment delivers gene-coated gold/tungsten particles into cells via compressed helium. Therefore, the combination of transient expression and viral vector systems is considered as a potentially robust tool for overcoming low protein expression levels achieved using stable transformation systems (Pogue and Holzberg 2012; Salazar-Gonzalez et al. 2015). Many virus-based expression systems have been developed to serve as potent tools for the manufacture of plant-derived biopharmaceuticals (Gleba et al. 2007; Hefferon 2014).

### 1.3 Plant virus-based expression system

*Tobacco mosaic virus* (TMV) is the pioneer who started the first chapter of virology and has also become an important player in modern day science. Since its discovery, TMV has contributed enormously into not only its own field of plant virology, but also other interdisciplinary subjects, such as biotechnology, evolution, and nanotechnology (Alonso et al. 2013; Scholthof 2004). The use of plant viruses for the production of recombinant proteins in plants has been demonstrated, with many good examples to support this statement. Beginning from a proof-of-concept idea, plant viral vectors have developed into a powerful tool in the industrial production of heterologous proteins, especially traditionally expensive ones such as antibodies and vaccines. As cellular parasites, plant viruses possess certain characteristics: (1) upon infection, they can exploit host machinery to produce a large amount of viral proteins as well as the viral genome; (2) they have the ability to suppress post-transcriptional gene silencing (PTGS) and protein degradation, by evolved mechanisms; (3) they can also establish systemic infection throughout the plant. These characteristics make plant viral vectors an alternative expression system to the traditional transgenic or transplastomic systems, with some remarkable advantages: (1) increase in expression speed and yield production; (2) reductions in cost and time; (3) a high throughput, flexible and scalable system. Many expression strategies have been employed for the development of powerful viral vectors during the last 30 years (Lico et al. 2008). These can be divided into four main categories, including gene insertion vectors, gene replacement vectors, modular or deconstructed vectors and peptide display vectors, in addition to a minor category, viral elements and combinations of different viral vectors.

#### 1.3.1 Gene insertion vectors

In this expression strategy, the heterologous sequence encoding the protein of interest is inserted into the viral genome at a certain site, e.g. coat protein (CP). The protein of interest is expressed as a fusion product, with viral CP, by the CP (subgenomic) promoter, or as an individual protein by a duplicated CP (subgenomic) promoter. A typical example is a vector derived from *Cowpea mosaic virus* (CPMV) (Gopinath et al. 2000). A jellyfish green

fluorescent protein (GFP) was fused to the last aa of the CPMV small CP in RNA2, via a 2A-cleavage sequence from *Foot-and-mouth disease virus* (FMDV), called F2A, to create the CPMV/S-2A-GFP construct. Following inoculation into cowpea, GFP expression was estimated at 1% of total soluble protein (TSP). In addition, systemic infection was detected at 10 days post inoculation (dpi) and the stability of the construct was maintained through five passages. A similar viral expression vector based on *Potato virus X* (PVX) has also been developed (Chapman et al. 1992). In this case, a bacterial  $\beta$ -glucuronidase (*GUS*) gene was inserted between a duplicated (artificial) CP subgenomic promoter (SGP) and the CP SGP to form pGC3. Transcripts of pGC3 were used to infect *Nicotiana clevelandii* and *N. tabacum* cv. Samsun NN. After infection, histochemical analysis results confirmed the expression of GUS in both inoculated and systemic leaves. Nevertheless, northern blot analysis showed that there were sequence deletions of GC3 RNA due to homologous recombination.

Along with the aforementioned achievements, some problems have also been identified from the first generation of viral vectors. Firstly, as the viral genome is kept integral, complete viruses are also expressed and able to spread systemically in host plants. This fact may raise some concerns in terms of biosafety and biosecurity. Secondly, large exogenous inserts (more than 1 kb) may hinder virus assembly, thus limiting the infection area and subsequent protein expression. Thirdly, the introduction of a cleavage site may result in a mixture of products (e.g. free proteins and fused proteins). Last, duplication of promoters often leads to homologous recombination of repeating sequences, resulting in the instability of these constructs. Extensive efforts have been made to obviate these problems.

### 1.3.2 Gene replacement vectors

An endogenous viral sequence is substituted by that of a heterologous gene of interest (GOI) to form a gene replacement vector. In the viral genome, not all viral elements are required for protein expression (e.g. CP). CP is essential for the systemic movement of most of viruses. However, advances in technology, such as vacuum infiltration, allow viral vectors to be dispersed throughout entire plants. Therefore, with systemic movement no longer necessary, removal of CP helps to eliminate bio-contamination threats. Also, with the exchange of viral CP to a gene of interest, viral vectors can bear a much larger insert when compared to gene

insertion vectors. Musiychuk et al. have created a “launch vector”, called pBID4, based on CP-depleted TMV (Musiychuk et al. 2007). pBID4 was engineered to express not only modified lichenase, but also its fusion products with other proteins of interest, e.g. plague, anthrax, influenza, etc. Lichenase is  $\beta$ -1,3-1,4-glucanase, a thermostable enzyme derived from *Clostridium thermocellum*. This enzyme was expressed as a carrier in fusions with different target proteins, in order to promote their expression, stability, and immunogenicity. In addition, lichenase remains stable at high temperatures (65°C), which is also adopted by the fusion products, facilitating fast and economical purification by heat, in downstream processes. Modified lichenase (LicKM), which is approximately 27 kDa, and proteins of interest (up to 100 kDa) were successfully produced using pBID4. For instance, antigen hemagglutinin (HA) of influenza type A/Vietnam/04 (H5N1) virus was expressed in two forms: (1) the globular domain (H5GD) fused with LicKM (LicKM-H5GD) and (2) the stem domain (H5SD) fused with LicKM (LicKM-H5SD). Both target proteins were extracted from *Nicotiana benthamiana* at 4-7 dpi and purified to over 80% purity using affinity chromatography. These plant-made vaccines also formed specific bindings to antibodies in sheep sera raised against HA of the influenza type A/Vietnam/04 virus.

The rise of agroinfiltration, using *A. tumefaciens*, has paved a novel way for the delivery of viral vectors into desired areas in plants, without the need for systemic virus movement. In addition, it has facilitated the design of viral vectors by giving an enhanced capacity for creativity and simplification. The ultimate result of this progress is modular systems based on deconstructed vectors.

### 1.3.3 Modular or deconstructed vectors

Accumulated evidence has shown that the gene insertion strategy has some flaws, as mentioned earlier. Moreover, a vector with the entire viral genome and GOI can result in a large-sized plasmid and thus, reduce the efficiency and flexibility of either genetic engineering, or cell expression machineries. In the modular system, the viral genome is divided into different genetic components, referred to as provectors or minimal vectors, which contain only the essential viral elements in favour of protein expression, e.g. RNA-dependent RNA polymerase (RdRp) (Giritch et al. 2006; Larsen and Curtis 2012). To form

an integrated system, these provectors must be delivered into one cell, via agroinfiltration, and subsequently co-expressed in order to function as a complete expression system. The most well-known deconstructed vectors are the MagniCON system, which is based on TMV. This system will later be described in detail in section 1.5 of this thesis. Here, I would like to give some details of others which are less well-known, but still successful enough.

Larsen and Curtis have demonstrated the comparisons between different viral expression vectors based on PVX, in cell suspension and hairy root systems (Larsen and Curtis 2012). PVX-GUS contains *GUS* inserted between the triple gene block (TGB) and CP, and driven by the CP SGP. The deconstructed vectors are PVX $\Delta$ CP-GUS and PVX $\Delta$ CP $\Delta$ TGB-GUS, in which the first vector lacks PVX CP, while the second has had both the CP and TGB deleted. Strikingly, PVX $\Delta$ CP $\Delta$ TGB-GUS (called a “minimal vector”) could yield GUS expression 6.6 fold higher than that of PVX-GUS. Co-expression of the minimal vector with a suppressor of PTGS from *Tomato bushy stunt virus* (TBSV), p19, in the pPSP19 vector, could further increase GUS expression by 44% and 990% in cell-suspension and infiltrated leaves, respectively. Furthermore, GUS expression increased dramatically, by 83%, in cell suspension when co-transformation with HC-Pro, a silencing suppressor of *Tobacco etch virus* (TEV), in place of p19. These experiments have shown that a minimal vector can produce an efficient infection and boost protein expression to higher levels.

In another example, Liu and Kearney designed a deconstructed vector, known as FET40/GFP, based on *Foxtail mosaic virus* (FoMV) (Liu and Kearney 2010). Like PVX, FoMV is also a member of the *Potexvirus* genus, whose characteristics will be described in more detail in section 1.6. Interestingly, this virus has a broad host range, which includes species in both the *Poaceae* family of monocots, and some dicotyledonous species. FET40/GFP contains only a partial viral genome, including the viral replicase, a putative SGP of subgenomic RNA1 (sg RNA1), *GFP* and the 3' end of the *CP* gene. The GFP sequence was driven by the SGP, which has 40 nucleotide (nt) of TGB1, with the ATG mutated to ATC. Co-expression of FET40/GFP and p19 could produce GFP as high as 1.7 mg/g of leaf tissue fresh weight. This yield was comparable with that of TRBO, a TMV based vector (Lindbo 2007). Notably, all of the above examples have highlighted not only

the importance of modular systems in protein expression, but also implicated the crucial role of agroinfiltration as an efficient method for the delivery of multiple constructs into plants (Peyret and Lomonosoff 2015).

#### 1.3.4 Peptide display vectors

In this strategy, the gene of interest is fused with the viral CP sequence in order to produce chimeric virus particles (CVPs), which are able to display the peptide of interest on their surface. Using the direct fusion method, the foreign peptide can be fused to CP at either its N- or C-terminus. Also, with the indirect method, the gene of interest is joined with CP via an extra sequence, e.g. a readthrough translational context (Sugiyama et al. 1995) or an F2A sequence (Uhde-Holzem et al. 2010). The top priority of gene fusion is to preserve viability of the chimeric virus. This means that the chimeric virus must be able to maintain replication, RNA and protein production, assembly into viral particles, and cell-to-cell or systemic movement, if required. These requirements sometimes restrict the size of inserts. Despite this drawback, peptide display vectors have been employed since 1986 (Haynes et al. 1986) and become a popular tool in the production of plant-made vaccines, benefitting from their many advantages. Subunit vaccines are formulated from epitopes, which are usually small peptides or proteins. Due to their small size, free epitopes are not easily recognized by antigen presenting cells and are unable to induce complete immune responses (Bachmann and Jennings 2010). CVPs have the ability to act as efficient carriers, by increasing the size of the antigen when presenting the epitopes to the host immune system and, hence, stimulate innate, antibody, and cell-mediated responses (Marconi et al. 2006; Marusic et al. 2001). In addition, CVPs are restricted within their host-plant range and thus, cannot replicate using their own molecular machinery and induce diseases in animal hosts. Lico et al. incorporated the nucleoprotein (NP) sequence of the influenza A virus strain A/PR/8/24/Mount Sinai (H1N1) to the N-terminus sequence of PVX, in order to generate NP-CVPs (Lico et al. 2009). The foreign peptide (Ala-Ser-Asn-Glu-Asn-Met-Glu-Thr-Met) was expressed together with PVX CP, by the CP SGP, and presented on the surface of NP-CVPs. Purified NP-CVPs from *N. benthamiana* were recovered at approximately 1.1 mg/g of fresh leaf weight. Mice immunized by the resulting CVPs, without adjuvants, were stimulated to produce interferon  $\gamma$



secreting antigen-specific CD8<sup>+</sup> T cells. Both CD8<sup>+</sup> and CD4<sup>+</sup> T lymphocytes play crucial roles in immune responses to intracellular infections and tumors. Therefore, epitope-displaying CVPs can be used as vaccines to activate cell-mediated immune responses.

In addition to vaccine technologies, the peptide-display virus system has been widely used in a variety of new fields, including nanotechnology and nanomedicine (Lico et al. 2015; Steinmetz et al. 2009a). More applications will be described thoroughly in section 1.4, 1.5 and 1.6, specific to *Comovirus*, *Tobamovirus* and *Potexvirus* genera.

### 1.3.5 Viral elements and combination of different viral systems

Many plant viral sequences have been working as essential elements in expression vectors. These include promoter sequences, translational enhancement sequences and gene-silencing suppressor sequences.

The *Cauliflower mosaic virus* (CaMV) 35S promoter was one of the first plant virus elements which have been extensively studied. CaMV belongs to the genus *Caulimovirus* in the *Caulimoviridae* family of para-retroviruses. It infects plants of the *Brassicaceae* family and some strains are able to infect species in the *Solanaceae* family. This promoter has been greatly used in transgene expression vectors to help secure strong expression of genes of interest. It is noteworthy that most transgenic plants, especially commercialised genetically modified plants, contain the CaMV 35S sequence, as a stable and long-term activating promoter. CaMV 35S is a strong constitutive promoter and able to drive high levels of RNA synthesis not only in viruses and plants, but also in a wide variety of prokaryotes, e.g. *Escherichia coli*, or other eukaryotes, yeast, mushroom, cell lines of human embryonic kidney cells, Chinese hamster ovary, and fishes like the Atlantic salmon (Assaad and Signer 1990; R  th et al. 1992; Seternes et al. 2016; Sun et al. 2002; Tepfer et al. 2004).

When entering host cells and uncoating their capsids, positive-sense, single-stranded RNA viruses hijack the host machinery to produce the necessary proteins involved in virus replication, such as RdRp. Evidence has shown that viruses are able to recruit host ribosomes to efficiently translate their RNA, owing to specific sequences in the 5' or 3' untranslated region (UTR) of viral genome. The TMV 5' omega ( $\Omega$ ) leader sequence and the *Tobacco*

*etch virus* (TEV) 5' leader sequence are examples (Gallie 2001; Gallie et al. 1987). Ofoghi et al. employed these leader sequences to enhance human calcitonin production in transgenic potato plants (Ofoghi et al. 2005). The TMV enhancer efficiency was 2-3 fold higher than that of TEV, when comparing the levels of human calcitonin in leaves, roots and tubers of transgenic potato plants.

Like humans, plants also possess defence mechanisms, and RNA silencing is a key component of the plant immune system against viruses. This mechanism is involved with post-transcriptional control of gene expression, in which the target transcripts go through a specific degradative pathway, recruiting small interfering RNA (siRNA, 21-23 nt) as mediators and indicators (Voinnet 2001). To counteract this mechanism, plant viruses have evolved the ability to suppress RNA silencing using their own specialized proteins, named silencing suppressors. Viral silencing suppressors include HC-Pro, p19, and 2b proteins, which are derived from potyviruses, tombusviruses and *Cucumber mosaic virus* (CMV), respectively. Each protein has its own mechanism for interference at different stages of RNA silencing pathways in plants. The suppressor p19 is well characterized and has a distinct mode of action. It is able to sequester viral small RNAs and inhibit their incorporation into the plants RNA-induced silencing complex (RISC) (Scholthof 2006). Furthermore, p19 also prevents the accumulation of Agronaut-1 (AGO1), by increasing the expression level of microRNA 168 while AGO1 is an essential component of anti-viral RISCs (Várallyay et al. 2010). Induction of mRNA accumulation is one step closer to high yields of protein expression. When co-infiltrated with other viral constructs, p19 was able to significantly enhance the expression levels of proteins of interest (Voinnet et al. 2003). Thus, the use of silencing suppressors is usually a primary consideration in the design of protein expression constructs.

In addition, the aforementioned viral elements and viral constructs also co-operated in a unique system to empower the competency, flexibility and scalability of virus-based constructs for the expression of specific peptides and proteins. It is noteworthy that the synergism of TMV and PVX has been exploited to create a viral system which expresses a full sized, functional immunoglobulin G (IgG) (Giritch et al. 2006) and hybrid chimeric PVX

particles (Dickmeis et al. 2015). In the first example, TMV and PVX-based expression vectors were co-infected into *N. benthamiana*, via agroinfiltration. TMV and PVX vectors carried the heavy chain and light chain sequences of monoclonal antibody (mAb) A5, respectively, in place of viral CPs. When PVX CP was introduced by another vector, the expression level of mAb was maximized at up to 0.5 g/kg of leaf tissue fresh weight. Also, TMV MP was able to rescue cell-to-cell movement of CP-deleted PVX to certain levels, albeit less efficiently than PVX CP, itself. Together, the combination of two different, non-competing viruses has facilitated the production of a full size mAb A5, subclass IgG1, which could not be achieved when using vectors based on the same virus, due to the dramatic reduction in expression. This research has built up a versatile platform which can express seven other functional mAbs of IgG1 and IgG2 classes in less than 14 dpi (Gleba et al. 2007). Recently, the same concept has been applied to co-express hybrid chimeric PVX vectors harbouring GFP and mCherry. The pPVX-GFP-2A-CP vector contained the full viral genome of PVX, GFP fused with F2A (GFP-2A) and flanked by the SGP of CP, and CP. The pTMV-mCherry-2A-CP PVX vector was constructed with the full genome of TMV, a fusion of mCherry, F2A and PVX CP flanked by duplicate TMV CP SGPs. Interestingly, PVX showed enhancement when co-infected with PVX and TMV, as documented previously (Goodman and Ross 1974). More than that, two large proteins (GFP and mCherry) were displayed on the hybrid chimeric PVX, as confirmed by fluorescence microscopy, electron microscopy and western blot analysis.

Taken together, all these examples remarkably demonstrate the capacities and opportunities to manipulate the viral genome for expression purposes. Positive-sense RNA viruses constitute the vast majority of plant viruses (approximately 80%) and this fact drives the need for more intensive studies to help understand these viruses for beneficial uses in biotechnology (Mandahar 2006). The following description of three prominent virus genera will give us insights into their contribution to virus expression systems in plants.

#### **1.4 Comovirus**

The genus *Comovirus* belongs to the family *Comoviridae*, in the order *Piconavirales*. This genus has 15 species, including *Bean pod mottle virus* (BPMV), and the type species *Cowpea*

*mosaic virus* (CPMV). CPMV was first isolated from cowpeas (*Vigna unguiculata*) fifty-eight years ago in Nigeria (Chant 1959). It can infect not only legume species, e.g. cowpea, bean, and soybean, but also the experimental plant, *N. benthamiana*. The virus genome is comprised of two positive-stranded RNAs, including RNA1 (5.9 kb) and RNA2 (3.5 kb), and encapsulated by an icosahedral-shaped capsid. Both RNAs are polyadenylated at the 3' termini and have a small protein (Vpg) covalently linked to their 5' ends. Both of the RNAs are translated as a single protein precursor and then proteolytically processed by viral protease to release mature proteins. RNA1 encodes five mature proteins: a proteinase cofactor (Co-pro), a helicase (Hel), a viral genome-linked protein, a proteinase (Pro), and the RNA-dependent RNA polymerase (RdRp), all of which are requisite to the replication of viral RNAs and polyprotein processing. Meanwhile, RNA2 codes for a replication cofactor (CR), a movement protein (MP), and two CPs, large CP (L-CP) and small CP (S-CP). As mentioned, the virus particle has an icosahedral shape, consisting of sixty copies each of the L-CP and S-CP. It has been shown that virus particles are involved in both local and systemic viral movement in plants and also in transmission by insect vectors (Wellink and van Kammen 1989).

The development of CPMV-based expression vectors started with its first use, as a peptide display system in the 1990s (Porta et al. 1994; Usha et al. 1993). In this system, the virus was modified to carry short antigenic peptides, known as epitopes, on the surface of its capsids. Due to its three-dimensionally symmetric structure, CPMV became a successful carrier, as it could present multiple copies of epitopes to the immune system, and thus, enhance the immunogenicity of these vectors to work effectively as vaccines. In addition to its structure, the high titer of CPMV particles in plants (1-2 mg/g of leaf tissue fresh weight), and its non-pathogenicity in mammalian cells, made CPMV a perfect candidate for an epitope presentation platform in terms of yield and biosafety. There are three sites where the epitope has been inserted into the CPMV coat protein, including the  $\beta$ B- $\beta$ C loop of the S-CP (the predominant insert site) (Langeveld et al. 2001; McLain et al. 1996; Rennermalm et al. 2001), the  $\beta$ C'- $\beta$ C'' loop of the S-CP (Taylor et al. 2000) and the  $\beta$ E- $\alpha$ B loop of the L-CP (Brennan et al. 1999). The first successful example of this system, in which no wild-type reversion was observed, was designed with the insertion at an optimal position in the  $\beta$ B- $\beta$ C

loop of the S-CP between alanine 22 and proline 23, to maximize the exposure of epitopes on the virus surface and eliminate the recombination of repeating sequences (Porta et al. 1994). The virions displaying the NIm1A site from VP1 of the *Human rhinovirus 14* (HRV-14) could provoke a response against the presenting epitope and showed the capability of modified CPMV chimeras as a novel vaccine. To date, accumulated experiments have confirmed that peptides containing less than 40 aa and having a pI lower than 9.0 could be successfully expressed by CPMV, with titres as high as that of wild-type viruses (Porta et al. 2003). In addition to plant-made vaccines, CPMV can be decorated with inorganic compounds, e.g. silica or an iron-platinum alloy, which serve for applications in nanotechnology (Steinmetz et al. 2006; Steinmetz et al. 2009b).

A variety of approaches, including gene substitution, gene insertion, deconstructed vectors and transgene combined virus vectors, have been used to modify CPMV as a gene expression system (Cañizares et al. 2006; Liu et al. 2005; Sainsbury et al. 2008a; Sainsbury et al. 2008b). As a result, a powerful CPMV-based expression system, pEAQ-HT, which is widely used today, has been developed (Sainsbury et al. 2009). The pEAQ-HT system was designed based on the hyper-translatable (HT) elements of CPMV RNA2 and a modified 5' UTR and 3' UTR. Deletion of the AUG codon at position 161, upstream of the AUG codon at position 512, which is the main translational initiation site, enhanced the level of translation of the insert gene, e.g. GFP, at up to 25% of the total soluble protein (TSP), which was equivalent to 1.2 g/kg of leaf tissue fresh weight in transiently transformed *N. benthamiana*. The CPMV 5' UTR, which is lacking AUG 161, or both AUG 161 and AUG 115, was named the hyper-translatable leader (HT leader) (Sainsbury and Lomonosoff 2008). The pEAQ-HT expression vector consists of an expression cassette which contains a region of insert genes and is flanked by the HT leader and the CPMV 3'UTR, and a p19 cassette. This single vector was then delivered into *N. benthamiana* by *A. tumefaciens*. Using this system, transient expression could produce a high level of proteins of interests in a matter of days, without the need for viral replication. Many series of this system have been created to achieve compatibility with both Gateway and traditional restriction enzyme-based cloning. This facilitates high-throughput cloning and protein expression within a short time frame. Moreover, this system was exploited to specifically express virus-like particles (VLPs) in *N.*

*benthamiana* for vaccine plants at up to 70 mg/kg of leaf biomass. For example, *Bluetongue virus* (BTV)-like particles were transiently expressed at 70 mg/kg leaf wet weight after two rounds of gradient centrifugation, when using pEAQ-HT combined with *CPMV-HT* (Thuenemann et al. 2013). BTV is the causal agent of bluetongue disease in ruminants, especially sheep and cattle. The symptoms include high fever, swelling face and tongue, cyanosis of the tongue, lameness, and infertility, all resulting in high morbidity and mortality. When tested in sheep, the plant-made BTV VLPs were able to elicit a strong immune response and provide protective immunity against a South African BTV-8 field isolate. These results showed that the pEAQ-HT system can efficiently produce VLPs for use as plant-made veterinary vaccines. This state-of-art system also provides a versatile tool for the production of CPMV capsids, without the RNA genome and this removes concerns of biocontainment. These capsids may also work as templates for synthetic biology purposes. Since CPMV possesses an icosahedral shaped nanostructure, its outer surface can be modified to incorporate different functional moieties, e.g. biotin, redox-active compound, gold nanoparticles, etc., by chemical modification. The application of the resulting molecules could lead to highly beneficial advances in bio-nanotechnology (Saunders and Lomonosoff 2013). For instance, gold nanoparticles anchored to a cysteine-substituted CPMV particle could be interconnected into a precise three-dimensional scaffold in order to generate nanoscale conductive networks (Blum et al. 2005). Together with other components, these particles could then form molecular electric circuits for nano-devices (Blum et al. 2007). More strikingly, even the empty CPMV, as nanoparticles alone, can contribute to cancer immunotherapy. A recent study demonstrated that CPMV inhalation could induce an antitumor response by activating neutrophils (Lizotte et al. 2016). Altogether, the pEAQ-HT system, which is based on the modified 5' UTR of CPMV, has proven its efficacy in the high yield production of not only heterologous proteins, but also virus-like particles for multiple purposes in interdisciplinary applications.

### **1.5 *Tobamovirus***

The genus *Tobamovirus* is classified into the family *Virgaviridae*. This is the only genus of plant virus which has rigid, rod-shaped particles and a monopartite, positive-stranded RNA

genome. This genus includes approximately 35 species, such as *Cucumber green mottle mosaic virus* (CGMMV), and the type member *Tobacco mosaic virus* (TMV). The discovery of TMV, when Martinus W. Beijerinck proved that “*contagium vivum fixum*” (virus) was a new infectious agent involved in the mosaic disease of tobacco (Beijerinck 1898), has become a milestone, which marked the beginnings of virology. TMV has been the subject of intensive research for over a century. It has been a popular tool and model for plant biology, virology, plant virus disease studies, biochemistry, structural biology, and genetics, etc. (Scholthof 2004). Here, I will focus on its utility in molecular farming and nanotechnology.

TMV possesses a wide host range, with at least 199 species from 30 plant families (Shew and Lucas 1991), including *N. tabacum*, *N. glutinosa*, and *N. benthamiana*, etc. The virus is transmitted mainly via the mechanical inoculation of sap from infected plants. The RNA genome of this virus is composed of approximately 6400 nucleotides (nt) (Goelet and Karn 1982). It encodes two replicases, including a 126-kDa protein and a 186-kDa protein, a 30-kDa movement protein (MP) and a 17.6-kDa coat protein (CP). The 186-kDa protein is produced by a translational readthrough past the amber stop codon (UAG), at the end of the 126-kDa protein (Pelham 1978). The 126-kDa protein has methyltransferase and helicase motifs, whereas, the 186 kDa contains the RdRp. The MP and CP are translated from co-terminal sg RNAs, sg RNA1 and sg RNA2, respectively. The 3' end of the 186-kDa protein sequence overlaps the open reading frame (ORF) of the MP by 15 nt. The 5' UTR of this genome is capped by a methyl guanine and contains a leader sequence, called the  $\Omega$  sequence, which is believed to enhance translation of the downstream sequence (Gallie et al. 1987). The 3' UTR can form a tRNA-like structure. TMV was the first virus that had been visualized by electron microscopy (Kausche et al. 1939) and studied by X-ray crystallography (Watson 1954). A novel self-assembly between RNA and capsid protein was also revealed and this became a model for virus structure research. The virus particle has a hollow cylindrical shape that is approximately 300 nm long, 18 nm in diameter, contains a 4 nm channel, and is composed of approximately 2130 subunits of coat protein (Caspar 1956; Franklin 1955; Watson 1954). Research has shown that the N-terminus, C-terminus and a 60s loop (between aa 55-60) are exposed on the viral surface and able to tolerate insertions.

However, the residues of aa 155-158 of the coat protein are somehow disordered (Namba and Stubbs 1986). These characteristics make TMV an attractive peptide display particle.

TMV was the first plant virus to be engineered for molecular farming. It was modified to display an epitope of a type 3 poliovirus (Haynes et al. 1986). The epitope, which contains eight aa (Glu-Gln-Pro-Thr-Thr-Arg-Ala-Gln), plus two glycine residues, serving as a spacer, were inserted into the C-terminus of TMV CP and expressed in *E. coli*. Purified TMV-polio 3 particles could induce anti-polio virus neutralizing antibodies when injected into rats. This success facilitated research for the improvement of TMV display system in plants, especially tobacco (*N. tabacum*). Turpen et al. successfully placed different epitopes of *Plasmodium* spp. into the surface loop region and at the C-terminus of the TMV CP, to produce malarial subunit vaccines in tobacco (Turpen et al. 1995). Epitopes of *Plasmodium vivax*, consisting of either four (BGC246) or 12 aa (BGC291), replaced two aa (Asp64 and Ser65), following Pro63, in the surface loop region of CP, by site-direct mutagenesis. Another epitope of *P. yoelii* (BGC261) was fused to the C-terminus of CP, after the readthrough stop codon context, "CARYYA". The virus particles exhibiting BGC291 and BCG261 reacted with their specific monoclonal antibodies, whereas, the BGC246 did not. Virus particles were recovered in high yields from infected tobacco (var. Xanthi), at 0.4 mg/g and 1.2 mg/g of leaf tissue fresh weight for BGC291 and BGC261, respectively. These examples demonstrate TMV as a competent epitope carrier for subunit vaccines. Simultaneous to the development of TMV peptide presentation system, the exploitation of TMV as a viral vector has also been rigorously investigated.

The first TMV-based vector for heterologous protein expression in plants was developed by Takamatsu et al. (Takamatsu et al. 1990). They created a gene substitution vector in which the CP gene was substituted by a bacterial chloramphenicol acetyltransferase gene (CAT). Although initially CAT expression was low (1 µg/g of leaf tissue), the low expression problem was later overcome through construct improvements. One of these was the modular system, MagnICON, which was based on TMV-deconstructed viral vectors developed by Icon Genetics (Halle, Germany). In this system, a series of constructs were made which contained different and separate modular genes, e.g. controlling elements, signal peptides,



gene of interest, purification tags, and cleavage motifs. These modules were delivered into plant cells by agroinfiltration, using different bacterial cultures containing each module of interest. For example, the 5' module included the viral *RdRp*, *MP*, and sequences for the CP SGP and a recombination site. The 3' module contained a recombination site sequence, the gene of interest (e.g. *GFP*), and the 3' end of the viral vector (Marillonnet et al. 2004). When entering into plant cells together, these modules would assemble into a fully functional infective replicon of interest, via site-specific recombination. This approach provided a basis for the fast development and optimization of viral expression vectors. Moreover, removal of putative cryptic sites in the 5' end of *RdRp* and the addition of introns in the *RdRp* and *MP* resulted in a 1000-fold increase in GFP expression (Marillonnet et al. 2005). This system was also competent when applied to whole plant transfection, named “magniffection”, by vacuum infiltration, which is being widely used in large-scale production of plant-made recombinant proteins. As the CP was completely omitted in MagnICON vectors, long distance movement of viruses was disabled, preventing systemic spread throughout plants and into the environment, which is safer in terms of biosafety concerns. As such, the MagnICON system has gained a reputation as a robust, versatile, time-saving and efficient modular viral platform to produce high yields of proteins of interest on an industrial scale. For example, the maximum yield of small *Hepatitis B virus* (HBV) surface antigen (S-HBsAg) was approximately 300 mg/kg of leaf tissue fresh weight in *N. benthamiana*, when using this platform in conjunction with vacuum infiltration (Huang et al. 2008). Interestingly, this protein was able to form VLPs displaying the correct conformation of the “a” antigenic determinant and used as an HBV vaccine. Similar to CPMV, the nanostructure of TMV has recently been manipulated for bio-nanotechnology. TMV has been used as a scaffold, where a variety of metals (e.g. gold, silver, nickel, etc.), glass, silicon wafers or biotin adhere to its interior or outer surfaces, resulting in the large-scale assembly of molecules which can form nanowires, thin film coatings, ordered fibers and labeling particles. Moreover, Czapar et al. have reported the use of TMV as a drug delivery system for cancer therapy (Czapar et al. 2016). Essentially, phenanthriplatin, which is a platinum-based anticancer agent, was chemically conjugated into the 4 nm wide central channel of TMV to generate TMV-encapsulated phenanthriplatin (PhenPt-TMV). Subsequently, the resulting PhenPt-TMV was

injected into mice with breast cancer. Mice treated with PhenPt-TMV showed up to a fourfold increase in tumor reduction when compared to control mice.

## 1.6 *Potexvirus*

As a genus of the family *Alphaflexiviridae*, *Potexvirus* includes more than 47 species, e.g. *Pepino mosaic virus* (PepMV), *Foxtail mosaic virus* (FoMV), *Bamboo mosaic virus* (BaMV), and *Potato virus X* (PVX). The type member of this genus is PVX, which mainly infects herbaceous plants, especially the *Solanaceace* family. PVX is characterized by a non-enveloped and flexible filamentous virion, approximately 470- 580 nm in length and 13 nm in diameter, which consists of 1270 CP subunits (Tollin and Wilson 1988). The genome is a monopartite, single stranded and positive-sense RNA that is 6.4 kb (Huisman et al. 1988), which encodes five open reading frames (ORFs): the RdRp, the triple gene block (TGB), which is formed by three overlapping ORFs (TGBp1-3), and the CP. The mature proteins of TGB and CP are translated from subgenomic RNAs (sg RNAs) in which the sg RNA1 encodes TGBp1, sg RNA2 for TGBp2-3, and sg RNA3 for CP, respectively. The genome and sg RNAs are capped with methyl-guanosine at the 5' end and polyadenylated at the 3' end. The RdRp is involved in viral replication and RNA capping (Huisman et al. 1988), whereas, the TGB has multiple functions, the majority of which involve virus replication, virus movement, translational activation, and gene silencing in plants (Angell et al. 1996; Davenport and Baulcombe 1997; Howard et al. 2004; Kalinina et al. 2002; Karpova et al. 2006; Voinnet et al. 2000). The CP is an essential requirement for long-distance viral movement, but is dispensable for cell-to-cell movement (Betti et al. 2012).

In contrast to CPMV and TMV, crystallographic data on the exposed regions of PVX CP, which display on the surface of virus particles, has yet to be fully detailed (Steinmetz et al. 2010). However, previous studies have shown that the N-terminus of CP was exposed on the external surface of PVX virions (Baratova et al. 1992). Thus, several epitopes have been fused to this region of the CP in order to form chimeric particles for vaccine production. For example, a neutralizing epitope (Glu-Leu-Asp-Lys-Trp-Ala), derived from glycoprotein 41 of the *Human immunodeficiency virus type 1* (HIV-1), was presented on PVX virions, via this method (Marusic et al. 2001). Mice immunized with the resulting chimeric virus

particles (CVPs) showed a neutralizing activity against HIV-1. Moreover, CVPs were able to evoke the mucosa-associated immune system into production of antigen-specific antibodies, when delivered intranasally without adjuvants. In the other words, chimeric PVX presenting a neutralizing epitope is a potential novel candidate in the fight against HIV.

The development of viral expression vectors based on PVX has drawn scientist's attention, not only as a tool for plant-made recombinant proteins, but also for studies of virus movement (Baulcombe et al. 1995; Chapman et al. 1992; Cruz et al. 1996). There have been two main strategies to generate PVX-based vectors: (1) Addition of an extra SGP of CP upstream of the gene of interest (GOI), both of which are then inserted between the MP sequence and the SGP of CP; (2) Introduction of the F2A between the GOI and CP, which are then placed downstream of the subgenomic promoter of CP. Both formats have been widely used. The first method is freely accessible for researchers through The Sainsbury Laboratory (<http://www.ayeaye.tsl.ac.uk/>) and serves as a valuable experimental tool for both over-expression and virus-induced gene silencing (VIGS). More interestingly, the second strategy, called the OVERCOAT system, has been exploited to produce chimeric virus particles presenting peptides and proteins of interest, the size of which can be as large as GFP (25 kDa) (Cruz et al. 1996). One obstacle to the virus presentation system is size limitations of the insert in order to maintain the correct virus assembly and prevent wild-type reversion (Bendahmane et al. 2000). The OVERCOAT system has been adapted to overcome this obstacle. In this system, F2A sequence has been introduced between the GOI and the CP. During translation, F2A-mediated cleavage help the detachment of foreign proteins from the CP. However, incomplete cleavage also happens at a low rate, and results in a fusion of the CP and a foreign protein, called heterologous CP. Subsequently, the heterologous CPs assemble with the CP to form fully functional CVPs. This allows foreign proteins to be presented at the outer surface of CVPs without steric hindrance. O'Brien et al. have successfully produced a PVX decorated with viral protein 6 (VP6) of rotavirus, using this system (O'Brien et al. 2000). The size of the VP6 in this study was approximately 46 kDa. In addition to chimeric PVX, unfused VP6 also formed icosahedral virus-like particles (VLPs), in a ratio of 1:50, when compared to the recombinant PVX. Another adapted system was also employed to express a long peptide (70 residues) of E2, the most immunogenic envelope

glycoprotein of *Classic swine fever virus* (CSFV), on the surface of PVX, based on F2A cleavage (Marconi et al. 2006). Partially purified chimeric PVX showed the capability to induce anti-E2 antibodies when rabbits were immunized. These two examples have shown the vast potential of PVX-based expression systems as a platform to facilitate the production of VLPs and chimeric PVX in plants for vaccine development.

The PVX-based system has also been explored for cancer vaccine development. The system has been shown with capacity to deliver both tumor antigen sequences and activating genes to enhance immune responses against tumor cells. Savelyeva et al. reported that p.scFv-PVXCP, in which single-chain Fv (scFv), a weak tumor antigen of B-cell malignancies, was fused with the PVX CP sequence (Savelyeva et al. 2001). The p.scFv-PVXCP was able to promote a distinct CD4<sup>+</sup> T-cell-mediated protective immunity, by attacking B-cell tumors in mice, whereas, p.scFv, containing only scFv, failed to generate a significant immune response. PVX has also been applied into biomedicines, as a prophylactic vaccine against breast cancer. Human epidermal growth factor receptor 2 (HER2) is an antigen associated with tumors found in HER2-breast cancer, which has been traditionally associated with poor prognoses and a high rate of metastasis. Passive immunotherapy with Trastuzumab, a monoclonal antibody which binds to HER2, cannot protect patients from metastasis or recurrence, whereas, a cancer vaccine has the potential to do this. Successful administration of prophylactic vaccines in high risk groups may also lighten the financial burden of intensive treatments (chemotherapy, hormone replacement therapy, etc.). Shukla et al. have developed a PVX-based HER2 platform for a breast cancer vaccine (Shukla et al. 2014). A B-cell epitope of HER2, called P4 (containing 21 residues), was coupled to the external surface of PVX, via chemical ligation, to create the chimeric PVX-P4. Mice immunized with purified PVX-P4 demonstrated the generation of HER2-specific antibodies and different IgGs (e.g. IgG2a, IgG2b and IgG1), in which IgG2a and IgG2b were believed to inhibit tumors and IgG1 could be involved in triggering immune responses. Through these studies, the PVX-derived system proves as powerful as its peers (e.g. CPMV, TMV) in the development of vaccines, giving promise for the development of efficient vaccines in the fight against a variety of diseases.

## 1.7 Research goals and objectives

As discussed briefly above, the costs associated with PRRS have been devastating the swine industry worldwide, and an effective therapy against this disease is highly demanded. In view of the advantages of plant-made vaccines and viral vectors, I proposed to develop a plant virus-based expression system for plant-made vaccine against PRRSV. In this thesis, different approaches were investigated for the construction of viral vectors expressing GP5-based subunit vaccines in several plant species, such as soybean, cucumber and *N. benthamiana*. The vaccine products from soybean and cucumber may be directly administered to pigs by oral route. The specific objectives of this project include:

1. Construction of BPMV-based expression vectors for GP5 production in soybean. This study aimed to produce and characterize the expression of GP5 in two moieties, GP5N and GP5C, using viral vectors.
2. Construction of a CGMMV-based expression vector for neutralizing epitope production in cucumber and *N. benthamiana*. This study aimed to investigate the characterization of chimeric CGMMV presenting GP5 neutralizing epitopes in cucumber and *N. benthamiana*.
3. Construction of a PepMV-based expression vector for GP5 expression in *N. benthamiana*. In this study, the modified GP5 was co-expressed with GFP in *N. benthamiana*.
4. Evaluation of recombinant protein expression in soybean, *N. benthamiana* and cucumber.

**Table 1.1:** Plant-made vaccines based on GP5 of PRRSV

Type	System	Vector	Protein	Level	Model	Dose	Response	Reference
<i>Transgenic plant</i>	Tobacco <i>N. tabacum</i>	pGKU-35PRRSV	GP5	0.011% TSP (~110 ng/g fresh weight of leaf tissue).	pigs	Feed 50 g leaves/pig for 4 times.	- Serum NA was detected at 48 DPIOV (3/6 pigs).  - Specific mucosal and systemic humoral and cellular responses were induced.	Chia et al. 2010
<i>Transgenic plant</i>	Tobacco <i>N. tabacum</i>	pGKU-LBT-ORF5	LTB-GP5	GP5: 155 ng/g fresh weight of leaf tissue.	pigs	Feed 35.2 g leaves LTB-GP5/pig for 3 times.	- NA was higher.  - Specific mucosal and systemic humoral and cellular responses were induced.  - Viral load decreased in 3 weeks post inoculation.	Chia et al. 2011

<b><i>Transgenic plant</i></b>	Potato	pBI121	GP5	Leaf: 2.5-4.7 $\mu\text{g/g}$ fresh weight. Tuber: 0.8-1.2 $\mu\text{g/g}$ fresh weight.	mice	Intragastrically deliver 0.5ml transgenic potato tuber extract /pig (~0.3 $\mu\text{g}$ GP5) for 4 times.	- NA was detected (2/8 mice).  - Specific mucosal and systemic humoral, and cellular responses were induced.	Chen and Liu 2011
<b><i>Transgenic plant</i></b>	Tobacco <i>N. tabacum</i>	pCaMteX AMV	CTB-GP5 and CTB-GP5-NEabc	CTB-GP5: Not detected by western blot. CTB-GP5NEabc 0.003-0.087% TSP.	-	-	- GM1 ELISA binding assay.	Wang et al. 2011
<b><i>Transgenic plant</i></b>	Banana leaf, Pei chiao AAA	pGKU-35PRRSV	GP5	0.021-0.037% TSP ~157-257 ng/g fresh weight of leaf tissue.	pigs	Feed 50g leaves/pig (~ 7.7-12.9 $\mu\text{g}$ GP5) for 3 times.	- Specific mucosal and systemic humoral and cellular responses were induced.	Chan et al. 2013

							- Viremia and tissue viral load was lower.	
<b><i>Transgenic plant</i></b>	Arabidopsis seed	pPphasGW	GP5, GP5-Tm and GP5-Tm:pFc	GP5 or GP5-Tm: 0.1% TSP. GP5-Tm:pFc: 0.83% TSP ~2.3 mg/g seed weight.	mice	Inject mice cocktail of 100 µg each GP5/GP4/GP3-Tm:pFc purified for 2 times.	- Antigen specific antibody but no neutralizing activity in sera was detected.	Piron et al. 2014
<b><i>Transient expression</i></b>	<i>N. silvestris</i>	pICH series	GP5-M and GP5-T VLP	qRT-PCR and TEM.	mice and pigs in future	-	-	Uribe-Campero et al. 2015
<b><i>Transient expression</i></b>	<i>N. benthamiana</i>	pEAQ-HT	GP5synt	5 mg/kg fresh weight of leaf tissue.	-	-	-	CORDIS 2015



“-“: Not reported

NA: Neutralizing antibody

LTB: Subunit B of *E.coli* heat-labile enterotoxin

CTB: Cholera toxin subunit B

DPIOV: Days post-initial oral vaccination

VLP: Virus-like particle

TEM: Transmission electron microscopy

qRT-PCR: Quantitative reverse transcription polymerase chain reaction

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## Chapter 2

# Developing a BPMV-based expression vector for production of recombinant protein in soybean

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## ***2.1 Introduction***

As discussed in Chapter 1, the viral protein GP5 is a promising subunit vaccine against PRRSV. To produce recombinant PRRSV GP5 in plants, in a previous study, my lab generated transgenic tobacco plants for the production of GP5 or its fusions (Wang et al. 2008; Wang et al. 2011). The cholera toxin B (CTB) of *Vibrio cholerae*, an efficient transmucosal carrier induction of peripheral immunological tolerance, was fused with either GP5 or its neutralizing epitopes (GP5-NE). In this strategy, CTB worked as an adjuvant to facilitate either GP5, or GP5-NE, delivery into mucosal sites and to promote mucosal immune response. The GP5 protein or GP5-CTB fusion was not detectable in the transgenic plants (Wang et al. 2011). The level of GP5-NE fusion was very low in the range of 0.003 to 0.087% of total soluble protein (TSP) and the fusion protein could bind to the mucosal GM1 receptor effectively (Wang et al. 2011). To circumvent the low expression of target proteins in the production of plant recombinant proteins, I attempted to use viral vectors. Plant viruses have the ability to reprogram plant cells to serve as an efficient factory for viral genome and protein production. Once the virus carrying a gene of interest is introduced to plants, it will replicate and the protein of interest can be expressed in large amounts (Gleba et al. 2007).

In this study, *Bean pod mottle virus* (BPMV), a member of the family *Comoviridae*, genus *Comovirus* (Lomonosoff and Ghabrial 2001), was manipulated to develop a viral-based expression vector for recombinant protein production in soybean plants. The genome of BPMV consists of two genomic RNAs including RNA1 and RNA2. Both of RNAs have a covalently linked protein, VPg, at the 5' termini and a poly A tail at the 3' termini. Viral proteins are expressed as a single protein precursor and subsequently proteolytically processed by the viral protease to release mature proteins. BPMV RNA1 (nearly 6 kb in length) codes for five mature proteins, protease cofactor (Co-pro), putative helicase (Hel), viral genome-linked protein (VPg), protease and putative RNA-dependent RNA polymerase (RdRp), which are necessary for viral replication. Meanwhile, BPMV RNA2 (nearly 3.6 kb) encodes four proteins consisting of a replication cofactor (CR), a putative movement protein (MP), a large coat protein (L-CP) and a small coat protein (S-CP). *Cowpea mosaic virus*

(CPMV), which is also a comovirus, has been developed as a viral vector for protein expression since 1998 (Gopinath et al. 2000; Verver et al. 1998) and viral elements are applied to enhance the expression of viral structure proteins which can form virus-like particles (Sainsbury et al. 2009b; Thuenemann et al. 2013a; Thuenemann 2013). In contrast, BPMV-based vectors has been developed mainly as a virus-induced gene silencing (VIGS) vector and to a much lesser extent for expression of non-therapeutics proteins (Zhang et al. 2010; Zhang and Ghabrial 2006; Zhang et al. 2013; Zhang et al. 2009). Since BPMV is one of the major viruses which infect soybean (Gu et al. 2002), it has a great potential as a viral system for expression of edible subunit vaccines without the need of purification. Here, I introduce a new BPMV based-vector which can be directly inoculated on soybean plants via biolistic bombardment to express green fluorescent protein (GFP) and a C terminus of GP5 fused with GFP.

## ***2.2 Materials and methods***

### **2.2.1 Plant materials**

*Nicotiana benthamiana* (*N. benthamiana*) were grown in controlled environment chambers under conditions of 16 hours (hr) in the light at 22°C, 8 hr in the dark at 18°C, and 70% relative humidity (RH). Light intensity during the 16-hr light period was approximately 100  $\mu\text{mol}$ .

Soybean (*Glycine max* L. Merrill) cultivar, Williams 82, was grown in a greenhouse at 22°C under a 16/8 hr photoperiod. After 10 days post germination, seedlings were used for biolistic bombardment.

### **2.2.2 Bacterial strains and growth conditions**

*Escherichia coli* (*E.coli*) strain DH5 $\alpha$  was used for general plasmid propagation and extraction. For specific DNA manipulation required for a methylation-deficient *E. coli* strain, such as involved in the use of some restriction sites including *StuI*, JM101 was used. Both strains were cultured with Luria-Bertani (LB) liquid medium (1% tryptone, 1% NaCl, 0.5% yeast extract plus 1 l distilled water) or solid medium supplemented with 1.5% (w/v) agar at

37°C. Appropriate antibiotics such as kanamycin (50 µg/ml) or ampicillin (100 µg/ml) were added to the LB media according to selection markers of the plasmids.

*Agrobacterium tumefaciens* (*A. tumefaciens*) strain GV3101 was grown at 28°C in LB liquid medium with kanamycin 50 µg/ml and rifampicin 50 µg/ml, or on LB solid medium supplemented with 1.5% (w/v) agar.

### 2.2.3 Virus

The genome of BPMV is bipartite, positive-stranded RNA genome, including RNA1 and RNA2. Vectors pGHoR1, pGG7R2M and pGG7R2V were obtained from Dr. Ghabrial (Zhang and Ghabrial 2006). pGHoR1 contains full length cDNA of BPMV RNA1 whereas, pGG7R2M and pGG7R2V contain BPMV RNA2 under the control of a T7 promoter (Gu et al. 2002). pGHoR1, pGG7R2M and pGG7R2V were subjected to Sanger DNA sequencing and their infectivity in soybean was confirmed via soybean bombardment. Sequencing results were perfectly matched to the published BPMV genome sequence (GeneBank accession number: AF394608, and M62738).

### 2.2.4 Construction of DNA infectious clones and plant expression vectors

Firstly, pCass4-Rz, a binary plant expression vector containing a double 35S promoter, a ribozyme self-cleavage site (*Rz*) and a terminator (*T*) (Annamalai and Rao 2005), was modified by replacing the *Rz* and *T* sites with the *Nos* terminator between the restriction sites of *Bam*HI and *Pme*I to generate vector pCass4-Nos. Full length genomic DNAs of either BPMV RNA1 or BPMV RNA2, were amplified from pGHoR1 and pGG7R2M, respectively, using Phusion High-Fidelity DNA Polymerase (NEB, Pickering, Ontario, Canada) and primers containing indicated restriction sites such as *Stu*I and *Bam*HI or *Stu*I and *Xba*I (Table 2.1). The resulting products were digested with the corresponding restriction enzymes and integrated into the same restriction sites (*Stu*I/*Bam*HI or *Stu*I/*Xba*I, respectively) of pCass4-Nos to create the cassettes of 35S promoter::BPMV RNA1::*Nos* terminator and the cassette of 35S promoter::BPMV RNA2::*Nos* terminator. All the above

restriction enzymes were purchased from NEB (New England Biolab, Canada). The inserted genes were confirmed by digestion and DNA Sanger sequencing.

To characterize the expression of GP5 *in planta*, the protein of interest was halved into two equal portions, the N-terminus (GP5N) containing the first 100 amino acid (aa) and the C-terminus (GP5C) comprising of the remaining 100 aa. Both GP5N and GP5C were tagged by GFP as GP5N-GFP or GP5C-GFP fusions. GFP is the enhanced GFP, which was described previously (GenBank accession number: U55762.1) (Cormack et al. 1995). The cDNA fragment of *GP5N* was amplified by the primers GP5N-F and GP5N-GFPlink-R while the *GFP* fragment was created by the primers GP5N-GFPlink-F and GFPatt-R. These two fragments were ligated by overlapping PCR using the primers GP5N-F and GFPatt-R. To generate *GP5C-GFP*, the same steps were followed using the primers, GP5C-F and GP5C-GFPlink-R for the *GP5C* fragment, GP5C-GFPlink-F and GFPatt-R for the *GFP* fragment and GP5C-F and GFPatt-R to overlap these two fragments. The resulting PCR products were analyzed by gel electrophoresis and purified using gel purification kit (QIAGEN Inc., Toronto, Ontario, Canada). Gateway cloning was performed by applying Gateway Technology (Invitrogen, Burlington, Ontario, Canada) following the manufacturer's protocol. *GP5N-GFP* or *GP5C-GFP* was introduced into a pDONR Zeo vector by a BP reaction (Hartley et al. 2000), and the resulting vector was then transformed into *E.coli* DH5 $\alpha$  to generate an entry clone. To confirm clone identity, the plasmid was sequenced with M13F and M13R primers. The entry vector was mixed with pCamGate-ER destination vector (Pereira et al. 2013) and LR Clonase enzyme (Invitrogen) for *in vitro* recombination to construct pCamGate GP5N-GFP and pCamGate GP5C-GFP. The resulting plasmids were confirmed by DNA sequencing using the primers pCamGateSeq-F and pCamGateSeq-R.

The pEAQ series of vectors were refined from the CPMV expression system by constructions of compatible systems for either traditional cloning or Gateway technology, combined with agroinfiltration (Sainsbury et al. 2009b). The expression cassette was designed to allow proteins of interest to be fused with a His-tag at their N-terminus or C-terminus for purification. In this experiment, a pair of restriction sites, *AgeI* and *SmaI*, was

exploited to insert GP5N-GFP or GP5C-GFP into pEAQ-HT to generate pEAQ GP5N-GFP and pEAQ GP5C-GFP, respectively. GP5N-GFP was amplified from pCamGate GP5N-GFP using primers AgeI-GP5N-F and GFP-R while GP5C-GFP was copied from pCamGate GP5C-GFP using AgeI-GP5C-F and GFP-R. His-tag was also attached at the C-terminus in each of these constructs. The sequences were eventually verified by PCR colony screening, restriction enzyme digestion and DNA Sanger sequencing.

pGG7R2V, which has the same sequence as pGG7R2M except for a multi-cloning site with a portion of *GFP*, flanked by duplicating viral proteinase sites, Glutamine-Methionine (QM), between coding sequences of MP and L-CP, was used for cloning of foreign genes (Zhang and Ghabrial 2006). Fusions of either *GFP* alone or *GP5N* and *GP5C* with *GFP* were created by PCR using primers containing recognition sites of *BamHI* and *MscI* enzymes, named BamHI-forward primers and MscI-reverse primers (Table 2.1) together with the templates of pCaMGate containing *GP5N-GFP* or *GP5C-GFP*, accordingly. Digested *GFP*, *GP5N-GFP* and *GP5C-GFP* were ligated into *BamHI/MscI*-digested pGG7R2V at 16°C overnight to produce pGG7R2V (GFP), pGG7R2V (GP5N-GFP) and pGG7R2V (GP5C-GFP) respectively. The presence of insert fragments in the resulting vectors was confirmed by colony PCR screening, digestion with *BamHI* and *MscI*, and DNA Sanger sequencing.

In order to generate a vector which can be inoculated onto soybean leaves without in vitro transcription, BPMV RNA2 was modified to fuse with genes of interest (GOI), *GFP*, *GP5N-GFP* and *GP5C-GFP* and inserted into an expression cassette including 35S promoter and *Nos* terminator in pCass4-Nos. pGG7R2V (GFP), pGG7R2V (GP5N-GFP) and pGG7R2V (GP5C-GFP) were amplified by Phusion High-Fidelity DNA Polymerase (NEB) using primers BPMVR2-F and BPMVR2-XbaI-R. The resulting products will be digested with *XbaI* and integrated into the same digestion sites (*StuI/XbaI*) of pCass4-Nos to form the constructs of pBPR2(GFP), pBPR2(GP5N-GFP) and pBPR2(GP5C-GFP). These constructs were used for biolistic bombardment after the inserted genes were confirmed by restriction enzyme digestion and DNA Sanger sequencing.

RNA1 is required to be translated correctly for RNA2 replication (Zhang and Ghabrial 2006). The 5' untranslated region (UTR) of RNA2 is necessary for RNA2 accumulation (Lin et al. 2013). Therefore, the sequence of viral genome in each construct was carefully scanned and matched with the natural sequence of BPMV, especially in the 5' and 3' ends of RNA1 and RNA2.

### 2.2.5 Bacterial transformation

Chemically competent *E.coli* strains DH5 $\alpha$  or JM101 (50  $\mu$ l) stored in a -80°C freezer were thawed on ice for 10 minutes (min). Plasmid or ligation reaction mixtures containing approximately 100 ng of DNA was added directly to competent cells in 1.5 ml tubes and mixed gently by stirring with a pipette tip. The tube was incubated on ice for 30 min. Cells were heat shocked at 42°C for 60 seconds (sec) and then immediately placed on ice for 2 min. 200  $\mu$ l of SOC medium (Invitrogen) was added and the resulting mixture was kept at 37°C for 1 hr in a shaking incubator (225 rpm) to maximize the transformation efficiency of *E.coli* (Hanahan 1983). An aliquot of each transformation (50  $\mu$ l) was spread on LB plates including appropriate antibiotics for selection. Plates were incubated overnight at 37°C and colonies were selected on the next day for PCR colony screening to confirm successful transformants.

*Agrobacterium* transformation was performed using a MicroPulser Electroporation apparatus by following the manufacturer's instructions (Bio-Rad). Competent cells of *A. tumefaciens* GV3101 (40  $\mu$ l) were thawed on ice for 10 min and gently mixed with approximately 100 ng of DNA plasmid. The mixture was transferred into a cold Gene Pulser cuvette (Bio-Rad), on ice, and then electroporated using the Bio-Rad *E.coli* Pulser (Bio-Rad) at 1.9 kV for 10 sec. Transformed cells immediately went through a short incubation with 200  $\mu$ l of SOC medium at 28°C for 2 hr in a shaking incubator (225 rpm). The cells were plated onto LB plates containing selection antibiotics and rifampicin, and then incubated at 28°C for 48 hr. A single colony was picked up for transformation analyses. Colonies with the correct insert sequence were cultured for agroinfiltration.

### 2.2.6 *In vitro* transcription

The preparation for *in vitro* transcription was described previously (Kachroo and Ghabrial 2012) with minor modifications. In brief, pGHoR1 and pGG7R2M were linearized by *SalI* and *NotI* or *SaII*, respectively. The fragments containing RNA1 (6 kb) and RNA2 (8 kb) were excised from agarose gel, and purified using the QIAprep Spin miniprep kit (QIAGEN). A part of purified fragments was tested by electrophoresis on 1.2% (w/v) agarose gel. When the sizes were confirmed, the rest was utilized as templates for *in vitro* transcription. *In vitro* synthesis of capped RNA was performed using mMESSAGING MACHINER kit (Ambion Inc., Austin, TX, USA), according to the manufacturer's protocol. To assess RNA yield and quality, 5  $\mu$ l of the *in vitro* transcription products was analyzed on 1.2% (w/v) agarose gels and the concentration of the products was measured by spectrophotometer (Nanodrop 1000, ABI). A mixture of RNA transcripts (including RNA1 and RNA2) was introduced into soybean leaf cells via biolistic bombardment during infectivity testing.

### 2.2.7 Biolistic bombardment

Since soybean cotyledons and leaves are recalcitrant to agroinfiltration, infectious clones of BPMV were introduced into the primary leaves of soybean at the vegetative cotyledon stage (Zhang et al 2009), by biolistic bombardment using the Helios Gene Gun System (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

To confirm the infectivity of the original infectious clones received from Dr. Ghabrial (Zhang and Ghabrial 2006), RNA transcripts of the templates, pGHoR1 and pGG7R2M, were bombarded into soybean plants. An equal amount of BPMV RNA1 (34  $\mu$ g) and BPMV RNA2 (34  $\mu$ g) was mixed with spermidine,  $\text{CaCl}_2$  and glycerol then followed by a precipitation onto 17 mg of 1.0  $\mu$ m gold particles (Bio-Rad), to produce an RNA loading ratio of 4  $\mu$ g RNA/shot following the manufacturer's instruction. The suspension of gold and RNA was loaded into the Gold-Coat tubing (Bio-Rad) using the Tubing Prep Station (Bio-Rad). Coated tubes were cut into 0.5 inch cartridges, which were installed in the cartridge holder of the Helios Gene Gun. Helium pressure was adjusted to 160 psi and three shots were distributed onto each primary leaf of the same plant. The leaves were supported by a



foam block to prevent them from being damaged during the bombardment procedure. Each plant was given five shots. For each plasmid mix, a total of six plants were bombarded per experiment. Leaf tissues of these plants were collected for detection of BPMV by double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA) 15 days after biolistic bombardment.

To test the infectivity of DNA-based BPMV infectious clones, DNA plasmids, pBPR1 and pBPR2 were introduced into soybean leaves via biolistic bombardment essentially as described above. The plants were also tested for the presence of BPMV by DAS-ELISA 20 days after biolistic inoculation (Zhang et al. 2013).

To transiently express different GOI in soybean, a mixture of pBPR1 and pBPR2(GOI) was introduced into soybean plants employing a similar method as mentioned in the infectivity test of DNA-based BPMV infectious clones. In addition, the pBPR2(GOI) construct included a GFP gene which can be transcribed, translated and exploited as an indicator of protein expression and virus accumulation. DAS-ELISA was performed 15 days after biolistic inoculation. When infection was confirmed, BPMV-infected plants were sampled for a time-course analysis, from days 20 downwards to 90 days post bombardment, to observe GFP using confocal microscopy or to quantify the level of protein expression by immunoblotting.

Four to six soybean plants were inoculated with each construct. Following biolistic bombardment, plants were maintained in a growth chamber at 20°C and 70% RH, with a photoperiod of 16 hr.

### 2.2.8 Electron microscopy

To detect virions by transmission electron microscopy (TEM), 0.5 g of BPMV-infected leaf was ground in 2 ml of general extraction buffer (Agdia, Elkhart, IN, USA) with a mortar and a pestle. The resulting homogenate was centrifuged at  $12\,000 \times g$  for 20 min at 4°C. The supernatant was removed to a new tube and subjected to a second centrifugation, under the same conditions, to help remove of plant debris. The resulting supernatant then was filtered

through a 45  $\mu\text{m}$  filter (VWR International, Mississauga, Ontario, Canada) to generate the working solution for TEM. Negative staining was performed using one drop of the working solution and 2% (w/v) potassium phosphor tungstate (PTA) as described (Kingsbury and Darlington 1968).

### 2.2.9 Rub inoculation

To test the stability of the BPMV expression vector, we performed serial mechanical infections. In this experiment, the infected materials from successful bombarded plants (the first passage) were employed to inoculate healthy plants. If these plants became infected, and showed symptoms, they were called the second passage. Tissue was then collected from these second passage plants to inoculate the new generation of plants. BPMV infected soybean leaves showing severe symptoms were chosen as the source of viral materials. Approximately 1 g of fresh leaf tissue was ground using a pestle in a mortar with 5 ml of 0.01 M phosphate buffer pH 8.0 (470  $\mu\text{l}$  of 1 M  $\text{K}_2\text{HPO}_4$ , 30  $\mu\text{l}$  of 1 M  $\text{KH}_2\text{PO}_4$  plus 50 ml distilled water) (Sambrook and Russell 2001). The primary leaves of 10 day-old-soybean were dusted with fine 320-grit carborundum powder (Alfa Aesar, Lancaster, UK) and gently rubbed with inoculum. The carborundum and rubbing facilitated virus entry to infect leaf cells. At least six soybean plants were inoculated for each construct.

### 2.2.10 Transient expression in *N. benthamiana* via agroinfiltration

For transient expression in *N. benthamiana*, the GOI, including either GP5N-GFP or GP5C-GFP, were recombined with either pCamGate-ER or pEAQ-HT and transformed into *A. tumefaciens* GV3101 via electroporation as described above.

*Agrobacterium* cultures were grown in LB liquid medium with kanamycin (50  $\mu\text{g}/\text{ml}$ ) and rifampicin (50  $\mu\text{g}/\text{ml}$ ) at 28°C, to an optical density of 1.5-2.0 at 600 nm ( $\text{OD}_{600}$ ). Bacterial cells were harvested by centrifugation at 5 000  $\times$  g for 5 min. The pellet was resuspended in agroinfiltration buffer (10 mM  $\text{MgCl}_2$ , 10 mM MES and 150  $\mu\text{M}$  4'-Hydroxy-3', 5'-dimethoxyacetophenone), incubated at room temperature for 2 hr and followed by a dilution with agroinfiltration buffer to a final  $\text{OD}_{600}$  of 1.0. The suspension was then infiltrated into

the abaxial leaf epidermis of 4-6 week old *N. benthamiana* with a 1 ml syringe (Kapila et al. 1997). Plants were maintained in a growth chamber under normal conditions after infiltration. Five plants were infiltrated for each construct. Transient expression was then monitored by either UV lamp illumination or confocal microscopy.

#### 2.2.11 Double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA)

DAS-ELISA with BPMV coat protein antibody (Agdia) was performed following the manufacturer's instructions, at 15-30 days after bombardment (Zhang et al. 2013). Capture antibody was added to a 96 well plate during the coating step. Subsequently, several washes applied to remove excess capture antibody. Coat proteins of BPMV were extracted from infected plants using the general extraction buffer, and then added to coated wells and bound to the capture antibody. Enzyme conjugated antibody was then used to bind the capture antibody-coat protein complex and form a "sandwich". A p-Nitrophenyl phosphate (PNP) solution was then added to the wells. The colorless PNP solution reacts with positive samples by developing a color change. To determine the absorbance value of color change, the plate was read using iMark Microplate Reader (Bio-Rad) at 415 nm. At least three biological replicates were performed for each construct. Only constructs that had ability to infect soybean plants were applied for further steps.

#### 2.2.12 Reverse transcription-polymerase chain reaction (RT-PCR)

Three leaf discs (6 mm diameter) equivalent to approximately 100 mg of leaf tissue were added to 2 ml tubes with three 2.3 mm ceramic beads (Biospec, Fisher Scientific LTD, Ottawa, ON, Canada) and homogenized using a TissueLyser II (QIAGEN) at 30 Hz for 60 sec. Tissue sample was immediately processed with 1 ml TRIzol Reagent (Invitrogen) following the manufacturer's procedure for RNA isolation. The RNA pellet was re-suspended in RNase-free water (Ambion Inc.). DNase I (Invitrogen) treatment was conducted to remove DNA contamination. The concentration and purification of total RNA was determined by measuring absorbance at 260 nm/280 nm and 230 nm/260 nm in a spectrophotometer (Nanodrop 1000, ABI). To confirm the infectivity of infectious clones,

RT-PCR was performed using the RNA extract. 1 µg of total RNA was reverse transcribed by Superscript III Reverse Transcriptase (Invitrogen) to synthesize first strand cDNAs. 1 µl of cDNA was served as a template for a sequential PCR reaction using GoTaq FlexiDNA polymerase (Promega, Madison, WI, USA). *GFP* was amplified by the primers of GFP-F and GFP-R.

### 2.2.13 Ultraviolet (UV) lamp

A portable Blak-Ray B-100AP UV lamp (Blak-Ray, Upland, CA, USA) was used for the detection of green fluorescence in infiltrated plants with constructs containing the GFP gene. Plants were brought to a dark room where long wave ultraviolet light (~ 365 nm) was used to excite fluorescence within leaf tissues. Pictures were taken by Nikon COOLPIX P500 digital camera (Nikon Canada, Mississauga, ON, Canada) under polarizing light for visible light images and under UV illumination.

### 2.2.14 Confocal microscopy

#### 2.2.14.1 *N. benthamiana*

For fluorescence visualization in *N. benthamiana* leaves, a 5 mm x 7 mm section was excised and mounted for detection using a Leica TCS SP2 confocal laser scanning inverted microscopy (Leica Microsystems, Wetzlar, Germany) with 63X water immersion objective. Leaf samples were immersed in a drop of water between a glass slide and a cover glass. Another drop of water was also added onto the objective lenses. For imaging GFP, a 488 nm argon laser was used for the excitation and emission fluorescence was detected at 500-525 nm.

Images from different color channels were collected simultaneously at a resolution of 512 x 512 pixels and analyzed with the Leica Application Suite for Advanced Fluorescence (LAS AF, version 2.3.5) (Leica Microsystem, Germany).

### 2.2.14.2 Soybean

For soybean plants, leaves from plants bombarded with constructs containing *GFP* and *GFP* fused with GOI were visualized for GFP signal. Leaf samples were taken as 3 mm leaf discs at 20, 30, 45 and 90 days post inoculation (dpi). The abaxial leaf epidermis of each leaf disc was imaged with a Leica TCS SP2 confocal equipped with either a 10X dry or a 63X water immersion objectives. The procedure was essentially as described for *N. benthamiana* except that there was no water needed on the 10X dry objective.

### 2.2.15 Fluorometry of GFP

To quantify the concentration of GFP in *N. benthamiana* leaf extracts, a GFP quantification kit, Fluorometric (Cells Biolabs Inc., Burlington, ON, Canada), was used, following the manual provided by the supplier. 100  $\mu$ l of tissue lysate containing GFP in Lysate Buffer was transferred into a 96 well, black, flat bottom polystyrene, tissue culture-treated microplate (Corning, MA, USA). Fluorescence intensity was measured using excitation and emission wavelengths of 485 nm and 516 nm, respectively, in a Synergy 2 microplate reader (BioTek, VT, USA). To account for background fluorescence in the plant tissue, the extract of plants infiltrated with an empty vector was used to normalize the data. All sample dilutions were compared to a standard curve that was generated using purified GFP provided in the kit, between the ranges of 20-120 ng. Three biological replicates were measured for each construct. TSP was calculated using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad) following the manufacturer's protocol, using bovine serum albumin (BSA) as a standard, and an iMark Microplate Reader (Bio-Rad) at 595 nm.

### 2.2.16 Immunoblotting

Three leaf discs of 15 mm in diameter were collected from different leaves showing fluorescence from a total of five plants. The sample was ground in liquid nitrogen using a mortar and a pestle. Total protein extract was processed by adding urea-sodium dodecyl sulphate (urea-SDS) extraction buffer, containing 50 mM Tris-HCl pH 6.8, 4.5% (w/v) SDS, 9 M urea, 1 mM EDTA and 7.5% (v/v)  $\beta$ -mercaptoethanol to the ground leaves. Protein

extraction from either control plants infected with an empty BPMV vector or, a mock plant, was also performed as described. The total protein of each sample was semi-quantitated by dot blot technique and GelCode Blue stain reagent (Thermo Fisher Scientific, Rockford, IL, USA), following the manufacturer's protocol, and compared using a colorimetric method with a known concentration of BSA and TotalLab TL100 software (Nonlinear Dynamics, Durham, USA).

For immunoblotting analysis, the extracted proteins were separated using 12% (w/v) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a polyvinylidene difluoride (PVDF) membrane. The primary antibody, polyclonal anti-GFP antibody produced in rabbit (Sigma-Aldrich, Oakville, ON, Canada) was applied to the membrane for detection of GFP or GP5C-GFP, respectively. Horseradish peroxidase (HRP) conjugated anti-rabbit IgG produced in goat (Sigma-Aldrich) was used as the secondary antibody to be bound to the primary antibody. The filter was visualized using an ECL detection kit (GE Healthcare, Mississauga, ON, Canada) and autoradiography, as described by the manufacturer. The amount of GFP or GP5C-GFP expressed was compared to a standard of purified GFP, to estimate the level of expression. The expression level was determined at 20, 30, 45 and 90 days after bombardment. The level of protein expression was assessed based on GFP expression using TotalLab TL100 software (Nonlinear Dynamics) to analyze band intensity.

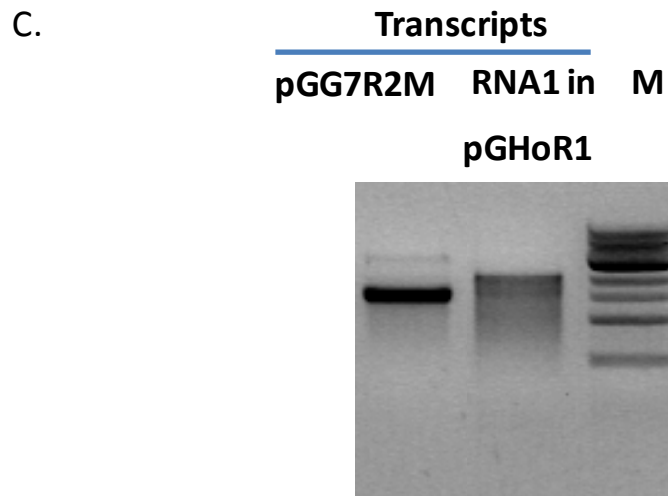
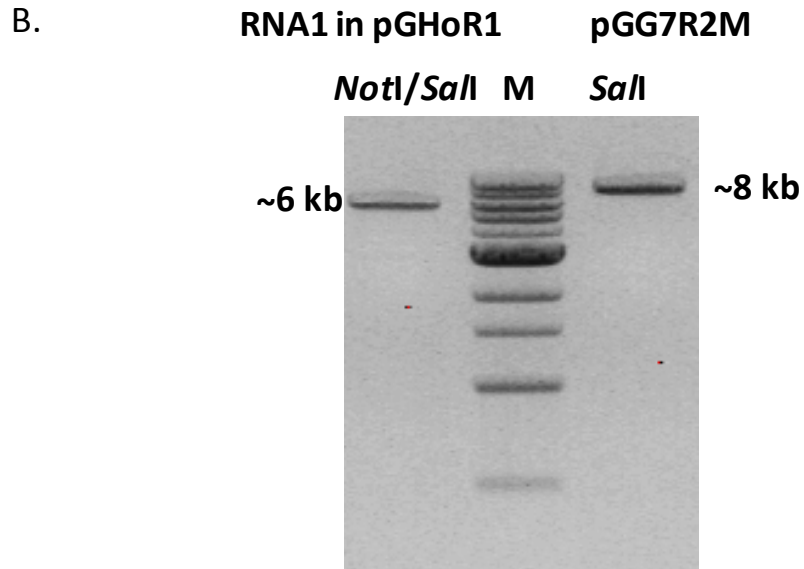
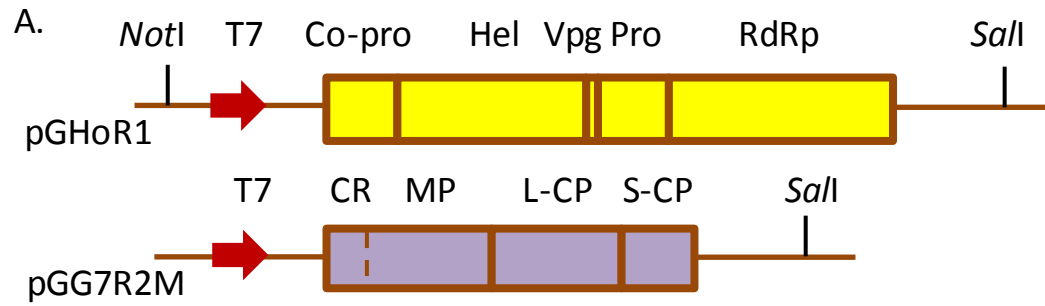
## **2.3 Results**

### **2.3.1 Infectivity of RNA infectious clones**

To validate constructs and determine the parameters for biolistic bombardment, soybean plants were infected using RNA transcribed from pGHoR1 and pGG7R2M. Both the plasmids contained T7 promoter which was placed upstream of the sequences of BPMV RNA1 and RNA2, respectively, and plasmid linearization was achieved by digestion with the restriction enzymes *NotI* and *SalI* or *SalI* only (Figure 2.1A). The purified RNA1 in pGHoR1 linearized by *NotI* and *SalI* had the approximate size of 6 kb, whereas pGG7R2M digested by *SalI* had a size of 8 kb (Figure 2.1B). To test the success of in vitro transcription, the

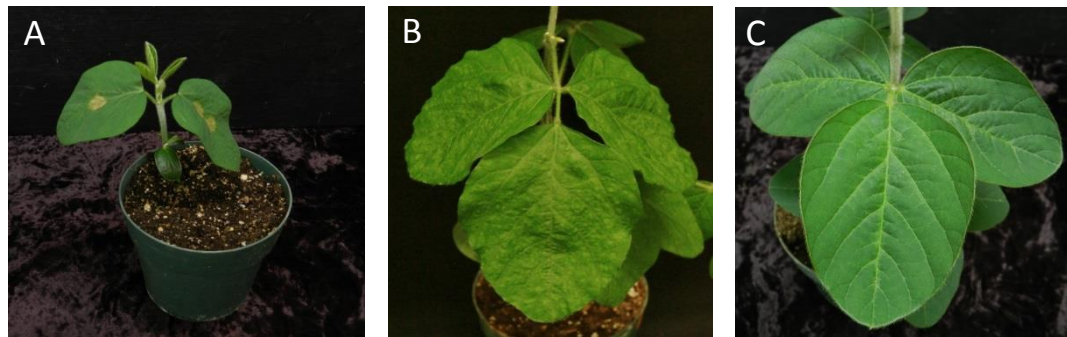
products were visualized by electrophoresis on an agarose gel. The results confirmed successful *in vitro* transcriptions (Figure 2.1C) and these transcripts were used for biolistic bombardment in soybean seedlings.

The transcripts of BPMV RNA1 and RNA2 were combined in a mixture and introduced onto the primary leaves of 10-day-old soybean seedlings via biolistic bombardment (Figure 2.2A). Several parameters, including the RNA loading ratio (DLR), Microcarrier Loading Quantities (MLQ) and discharge pressure, were adjusted to get the highest rate of infectivity, as well as maintain plant vitality. Intriguingly, the bombarded plants began to show symptoms at 12 dpi with mosaic patterning and wrinkled leaves (Figure 2.2B), compared to healthy plants (Figure 2.2C). DAS-ELISA confirmed the presence of BPMV in all soybean plants bombarded with the transcripts. Overall, these data suggest that pGHoR1 and pGG7R2M are infectious and thus, can be exploited as templates for the construction of BPMV DNA infectious clones.





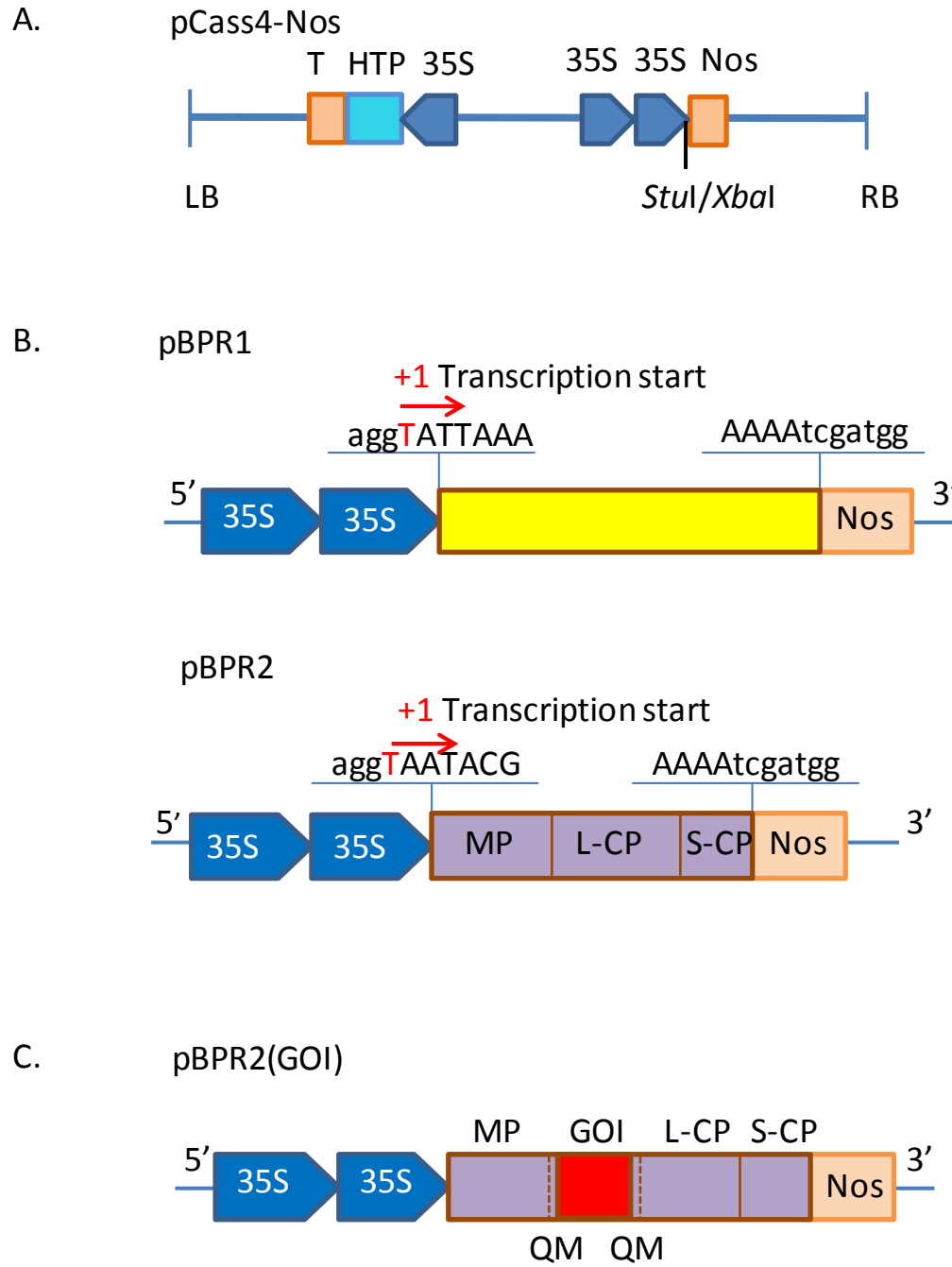
**Figure 2.1** BPMV RNA infectious clones. **A.** Schematic representation of BPMV RNA1 in pGHoR1 and BPMV RNA2 in pGG7R2M that were employed to produce infectious RNAs (not drawn to scale). The full length cDNA of BPMV RNA1 and RNA2 were placed downstream of T7 promoters. The names of mature proteins were designated above their respective coding regions. BPMV RNA1 encodes five proteins including protease cofactor (Co-pro), helicase (Hel), viral genome-linked protein (Vpg), RNA-dependent RNA polymerase (RdRp). BPMV RNA2 translates into four proteins; RNA2 replication cofactor (CR), movement protein (MP), large coat protein (L-CP) and small coat protein (S-CP). The vertical lines show restriction enzyme sites for plasmid linearization. pGHoR1 was digested by *NotI* and *SalI*, whereas, pGG7R2M was linearized by *SalI* only. **B.** Purified products from the digestion of pGHoR1 and pGG7R2M prior to *in vitro* transcription. The linearized templates of RNA1 in pGHoR1, and RNA2 in pGG7R2M were approximately 6 kb and 8 kb in length, respectively, as shown on a 1.2% (w/v) agarose gel. **C.** RNA transcripts of the linearized templates on a 1.2% (w/v) agarose gel.



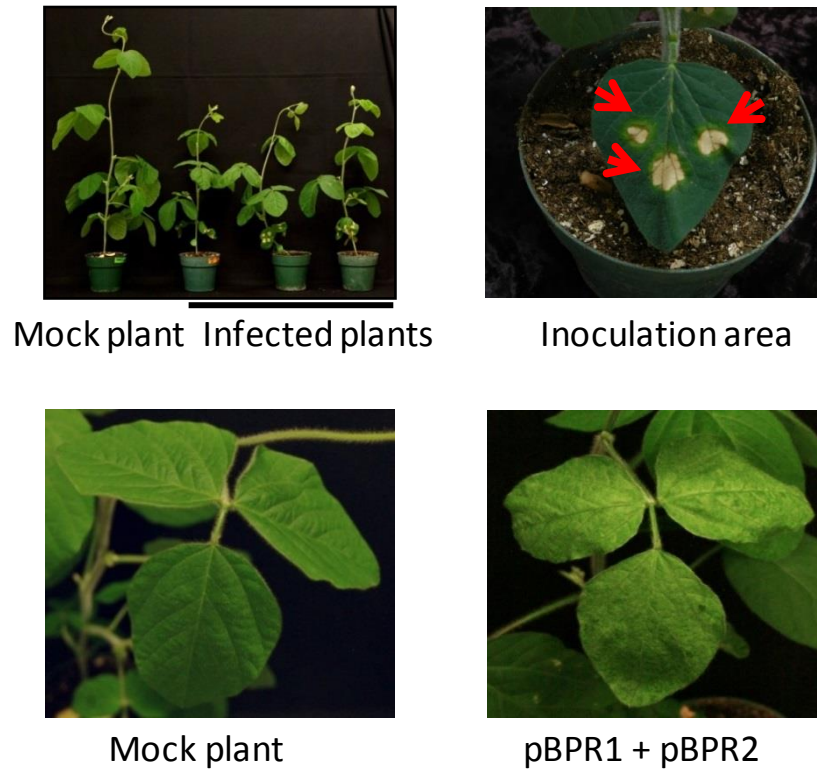
**Figure 2.2** Biolistic bombardment on soybean using RNA transcripts. **A.** A soybean seedling at 10 days old was infected by biolistic bombardment. **B.** Infected soybean leaves, which showed mosaic and blistering symptoms. **C.** Healthy soybean leaves.

### 2.3.2 Construction of the full-length cDNA infectious clones and expression vectors

To directly use the vector for infection without *in vitro* transcription, I replaced the T7 promoter with the *Cauliflower mosaic virus* 35S promoter (*CaMV* 35S) and introduced a nopaline synthase (*Nos*) terminator. The full-length cDNAs of RNA1 and RNA2 were amplified from pGHoR1 and pGG7R2M (Zhang and Ghabrial 2006), respectively, and cloned into pCass4-Nos vector (Figure 2.3A) to generate pBPR1 and pBPR2 (Figure 2.3B). The BPMV-based expression vectors were constructed based on pGG7R2V (Zhang and Ghabrial 2006) to generate pBPR2(GOI), which contains genes of interest such as *GFP*, *GP5N-GFP*, and *GP5C-GFP* (Figure 2.3C). The resulting plasmids were mixed and bombarded into primary leaves of soybean plants. Infected plants started to exhibit symptoms on young leaves 15 dpi. BPMV symptoms included stunted height and mosaic, blistered and mottled leaves, which were identical to soybean plants infected by wild-type virus or RNA transcripts. The symptoms were fully developed 20 days after bombardment when there were no symptoms on mock-treated plants (bombarded with pCass4-Nos) (Figure 2.4). DAS-ELISA confirmed the presence of BPMV in samples of infected leaves at 20 dpi. The results indicated that the modified full-length cDNA clones of BPMV were infectious on soybean plants.



**Figure 2.3** Construction of plant expression vectors (not drawn to scale). **A.** pCass4-Nos containing multi-cloning sites, hygromycin phosphotransferase gene (*HPT*) and double *CaMV 35S*, followed by cloning sites and a *Nos* terminator between LB (left border) and RB (right border). **B.** Constructs of cassettes in T-DNA plasmids harboring BPMV genomic RNAs. The transcription start site is designed right after a double *35S* promoter and a *Nos* terminator is attached to the poly A tail of the virus genome to ensure replication of the virus genome and protein expression in each construct. **C.** Genes of interest (GOI) are fused between movement protein (*MP*) and large coat protein (*L-CP*) in RNA2 of BPMV and flanked by two cleavage sites of Glutamine-Methionine (*QM*). GOI includes *GFP*, and *GP5C* or *GP5N* fused with *GFP*.



**Figure 2.4** Soybean plants infected by BPMV DNA infectious clones at 20 dpi. Red arrows show spots of biolistic bombardment.

### 2.3.3 Exploiting BPMV-based vector for GFP expression in soybean

To test whether the BPMV based expression vector is able to infect and produce proteins of interest in soybean plants, a mixture of pBPR2(GFP) and pBPR1 was introduced into soybean plants via biolistic bombardment. Plasmid pBPR2(GFP) contains a cassette of double 35S promoter::RNA2(GFP)::*Nos* terminator which can be transcribed into the RNA2-GFP transcript to produce RNA2 proteins and GFP in plants (Figure 2.5A). The results showed that pBPR2(GFP) was infectious when co-bombarded with pBPR1. Inoculated soybean plants developed mosaic and mottling symptoms at 15 dpi, similar to the BPMV empty vector-infected plants. These symptoms were not evident in mock-treated plants which were bombarded with pCass4-Nos (Figure 2.5B). DAS-ELISA also indicated the infection of BPMV in bombarded soybean plants.

Furthermore, I also proved that GFP were expressed on systemic leaves of GFP infected plants. Samples of systemic leaves showed green fluorescence under confocal microscopy starting at 20 days after bombardment. To characterize the GFP expression, green fluorescence was observed in newly infected leaves at 15, 20, 30, 45 and 90 days post bombardment. The signal began to show in a mosaic pattern on leaves at 20 dpi, increased remarkably at 30-45 dpi and subsequently decreased at 90 dpi (Figure 2.6A). RT-PCR analysis of RNA purified from upper non-bombarded leaves with primers covering GFP coding region amplified a cDNA fragment of approximately 720 base pairs (bp) which matched the GFP positive control (Figure 2.6B). Interestingly, none of these plants showed fluorescence when scanned by a hand-held UV lamp at mentioned time points, possibly due to the thick layer of epicuticular wax on leaf surface.

To evaluate the GFP expression, leaf samples were subjected to western blot analysis. Total protein was extracted from leaves and showed green fluorescence using a urea-SDS extraction buffer. The protein extract was quantified by dot blot analysis with a polyclonal anti GFP-antibody, and GFP was measured using GFP fused with hydrophobin I (GFP-HFBI) as a standard. The results indicated that GFP expression could reach 37.29  $\mu\text{g/g}$  of leaf fresh weight at 30-45 dpi, equivalent to 0.5% of total protein in soybean while the amount decreased dramatically, to 2.5  $\mu\text{g/g}$  of leaf fresh weight, at 90 dpi. The data of both

confocal microscopy and western blot were in agreement that GFP was produced in soybean leaves at highest level in 30-45 dpi (Figure 2.7A). Therefore, the best harvest time should be within 30-45 dpi. The size of GFP produced by the BPMV vector in soybean was slightly bigger than 25 kDa, the calculated molecular mass of GFP due to the addition of 27 amino acids (aa), derived from MP and L-CP of BPMV, in the expression construct (Figure 2.7B).

Taken together, all data support that the BPMV based expression vector can be exploited to infect soybean plants and produce GFP in whole soybean plants. The GFP expression was 37.29 µg/g fresh weight of leaf tissue at 30-45 days after bombardment. Moreover, the fact that GFP could be transiently expressed in soybean leaves by employing these viral vectors encouraged us to investigate whether this system would be suitable for production of plant-made vaccine against PRRSV.

### 2.3.4 Exploiting BPMV-based vector for GP5 expression

#### 2.3.4.1 Characterization of GP5 and its expression in *N. benthamiana*

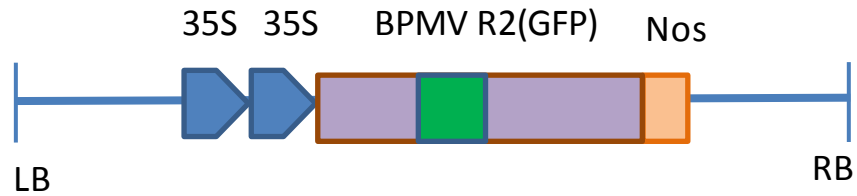
The GP5 protein of PRRSV possesses four main regions including a signal peptide (aa 1-32), an ectodomain (aa 33-63), a transmembrane domain (aa 64-134) and an endodomain (aa 135-200), (Music and Gagnon 2010; Peyret and Lomonosoff 2013; Thuenemann et al. 2013a). As indicated earlier, GP5 or its fusion GP5-CTB expression in transgenic plants was not detectable (Wang et al. 2011). To test if this can be improved by expression of partial GP5, GP5 was halved into two parts, the first 100 aa of GP5 (GP5N) and the remaining 100 aa of GP5 (GP5C), and each was tagged by GFP (Figure 2.8). *GP5N-GFP* and *GP5C-GFP* fusions were inserted into pCaMGate-ER via Gateway cloning (Figure 2.9A). pCaMGate-ER containing several components which enhances protein expression and accumulation in plants. The double *CaMV 35S* promoter and *tCUP* (Wu et al. 2001) translational enhancer from tobacco can promote the transcription and translation of GOI. PR1b, a secretory signal peptide from tobacco (Cutt et al. 1988) and KDEL, an endoplasmic reticulum (ER) retrieval tetra peptide, were used to retain the protein of interest to the ER. Elastin-like polypeptides (ELP), a pentapeptide repeat polymer (Val-Pro-Gly-Xaa-Gly), could allow the purification process based on its ability to form aggregates above its transition temperature. Moreover,



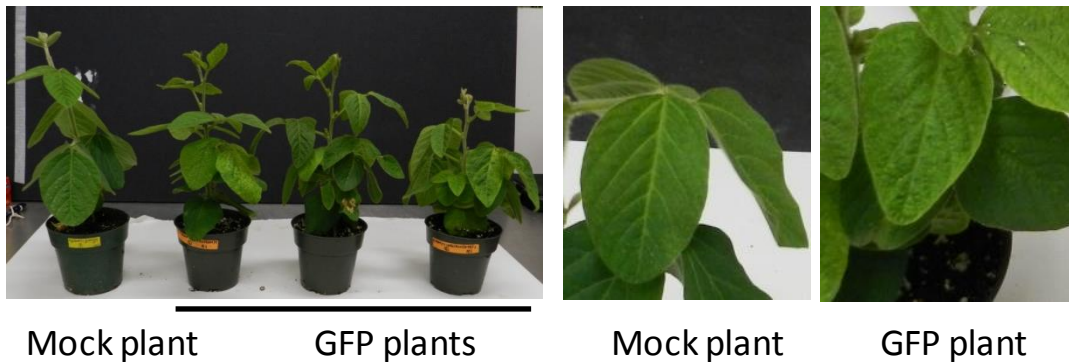
ELP has proved to increase the accumulation of recombinant proteins *in planta* (Conley et al. 2009; Joensuu et al. 2010). Quantitation of GFP indicated that GP5N-GFP and GP5C-GFP expression levels were slightly different at 4 dpi, with the first producing less protein, 0.023% of TSP, when compared to 0.031% of TSP, for GP5C-GFP.

To further investigate the expression pattern of GP5 in two moieties GP5N and GP5C, pEAQ GP5N-GFP and pEAQ GP5C-GFP were created and infiltrated into *N. benthamiana* (Figure 2.10A). The backbone vector is pEAQ-HT, a binary vector derived from *Cowpea mosaic virus* (CPMV). HT is in abbreviation for hypertranslation since this vector uses a modified 5' UTR and 3'UTR of CPMV to enhance the translation of GOI, which are inserted between them. In order to suppress gene silencing, a cassette of p19, known as a viral gene silencing suppressor, is placed downstream of 3'UTR. None of the plants infiltrated with pEAQ GP5N-GFP or pEAQ GP5C-GFP exhibited fluorescence when illuminated by UV light. However, the plants infiltrated with pEAQ GFP as a positive control showed tremendous fluorescence in the same condition. Confocal microscopy was employed to observe green fluorescence in these plants. Interestingly, green fluorescence was evident in *N. benthamiana* leaves infiltrated with either GP5N-GFP or GP5C-GFP plasmids (Figure 2.10B). The level of GP5N-GFP was much higher than that of GP5C-GFP at 3 dpi (Figure 2.10C). *N. benthamiana* infiltrated with either GP5N-GFP or GP5C-GFP started to show necrosis at 3 dpi and the infiltrated leaves were totally wilted and dead after 1 week, as shown in Figure 2.11A and B, respectively. The plants infiltrated with either pEAQ GFP (Sainsbury et al. 2009b), or buffer, developed normally (Figure 2.11A and B). Overall, the results confirm that there was a variance in the expression of GP5N and GP5C when fused with GFP in *N. benthamiana* and both could cause necrosis when expressed in *N. benthamiana*.

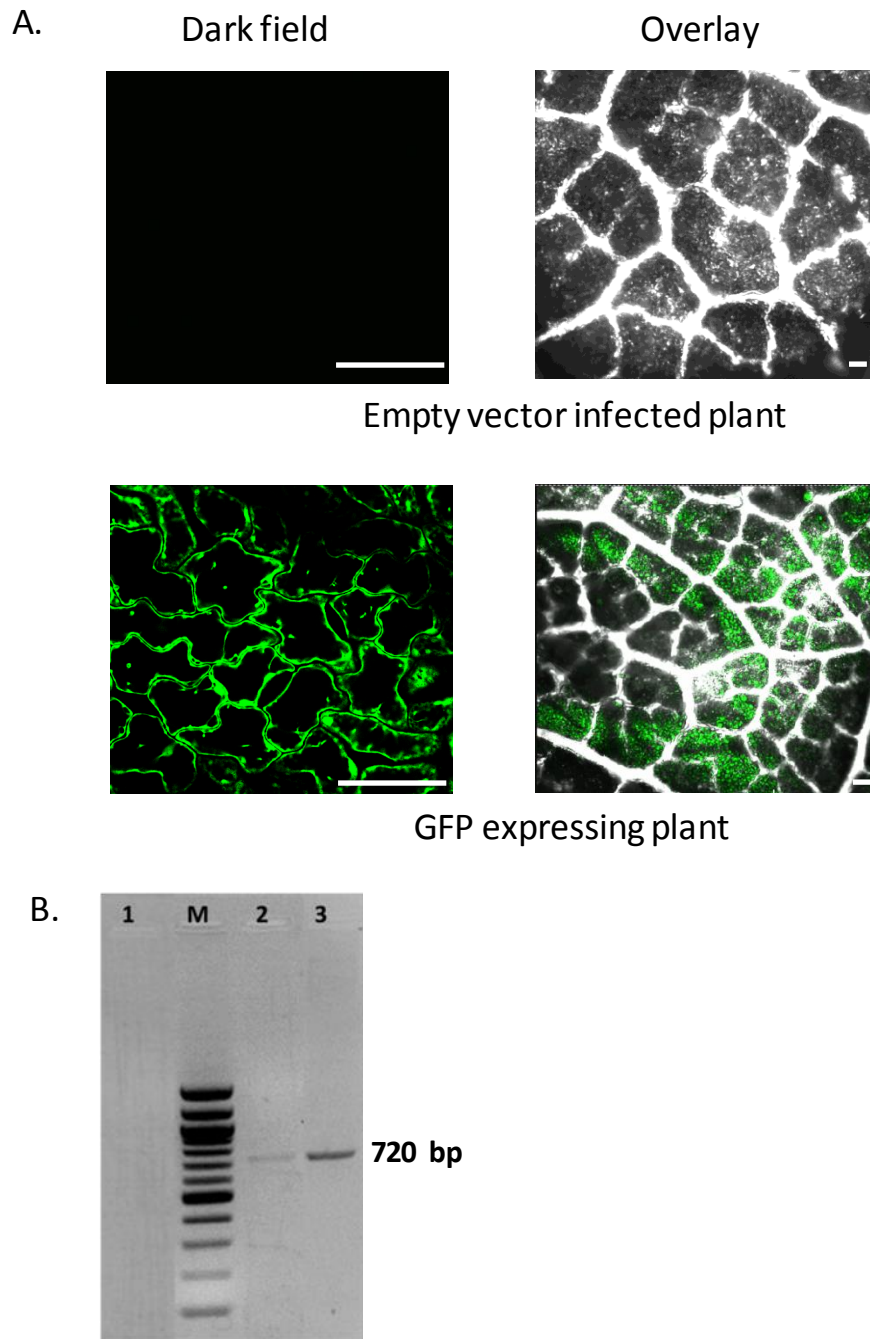
A. pBPR2(GFP)



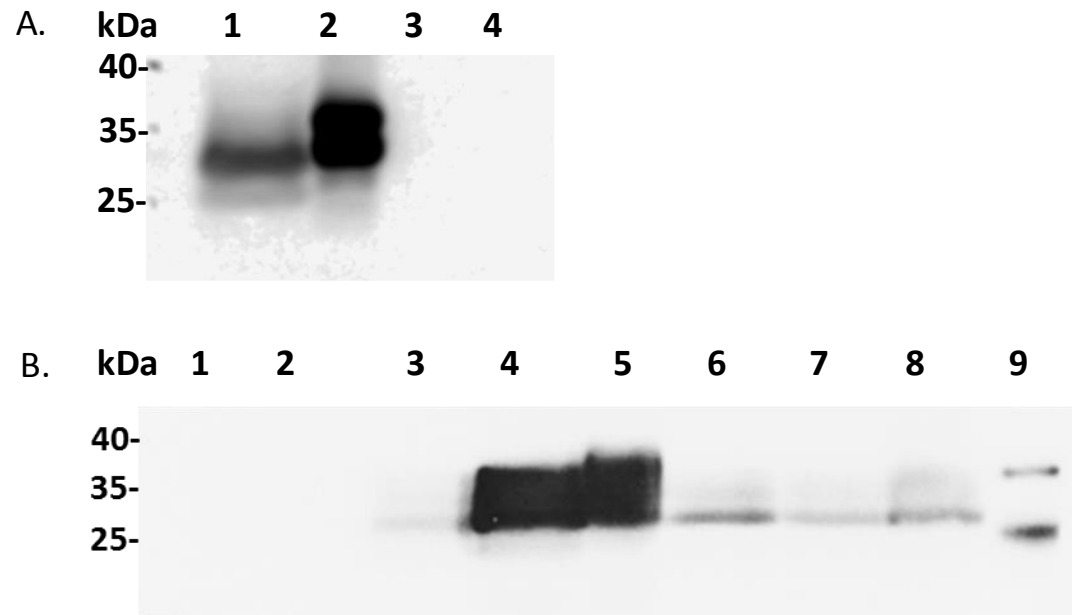
B.



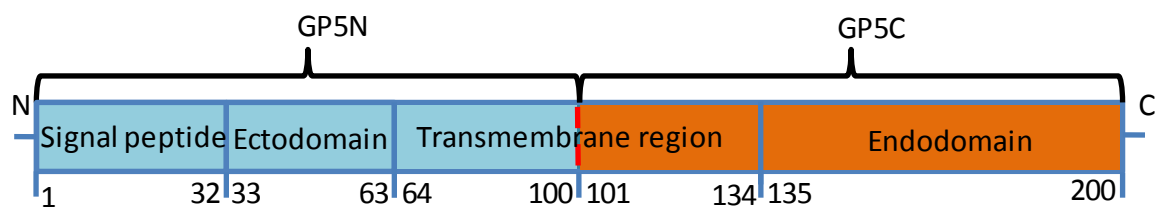
**Figure 2.5** Construct of pBPR2(GFP) and its infectivity to soybean plants (not drawn to scale). **A.** Construct of the BPMV-based expression vector containing *GFP* which was placed between the left border (LB) and the right border (RB) in pBPR2(GFP). **B.** Soybean plants bombarded with this construct at 20 dpi. The GFP plants showed mosaic and wrinkling symptoms in leaves when compared to mock inoculated plants.



**Figure 2.6** GFP expression in soybean plant using pBPR2(GFP). **A.** GFP observation by confocal microscopy at 30 dpi. Scale bar is 100  $\mu$ m. **B.** RT-PCR results showed a 720 bp band of *GFP* in GFP expressing plants at 30 dpi. Lane M: 100 bp ladder. Lane 1: Empty vector infected plant. Lane 2: GFP expressing plant. Lane 3: Positive control with a plasmid containing *GFP*.



**Figure 2.7** Western blot using anti-GFP antibody for GFP extracted from soybean leaves infected with BPMV expression vectors containing *GFP*. **A.** Western blot of GFP in soybean leaves at 30 dpi. 50  $\mu$ g total protein of extract was loaded per well. Lane 1: Extract from leaves infected with BPMV expression vector containing *GFP*. Lane 2: GFP-HFBI as a positive control. Lane 3: Extract from leaves infected with BPMV empty vector. Lane 4: Extract from mock inoculated plant. **B.** A time course of GFP expression in soybean leaves. 70  $\mu$ g total protein of extract was loaded per well. Lane 1: Mock inoculated plant. Lane 2: Empty vector. Lane 3: 20 dpi. Lane 4: 30 dpi. Lane 5: 45 dpi. Lane 6- 8: 90 dpi. Lane 9: 50 ng GFP-HFBI as a standard for quantification of GFP.



**Figure 2.8** Characteristics of glycoprotein 5 (GP5) in PRRSV. GP5 was halved into GP5N and GP5C shown by the brackets. The numbers under the bar indicate the position of amino acids in this protein relevant to the coding regions; signal peptide, ectodomain, transmembrane domain and endodomain. “N” and “C” are abbreviated for N-terminus and C-terminus of GP5. The dashed line in red separates the protein into two halves.

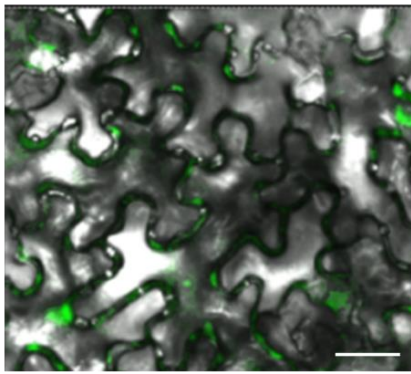
## A. pCamGate GP5N-GFP



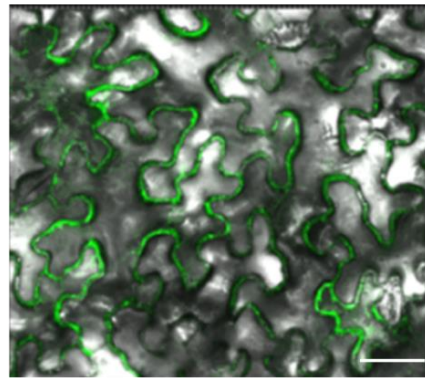
## pCamGate GP5C-GFP



## B.

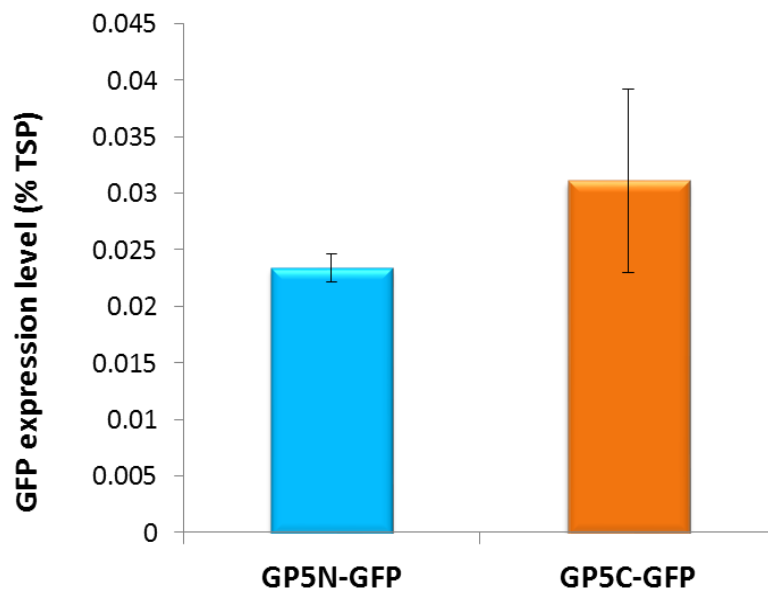


GP5N-GFP

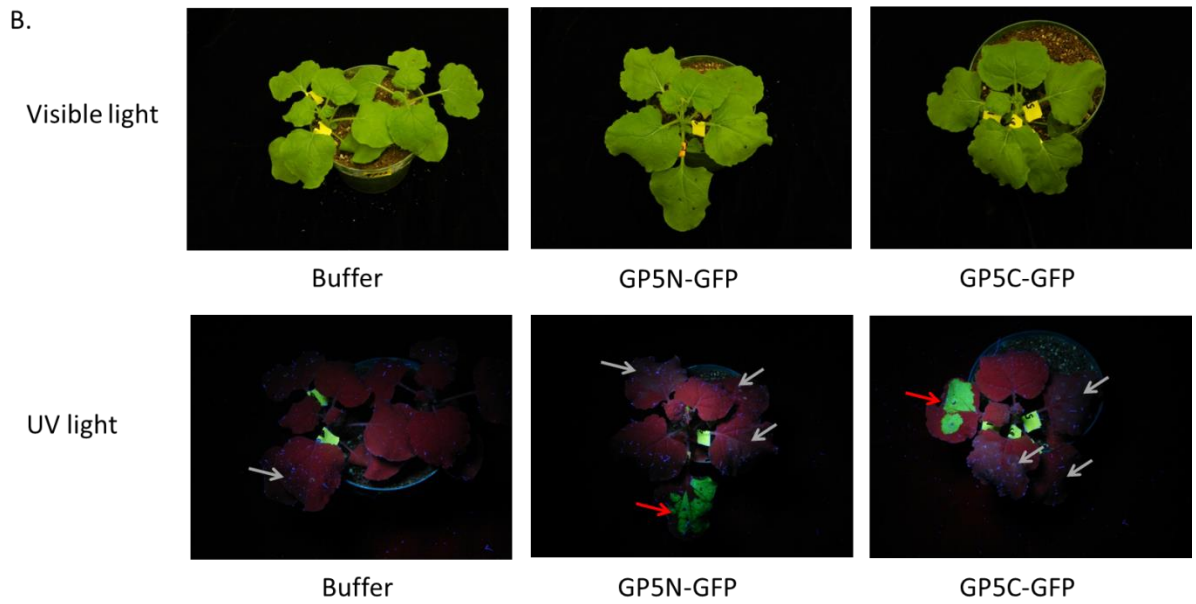
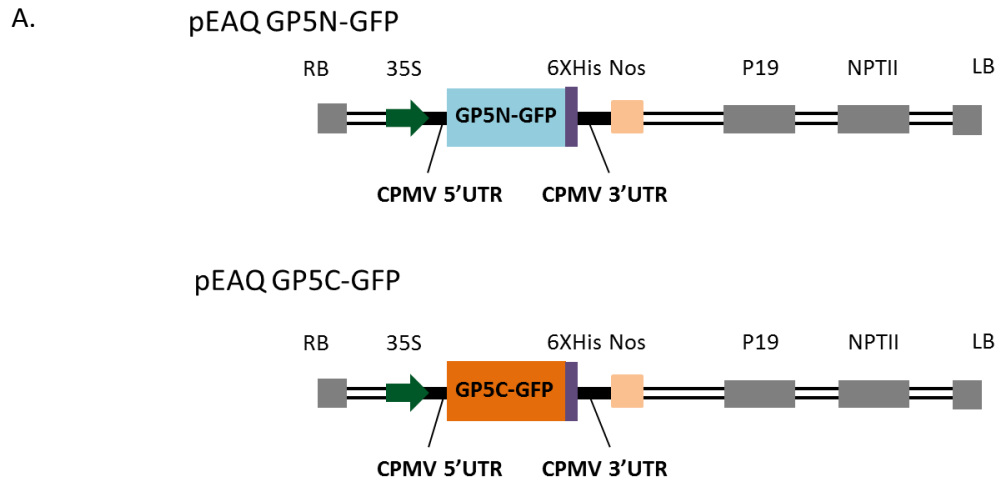


GP5C-GFP

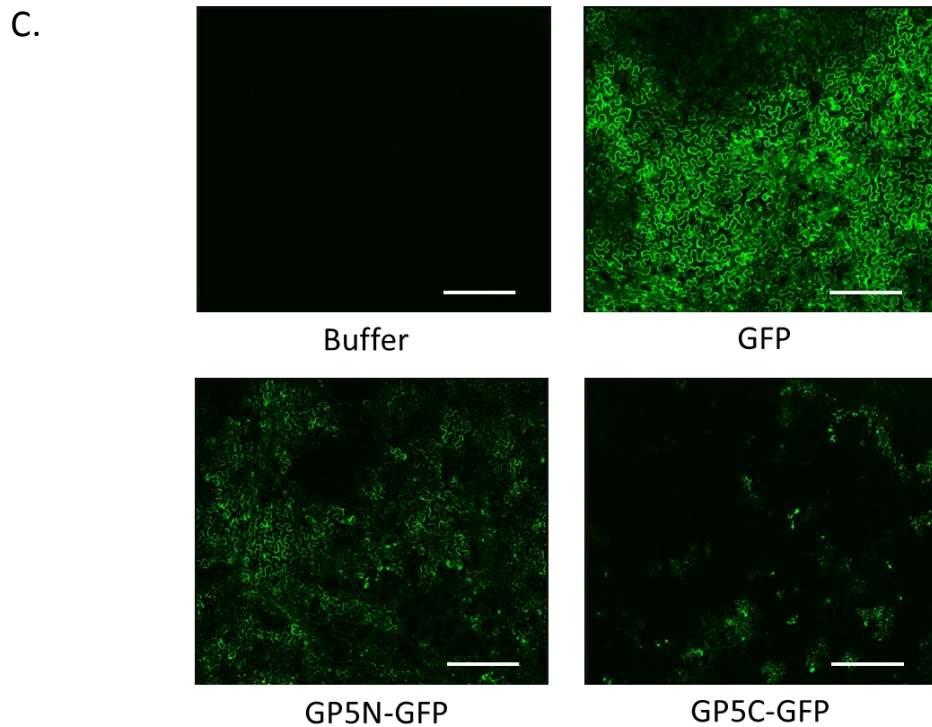
## C.



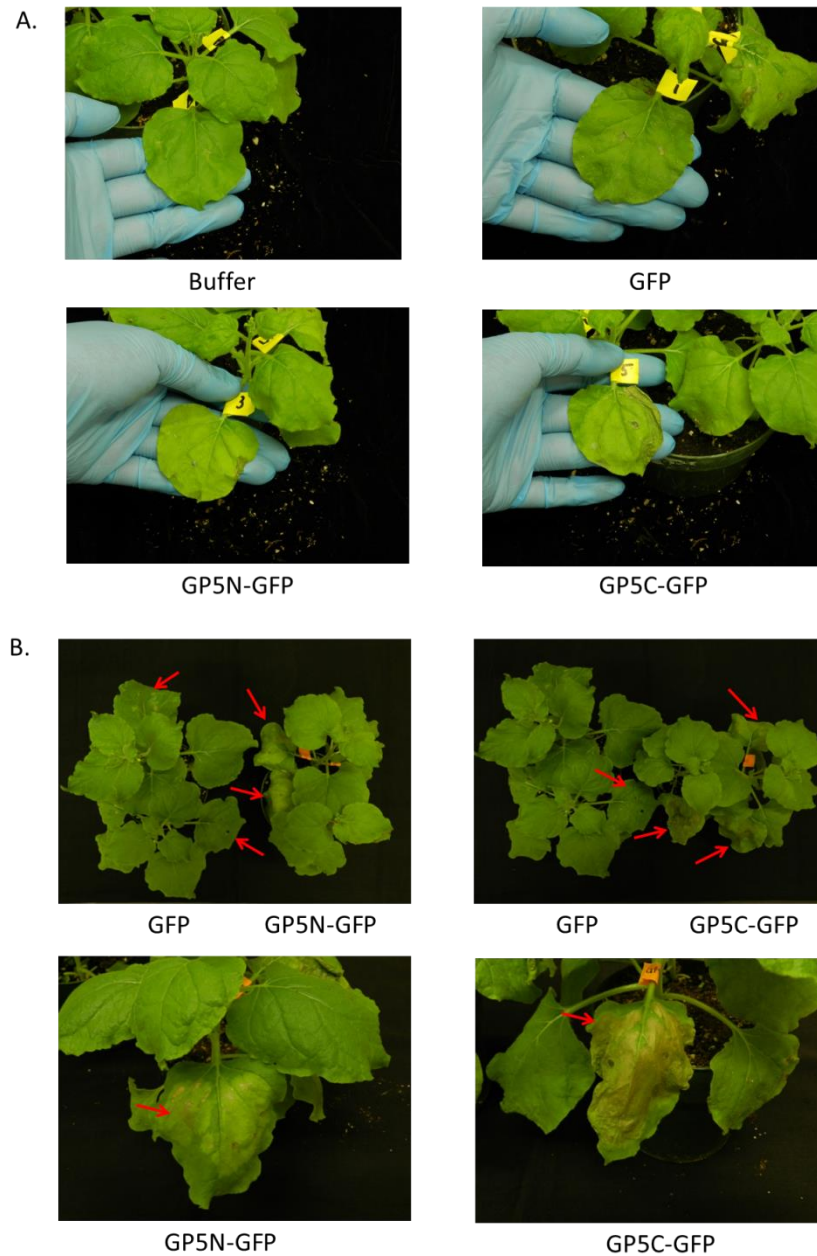
**Figure 2.9** The expression of GP5N-GFP and GP5C-GFP in *N. benthamiana* using pCamGate-ER at 4 dpi. **A.** Schematic representations of pCamGate GP5N-GFP and pCamGate GP5C-GFP (not drawn to scale). 2 x 35S - double *CaMV 35S* promoter; tCUP - tobacco translational enhancer; PR1b - Pathogenesis-Related-1b, a secretory signal peptide from tobacco; Xpress and cmc - detection/purification tags; attB1 and attB2 - Gateway recombination sites; KDEL - an endoplasmic reticulum (ER) retrieval tetrapeptide; ELP - elastin-like polypeptides; Nos - nopaline synthase terminator. **B.** Observation of fluorescence of these constructs by confocal microscopy. Scale bar is 40  $\mu\text{m}$ . **C.** The expression of each construct via GFP quantitation. Error bars represent the standard deviation of the mean (n=3).







**Figure 2.10** The expression of GP5N-GFP and GP5C-GFP in *N. benthamiana* using pEAQ-HT at 3 dpi. **A.** Schematic representations of pEAQ GP5N-GFP and pEAQ GP5C-GFP (not drawn to scale). RB - right border; 35S - *CaMV* 35S promoter; CPMV 5'UTR - translational enhancer sequences from *Cowpea mosaic virus* (CPMV); GP5N-GFP - GP5N fused with GFP; GP5C-GFP - GP5C fused with GFP; 3'UTR - translational enhancer sequences from CPMV; 6XHis - detection/purification tag; Nos - nopaline synthase terminator; p19 - expression cassette of p19 protein, a gene silencing suppressor from *Tomato bushy stunt virus*; NPTII - neomycin phosphotransferase II as a selective marker; LB - left border. **B.** The observation of fluorescence of these constructs under visible light and UV light. The red arrow indicates the positive control pEAQ GFP construct. The grey arrows show the leaves infiltrated with the constructs labelled below each photo. **C.** The observation of fluorescence of these constructs under confocal microscopy. Scale bar is 300  $\mu\text{m}$ .

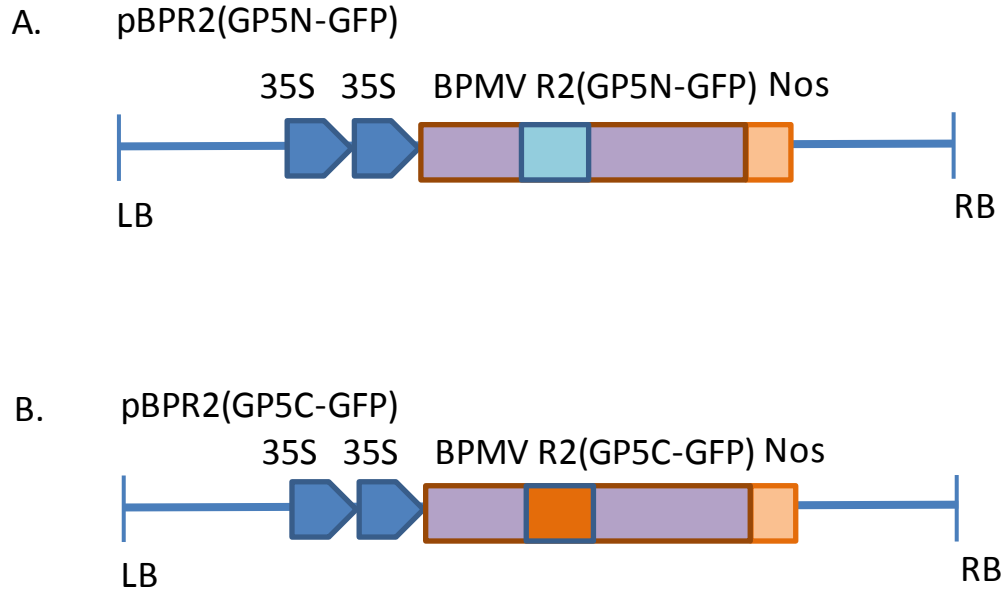


**Figure 2.11** Necrosis symptoms of plants infiltrated by pEAQ GP5N-GFP and pEAQ GP5C-GFP. **A.** Necrosis started in infiltrated leaves at 3 dpi. **B.** Necrosis developed in the whole of infiltrated leaves, as the leaves became wilted and died, after one week while those leaves infiltrated with either pEAQ GFP, or buffer, did not show necrosis and grew ordinarily well. The red arrows point to infiltrated leaves.

#### 2.3.4.2 Expression of GP5 in soybean using BPMV based vector

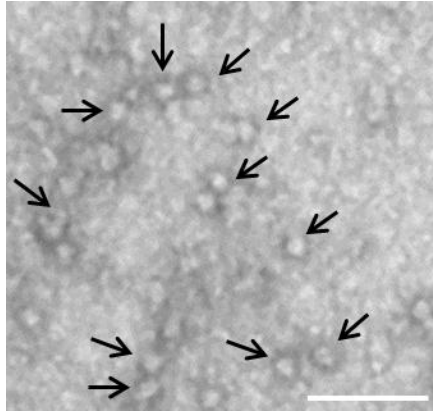
To circumvent the necrosis phenomenon and the low expression of GP5N and GP5C in *N. benthamiana*, soybean was tested as an alternative plant for GP5 expression. The constructs of pBPR2(GP5N-GFP) and pBPR2(GP5C-GFP) were generated as expression vectors and introduced into soybean plants together with pBPR1 via biolistic bombardment (Figure 2.12). All plants were infected with the combination of pBPR1 and pBPR2(GP5C-GFP), whereas none of the plants inoculated with the constructs of pBPR1 and pBPR2(GP5N-GFP) showed symptoms of BPMV infection. The infected plants displayed symptoms with mosaic, wrinkling and mottling leaves, similar to the BPMV infection described. DAS-ELISA confirmed the infectivity of these clones in which, pBPR2(GP5C-GFP) was infectious and pBPR2(GP5N-GFP) was not. To further examine the presence of BPMV in infected plants with pBPR2(GP5C-GFP), extracts of leaves with severe symptoms was observed by TEM. The result revealed many icosahedral-like particles which had diameters of approximately 30 nm perfectly matching with the predicted size of BPMV (Figure 2.13A). These results indicate that pBPR2(GP5C-GFP) can be co-bombarded into soybean plants to produce GP5C-GFP.

Moreover, the data also demonstrate that GP5C-GFP was expressed in systemic leaves and the total amount of GP5C-GFP can be as high as 4.36  $\mu\text{g/g}$  fresh weight of leaf tissue. GFP in the fusion protein was observed by confocal microscopy in a time course of 15-20-30-45-90 dpi. As expected, GP5C-GFP infected plants showed their high peak of fluorescence at 30-45 dpi (Figure 2.13B), which was correlated with GFP expression in this study. Total protein was extracted from leaves which showed fluorescence under confocal microscopy at 45 dpi and subjected to dot blot and western blot. Western blot results showed that GP5C-GFP had the size of about 39 kDa, however, another band was detected at 27 kDa which was possibly GFP cleaved from the fusion protein (Figure 2.13B). Both bands were analyzed by TotalLab TL100 software and compared with the standard GFP. Assuming there was an equal amount of GP5C and GFP in the fusion protein GP5C-GFP, the total amount of GP5C-GFP produced in soybean leaves could be as high as 4.36  $\mu\text{g/g}$  of fresh weight, equivalent to 0.06% of total protein.



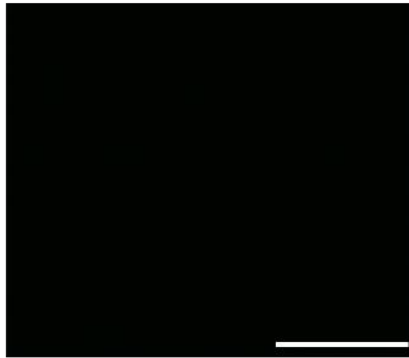
**Figure 2.12** Construction of the T-DNA between the left border (LB) and the right border (RB) in BPMV expression vectors (not drawn to scale). **A.** pBPR2(GP5N-GFP) **B.** pBPR2(GP5C-GFP).

A.

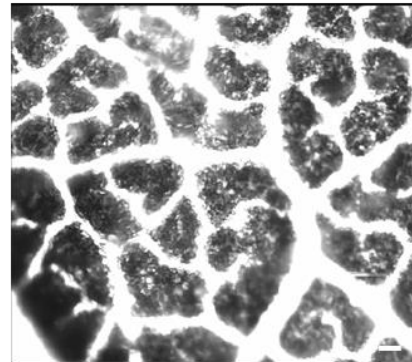


B.

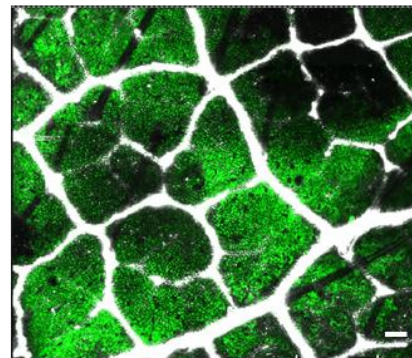
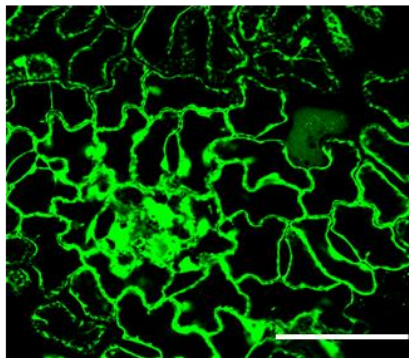
Dark field



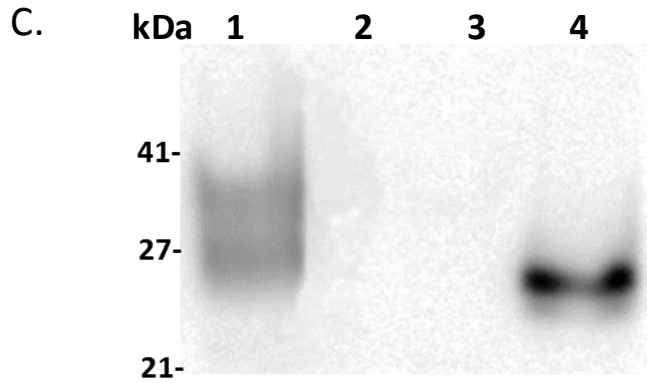
Overlay



Empty vector infected plant



GP5C-GFP infected plant



**Figure 2.13** Analysis of GP5C-GFP expression in soybean plants infected by BPMV expression vectors. **A.** BPMV virions viewed by TEM. The virions of icosahedral shapes were indicated by black arrows, with their diameters which varied between 20-35 nm. Scale bar 100 nm. **B.** Confocal microscopy of soybean leaves expressing GP5C-GFP at 45 dpi. Scale bar 100  $\mu$ m. **C.** Western blot of GP5C-GFP expression using anti-GFP antibody, 50  $\mu$ g total protein of GP5C-GFP extract was loaded on gel. Lane 1: GP5C-GFP plant. Lane 2: Empty vector. Lane 3: Mock plant. Lane 4: 10 ng of GFP as a standard of quantification.

### 2.3.5 Stability of the foreign genes expressed by BPMV based vectors

Since the production of heterologous proteins using this system relies on virus replication and translation, the stability of viral vectors plays a critical role during these stages, to ensure the functions, as well as, protein production. Many reports have indicated that heterologous genes were deleted from expression vectors, due to homologous recombination, resulting in reversion to the wild type of a modified virus (Zhang and Ghabrial 2006; Zhang et al. 2007; Zhang et al. 2009). To test the stability of these constructs, we employed the use of GFP and confocal microscopy. Leaf tissues were harvested from soybean plants infected with either BPMV-GFP or BPMV-GP5CGFP, as materials to mechanically inoculate new plants. When these new plants were infected, and produced proteins of interest, the leaves were used to infect a subsequent batch of soybean plants. The fluorescence of GFP could be detected by confocal microscopy up to 6 passages, with a similar intensity to that observed in the first passage.

## 2.4 Discussion

In the 1990s, a variety of BPMV isolates was collected from the fields of soybean in the United States which demonstrated the diversity of this virus in nature. To study this virus for disease management, the infectious clones of three different strains, K-G7, K-Ha1 and K-Ho1 were constructed and their transcripts were inoculated in soybean plants (Gu and Ghabrial 2005). Later on, the infectious clone of RNA2, belonging to K-G7 isolate, was modified as a VIGS vector (Zhang and Ghabrial 2006). The full length cDNAs of BPMV RNAs were placed under the control of a T7 promoter in all of these infectious clones which required an extra step of *in vitro* transcription to produce RNA transcripts for mechanical infection. Recently, modifications were made by replacing the full length cDNAs of RNA1 and RNA2, of the IA-D1 isolate, under the control of a *CaMV 35S* promoter and *Nos* terminator for both protein expression and gene silencing purposes (Zhang et al. 2010). In this study, I also used the same method to switch the T7 promoter of infectious clones, pGHoR1, pGG7R2M and pGG7R2V, to *CaMV 35S* promoter, and to add *Nos* terminator to eliminate the inconvenience of *in vitro* transcription and enhance the versatility of the

expression system. I further demonstrate here that the modified clones were infectious and successfully directed the expression of heterologous proteins, GFP and GP5C-GFP in soybean plants. To date, many different BPMV-based vectors have been developed and utilized as an effective tool for functional genomic studies via VIGS (Chen et al. 2016; Chen et al. 2015; Rao et al. 2014; Singh et al. 2011). Nevertheless, their application for heterologous protein expression is limited to several proteins, GFP, Bar, DsRed and silencing suppressors (Zhang et al. 2010). To my best knowledge, this is the first report on the application of BPMV-based expression vectors for vaccine production in soybean leaves.

The concept of plant-made vaccines for oral administration has drawn a lot of interest recently since the therapeutic proteins produced in plants showed their efficiency in clinical trials and treatments. A remarkably successful story is that ZMapp, a mixture of three chimeric monoclonal antibodies produced in tobacco plants, was administered to seven patients and five of them were recovered during the 2014 Ebola outbreak (Budzianowski 2015). The drugs are now produced rapidly in *N. benthamiana* via transient expression under on-going development for commercialization. The potentials of fast production in plants and versatile administration via oral routes without needles have made plant-based vaccines more appealing (Liew and Hair-Bejo 2015) as these help to reduce the administration costs when a large population is involved. Since the first plant-based vaccine, *Hepatitis B virus* surface antigen (HBsAg), was produced in transgenic tobacco (Mason et al. 1992), a variety of plants including *Arabidopsis thaliana*, alfalfa, spinach, potato, rice, bean, maize, tomato, strawberry and carrot have been used for vaccine production (Liew and Hair-Bejo 2015). In this study, soybean leaves were selected as a plant bioreactor to express an oral vaccine against PRRSV in pigs. When compared to tobacco plants, soybean plants contain a low amount of alkaloids and no nicotine, thus, is suitable for animal feeding without the concern of toxicity. Moreover, my results showed that the expression of GP5 using pCamGate and pEAQ-HT caused necrosis in *N. benthamiana* starting at early days post agroinfiltration resulting in a low yield of proteins of interest. It has been reported that GP5 is involved in the apoptosis phenomenon induced by PRRSV in animal cells and the first N-terminal 119 aa of GP5 has been mapped for apoptosis induction (Fernández et al. 2002; Suárez et al. 1996). Lomonosoff group also reported the necrosis of GP5 infiltrated leaves in *N. benthamiana*



(Thuenemann et al. 2013) and suggested that the protein need to be engineered to eliminate its toxicity to plants such as a removal of transmembrane domains. Here, I chose soybean plants which are considered physically more durable and biolistic bombardment, instead of agroinfiltration, to alleviate this necrosis. Overall, soybean is a worldwide crop which can grow on almost every continent and supply high amount of proteins. Exploiting this BPMV based expression system to produce a ready-to-use vaccine in soybean leaves will increase the value of the plant and provide a low-cost medicine in developing countries.

Transgenic plants have been employed for the production of recombinant proteins since 1986 (Ma et al. 2003). Within three decades, transgenic plants became the most commonly used platform to express heterologous proteins, several of which are commercialized products. However, the procedure requires a long time investment, often 6 months to a year, and intensive labour to generate stable transgenic plants that produce proteins of interest. It may also require several generations to obtain homozygous lines. Due to its time and labour consumption, this system is inappropriate for the timely production of vaccines against emerging viral epidemics. From past epidemics, such as influenza, it has become clear that earlier action is taken, the easier it is to control and vaccines play a critical role in this earlier time point to prevent outbreaks. In addition, transgenes can be subjected to gene silencing in plant nuclear transformation and result in a low level of protein expression (Chan and Daniell 2015; Yao et al. 2015). Here, I introduced a transient expression system together with a viral vector as an alternative method to traditional stable transformation, as it is less time demanding and produces higher expression yields. Furthermore, BPMV-based expression vectors can allow heterologous proteins to be expressed throughout the plant, with high accumulation in leaves and vascular systems during virus infection based on the movement ability of this virus. This is more efficient than regular agroinfiltration-mediated transient expression, in which proteins of interest accumulated only in the infiltrated areas. Although the GP5N-GFP could not be produced in soybean using this system, the expression of GP5C-GFP was approximately 4.36  $\mu\text{g/g}$  fresh weight of leaf tissue, equivalent to 0.06% of total protein. Previously, it has been reported that GP5 was expressed at a lower level such as 110-257  $\text{ng/g}$  fresh weight of leaf tissue (0.011-0.037% TSP) in transgenic tobacco and transgenic banana (Chan et al. 2013; Chia et al. 2010; Chia et al. 2011). It is impossible to precisely

compare these expression systems since my system expressed the C-terminus of GP5, and theirs expressed the entire GP5. However, the results confirm that the BPMV-based expression system can be promisingly exploited for a higher level of protein production in soybean plants.

In this study, the level of GFP expression was 0.5% of total protein, which is lower than the 1% of total protein previously reported when RNA transcripts were used to inoculate in soybean (Zhang and Ghabrial 2006). I believe that the difference of expression level may be caused by the methods of inoculation. In my experiment, the DNA expression vectors were introduced into plants via biolistic bombardment and underwent transcription in the nucleus. This was followed by an export of RNA to the cytoplasm then virus replication happened and proteins of interest were translated along with viral proteins. The procedure in the nucleus and exporting process may increase negative impacts on the synthesis of mRNA and proteins, due to gene silencing and improper protein interactions (Marillonnet et al. 2005). In contrast, neither the nucleus nor exporting-to-cytoplasm procedure was necessary when plants were infected by RNA transcripts, thus, less exposure to gene silencing as well as inefficient interactions result in a higher level of GFP expression. On the other hand, I can use the infected tissue after biolistic bombardment for subsequent infections by mechanical inoculation.

GFP has been employed as a marker for many experiments due to its ability to produce fluorescence excitation under UV light (Cui and Wang 2016; Oparka 1997; Saberianfar et al. 2016; Sattarzadeh et al. 2015). In this study, GFP was used as a reporter to track virus accumulation and quantify the level of protein expression. One of virus characteristics is the mosaic pattern which can be explained by variations in virus accumulation distributed in different parts of plants. The use of GFP facilitated observation of virus movement as well as viral protein accumulation. Furthermore, GFP was fused with either GP5N or GP5C in order to increase the amount of protein expression, by adding a stabilizing protein domain (Piron et al. 2014) and keep track of protein distribution (Zhang and Ghabrial 2006).

I also demonstrated that both BPMV-GFP and BPMV-GP5C-GFP vectors were stable after six serial passages in soybean. The green fluorescence was maintained throughout the

soybean plants, especially in leaves and vascular systems in each passage. These results suggest that mechanical inoculation by rubbing BPMV-infected leaf tissue onto new plants can become an alternative conventional method since soybean leaves are recalcitrant to agroinfiltration. I stored infected leaves at  $-80^{\circ}\text{C}$  up to 6 months and used these materials to effectively infect healthy plants. The new plants showed a hundred percent rate of infection using this method. The stability of this expression system facilitates the production of recombinant proteins on a large scale with automatically mechanical inoculation.

## ***2.5 Conclusion***

In summary, my data suggest that BPMV-based expression vectors can be employed to produce heterologous proteins in soybean plants. The level of GFP expressed in soybean using this system was  $37.29\ \mu\text{g/g}$  fresh weight of leaf tissue (0.5% of total protein) at 30-45 dpi. In addition, the C-terminus of GP5 fused with GFP was produced up to  $4.36\ \mu\text{g/g}$  leaf fresh weight (0.06% of total protein) in soybean leaves. The possibility of this protein to be an oral vaccine for pigs against PRRSV, and whether the level of protein expression can be increased by employing several strategies, including co-expression with a silencing suppressor p19 (Liu and Kearney 2010; Sainsbury et al. 2009b), fusing the protein of interest with a stabilizing domain (Piron et al. 2014) or generating a BPMV RNA1 transgenic soybean, which is then bombarded with modified BPMV RNA2, will be interesting topics for future studies. Despite the fact that much research has been done to express GP5 in either transgenic plant system (Chan et al. 2013; Chen and Liu 2011; Chia et al. 2010; Chia et al. 2011; Piron et al. 2014) or transient system (Uribe-Campero et al. 2015), this is the first report of using viral expression vectors to successfully produce the GP5C-GFP in soybean leaves for an oral vaccine in pigs. Moreover, since there are not many viral expression vectors which can infect legumes, the BPMV based expression vectors offers an opportunity to produce soybean-made vaccine for oral administration.

**Table 2.1** Primers used to construct expression vectors.

<b>Name</b>	<b>Sequence (5'-3')</b>
<b>BamHI-GFP-F</b>	ATCCGGATCCGTGAGCAAGGGCGAGGAGCTGTT
<b>MscI-GFP-R</b>	ACCTTGGCCACTTGTACAGCTCGTCCATGCCGAGAG
<b>BamHI-GP5N-F</b>	ATCCGGATCCTTGGGGAAATGCTTGACCGCG
<b>BamHI-GP5C-F</b>	ATCCGGATCCTATTACCATGGGCGGTATGTTTTG
<b>BPMVR1-F</b>	TATTAATAATTTTCATAAGATTTGAAATTTTGAT
<b>BPMVR1-BamHI-R</b>	CGCGGATCCTTTTTTTTTTTTTATATTTAAACAC
<b>BPMVR2-F</b>	TAATACGACTCACTATAGTATTAATAATTTTCATAAGATTTG
<b>BPMVR2-XbaI-R</b>	GCGTCTAGATTTTTTTTTTTTTTTTTTTTTTTTAAAAT
<b>Nos-BamHI-F</b>	TTAGGATCCAGCTCGAATTTCCCCGATCGT
<b>Nos-PmeI-R</b>	CGCGTTTAAACCGCCAATATATCCTGTC
<b>GFP-F</b>	GTGAGCAAGGGCGAGGAGCTGTT
<b>GFP-R</b>	CTTGTACAGCTCGTCCATGCCGAGAG
<b>GP5N-F</b>	GGGgacaagttgtacaaaaagcaggcttgATGTTGGGGAAATGCTTGAC CGCG
<b>GP5C-F</b>	GGGgacaagttgtacaaaaagcaggcttgTATTACCATGGGCGGTATGT TTTG
<b>GP5N-GFPlink-F</b>	GATCACTGTGTCTGCCGCCGGAATGGTGAGCAAGGGCGAG GAGC
<b>GP5N-GFP link-R</b>	GCTCCTCGCCCTTGCTCACCATTCCGGCGGCAGACACAGTG ATC
<b>GFPatt-R</b>	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTGTACAGCT CGTCCATGCC
<b>GP5C-GFPlink-F</b>	CAGCGGAACAATGGGGTCGTCCATGGTGAGCAAGGGCG AGGAGC
<b>GP5C-GFPlink-R</b>	GCTCCTCGCCCTTGCTCACCATAGGACGACCCCATGTTCCG CTG
<b>pCamGateSeq-F</b>	CCTCACCTCAAACCCACCA
<b>pCamGateSeq-R</b>	CCACGCCAGGTACTCCTACT
<b>M13 foward</b>	GTAAAACGACGGCCAG
<b>M13 reverse</b>	CAGGAAACAGCTATGAC
<b>AgeI-GP5N-F</b>	ATCGGACCGGTATGTTGGGGAAATGCTTGACCGC

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**AgeI-GP5C-F**      ATCGGACCGGTATGTATTACCATGGGCGGTATGT

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\*Letter in lower cases stand for Gateway compatible primers including attB1 sites.

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## Chapter 3

# **Production of PRRSV neutralizing epitopes on the surface of viral particles of recombinant CGMMV in cucumber**

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### 3.1 Introduction

*Cucumber green mottle mosaic virus* (CGMMV) is an economically important seed-borne virus which affects cucurbitaceous crops worldwide. 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; Wijethunga et al. 2014; Yoon et al. 2008). In recent years, several reports have alerted the existence of this virus in Australia and North America, including United States and Canada (Li et al. 2015; Tesoriero et al. 2016; Tian et al. 2014) resulting in devastating economic losses in melon and greenhouse cucumber crops. CGMMV belongs to the subgroup II of the genus *Tobamovirus* in the family *Virgaviridae*. Like other members of this genus, CGMMV assembles rod shaped particles, which are approximately 300 nm x 18 nm in size and encapsidate a single-stranded, positive-sense RNA of 6423 nucleotides (nt) in length as its genome. 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). The movement and coat proteins are translated from individual 3' co-terminal subgenomic RNAs. The coding sequence for MP is overlapped with that of the 186-kDa protein and CP. The 3' UTR of the RNA genome consists of approximately 174 nt and forms a tRNA like structure. Similar to *Tobacco mosaic virus* (TMV), which is in the same genus, CGMMV can easily infect its host plants and yields a large amount of viral particles within a short time of initial infection. The simple monopartite genome of CGMMV makes genetic engineering relatively easy for expression of exogenous proteins. Therefore, CGMMV has great potential for the development of a virus-based expression system. However, a CGMMV vector developed recently was unstable due to the recombination of repeating promoters. In this vector, *GFP* was placed in frame with MP and CP sequences and transcribed by a subgenomic promoter of CP. GFP was initially detected in new leaves of bottle gourd, but deletion of *GFP* also occurred (Zheng et al. 2015). Apparently, CGMMV is not suitable for expression of long polypeptides. Based on their naturally symmetrical nanostructures, virus particles have demonstrated a proficiency to carry and display small peptides attached to the outer part of their capsids. Such small peptides could be a component of an enzyme inhibitor (Hamamoto

et al. 1993) or, an antigen, which binds to antibodies in immune systems (Brennan et al. 1999; Sugiyama et al. 1995). For example, Dalsgaard et al. have employed CPMV to deliver the neutralizing epitope of the VP2 capsid protein, from *Mink enteritis virus* (MEV), as a subunit vaccine for mink, dogs and cats. The virus particles of CPMV have an icosahedral shape, which is comprised of 60 copies each of large (L) and small (S) protein subunits. The viral epitope, which includes 17 amino acids, was inserted into the  $\beta$ B- $\beta$ C loop of the S coat protein subunit and produces viable virus particles, each containing 60 copies of the epitope. Approximately 100  $\mu$ g, or 1 mg, of purified chimeric virus particles can protect mink from clinical disease, but only the 1 mg dose is able to prevent viral shedding (Dalsgaard et al. 1997). This example supports the idea that chimeric virus particles could act as efficient carriers in the development of plant-based vaccines against animal diseases. CGMMV is distinguished from CPMV by its helical shape, which is characteristic of the *Tobamovirus* genus, which includes TMV, *Odontoglossum ringspot virus* (ORSV), *Ribgrass mosaic virus* (RMV) and *Tobacco mild green mosaic virus* (TMGMV). As evidenced when visualization with fibre diffraction methods, the topography of all viruses in this family, including CGMMV, share similar coat protein structures, with a four- $\alpha$  helix coat protein core and the N-terminus and C-terminus exposed on the surface of virus particles (Lobert et al. 1987; Namba and Stubbs 1986). The amino acid sequence of the coat protein of CGMMV, however, is just 36% identical to that of TMV (Meshi et al. 1983). The origin-of-assembly (OAS) is located approximately 300 nt from the 3' end of the CGMMV genome, whereas, it is located approximately 900 nt from the 3' end of the TMV genome. These differences divided tobamoviruses into two categories, subgroup I and II, based on coat protein sequences and OAS. TMV belongs to subgroup I and CGMMV in subgroup II (Fukuda and Okada 1982; Fukuda et al. 1980). Despite this, several studies have shown that CGMMV is also capable of peptide expression on the surface of chimeric virus particles and could be utilized for vaccine development, similar to TMV (Ooi et al. 2006; Teoh et al. 2009).

In this study, the full-length cDNA sequence of CGMMV-ON, derived from infected cucumber plants in an Ontario greenhouse, was cloned into a binary vector. The clone was delivered to plants through agroinfiltration. The clone successfully infected cucumbers, watermelons, cantaloupes and *N. benthamiana* in pathogenicity tests, with different levels of

virulence severity. The infectious clone was further modified to express chimeric CGMMV, bearing the neutralizing epitope (NE) of PRRSV, in cucumber plants. The NE, which induces the production of neutralizing antibodies against the disease in pigs, is a nine-amino-acid peptide (Ser<sup>37</sup>-Gln-Leu-Gln-Ser-Ile-Tyr-Asn-Leu<sup>45</sup>) of GP5, the major envelope protein of PRRSV. Here, I describe the construction of this expression vector and the expression of chimeric CGMMV, via a readthrough translational mechanism, in cucumber for a plant-made vaccine against PRRSV.

### ***3.2 Materials and methods***

#### **3.2.1 Construction of agro-infectious CGMMV clone**

To produce CGMMV genomic cDNA, total RNA was extracted from CGMMV-ON infected cucumber leaves using TRIzol reagent (Invitrogen, Burlington, Ontario, Canada). First strand cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen) with Random Primers 6 (New England Biolabs, Pickering, Ontario, Canada), followed by RNase H (Invitrogen) treatment at 37°C for 20 min. The full-length viral genome was amplified using the forward primer, CGMMV5UTR-F, and the reverse primer, CGMMV3UTR-R. PCR was performed using Phusion High-Fidelity DNA Polymerase (New England Biolabs) and the following cycle: (1) a pre-denaturation at 98°C for 3 min; (2) 25 cycles of denaturation at 98°C for 30 sec, annealing at 50°C for 1 min and extension at 72°C for 7 min; and (3) an extra 5 min of extension at 72°C. The resulting PCR product, of 6.5 kb, was then gel separated and purified using GenepHlow Gel Extraction Kit (FroggaBio, Toronto, Ontario, Canada) and subsequently cloned into pCR-Blunt with a Zero Blunt PCR Cloning Kit (Invitrogen), to create pCR-Blunt CGMMV. Selected clones were confirmed by colony PCR using M13 reverse and CGMMVR3-R primers and by DNA sequencing with M13 reverse and M13 forward primers.

The binary vector, pCB 301 (Xiang et al. 1999) was modified to contain a double 35S promoter and a ribozyme sequence at the 3' end, right upstream of the terminator (*Rz-T*), and was used for construction of an infectious CGMMV clone. The double 35S promoter and *Rz-T* fragments were amplified from pCass4-Rz (Annamalai and Rao 2005) using two primer

sets, *SacI*-35S-F and *SpeI*-*StuI*-35S-R, and *BamHI*-RzT1-F and *KpnI*-RzT1-R, respectively. The amplified PCR products were digested with *SacI* and *SpeI* (for the double 35S product) and *BamHI*-HF and *KpnI* (for the *Rz-T* product), and cloned into the corresponding sites (*SacI/SpeI* or *BamHI/KpnI*) of the pCB 301 vector. The resulting pCB-Rz vector was then introduced into *Escherichia coli* DH 5 $\alpha$  (Invitrogen). The insert cDNAs were confirmed by DNA sequencing.

The full-length cDNA sequence of the CGMMV genome was obtained in two steps using Phusion High-Fidelity DNA Polymerase (New England Biolabs). The first 1348 nt of the CGMMV cDNA were amplified by the forward primer, CGMMV5UTR-F and the reverse primer, *BamHI*-*AgeI*-R. PCR was performed using the following cycle: (1) a pre-denaturation at 98°C for 3 min; (2) 25 cycles of denaturation at 98°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 1 min; and (3) an extra 5 min of extension at 72°C. The purified PCR product was digested with *BamHI* and ligated into the corresponding sites (*StuI/BamHI*) of pCB-Rz, such that the 5' untranslated region (UTR) of the CGMMV genome was just downstream of the transcription start site of the double 35S promoter, to create pCB-CG-1. The remainder cDNA of the CGMMV genome was amplified using the forward primer, CGMMV*AgeI*-F, and the reverse primer, *BamHI*-3UTR-R, using the following PCR cycle, (1) a pre-denaturation at 98°C for 3 min; (2) 25 cycles of denaturation at 98°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 4 min; and (3) an extra 5 min of extension at 72°C. The PCR product was purified, digested with *AgeI/BamHI* and cloned into pCB-CG-1 in order to generate the full-length cDNA clone of CGMMV, pCB-CG. pCB-CG was confirmed by PCR and DNA sequencing.

All the primer sequences are described in Table 3.1.

### 3.2.2 Construction of chimeric CGMMV presenting the NE of GP5

My first attempt was made by introducing NE into the C-terminus of the CGMMV CP before the stop codon. The sequence of NE, encoding nine amino acids (Ser<sup>37</sup>-Gln-Leu-Gln-Ser-Ile-Tyr-Asn-Leu<sup>45</sup>), was amplified together with the sequence of CGMMV CP, by fusion PCR, using pCB-CG as a template and primers, *NheI*-NE-F and *BamHI*-3UTR-R. The PCR



product was digested and ligated into pCB-CG to create pCG-NE. The construct was confirmed by DNA sequencing, transformed into agrobacteria (strain GV3101) and subsequently infiltrated into cucumber cotyledons.

For production of NE-containing CP, another attempt was made using a readthrough strategy (Ooi et al. 2006; Teoh et al. 2009). The CGMMV clone was engineered to include the readthrough (RT) sequence, either “TCT-AAA-TAG-CAA-TTA” (RT1) or “TCC-AAA-TAG-CAA-TTA” (RT2). First, a fragment of the CP coding region in pCB-CG was digested by *MluI* and *BamHI* and then ligated in to a *MluI/BamHI*-digested pCR-Blunt vector (Invitrogen) to generate pCR-CP. The RT1-NE-3’UTR was amplified from pCG using HindIII-AmberNE-F and BamHI-3UTR-R and then introduced into pCR-CP via *HindIII/BamHI* sites to create pCR-NE. The entire CP coding sequence and 3’ UTR of CGMMV were digested from pCR-NE and ligated into pCB-CG via *MluI/BamHI* sites to form pCG-RT1NE. Second, the RT2-NEcmyc-3’UTR was amplified using HindIII-NEcmycC-F and BamHI-3UTR-R, and inserted into pCR-CP, via *HindIII/BamHI*, to form pCR-NEcmyc. The entire CP and 3’ UTR fragment was digested from pCR-NEcmyc and ligated into the *MluI/BamHI* sites of pCB-CG, to generate pCG-RT2NEcmyc.

All the primer sequences are described in Table 3.1.

### 3.2.3 Agroinfiltration of CGMMV infectious clone and chimeric CGMMV clones

The CGMMV based constructs were agroinfiltrated into plants using *Agrobacterium tumefaciens* (strain GV3101). Agrobacterial cultures were grown in liquid LB medium with kanamycin (50 µg/ml) and rifampicin (50 µg/ml) at 28°C, to an optical density at 600 nm (OD<sub>600</sub>) of 1.5-2.0. Cells were harvested by centrifugation at 5000 x g for 5 min. The pellet was resuspended in agroinfiltration buffer (10 mM MgCl<sub>2</sub>, 10 mM MES and 150 µM 4’-Hydroxy-3’, 5’-dimethoxyacetophenone) and incubated at room temperature for 2 hr. This was followed by a further dilution with agroinfiltration buffer, to reach a final OD<sub>600</sub> of 1.0. The suspension was then infiltrated into either the abaxial leaf epidermis of *Nicotiana*

species, or the cotyledons of cucumbers, watermelons and cantaloupes, with a 1 ml syringe (Kapila et al. 1997)

For the infectivity test of the CGMMV infectious clone, leaves of 3-week-old *N. benthamiana* and *N. tabacum* seedlings were agroinfiltrated with 1 ml of bacterial suspension (Yoon et al. 2011). For cucumbers (*Cucumis sativus* var. Straight 8), watermelons (*Citrullus lanatus* var. Greybelle) and cantaloupes (*Cucumis melo* var. Hami), fully expanded cotyledons of 10-day-old plants were agroinfiltrated (Cui et al. 2013). For each variety, six seedlings (two leaves or cotyledons each) were used. Infectivity was identified by either double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA) or reverse transcription-polymerase chain reactions (RT-PCR).

#### 3.2.4 DAS-ELISA and RT-PCR

CGMMV infection of agroinfiltrated plants was confirmed using either ELISA or RT-PCR. DAS-ELISA was performed using a CGMMV ELISA kit (catalog number SRA 45701; Agdia, Elkhart, IN, USA) and following the manufacturer's protocol. Three 4 mm leaf disks were sampled from systematic leaves and homogenized using a TissueLyser II (QUIAGEN), mixed well with 500 µl of general extraction buffer (GEB) and then 100 µl of the resulting mixture was added to a 96 well plate. Following the ELISA procedure, virus positive samples were detected by a significant color development in plate wells. To determine the absorbance value of the color change, plates were read using an iMark Microplate Reader (Biorad) at 415 nm. Three biological replicates were performed for each treatment. ELISA values were compared between infected plants and mock-treated plants using the two-tailed Student's t-test. Statistical analysis was performed using Microsoft Excel software. A p-value of 0.05 or less indicates a significant difference.

Total RNA was extracted from young leaves using TRIzol (Invitrogen) and treated with DNase I (Invitrogen), as described in 2.2.12. First-strand cDNA was generated by reverse-transcription reactions with Random Primer 6 (New England Biolabs) and SuperScript III Reverse Transcriptase (Invitrogen). To test the infectivity of pCB-CG, RT-PCR was conducted to amplify the full length of the MP of CGMMV, using primers CGMMVMP-F

and CGMMVMP-R, at 15 dpi. To identify the infectivity of expression constructs including pCG-NE, pCG-RT1NE and pCG-RT2NEcm<sup>yc</sup>, RT-PCR was performed with primers of CP-F and BamHI-3UTR-R, at 21 dpi. Similar primers were also used for RT-PCR and sequencing at 45 dpi to examine the stability of pCG-RT2NEcm<sup>yc</sup>. The resulting sequence was aligned with the insert and wild-type GGMMV sequences using the online T-coffee program (<http://tcoffee.crg.cat>). All PCR reactions were performed using 2X Taq Frogga Mix (FroggaBio, Toronto, ON, Canada) using the following cycle: (1) a pre-denaturation at 94°C for 3 min; (2) 32 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 1 min; and (3) an extra 10 min of extension at 72°C. Internal control of RT-PCR for pCG-NE was performed using primers Actin-F and Actin-R. All the primer sequences are described in Table 3.1.

### 3.2.5 Chimeric coat protein extraction and quantification

To study the protein production of chimeric CGMMV in cucumber, western blot analysis was performed using systemic leaf tissue samples. Ten leaf discs (3 mm in diameter) were sampled for a time-course experiment, with collections at 21 and 50 dpi, and homogenized using a TissueLyser II (QUIAGEN). One ml of extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 0.1% (v/v) TritonX-100, 5 mM dithiothreitol, 1.5X protease inhibitor) was then added to the ground leaves in order to obtain the total soluble protein (TSP). Protein extraction from buffer-only infiltrated plants and CGMMV infiltrated plants was also performed under the same conditions and used as negative and positive controls, respectively. Total soluble protein concentration levels were determined using a Bradford assay (Bradford 1976), with bovine serum albumin (BSA) as a standard, and using an iMark Microplate Reader (Biorad). Protein extracts were separated by electrophoresis in a 15% (w/v) sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. To identify CP, primary anti-CGMMV CP monoclonal antibody, produced in mouse (Zheng et al. 2015), was incubated with the membrane and subsequently detected using horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich, Oakville, ON, Canada). To determine levels of chimeric CP tagged with cm<sup>yc</sup>, the primary antibody was changed to mouse anti-cm<sup>yc</sup>

monoclonal antibody (Sigma- Aldrich). Western blots were visualised by the Immobilon Western (Millipore Corporation, Billerica, MA, USA), following the manufacturers' protocol. The resulting images were analysed with TotalLab TL100 software (Nonlinear Dynamics, Durhan, USA) by comparing the densitometry of bands with known amounts of a synthetic cellulose-binding domain (CBD) protein tagged with cmyc (GenScript, Piscataway, USA). Three biological replicates were performed for each time-course extraction.

### 3.2.6 Characterization of the chimeric CGMMV displaying NE by transmission electron microscopy (TEM)

30  $\mu$ l of the anti-cmyc agarose conjugate (Sigma-Aldrich) was washed 5 times with 1 ml washing buffer (20 mM Tris-HCl pH 7.5, 300 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.5% (v/v) Triton X-100). CGMMV particles were extracted following the procedure as described (Seo et al. 2013). Chimeric CGMMV-infected leaf tissue (0.5 g) was ground with 1 ml of extraction buffer (20 mM Tris-HCl pH 7.5, 300 mM NaCl, 5 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 0.5% (v/v) Triton X-100) using a mortar and pestle. The extraction was centrifuged at 12 000  $\times$  g and 4°C for 10 min and then filtered through a 45  $\mu$ m cell strainer (BD Biosciences, Franklin Lakes, NJ, USA) to eliminate cell debris. The resulting supernatant was added to the washed resin in a 1.5 ml Eppendorf tube and incubated for 2 hr on an orbital shaker at 4°C, following the manufacturers' protocol. After incubation, the virus-resin complexes were then precipitated by centrifugation for 1 min at 8 000  $\times$  g and washed six times in 500  $\mu$ l of wash buffer to remove nonspecific bindings. The resin was resuspended in 100  $\mu$ l of wash buffer in the final step. Wild-type CGMMV-infected leaves were processed in the same procedure as a negative control. The resulting suspension was subjected to negative staining with 2% (w/v) uranyl acetate (pH 4.5) and visualization using TEM (Haschemeyer and Meyers 1973).

## 3.3 Results

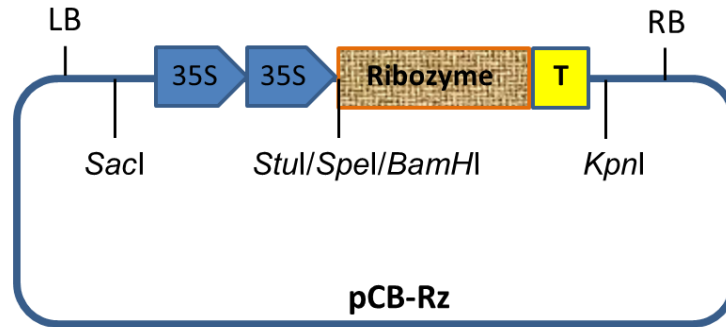
### 3.3.1 Characteristics of CGMMV infectious clone for agroinfiltration

The full-length cDNA of CGMMV was successfully cloned into plasmid pCB-Rz (Figure 3.1A) between the double 35S promoter and the ribozyme sequence (Rz), to generate

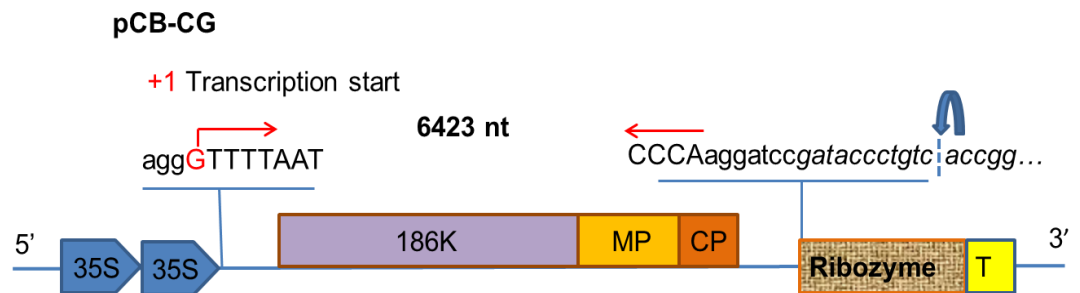
pCB-CG. Since the 5' UTR sequence is important for efficient replication and biological activities of the positive strand RNA virus (Boyer and Haenni 1994; Janda et al. 1987), the transcription start site was designed precisely at the nucleotide “G” of the CGMMV genome to produce the authentic viral 5' end. Transcription of the full-length CGMMV cDNA, followed by ribozyme self-processing would produce the entire viral genome with an additional 18 nt extension at the 3' UTR (Figure 3.1B).

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*. Plants were observed for symptoms at 10-15 dpi. Cucurbit plants showed obvious mosaic, chlorosis, necrosis and crinkle symptoms on new emerging leaves at 10 dpi, identical to the symptoms of wild-type CGMMV-infected plants (Figure 3.2A, B, and C). Mild mottling and distortion appeared on the upper leaves of *N. benthamiana* at 15 dpi (Figure 3.2D). No typical symptom was displayed in *N. tabacum*. All mock plants treated with buffer infiltration did not exhibit any of these symptoms (Figure 3.2). 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 cucumbers, watermelons, cantaloupes and *N. benthamiana* infiltrated with the pCB-CG clone, but not mock-treated control plants (Figure 3.3A, B and C). Although DAS-ELISA results showed a significant difference between pCB-CG-infiltrated *N. tabacum* and mock-treated plants, RT-PCR did not show a strong band of interest. 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*, and that symptoms were various, depending on the host family.

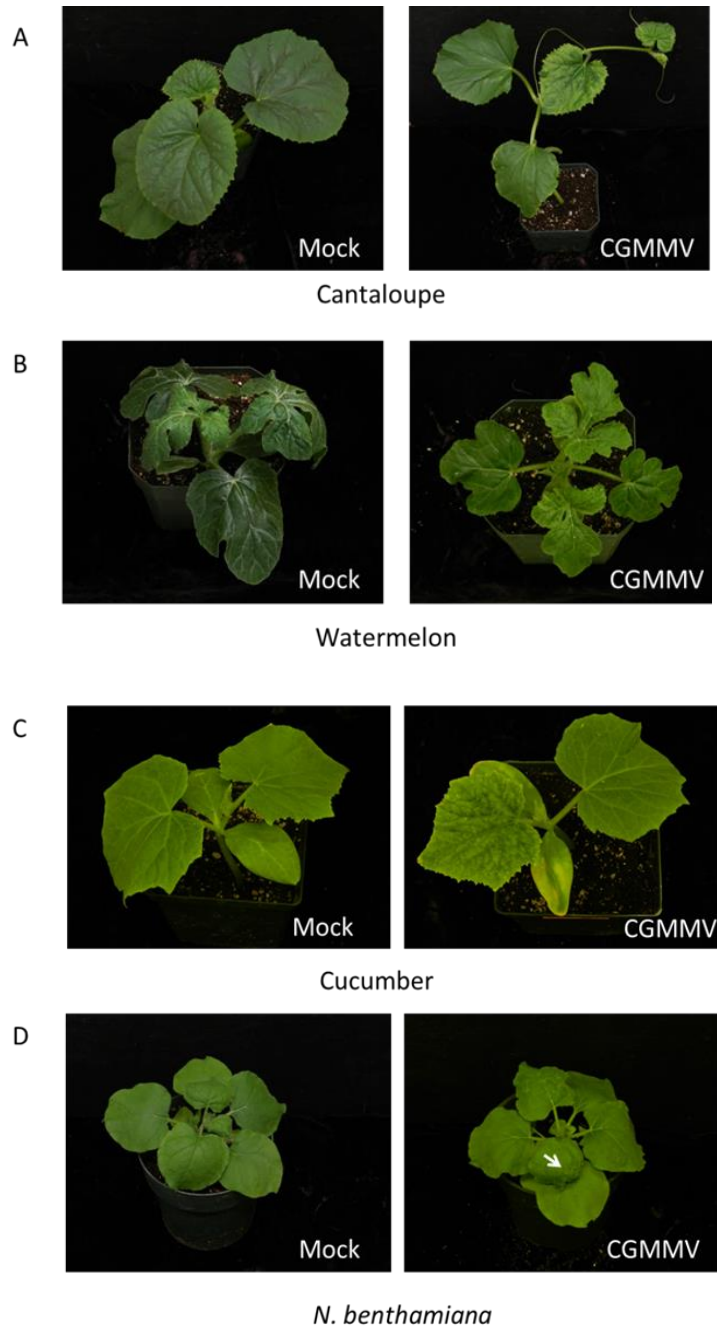
A.



B.



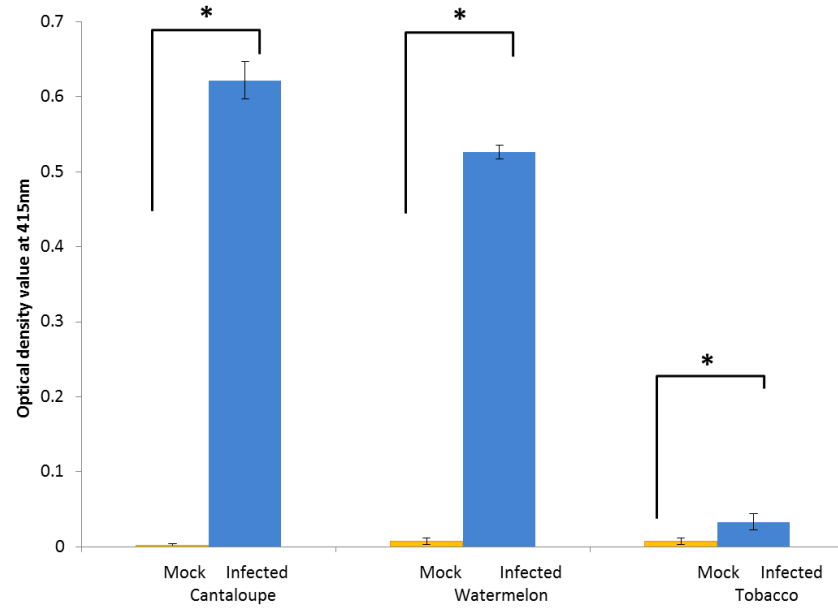
**Figure 3.1** Construction of the CGMMV infectious clone. **A.** Schematic representation of T-DNA vector, pCB-Rz, which was used to clone the cDNA of CGMMV genomic RNA between the left border (LB) and right border (RB) (not drawn to scale). The vector contains a double *CaMV 35S* promoter (*35S*), a ribozyme sequence (*Rz*) and a *CaMV 35S* terminator (*T*) derived from pCass4-Rz. Cloning region with three restriction sites, *StuI*, *SpeI* and *BamHI* was used to introduce cDNA of the CGMMV genome. **B.** Characteristics of T-DNA plasmid containing the CGMMV genome used for agroinfiltration. Single lines and open box represent noncoding and coding regions, respectively. Polycistronic CGMMV RNA encodes for a 186-kDa protein (186K), movement protein (MP) and coat protein (CP). The dashed line represents readthrough translation in the 186K coding sequence. The 3' end of the double *35S* promoter is represented in lower case, whereas, the 5' sequence of CGMMV cDNA was shown in uppercase. Transcription start site is shown with a bent arrow. The 3' end of CGMMV cDNA is shown in uppercase and the non-viral sequence, following the ribozyme site, is in lowercase and italic. The self-cleavage site of ribozyme is indicated by a curved arrow.



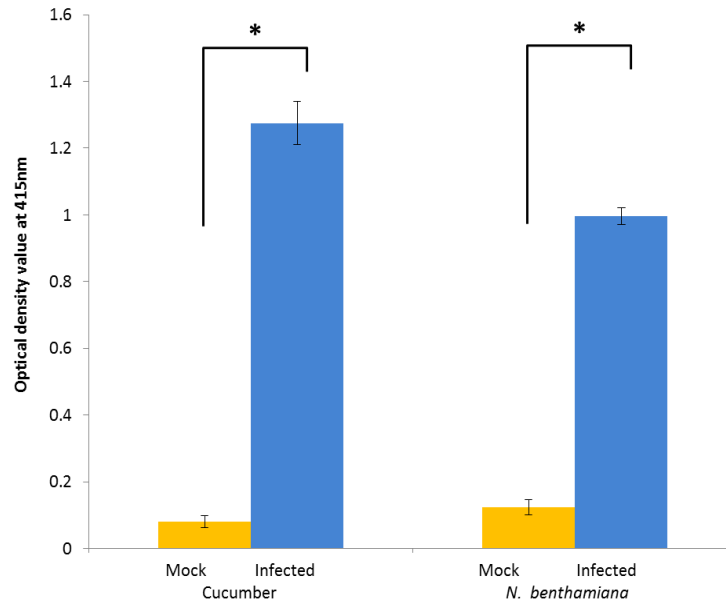


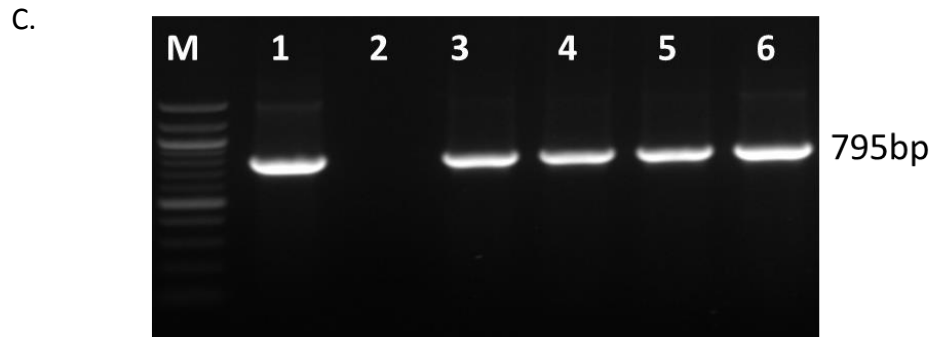
**Figure 3.2** Pathogenicity test on natural and experimental hosts with the CGMMV infectious clone. Symptoms in: **A.** Cantaloupe (*Cucumis melo* var. Hami) at 15 dpi with mosaic and crinkled leaves, **B.** Watermelon (*Citrullus lanatus* var. Greybelle) infiltrated with the infectious clone showed mosaic and clearing vein symptoms on leaves, **C.** Cucumber (*Cucumis sativus* var. Straight Eight) which was infected with CGMMV showed mosaic and crinkled leaves, and **D.** *N. benthamiana* showed mild symptoms with crinkle and mosaic leaves at leaf edges, as indicated by the white arrow.

A.



B.





**Figure 3.3** DAS-ELISA and RT-PCR results of infiltrated plants at 15 dpi. Error bars represent the standard deviation of the mean (n=3). Asterisks indicate significant differences from mock-treated plants (Student's t-test,  $p < 0.05$ ). **A.** Infiltrated cantaloupes, watermelons and *N. tabacum*. **B.** Infiltrated cucumbers and *N. benthamiana*. **C.** RT-PCR results of infiltrated plants at 15 dpi, amplifying the coding sequence of the viral movement protein. Lane M: 100 bp DNA marker. Lane 1: CGMMV infected cucumber (greenhouse) as a positive control. Lane 2: Negative control mock plant infiltrated with buffer. Lane 3: Infiltrated cucumber. Lane 4: Infiltrated watermelon. Lane 5: Infiltrated cantaloupe. Lane 6: Infiltrated *N. benthamiana*.

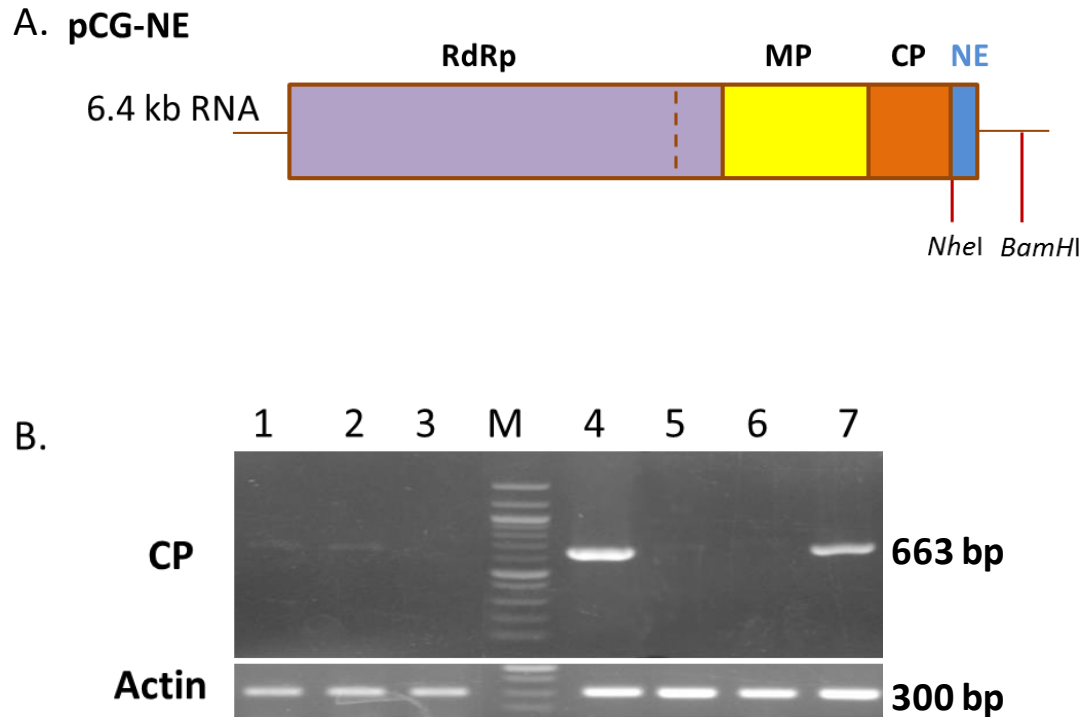
### 3.3.2 Infectivity of chimeric CGMMV

#### 3.3.2.1 Direct fusion downstream of CP

Firstly, NE, which includes nine amino acids (Ser<sup>37</sup>-Gln-Leu-Gln-Ser-Ile-Tyr-Asn-Leu<sup>45</sup>) of the PRRSV GP5, was inserted right before the stop codon of CGMMV-CP, to generate the construct pCG-NE (Figure 3.4A). To test the infectivity of this construct in cucumber and *N. benthamiana*, the upper non-infiltrated leaves of plants infiltrated with pCG-NE was sampled at 21 dpi and total RNA purified from the samples was analyzed by RT-PCR. Viral RNA was hardly detected in cucumber and *N. benthamiana* infiltrated with pCG-NE (Figure 3.4B), unlike samples from pCB-CG infiltrated plants which served as a positive control. These results indicated that the direct fusion strategy did not work well.

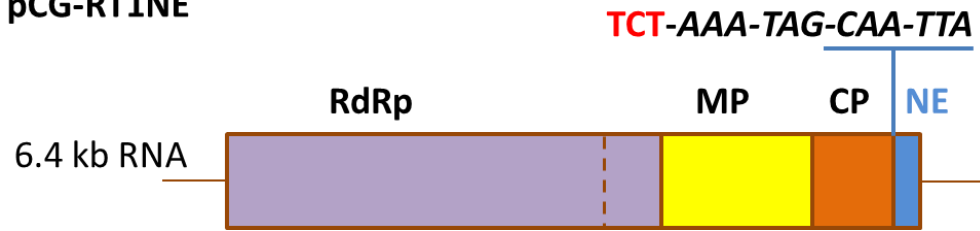
#### 3.3.2.2 Readthrough stop codon

The modified CGMMV constructs generated using the readthrough strategy were also tested for their infectivity. In contrast to the direct fusion mentioned above, both constructs displayed high infectivity in cucumber, but the infectivity was varied on *N. benthamiana*. The pCG-RT1NE clone could infect both cucumber and *N. benthamiana*, whereas the pCG-RT2NE<sub>cmcy</sub> could only infect cucumber (Figure 3.5A and 3.6A). The severity of symptoms caused were varied, depending on construct and host plant combinations. *N. benthamiana* infected by pCG-RT1NE showed very mild symptoms, with clearing veins and blistering on leaf edges (Figure 3.5B bottom), and pCG-RT1NE-infected cucumber demonstrated wrinkled and mosaic leaves (Figure 3.5B top). In contrast, pCG-RT2NE<sub>cmcy</sub>-infected cucumber displayed blistering and cupped leaves (Figure 3.6B). Consistently, DAS-ELISA and RT-PCR confirmed the presence of chimeric CGMMV in all cucumber plants infiltrated with pCG-RT1NE and pCG-RT2NE<sub>cmcy</sub>, and in *N. benthamiana* infiltrated with pCG-RT1NE (Figure 3.5C and 3.6C).



**Figure 3.4** Construction of pCG-NE and its infectivity in *N. benthamiana* and cucumber. **A.** Schematic representation of pCG-NE in which 2 restriction sites of *NheI* and *BamHI* were employed to introduce NE upstream before the stop codon of CP (not drawn to scale). **B.** RT-PCR results of pCG-NE infiltrated plants at 21 dpi, amplifying sequences of coat protein (CP) and 3' UTR, and actin. Lane 1-3: pCG-NE infiltrated cucumbers. Lane M: 100 bp DNA marker. Lane 4: pCG infiltrated cucumber as a positive control (663 bp). Lane 5-6: pCG-NE-infiltrated *N. benthamiana*. Lane 7: pCG infiltrated *N. benthamiana* as a positive control (663 bp).

## A. pCG-RT1NE



## B.

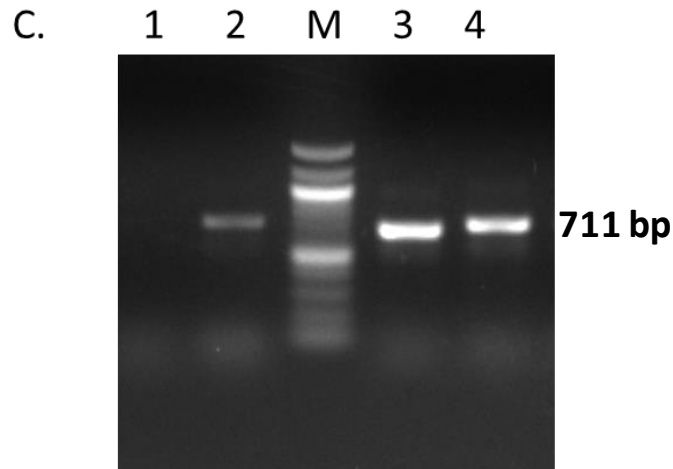
Cucumber



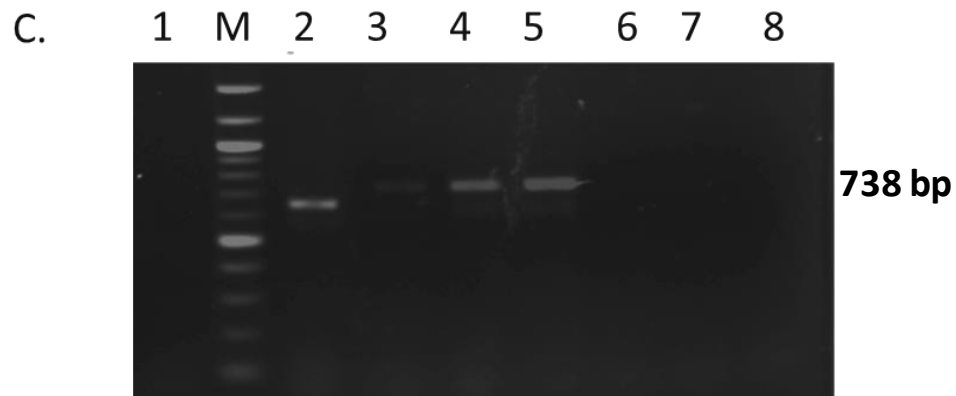
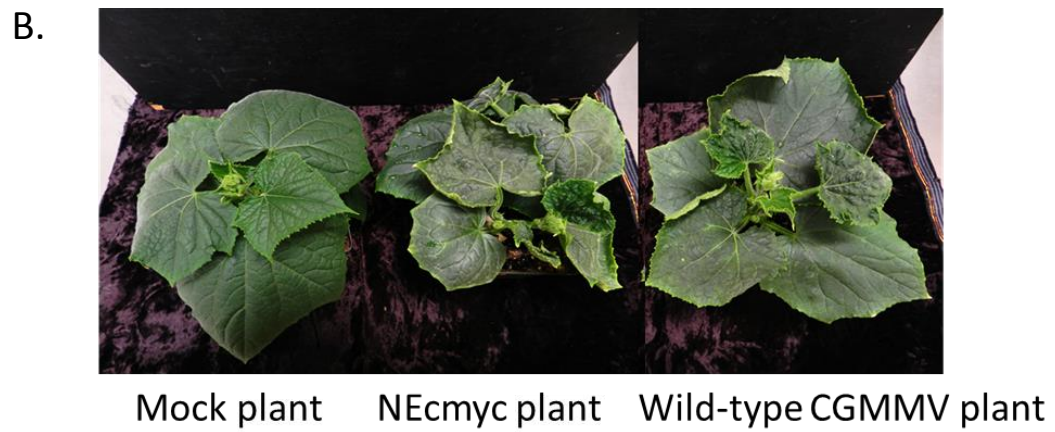
Buffer infiltrated plant Mock plant NE plant

*N. benthamiana*

Buffer infiltrated plant Mock plant NE plant



**Figure 3.5** Construction of pCG-RT1NE and its infectivity in *N. benthamiana* and cucumber. **A.** Schematic representation of pCG-RT1NE (not drawn to scale). **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, amplifying the coding sequence of CGMMV CP fused with NE and 3' UTR (711 bp). Lane 1: Negative control. Lane 2: Infiltrated *N. benthamiana*. Lane 3: Positive control of wild-type CGMMV-infected plant. Lane M: 100 bp DNA ladder. Lane 4: Infiltrated cucumber.





**Figure 3.6** Construction of pCG-RT2NEcmcy and its infectivity in *N. benthamiana* and cucumber. **A.** Schematic representation of pCG-RT2NEcmcy (not drawn to scale). **B.** Symptoms in cucumber infiltrated with pCG-RT2NEcmcy at 21 dpi. **C.** RT-PCR results from samples of cucumbers and *N. benthamiana* infiltrated with pCG-RT2NEcmcy at 21 dpi, amplifying the extended CP sequence (738 bp). Lane 1: Negative control. Lane M: 100 bp DNA ladder. Lane 2: Wild-type CGMMV-infected plant. Lane 3-5: pCG-RT2NEcmcy infiltrated cucumber. Lane 6-8: pCG-RT2NEcmcy-infiltrated *N. benthamiana*.

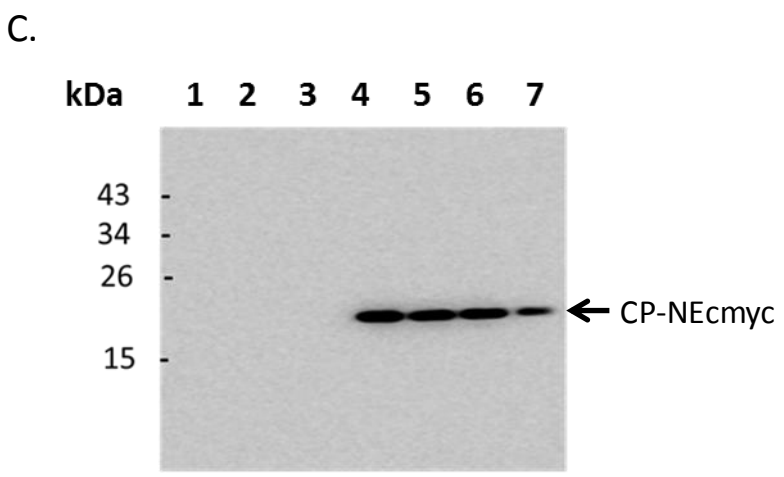
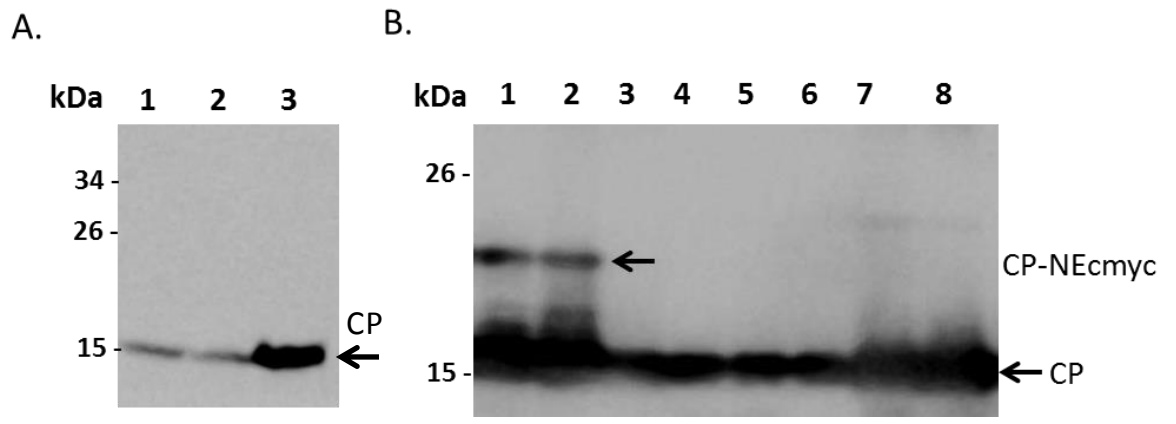
### 3.3.3 Peptide expression on the coat protein of chimeric CGMMV via readthrough translation

Since the pCG-RT1NE could infect both cucumber and *N. benthamiana*, protein extracts from these plants were subjected to western blot analysis, using anti-CP antibody, to detect NE-containing CP. Unfortunately, none of these plants produced chimeric CP. The results of western blot analysis showed only one band, approximately 15 kDa in size, which matched with the predicted molecular mass for CGMMV CP (used as a negative control) (Figure 3.7A)

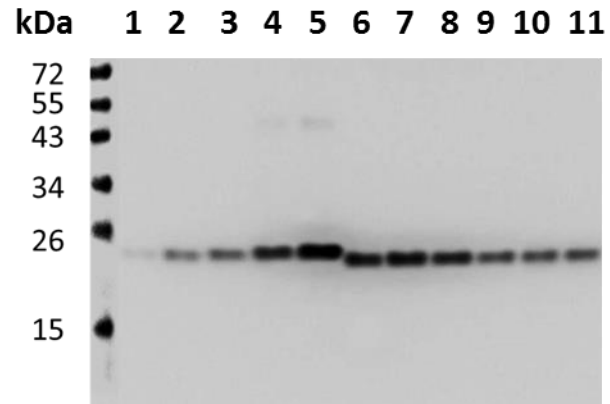
On the contrary, the chimeric CP containing NEcmv (CP-NEcmv), which has a size of approximately 20 kDa, was detected in cucumber infiltrated with pCG-RT2NEcmv using both anti-CGMMV CP antibody (Figure 3.7B) and anti-cmv antibody (Figure 3.7C). The expression level of CP-NEcmv was estimated to be 35.84  $\mu\text{g/g}$  of fresh weight (0.067% of TSP) at 21 dpi, and decreased to 16.60  $\mu\text{g/g}$  of fresh weight (0.038% of TSP) at 50 dpi (Figure 3.7D and E).

### 3.3.4 Presentation of the NE on the surface of chimeric CGMMV coat protein

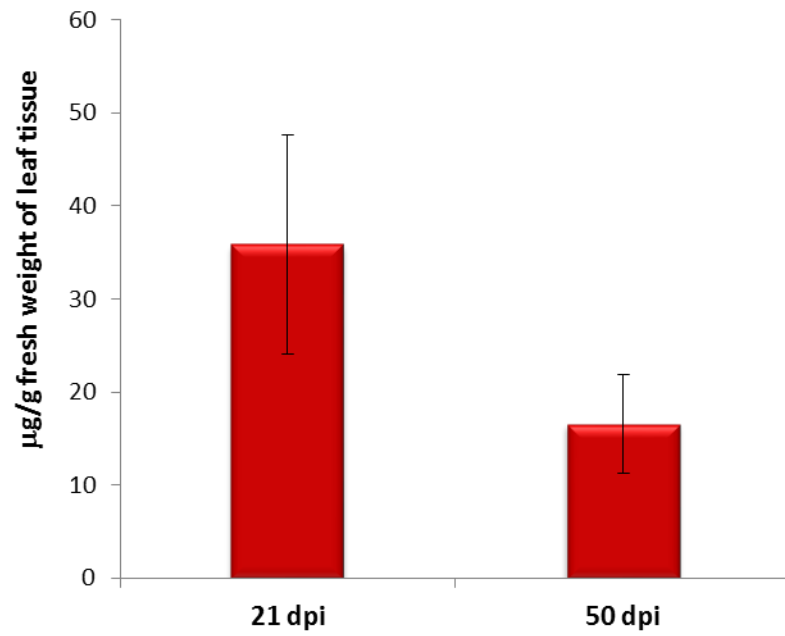
To determine whether the NE of PRRSV GP5 was displayed on the surface of CGMMV capsids, the chimeric virions extracted from pCG-RT2NEcmv-infiltrated leaves underwent immunoprecipitation using anti-cmv beads and were imaged using transmission electron microscopy, as described in Figure 3.8A. The amount of chimeric CGMMV bound to anti-cmv beads was remarkably more abundant when compared to wild-type CGMMV, as a negative control, at 19 000X magnification (Figure 3.8B 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 distinct for CGMMV (Figure 3.8B bottom). The results demonstrated that the affinity of chimeric virions to anti-cmv beads was much higher than that of the wild-type CGMMV virions, due to the immuno-attraction between cmv antigen and the anti-cmv beads. These data affirmed that NE, as well as cmv, was successfully displayed on the surface of chimeric CGMMV capsids.



D.

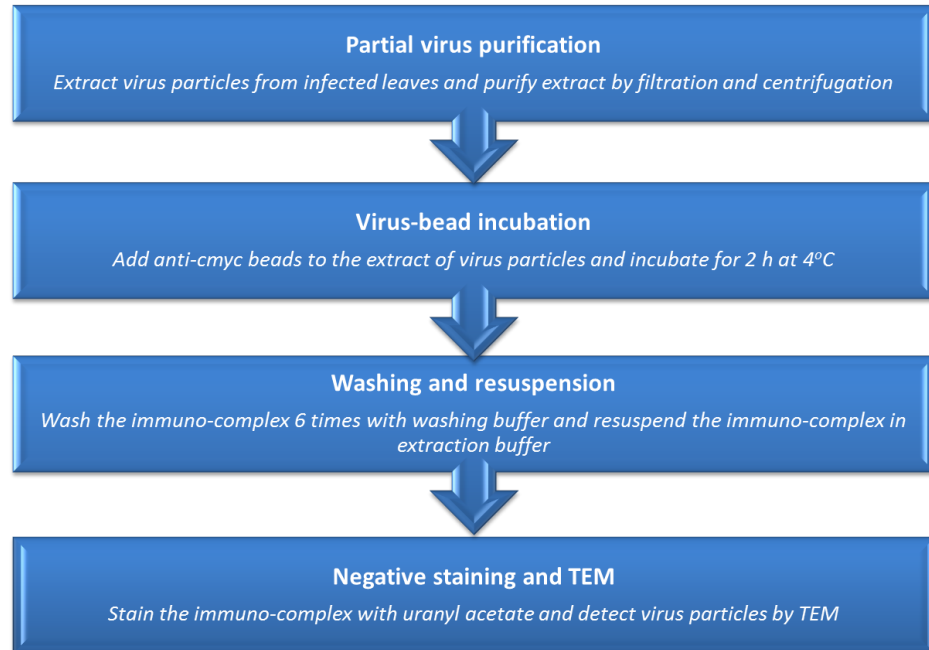


E.



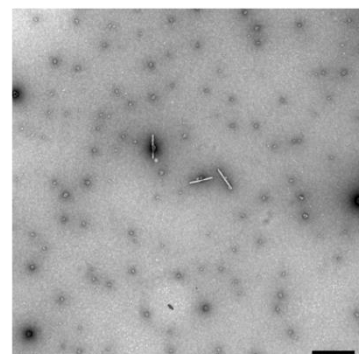
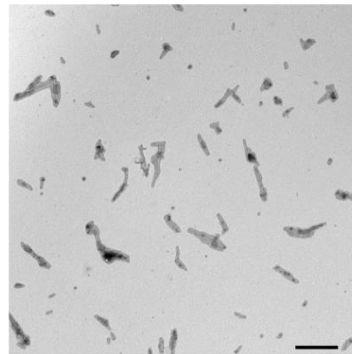
**Figure 3.7** Western blot analysis of protein extracts from chimeric virus-infected leaves. **A.** Western blot analysis of pCG-RT1NE-infected leaves using anti-CP antibody. Lane 1: Chimeric virus-infected cucumber. Lane 2: Chimeric virus-infected *N. benthamiana*. Lane 3: Wild-type virus-infected *N. benthamiana*. **B.** Western blot analysis of pCG-RT2NE-infected leaves using anti-CP antibody. Lane 1-2: Chimeric virus-infected cucumber. Lane 3-6: Wild-type virus-infected *N. benthamiana*. Lane 7-8: Wild-type virus-infected cucumber. **C.** pCG-RT2NE-infected plants displayed a chimeric CP (containing NEcmcy) band, at about 20 kDa on a western blot with anti-cmyc antibody. Lane 1: Non-infiltrated plant. Lane 2: Buffer infiltrated plant. Lane 3: Wild-type virus-infiltrated plant. Lane 4-7: pCG-RT2NEcmcy-infiltrated cucumber. **D.** Quantification of chimeric CP at different harvest time using western blot with anti-cmyc antibody. Lane 1-5: 1, 4, 8, 16, 32 ng of cellulose-binding domain (CBD) protein tagged with cmyc as a standard, respectively. Lane 6-8: pCG-RT2NEcmcy-infiltrated cucumber at 21 dpi. Lane 9-11: pCG-RT2NEcmcy-infiltrated cucumber at 50 dpi. **E.** Level of chimeric CP expression measured by  $\mu\text{g}$  per g fresh weight of leaf tissue. Error bars represent the standard deviation of the mean (n=3).

A.

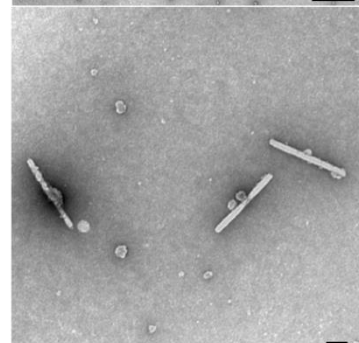
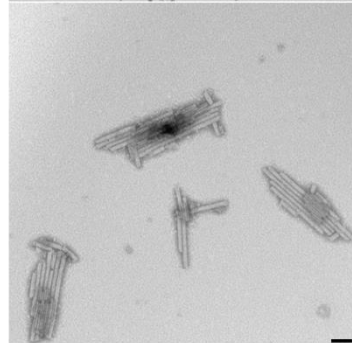


B.

Bar = 500 nm  
19 000X magnification



Bar = 100 nm  
64 000X magnification



**Chimeric CGMMV**

**Wild-type CGMMV**

**Figure 3.8** The presentation of NEcmv on the surface of chimeric CGMMV.

**A.** Flow chart of the immunoprecipitation procedure for detection of chimeric CGMMV.

**B.** TEM results of chimeric CGMMV under different magnification, 19 000X and 64 000X. The number of chimeric virus particles bound to anti-cmyc beads was remarkable when compared to the wild-type viruses. At 64 000X magnification, these virus particles displayed the rod shapes which are characteristics for CGMMV.

### 3.3.5 The stability of the pCG-RT2NEcmyc

To investigate the stability of pCG-RT2NEcmyc in cucumber, RT-PCR and DNA sequencing was conducted using newly emerging young leaves from infiltrated plants at 45 dpi. The results from RT-PCR confirmed that the amplicon of chimeric CP and 3'UTR, 738 bp, was preserved and larger than that of wild-type CP, which is 663 bp (Figure 3.9A). The NEcmyc sequence on the chimeric coat protein was also confirmed at 45 dpi (Figure 3.9B). Moreover, no wild-type CP was detected in plants infiltrated with the pCG-RT2NEcmyc construct. The results confirmed that the pCG-RT2NEcmyc was stable in infiltrated cucumber for at least 45 dpi.

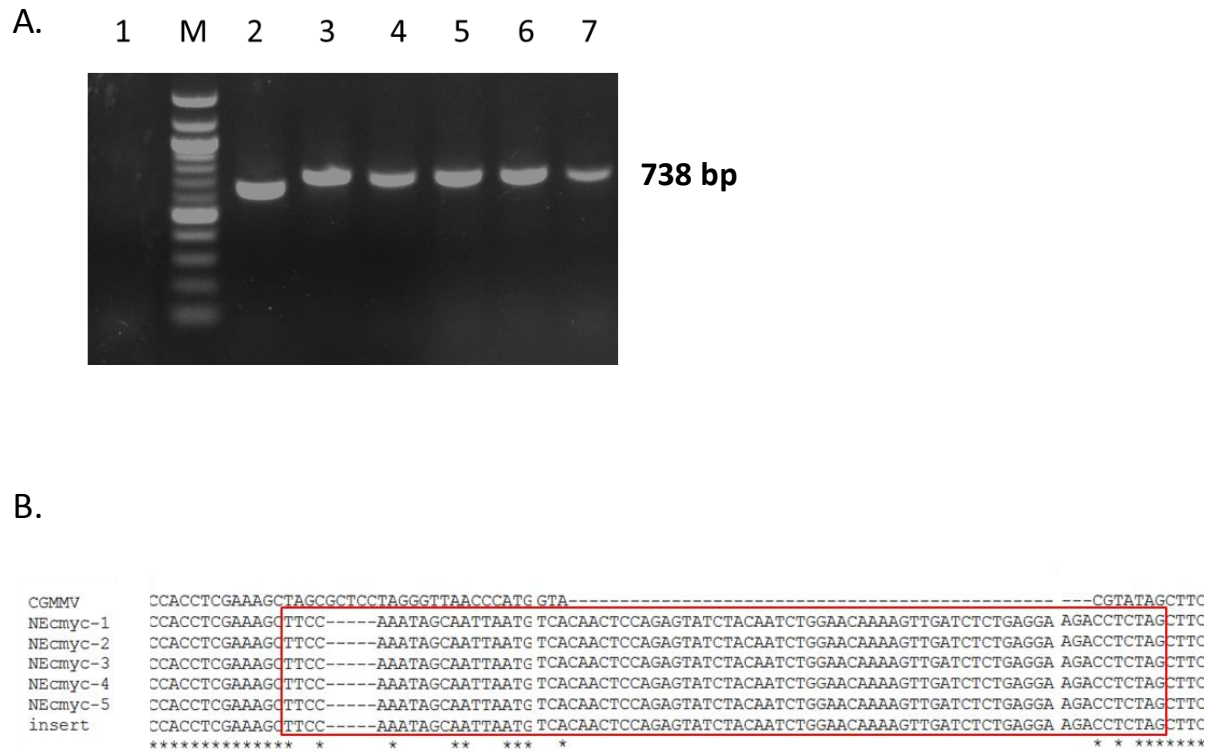
## ***3.4 Discussion***

CGMMV is an important viral pathogen that impedes Cucurbita crop production in the world. In this study, I developed a full-length cDNA infectious clone based on CGMMV-ON, a CGMMV isolate from a greenhouse in Ontario, Canada and further modified it for expression of PRRSV GP5. In addition to being employed as expression vectors for biotechnological applications, such as the production of proteins of interest in plants (Lindbo 2007b; Marillonnet et al. 2005), infectious clones also play an essential role in studies of RNA plant viruses such as replication and virus-host interactions (Ahlquist et al. 1984; Ahlquist et al. 2003; Cui et al. 2013; Cui and Wang 2016). This explains why the generation of infectious clones is often the initial step of virus investigations. Construction of infectious clones may encounter many technical difficulties in order to maintain the biological characteristics of wild-type viruses. For example, the addition of non-viral nucleotides at either the 5' end or the 3' end of transcripts has a deleterious impact on viral replication and infectivity (Annamalai and Rao 2005; Chapman 2008). The process of developing an infectious clone into a binary vector also involves the potential introduction of mutants, which can lead to infectivity failures (Boyer and Haenni 1994; Junqueira et al. 2014; Lai 2000; Lindbo 2007a; Liu and Kearney 2010). Keeping these in mind, I minimized PCR cycles and subcloning steps to prevent mutations during construction of the CGMMV infectious clone. Moreover, the CGMMV genome was placed right downstream of a double



*CaMV 35S* promoter in order to eliminate any non-viral nucleotide between the transcription start and the 5' end of virus genome, thus, ensured a successful *in vivo* transcription in plant (Turpen et al. 1993). The CGMMV vector developed in this study can be introduced into plants through agroinfiltration, a versatile method compared to biolistic bombardment or inoculation of RNA transcripts (Chapman 2008).

CGMMV is a member of the genus *Tobamovirus*, which also includes TMV. Tobamoviruses are well-known for their stability and persistence for long periods on non-host surfaces, such as plant debris, soil and tools, which facilitates the transmission of these viruses by mechanical means (Broadbent 1976; Broadbent et al. 1965; Lanter et al. 1982). As a member of this genus, CGMMV employs these characteristics to spread among cucurbit crops worldwide (Ainsworth 1935; Al-Shahwan and Abdalla 1992; Fletcher et al. 1969; Liu et al. 2009; Wijethunga et al. 2014; Yoon et al. 2008). Natural hosts of CGMMV are restricted to *Cucurbitaceae*, including cucumber, squash, melon, muskmelon, bottle gourd and watermelon (Lovisolo 1981). The infectious clone derived from CGMMV infected cucumber plants found in these greenhouses can infect not only natural hosts, such as cucumbers (*Cucumis sativus* var. Straight Eight), cantaloupes (*Cucumis melo* var. Hami) and watermelons (*Citrullus lanatus* var. Greybelle), but also an experimental host, *N. benthamiana* with disease symptoms ranging from latent to mild (Figure 3.2). In *N. benthamiana*, systemically infected leaves, although without obvious symptoms, did carry the virus (Figure 3.3B and C). The results suggested that there was a remarkable difference in host responses to CGMMV, and *N. benthamiana* may serve as a model plant for the studies of CGMMV pathogenesis and virus-host interactions.



**Figure 3.9** Stability of chimeric construct pCG-RT2NEcmv at 45 dpi. **A.** RT-PCR results using primers for chimeric coat protein and 3' UTR sequence. Lane 1: Negative control. Lane M: 100 bp ladder. Lane 2: Wild-type CGMMV. Lane 3-7: Chimeric CGMMV. **B.** Sequence alignment of chimeric virus. The insert sequence of NEcmv was detected in all of infiltrated cucumber plants at 45 dpi.

In this study, CGMMV was utilized as a viral expression system for peptide display on the surface of CGMMV capsids, by the readthrough strategy. Several 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. 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 plants. In this study, I modified the full length cDNA of CGMMV-ON to express the NE of PRRSV GP5 in cucumber. In my study, *in vitro* transcription was eliminated and agroinfiltration was exploited as a one-step, low-cost inoculation method. The infectivity rate was 100% of infiltrated cucumber plants. These data suggests that this vector may be inoculated via agroinfiltration for scale-up production.

Systemic movement in infected host 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, expressing PRRSV-NE was designed to preserve the ability of wild-type CGMMV to spread systemically in cucumber. In this study, the NE was initially fused directly upstream of the CP stop codon. However, this chimeric CGMMV demonstrated inefficient systemic movement in both cucumber and *N. benthamiana* (Figure 3.4), probably due to the incompetence of virus assembly. CGMMV long distance movement is through the xylem or phloem in the form of virus particles (Moreno et al. 2004; Simón-Buela and García-Arenal 1999) which requires an intact CP. Direct fusion of the nine-amino-acid NE to CP may impair CP structure and disrupt CP assembly, thus, leading to the failure of systemic infection as previously described with TMV (Dawson et al. 1989; Saito et al. 1990; Takamatsu et al. 1990).

To maintain systemic virus movement and express NE on CP, I exploited ribosomal readthrough translation in my second approach. Several families of plant viruses including tobamoviruses, luteoviruses, benyviruses and tombusviruses have been reported to use the stop codon readthrough strategy to regulate gene expression (Harrell et al. 2002). For example, a 126-kDa protein was translated from the first open reading frame in the sequence of TMV, and a 183-kDa protein was encoded by an extension of the first open reading frame

through an in-frame UAG stop codon. By using this readthrough translation, TMV controlled the ratio of 126-kDa and 183-kDa synthesis, which plays a critical role as helicase and RdRp in viral replication, at a relative level of approximately 20 to 1 (Skuzeski et al. 1991). CGMMV also possesses this mechanism for RdRp synthesis. However, the essential sequence required for readthrough of CGMMV, “AAA-UAG-CAA-UUA”, was distinct from that of TMV, “CAA-UAG-CAA-UUA”, and using the TMV readthrough sequence could not produce readthrough translation of CGMMV in musk melon (Teoh et al. 2009). In this study, two sequences named RT1 and RT2, were used to express NE and NEcmv, 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 in cucumber, in contrast RT2, “TCC-AAA-TAG-CAA-TTA” successfully led to expression of the readthrough protein CP-NEcmv (Figure 3.7A, B and C). Moreover, the construct pCG-RT1NE could establish systemic infection in both cucumbers and *N. benthamiana* (Figure 3.5), whereas pCG-RT2NEcmv could infect cucumber only (Figure 3.6). These results suggest that the minimal context of leaky stop codon in CGMMV should be extended to “UCC-AAA-UAG-CAA-UUA” for an efficient readthrough translation, and that the infectivity of chimeric virus is dependent on the foreign peptides introduced on CGMMV CP, especially their sizes.

Despite the intense research on peptide-displaying TMV, there are limited reports on the exploitation of CGMMV for antigen presentation. In previous studies, the level of chimeric CGMMV containing a HBsAg or a truncated dengue virus type 2 envelope protein binding region (EB4), was estimated at 50% of the total virus particles produced in musk melon at 14 dpi (Ooi et al. 2006; Teoh et al. 2009). In this study, the chimeric CP containing NEcmv was measured at 35.84 µg/g of fresh weight (0.067% of TSP) in cucumber at 21 dpi, and the ratio of chimeric CP to wild-type CP was approximately 1:20 (Figure 3.7B). In the case of TMV, the amount of TMV CP with ACEI, a 12 amino acid peptide which has anti-hypertensive effects in humans, was 100 µg/g fresh weight of leaf tissue in tobacco and tomato, with a readthrough efficiency of 5% (Hamamoto et al. 1993). The readthrough efficiency of TMV in tobacco has been reported at 5-10% (Skuzeski et al. 1991; Sugiyama et

al. 1995). Therefore, translation via a leaky stop codon of CGMMV in cucumber occurred at a similar rate to TMV as described previously, and that the expression of chimeric CGMMV in musk melon may be higher than in cucumber, since readthrough mechanism is host-dependent (Ooi et al. 2006). The stability of a virus-based expression vector also plays a critical role in yield determination. Because viruses have a significant rate of mutation and recombination via replication, a foreign gene is usually deleted from the virus genome and, as a result, chimeric viruses will revert back to the wild type. Chimeric CGMMV, which displayed 45 amino acids of EB4 on its surface, was found to lose the EB4 gene after 21 dpi (Teoh et al. 2009). In this study, no deletion was found in cucumber after 45 dpi, as shown by RT-PCR, DNA sequencing and western blot analysis (Figure 3.7D and Figure 3.9). These results indicate that the CGMMV-based vector is stable and can be used to express PRRSV-NE in cucumber for at least 6 weeks.

Viruses are a highly promising platform for antigen display. CGMMV possesses a symmetrical helical structure composed of about 2100 molecules of coat protein along its rod shape. Ideally, if each of these molecules is fused with one molecule of PRRSV-NE, each virion could carry approximately 2100 copies of PRRSV-NE on its surface. To further test if these chimeric virus particles displayed NE on their surfaces, as antigen carriers, immunoprecipitation and TEM were performed. The results confirmed that NE<sub>cm</sub> protruded outward from the CGMMV virion, together with the C-terminal portion of coat protein and was able to bind to anti-cmyc beads, leading to the formation of immune complexes, as shown in Figure 3.8. These results are consistent with other reports on TMV or CGMMV (Ooi et al. 2006; Sugiyama et al. 1995; Turpen et al. 1995). The multivalent presentation of surface PRRSV-NE on chimeric CGMMV has the potential to enhance immune response and, simultaneously, enable exceptionally high payload capacities. Indeed, chimeric CGMMV expressing HBsAg was able to stimulate specific antibody production by lymphocytes *in vitro* (Ooi et al. 2006). Virus-like particles displaying NE and other T-epitopes of PRRSV were reported to be capable of neutralizing viruses in MARC 145 cells (Murthy et al. 2015). Further *in vivo* experiments are needed to examine the ability of the chimeric CGMMV-NE<sub>cm</sub> as an effective vaccine against PRRSV.

### ***3.5 Conclusion***

In summary, the infectious clone of CGMMV-ON was successfully generated and modified to express PRRSV-NE on the surface of chimeric CGMMV. The infectious clone, itself, was able to infect several cucurbit crop species, such as cucumbers, watermelons and cantaloupes and the experimental host *N. benthamiana*. However, the expression clone could only produce the fusion protein in its natural host, cucumber, via readthrough translation. Chimeric CGMMV presenting NEcmyc on CP surface was quantified to be 35.84  $\mu\text{g/g}$  fresh weight of leaf tissue (0.067% of TSP) at 21 dpi. The foreign gene and peptide were detected in cucumber until 50 dpi. These data demonstrate that the CGMMV vector holds great promise to expression an edible vaccine against PRRSV in pigs.

**Table 3.1** Primers used to construct the CGMMV infectious clone and CGMMV-based expression vectors

<b>Name</b>	<b>Sequence (5'-3')</b>
<b>CGMMV5UTR-F</b>	GTTTAAATTTTATAATTAACAACAAC
<b>CGMMV3UTR-R</b>	TGGGCCCTACCCGGGAAA
<b>M13 reverse</b>	CAGGAAACAGCTATGAC
<b>M13 forward</b>	GTTTTCCAGTCACGAC
<b>CGMMVR3-R</b>	CCTCTCTCGAATGGTCCCAGTGTA
<b>SacI-35S-F</b>	ATACGAGCTCAAGCTTGCATGCCTGCAGGT
<b>SpeI-StuI-35S-R</b>	ATACACTAGTAGGCCTCTCCAAATGAAATG
<b>BamHI-RzT1-F</b>	ATCCGGATCCTAGAGGATCCGATACCCTGT
<b>KpnI-RzT1-R</b>	ATACGGTACCTACGCCAGCTGGCGAAAGGG
<b>BamHI-AgeI-R</b>	ATCCGGATCCTCGACCAGGGCCCCTTTA
<b>CGMMVAgeI-F</b>	AACGTACCGTTGATCAGCT
<b>BamHI-3UTR-R</b>	ATCCGGATCCTGGGCCCTACCCGGGG
<b>CGMMVMP-F</b>	ATGTCTCTAAGTAAGGTGTC
<b>CGMMVMP-R</b>	CTAGGTGTGATCGGATTGTA
<b>NheI-NE-F</b>	CGCCGCTAGCTCACAACCTCCAGAGTATCTACAATCTGT AGCTTCGAGGGTCTTCTGATGG
<b>HindIII-AmberNE-F</b>	TCGAAAGCTTCTAAATAGCAATTATCACAACCTCCAGA GTATCTACAATCTGCAACCATAGCTTCGAGGGTCTTCT GATGG
<b>HindIII-NEmycC-F</b>	CCAAGCTTCCAAATAGCAATTAATGTCACAACCTCCAG AGTATCTACAATCTGGAACAAAAGTTGATCTCTGAGG AAGACCTTAGCTTCGAGGGTCTTCTGATGG
<b>Actin-F</b>	GGGATGTGAAGGAGAA GTTGGC
<b>Actin-R</b>	ATCAGCAATGCCCGGGAACA

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## Chapter 4

# Construction of a PepMV-based expression vector and its application for vaccine production against PRRSV

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## 4.1 Introduction

*Pepino mosaic virus* (PepMV) is a member of the genus *Potexvirus* in the family *Alphaflexiviridae*. It was first isolated from field pepino (*Solanum muricatum*) in 1974 in Peru (Jones et al. 1980). Since then, it has been found in Europe (Cotillon et al. 2002; Hasiow-Jaroszewska et al. 2009; Pagan et al. 2006; Roggero et al. 2001; van der Vlugt et al. 2000), North and South America (Ling et al. 2008; Ling 2007; Maroon-Lango et al. 2005), South Africa (Carmichael et al. 2011), and Asia (Ge et al. 2013). In Canada, the virus was first detected in tomatoes (*Solanum lycopersicum*) in southern Ontario greenhouse during the winter of 2000 (French et al. 2000). Phylogenetic analysis revealed five genotypes of PepMV, i.e. European (EU), Peruvian (LP), southern Peruvian (PES), American (US1) and Chilean 2 (CH2), that differ each other in the genomic sequence and host range (Hanssen and Thomma 2010; Minicka et al. 2016; Moreno-Perez et al. 2014). The virus has a filamentous shaped virion of approximately 508 nm in length (Jones et al. 1980). The genome of PepMV consists of a positive, single-strand RNA of approximately 6.4 kb and contains five open reading frames (ORFs), which encode a 164-kDa RNA-dependent RNA polymerase (RdRp), triple gene block proteins (TGB) of 26, 14, and 9 kDa (TGBp1, 2, and 3 in abbreviation, respectively) and a 25-kDa coat protein (CP) (Aguilar et al. 2002). RdRp is the only viral protein required for virus replication. All TGB as well as the CP are involved in viral movement and the development of symptoms. In addition, TGB1 also functions as an RNA silencing suppressor and RNA helicase. Like other potexviruses, RdRp is expressed from the genomic RNA, while TGB and CP are translated from subgenomic RNAs. In general, the host range of PepMV is restricted to plants in the family *Solanaceae*, e.g. tomato, potato, eggplant and *Nicotiana benthamiana* (Blystad et al. 2015; Minicka et al. 2016). When entering host cells, the virus can replicate rapidly, resulting in a high level of viral proteins within its host (Minicka et al. 2015). This outstanding expression ability makes PepMV a promising candidate to be a virus-based expression system in solanaceous plants, particularly *N. benthamiana*.

Transient expression systems are a versatile platform, using *A. tumefaciens* to deliver genes of interest into plant leaves in order to produce recombinant proteins within a few days.

Currently, this system has been a popular and powerful tool, with the ability of upgrading to large scale production of desired proteins when applied in *N. benthamiana* (Hefferon 2013; Lomonosoff and D'Aoust 2016). *N. benthamiana* belongs to the genus *Nicotiana*, in the family *Solanaceae*. The plant was first identified and collected in Australia about 160 years ago. However, interest in this species has just recently accelerated in both research and application fields (Goodin et al. 2008). *N. benthamiana*, a widely used laboratory model plant, has become one of a few most popular plant species in molecular farming, due to several factors. Firstly, it is very susceptible to agroinfiltration, which facilitates conventional transient expression. Secondly, the plant is easily grown either in controlled growth chambers, for research, or in greenhouses, for industrial purposes. It is also a common host of many pathogens such as viruses including PepMV, bacteria and fungus. Moreover, many reports have shown that *N. benthamiana* is able to produce protein bodies and increase the level of recombinant proteins within a short time, when exploited for transient expression with viral vectors (Goodin et al. 2008; Peyret and Lomonosoff 2015; Twyman et al. 2003). Given these advantages, *N. benthamiana* was chosen as a bioreactor for protein expression in this study.

Polyprotein or proteolytic processing is a crucial expression strategy for many viruses, in order to produce different functional proteins from a polycistronic genome. The viral polyprotein is cleaved by viral proteinase(s) to create individual mature proteins. The 2A protein derived from *Foot-and-mouth disease virus* (FMDV-2A or F2A), consisting of 18 amino acids (-LLNFDLLKLAGDVESNPG-), is an autonomous element that mediates a co-translational self-cleavage at its own C-terminus (Donnelly et al. 2001; Ryan et al. 1991). This cleavage site is located between the last glycine of the F2A and the first proline of the downstream F2B protein. Due to this autonomous self-cleaving ability, F2A has been widely employed to perform an artificial “self-cleavage” and, hence, to release co-expressed multiple proteins encoded by the same expression cassette in plant cells (Luke et al. 2015).

In this work, a PepMV-based vector was constructed using an Ontario PepMV isolate (PepMV-ON) and was used for transient expression of recombinant proteins in *N. benthamiana*. A reporter gene, the green fluorescent protein (*GFP*) and an ectodomain of

PRRSV GP5-fused with GFP (designated as *GP5e-GFP*) were tested using this viral vector. Since PRRSV infects pigs worldwide and causes tremendous losses in the swine industry, my goal was to produce GP5e-GFP as a plant-made subunit vaccine against PRRSV.

## ***4.2 Materials and methods***

### **4.2.1 Construction of PepMV-based expression clones**

The full-length cDNA of a PepMV isolate (PepMV-ON), obtained from infected tomatoes in an Ontario greenhouse, was amplified by RT-PCR and inserted into a binary vector, pGR 107 (Jones et al. 1999), to generate the full-length cDNA clone, pGR-PepMV-ON-3. All the PepMV-based vectors were constructed using standard restriction enzyme digestion and fusion PCR methods. First, a fragment, which included 36 nucleotides (nt) of CP sequence and the multi-cloning sites *ScaI*, *XhoI*, *XmaI* and *SmaI*, was amplified by primers of *SfoI*-*XmaI*-CP36R and *BbvCI*-TGB2-F using pGR-PepMV-ON-3 as a template. The resulting amplicon was digested with *SfoI* and *BbvCI* and then introduced into digested pGR-PepMV-ON-3, via the same restriction enzyme sites, to create pGR-Pep-36CP. To generate pGR-Pep-F2A, the resulting amplicon following fusion PCR with primers *XmaI*-F2A-CP-F and *SfoI*-TDNA, was inserted into pGR-Pep-36CP via *XmaI* and *SfoI*. The primer *XmaI*-F2A-CP-F contains the sequence of the 2A self-cleaving peptide of FMDV. To construct pGR-Pep-GFP, the *GFP* was amplified using primers *ScaI*-GFP-F and *XmaI*-myc-R, with pCaMGateER-GP5ectoGFP (kindly provided by Hong Zhu from Dr. Rima Menassa's lab) as a template, and then introduced into digested pGR-Pep-F2A via *ScaI* and *XmaI* sites. GFP is the enhanced GFP, which was described previously (GenBank: U55762.1) (Cormack et al. 1995). GP5e-GFP was amplified using primers *ScaI*-GP5e-F and *XmaI*-myc-R and inserted into digested pGR-Pep-F2A by the same method, to create pGR-Pep-GP5e-GFP. PCR was performed using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Pickering, Ontario, Canada) and the following cycle: (1) a pre-denaturation at 98°C for 3 min; (2) 25 cycles of denaturation at 98°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 90 sec; and (3) an extra 5 min of extension at 72°C. All the primer sequences are listed in Table 4.1. The resulting constructs were confirmed by PCR and DNA sequencing.

#### 4.2.2 Agroinfiltration of *N. benthamiana* with PepMV-ON-derived clones

Agrobacterium transformation was performed, as described in 2.2.5. Agroinfiltration was carried out on two expanded leaves of *N. benthamiana*, as previously described in 2.2.10. However, the bacterial strain used was *A. tumefaciens* GV3101 containing the pSoup plasmid (Hellens et al. 2000). Transformed bacteria were selected and cultured in LB medium with kanamycin (50 µg/ml), rifampicin (50 µg/ml) and tetracycline (10 µg/ml) at 28°C. Following transformation, bacteria recovered in a 28°C incubator for 2 hr, before being plated on selection media.

For infectivity testing of the PepMV infectious clone, young seedlings of *N. benthamiana* were agroinfiltrated with 1 ml of suspension into two leaves at 3-4 weeks of age (Yoon et al. 2011). A total of six seedlings were used. Infectivity was identified by either DAS-ELISA or RT-PCR at 15 dpi, using systemically infected leaves.

For protein expression, plant materials, agroinfiltration and detection were essentially as described above. For co-infiltration of p19 (Silhavy et al. 2002) and pGR-Pep-GP5e-GFP, suspensions of agrobacteria containing each construct were adjusted using agroinfiltration buffer to obtain a final density of OD<sub>600</sub> and then mixed together in a 1 to 1 ratio. The resulting suspension was infiltrated into *N. benthamiana* leaves, as described above.

#### 4.2.3 RT-PCR

Total RNAs were extracted from young leaves using TRIzol (Invitrogen, Burlington, Ontario, Canada) and treated with DNase I (Invitrogen). First-strand cDNA was generated by reverse-transcription reactions using Oligo dT and SuperScript III Reverse Transcriptase (Invitrogen). To determine the infectivity of pGR-PepMV-ON-3, RT-PCR was conducted to amplify the sequence of PepMV TGB and CP, using coupled primers Pep5-F and Pep6-R. Internal control with actin was carried out using Actin-F and Actin-R. To determine the infectivity of expression constructs, including pGR-Pep-GFP and pGR-Pep-GP5e-GFP, RT-PCR was performed using primers TGB3-F and GFP-R and systemically infected leaves collected at 15 dpi. PCR was performed using 2X Taq FroggaMix (FroggaBio, Toronto, ON,



Canada) with the following cycles: (1) a pre-denaturation at 94°C for 3 min; (2) 32 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 90 sec; and (3) an extra 10 min of extension at 72°C. All the primer sequences are described in Table 4.1.

#### 4.2.4 Visualization of green fluorescence with a UV lamp

A handheld UV lamp was used to detect green fluorescence in plants infiltrated with constructs containing GFP in a dark room, as previously described in 2.2.13.

#### 4.2.5 Confocal microscopy

To visualize fluorescence at the cellular level, confocal laser scanning inverted microscopy was employed as describe in 2.2.14.1.

#### 4.2.6 Protein extraction and quantification

To analyze prote in production in *N. benthamiana*, western blot analysis was performed using infiltrated leaves. Three leaf discs (3 mm in diameter) were sampled for a time-course experiment set at 3 to 9 dpi, and homogenized in liquid nitrogen using 2.3 mm ceramic beads (BioSpec Inc., Bartlesville, USA) and a TissueLyser II (Qiagen). Extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 0.1% (v/v) TritonX-100, 5 mM dithiothreitol, protease inhibitor) of 250 µl was added to the homogenized leaf tissue to obtain the total soluble protein (TSP) from ground leaves. Protein extraction using a p19 infiltrated plant and a PepMV infiltrated plant was also performed under the same conditions, as negative controls. Total soluble protein concentration was determined by Bradford assay (Bradford, 1976), with bovine serum albumin as a standard, using an iMark Microplate Reader (Biorad). Protein extract was separated by electrophoresis in a 12% (w/v) sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Biorad, Hercules, USA). The primary antibody, either anti-GFP monoclonal antibody produced in mouse (Sigma-Aldrich Oakville, ON, Canada) or anti-PepMV CP polyclonal antibody produced in rabbit (Agdia, Elkhart, IN, USA), was incubated with the membrane. The membrane, with the primary antibody produced in mouse, was

incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich), whereas, the membrane with primary antibody produced in rabbit was incubated with HRP-conjugated goat anti-rabbit IgG antibody (Sigma-Aldrich). Western blots were visualised using Immobilon Western (Millipore Corporation, Billerica, MA, USA), following the manufacturers' protocol. The resulting images were analysed with Total Lab TL100 software (Nonlinear Dynamics, Durhan, USA) by comparing the densitometry of bands with known amounts of a standard GFP fused with hydrophobin-I (GFP-HFBI). Three biological replicates were performed for each time-course extraction.

### **4.3 Results**

#### **4.3.1 Infectivity of infectious clone**

The full-length PepMV cDNA clone pGR-PepMV-ON-3 (GenBank accession number: KY031324), was constructed using a Canadian PepMV isolated from infected tomatoes in Ontario greenhouses (Figure 4.1A). This isolate showed approximately 99% similarity to the American genotype US1 and the clone pGR-PepMV-ON-3 was infectious and induced similar symptoms on tomato as its virus (Wu and Wang unpublished data). The natural host range of PepMV is limited within the family of *Solanaceae* (Minicka et al. 2016). To test if this isolate can infect *N. benthamiana*, the infectious clone was introduced into *N. benthamiana* leaves via agroinfiltration. RT-PCR was performed to amplify TGB of PepMV. Results confirmed that the clone was infectious, with a 100% infection rate (Figure 4.1B). Viral symptoms began to appear at 7-8 dpi. Infected plants showed mild mosaic, leaf blistering and yellow spots in newly emerged leaves (Figure 4.1C). With the ability to induce fast infection, this clone was an eligible candidate for the construction of a transient expression vector in *N. benthamiana*.

#### **4.3.2 Construction of PepMV-based expression vectors**

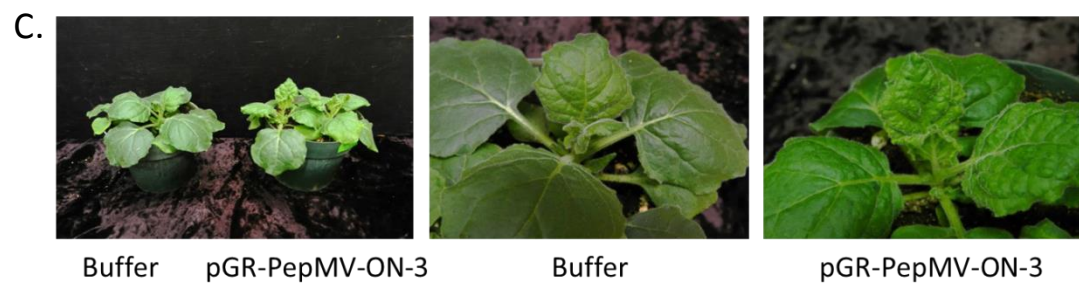
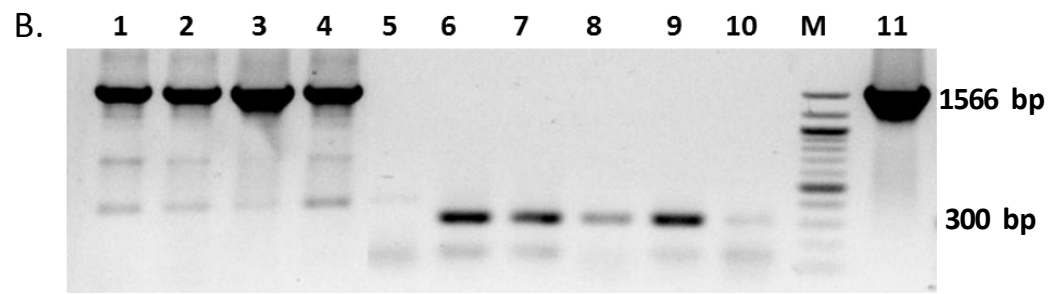
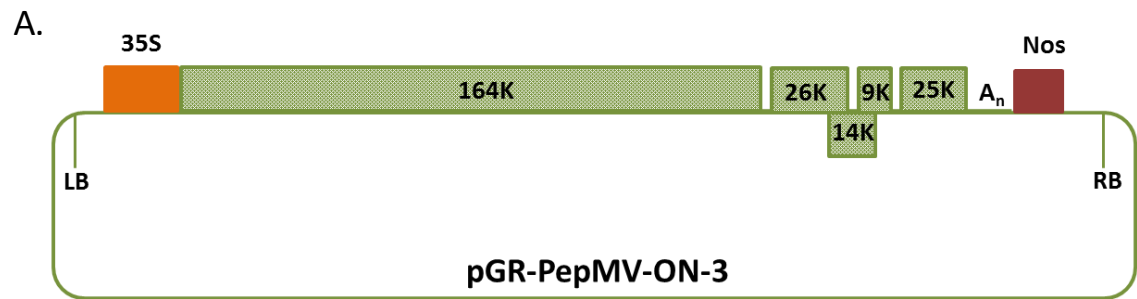
To construct a PepMV-based expression vector, the 2A self-cleaving peptide of FMDV (F2A) (Kim et al. 2011), NFDLLKLAGDVESNPGP, was inserted after the putative sub-genomic promoter of CP (Sempere et al. 2011). Together with F2A, a multi-cloning site

(MCS) including *ScaI*, *XhoI*, *XmaI* and *SmaI* was also fused to the N-terminal of F2A to generate pGR-Pep-F2A. In order to maintain the integrity of CP, the first 36 nucleotides of the *CP* gene was repeated immediately after the MCS (Figure 4.2A). *GFP* was inserted via *ScaI* and *XmaI* restriction sites into pGR-Pep-F2A to construct pGR-Pep-GFP (Figure 4.2B). When this vector is introduced into plant cells, GFP would be translated as a 12aaCP-GFP-F2A-CP fusion which simultaneously undergoes auto-proteolysis by F2A to produce two proteins, GFP-F2A with 12 amino acids of the N-terminal CP (12aaCP-GFP-F2A) and CP. The chimeric gene *GP5e-GFP* was also inserted into pGR-Pep-F2A, via *ScaI* and *XmaI* in the MCS, to create pGR-Pep-GP5e-GFP (Figure 4.2C). The expected translation product for this viral expression vector would be processed into GP5e-GFP-F2A with 12 amino acids of the N-terminal CP (12aaCP-GFP5e-GFP-F2A) and CP.

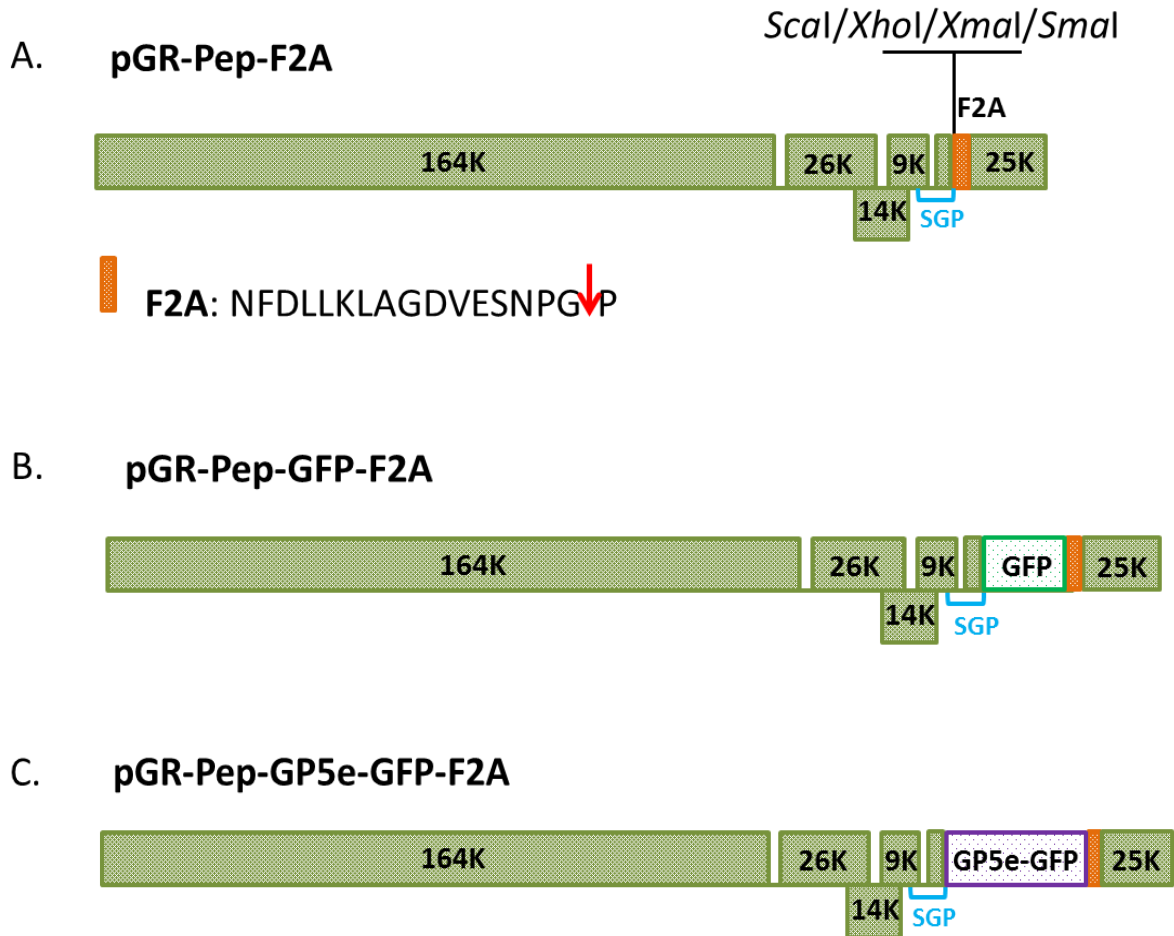
#### 4.3.3 Agroinfiltration of *N. benthamiana* with pGR-Pep-GFP

pGR-Pep-GFP was transformed into *A. tumefaciens* GV3101 via electroporation and delivered in 4-week-old *N. benthamiana*. Following agroinfiltration, infiltrated leaves showed mild green fluorescence, when illuminated by a handheld UV lamp, at 2 dpi (Figure 4.3A). Intense green fluorescence was visualized in the infiltrated leaves at 4 dpi (Figure 4.3A). At 8 dpi, green fluorescence appeared strongly in newly emerging leaves, as a result of viral systemic movement (Figure 4.3A). Symptoms caused by PepMV infection arose in these infiltrated plants with mild mosaic and bubbling leaves at 8 dpi. No symptoms and no green fluorescence were detected in the plants infiltrated with buffer during the observation period (Figure 4.3A).

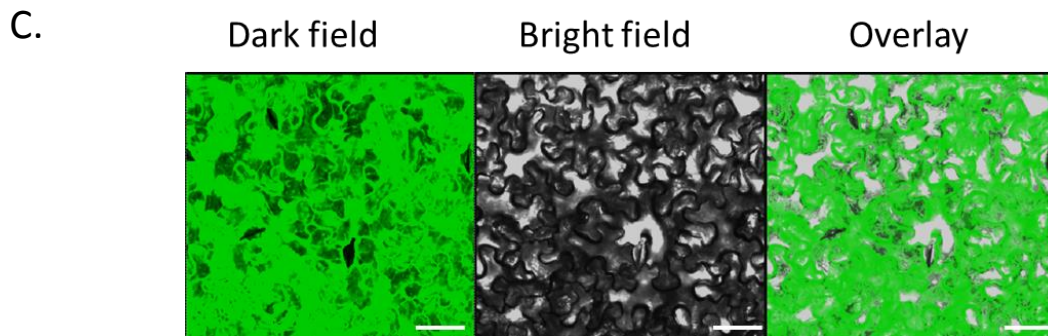
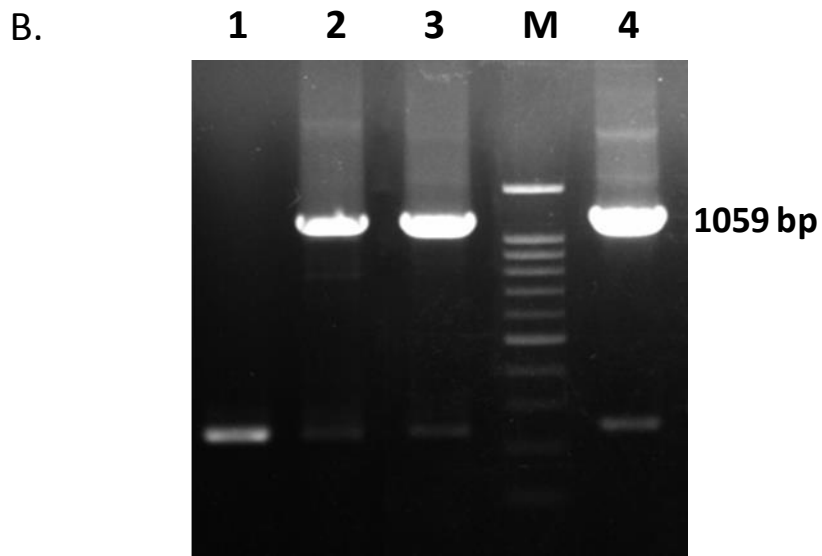
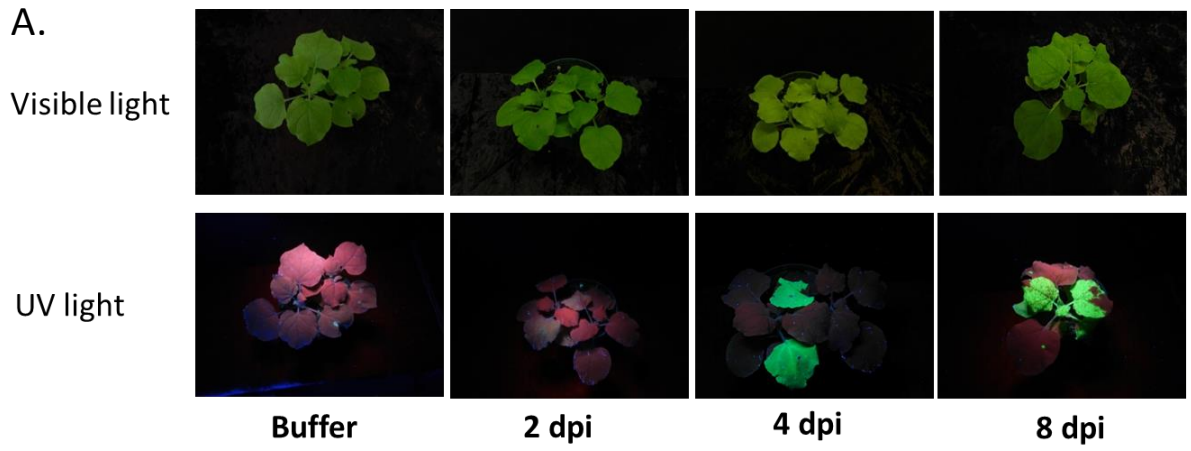
To further confirm GFP expression in the upper newly emerging leaves of the agroinfiltrated plants, RT-PCR and confocal laser scanning microscopy were performed. RT-PCR detected a 1059-bp band of TGB3 and GFP in the upper leaves at 15 dpi (Figure 4.3B) Confocal images showed intense green fluorescence in these leaves at 9 dpi (Figure 4.3C). Taken together, these results affirmed the ability of the recombinant virus derived from pGR-Pep-GFP to move long-distance and establish systemic infection (Figure 4.3B and C).



**Figure 4.1** Schematic representation of the PepMV infectious clone, pGR-PepMV-ON-3 (not drawn to scale). **A.** Characteristics of the T-DNA plasmid harboring the PepMV genome used for agroinfiltration. Single lines and open box represents noncoding and coding regions, respectively. Polycistronic PepMV RNA encodes for a 164-kDa protein (164K), triple gene block (26K, 14K and 9K) and a 25-kDa coat protein (25K). At the 5' end, the nucleotide sequence of the double *CaMV* 35S promoter (35S) is indicated with an orange box, whereas, the 3' poly A tail is indicated with A<sub>n</sub>. The nopaline synthase terminator (*Nos*) is presented by a brown box at the downstream end of the 3' tail. The LB stands for left border and RB for right border. **B.** RT-PCR results from PepMV infected *N. benthamiana* at 15 dpi. The bands of TGB3-CP, and actin are 1566 bp, and 300 bp, respectively. Lane 1-4: pGR-PepMV-ON-3-infiltrated plants. Lane 5: Buffer infiltrated plant. Lane 6-9: Actin control from pGR-PepMV-ON-3 infiltrated plants. Lane 10: Actin control from buffer infiltrated plants. Lane M: 100 bp DNA marker. Lane 11: Positive control from PepMV-infected plant. **C.** Symptoms of infected plants at 15 dpi.



**Figure 4.2** Schematic representation of expression vectors containing the 2A peptide of *Foot-and-mouth disease virus* (F2A) (not drawn to scale). F2A peptide has amino acid sequence as LLNFDLLKLAGDVESNPG. **A.** pGR-Pep-F2A contains a multi-cloning site (*ScaI/XhoI/XmaI/SmaI*) and F2A which are inserted between the putative subgenomic promoter (SGP) of coat protein (CP) and CP (25K). F2A is represented by an orange box. The cleavage site is located between glycine (G) and proline (P), and designated by a red arrow. **B.** Green fluorescent protein gene (*GFP*) was inserted between the *SGP* and *F2A*, by restriction enzyme digestion (*ScaI* and *XmaI*) and ligation to generate pGR-Pep-GFP. **C.** The sequence containing the neutralizing epitope of glycoprotein 5 (*GP5e*) was fused with *GFP* to create *GP5e-GFP*. Subsequently, *GP5e-GFP* was placed between the *SGP* and *F2A* using the same method as pGR-Pep-GFP, to create pGR-Pep-GP5e-GFP.



**Figure 4.3** *N. benthamiana* infiltrated with pGR-Pep-GFP. **A.** A time-course observation of pGR-Pep-GFP infiltrated plants under visible and UV light at 2, 4, 8 dpi, respectively, and buffer infiltrated plant as a negative control. **B.** RT-PCR results from pGR-Pep-GFP infiltrated plants at 15 dpi. The band of TGB3-GFP is 1059 bp. Lane 1: Negative control using buffer infiltrated leaf. Lane 2-3: pGR-Pep-GFP infiltrated plants. Lane M: 100 bp DNA marker. Lane 4: Positive control using pGR-Pep-GFP. **C.** Observation of fluorescence in newly emerged leaves by confocal microscopy. Scale bar 68  $\mu\text{m}$ .



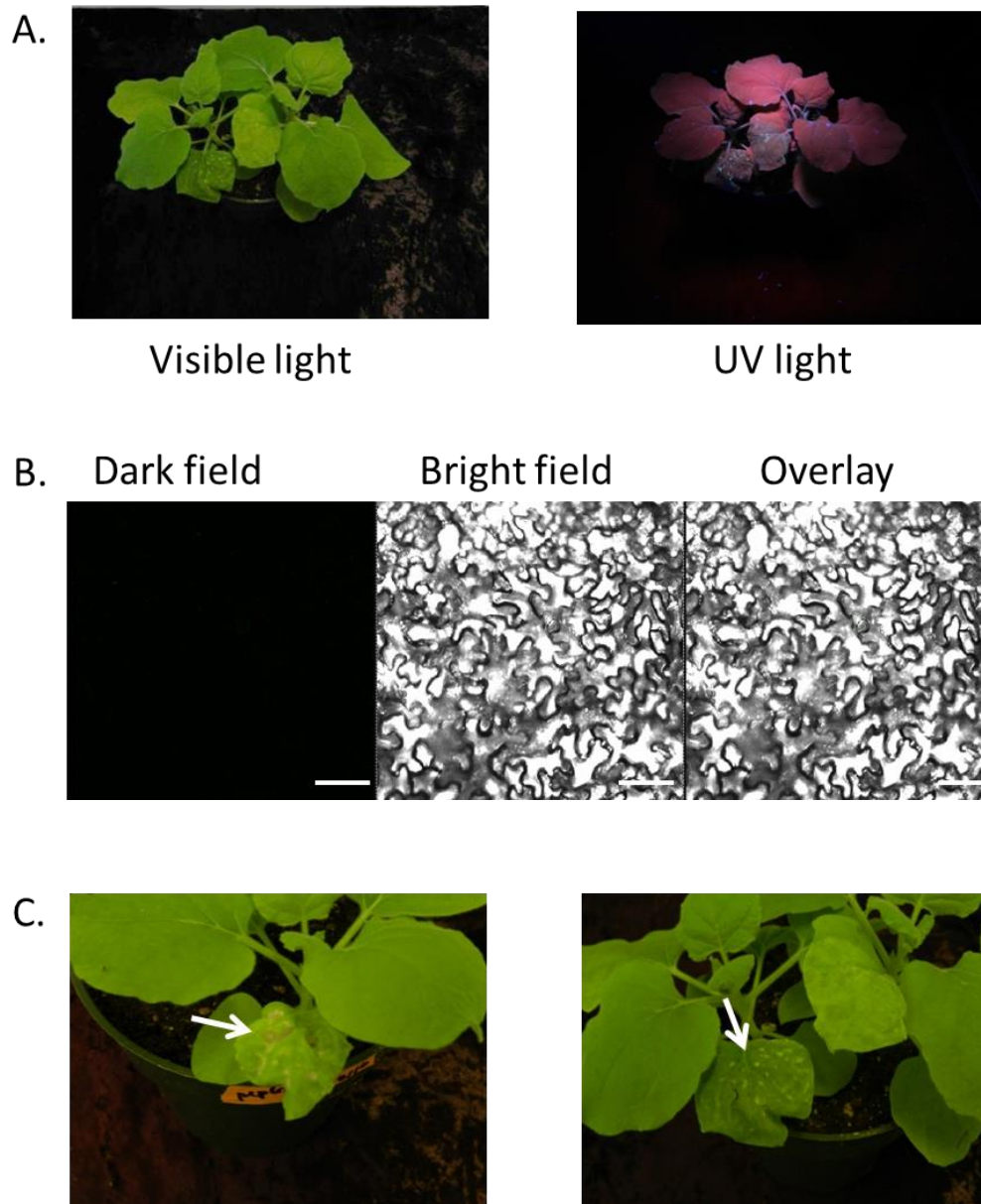
#### 4.3.4 Agroinfiltration of pGR-Pep-GP5e-GFP

##### 4.3.4.1 No expression without p19 co-infiltration

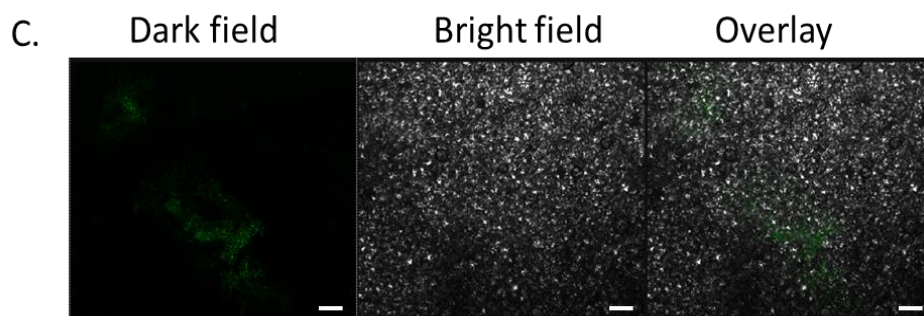
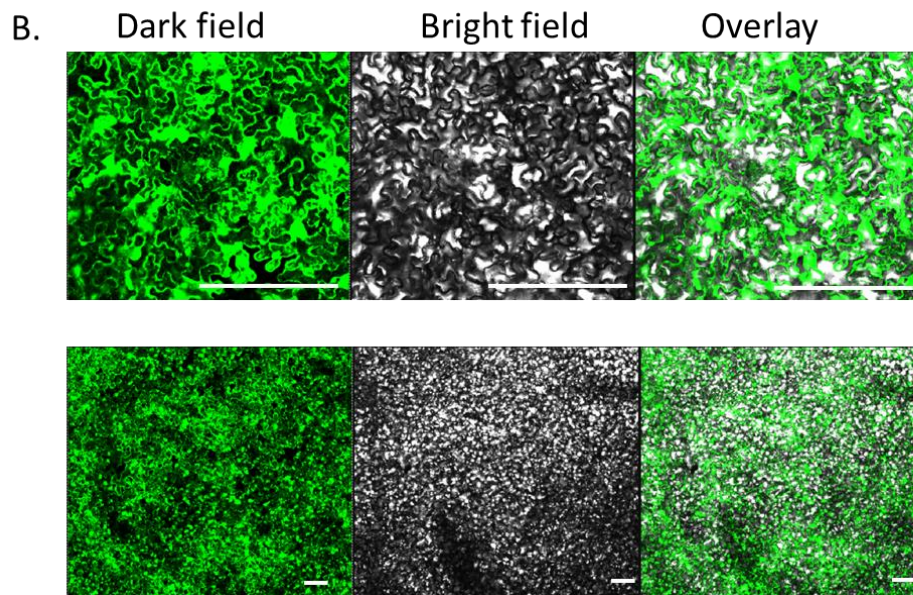
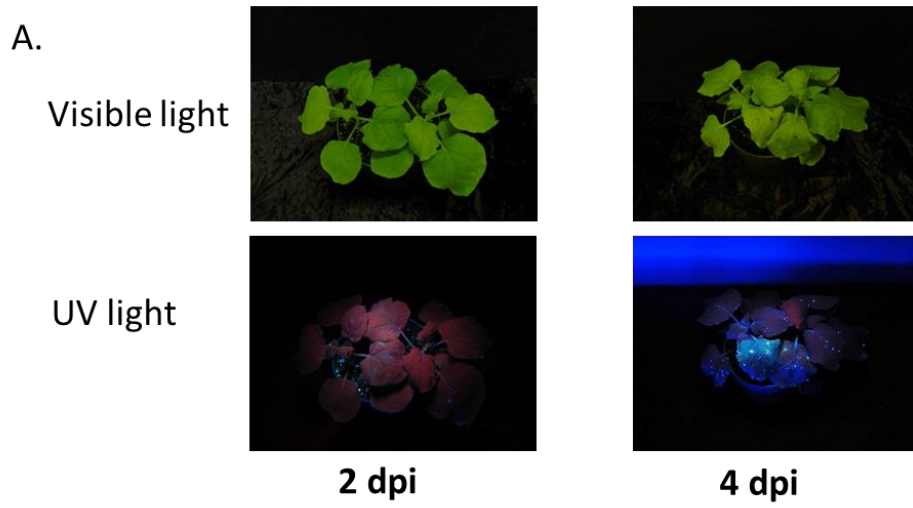
Encouraged by the GFP expression results, I decided to use the same method to express the GP5 epitope fused with GFP (GP5e-GFP) in *N. benthamiana*. The pGR-Pep-GP5e-GFP was introduced into *Agrobacterium* and infiltrated into *N. benthamiana* leaves. Unexpectedly, green fluorescence was hardly observed in both the infiltrated or systemic leaves up to 9 dpi, using either a handheld UV lamp or confocal laser scanning microscopy (Figure 4.4A and B). Furthermore, necrosis appeared early, at 4-5 dpi, in the infiltrated areas and had spread to the whole leaf at 7 dpi in plants agroinfiltrated with pGR-Pep-GP5e-GFP (Figure 4.4C). On the other hand, no necrosis was observed in mock-infiltrated plants (with buffer only) (Figure 4.3A).

##### 4.3.4.2 Positive GFP signals observed in plants co-infiltrated with pGR-Pep-GP5e-GFP and p19

Previous studies reported that co-expression of a suppressor of post-translational gene silencing (PTGS) could increase the accumulation levels of exogenous proteins (Conley et al. 2009; Liu and Kearney 2010). Thus, a binary vector, harboring the silencing suppressor, p19 of *Cymbidium ringspot tobusvirus* (CymRSV) (Silhavy et al. 2002), was agro-infiltrated with pGR-Pep-GP5e-GFP into *N. benthamiana*. Mild green fluorescence was observed at 2 dpi and increased at 4 dpi (Figure 4.5A). Necrosis appeared at 8 dpi, which was similar to pGR-Pep-GFP on the infiltrated leaves. To further investigate the influence of p19 on GP5e-GFP expression, infiltrated and the upper new leaves were examined under a confocal laser scanning microscope. The results revealed an abundance of *N. benthamiana* cells expressing GFP in infiltrated leaves (Figure 4.5B). However, only very weak GFP signals were detected in the upper leaves (Figure 4.5C). These data suggest that the silencing suppressor, p19, could increase the expression of GP5e-GFP in infiltrated leaves only and that GP5e-GFP was toxic to the plants which could result in necrosis.



**Figure 4.4** Expression of GP5e-GFP without p19 co-infiltration. **A.** Fluorescence observation at 6 dpi under visible light and a handheld UV lamp. **B.** Fluorescence observation at 9 dpi using confocal microscopy. Scale bar 68  $\mu$ m. **C.** Necrosis in pGR-Pep-GP5e-GFP infiltrated leaves at 5-7 dpi. The white arrows point to necrosis leaves.



**Figure 4.5** *N. benthamiana* infiltrated with a mixture of pGR-Pep-Gp5e-GFP and p19. **A.** Fluorescence observed in infiltrated plants at 2 and 4 dpi under visible light and a handheld UV lamp. **B.** Fluorescence observed in infiltrated leaves at 6 dpi by confocal microscopy. Scale bar 68  $\mu\text{m}$ . **C.** Fluorescence detected in the secondary upper leaves at 9 dpi by confocal microscopy. Scale bar 68  $\mu\text{m}$ .

#### 4.3.5 Immunoblotting analysis of GFP and GP5e-GFP

To detect the presence of GFP and GP5e-GFP in infiltrated leaves, western blot analysis was carried out using anti-GFP antibody and anti-PepMV CP antibody at 4 dpi. 12aaCP-GFP-F2A or 12aaCP-GP5e-GFP-F2A was expected to be released from 12aaCP-GFP-F2A-CP and 12aaCP-GP5e-F2A-CP expressed in plants agroinfiltrated with pGR-Pep-GFP and pGR-Pep-GP5e-GFP, respectively. As shown in Figure 4.6A, a 25-kDa band of PepMV CP and an approximately 55-kDa band, which was possibly a processed product containing GFP-F2A-CP, were detected in both GP5e-GFP and GFP plants by the anti-PepMV CP antibody. Aside from this similarity, the patterns of GP5e-GFP and GFP expression were distinguished in the same blot. For pGR-Pep-GFP, the blot showed bands of 12aaCP-GFP-F2A-CP (~58 kDa) and 12aaCP-GFP-F2A (33 kDa). Meanwhile, a band of 12aaCP-GP5e-GFP-F2A-CP (~66 kDa) was shown for pGR-Pep-GP5e-GFP (Figure 4.6A). On the other hand, another blot probed by anti-GFP antibody revealed 12aaCP-GP5e-GFP-F2A (41 kDa) and 12aaCP-GP5e-GFP-F2A-CP (~66 kDa) in plants agroinfiltrated with pGR-Pep-GP5e-GFP, whereas, 12aaCP-GFP-F2A (33 kDa), and 12aaCP-GFP-F2A-CP (~58 kDa) appeared in pGR-Pep-GFP-infiltrated samples (Figure 4.6B). These detected proteins were not detected in p19-infiltrated plants (Figure 4.6A and B) or in pGR-PepMV-ON-3 infiltrated plants. Results indicate that fusion proteins were efficiently processed by the F2A protein to produce GP5e-GFP and GFP, despite a small amount of unprocessed fusions.

A time-course protein quantification from 3-9 dpi revealed that the accumulation of proteins of interest was optimal at 4-6 dpi. The level of GFP recombinant proteins including GFP-F2A fused with 12 amino acids from the N-terminus of CP (12aaCP-GFP-F2A) and 12aaCP-GFP-F2A fused with the entire CP (12aaCP-GFP-F2A-CP) was as high as 2.03 mg/g of fresh weight in pGR-Pep-GFP-infiltrated leaf tissue at 5 dpi. Specifically, 12aaCP-GFP-F2A and 12aaCP-GFP-F2A-CP accounted for 1.30 mg/g and 0.73 mg/g fresh weight of leaf tissue, respectively (Figure 4.7A). In contrast, GP5e-GFP recombinant proteins were estimated to be 0.036 mg/g of fresh weight in pGR-Pep-GP5e-GFP-infiltrated leaf tissue (Figure 4.7B), where the levels of 12aaCP-GP5e-GFP-F2A and 12aaCP-GP5e-GFP-F2A-CP were approximately 0.014 and 0.022 mg/g of fresh weight, respectively (Figure 4.7B). These

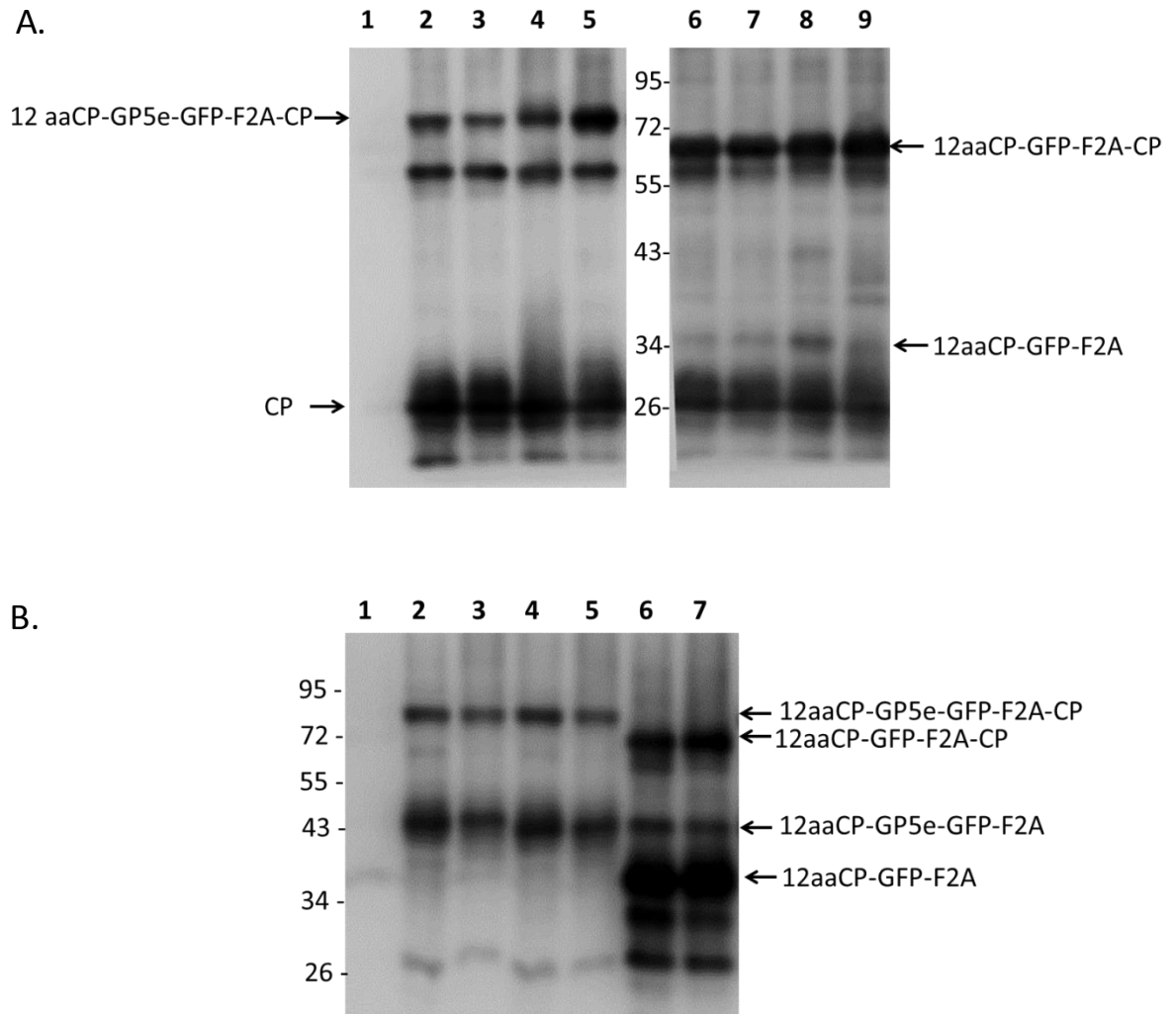
results confirmed that the PepMV-based expression vector could be used for protein expression within a short time period (5 days) in *N. benthamiana*. However, more refinements need to be made to enhance GP5e expression.

#### **4.4 Discussion**

In this study, an infectious clone was developed based on an Ontario greenhouse-tomato isolate of PepMV, pGR-PepMV-ON-3, and further modified to expression recombinant proteins in *N. benthamiana*. PepMV has diverse strains that significantly differ in host range and disease symptoms (Blystad et al. 2015; Duff-Farrier et al. 2015; Hasiow-Jaroszewska et al. 2011; Hasiow-Jaroszewska et al. 2010; Minicka et al. 2016; Sempere et al. 2016). Thus, pGR-PepMV-ON-3 was first examined and observed for the development of symptoms in *N. benthamiana*. Plants exhibited a 100% infection rate following agroinfiltration into young leaves. Compared to the European strain, PepMV-ON-3 caused moderate blistering and mosaic leaf symptoms in *N. benthamiana* at 7-8 dpi. These symptoms are easily observed and may serve as an indicator of infection. PepMV-ON-3 does not induce any local or systemic necrosis as a hypersensitive response of plants that may restrict viral spread and significantly reduce the yield of protein production (Aguilar et al. 2015; Dickman et al. 2001; Love et al. 2005). For instance, the Polish necrotic isolate of PepMV not only induce systemic necrosis on tomato but also on *N. benthamiana* (Minicka et al. 2015; Sempere et al. 2016). Thus, pGR-PepMV-ON-3 is a powerful viral vector for the development of a transient expression system in *N. benthamiana*.

To circumvent homologous recombination, a problem often associated with subgenomic promoter duplication which is needed for expression of the heterologous protein in viral vectors (Dawson et al. 1989; Donson et al. 1991; Zheng et al. 2015), I fused GFP or GP5e with a F2A peptide and inserted the chimeric gene in frame into the N-terminus CP to avoid addition of another copy of the CP promoter. F2A's co-translational self-cleavage could free CP from the fusion protein, which is needed for virion assembly and viral long distance movement. The F2A which I used in this study was from FMDV, belonging to the family *Picornaviridae*. Other 2A peptides, such as *Equine rhinitis A virus 2A* (E2A), *Porcine teschovirus-1 2A* (P2A), *Thosea asigna virus 2A* (T2A), *Cytoplasmic polyhedrosis virus 2A*

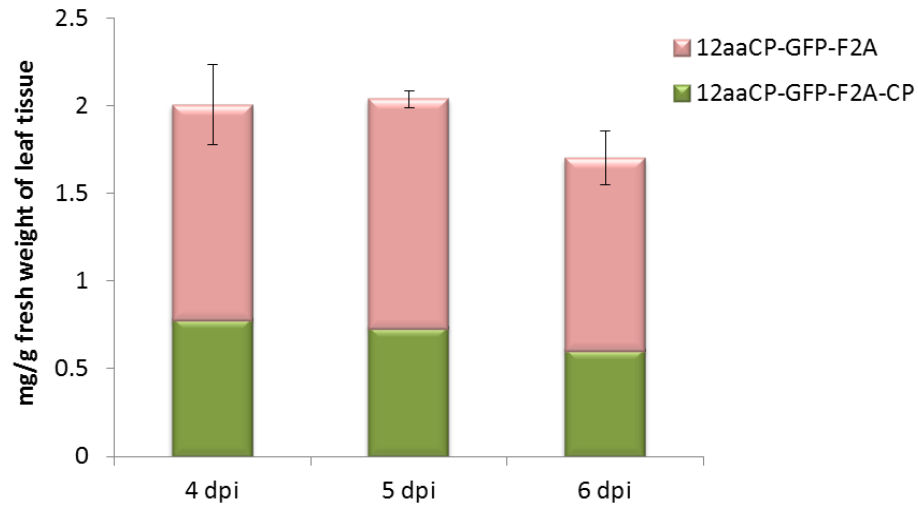
(BmCPV 2A), and *Flacherie virus* (BmIFV 2A), have also been characterized and applied in biological research (Kim et al. 2011; Wang et al. 2015). Cleavage efficiency of these peptides has been well-studied in different cell lines. In human cell lines, zebrafish embryos and mouse liver cells, P2A showed higher cleavage efficiency, when compared to F2A, E2A, and T2A (Kim et al. 2011). This was also the case in silkworm (*Bombyx mori*) where P2A carried a glycine-serine-glycine spacer (GSG), when compared to F2A, E2A, T2A, BmCPV 2A and BmIFV 2A (Wang et al. 2015). Nevertheless, a number of studies have demonstrated that F2A can efficiently process for multiprotein expression in plants (Sempere et al. 2011; Zhang et al. 2010). In this study, the F2A sequence was flanked by the first 36 nt sequence of CP and the entire CP sequence (without the start codon) in order to maintain optimal activity of the CP subgenomic promoter (Sempere et al. 2011). Western blot analysis showed that F2A functioned efficiently to process fusion proteins to release 12aaCP-GFP-F2A or 12aaCP-GP5e-GFP (Figure 4.6B), and viral CP (Figure 4.6A). Although a small amount of fusions (either 12aaCP-GFP-F2A-CP or 12aa-GP5e-GFP-F2A-CP) were detected, the systemic movement of the modified PepMV was preserved, as shown by GFP expression in newly emerged leaves (Figure 4.6A and Figure 4.3A). The level of GFP expression in infiltrated leaves was approximately 1.30 mg/g fresh weight in the form of 12aaCP-GFP-F2A, and 0.73 mg/g fresh weight of 12aaCP-GFP-F2A-CP at 5 dpi (Figure 4.7A), which is comparable to the pEAQ-HT expression system (Sainsbury et al. 2009). The results show that F2A works efficiently at protein cleavage in order to concurrently acquire a high level of GFP expression and maintain viral movement and that pGR-Pep-F2A can be exploited as a virus-based expression vector to produce recombinant proteins in *N. benthamiana*.



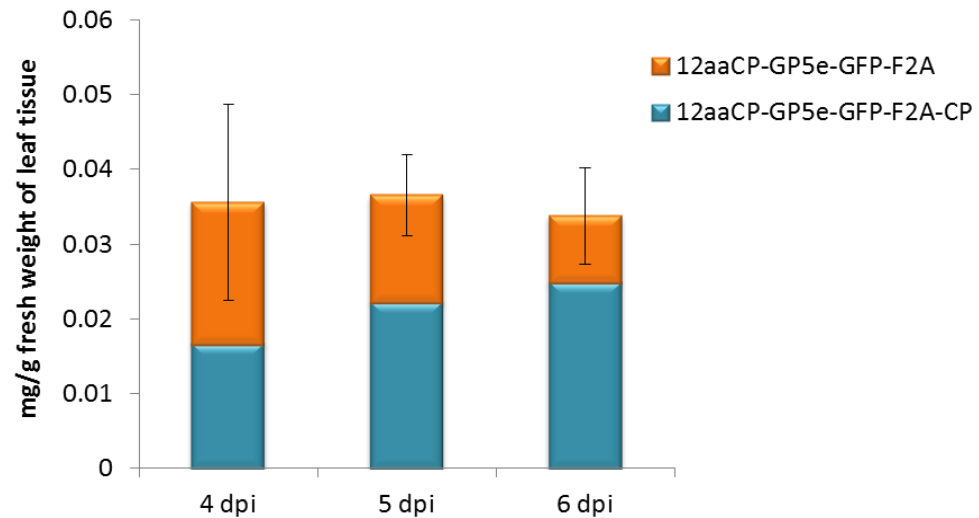
**Figure 4.6** Western blot of infiltrated leaves at 4 dpi. **A.** Western blot with anti-PepMV CP antibody. Lane 1: p19 infiltrated leaves. Lanes 2-5: pGR-Pep-GP5e-GFP-F2A infiltrated leaves. Lanes 6-9: pGR-Pep-GFP-F2A infiltrated leaves. **B.** Western blot with anti-GFP antibody. Lane 1: p19 infiltrated leaves. Lanes 2-5: pGR-Pep-GP5e-GFP-F2A infiltrated leaves. Lanes 6-7: pGR-Pep-GFP-F2A infiltrated leaves.



A.



B.



**Figure 4.7** Quantification of protein expression during a time course of 4-6 dpi. Error bars indicate the standard deviation of the mean (n=3). **A.** Expression of green fluorescent protein (GFP) in *N. benthamiana*. **B.** Expression of glycoprotein 5 (GP5) containing epitope and fused with GFP (GP5e-GFP) in *N. benthamiana*.

Since the results from the GFP construct were promising, I sought to determine if the viral vector, pGR-Pep-F2A, could be used to express a subunit vaccine against PRRSV in *N. benthamiana*. The GP5e sequence is the first 180 nucleotides of the GP5, which was codon optimized for plants and had the transmembrane domain removed. This sequence contains the neutralizing epitope (NE) of GP5, which induces neutralizing antibodies against PRRSV in pigs (Music and Gagnon 2010). In contrast to pGR-Pep-GFP, GFP was not detected, by either a handheld UV lamp or confocal microscopy, in a time course of 9 dpi when *N. benthamiana* was infiltrated with pGR-Pep-GP5e-GFP. Moreover, infiltrated leaves turned brownish quickly, a sign of necrosis, at 5-7 dpi, possibly due to the toxicity of GP5e. To help overcome the difficulties involved in GP5e-GFP expression, a silencing suppressor, p19, from CymRSV (Silhavy et al. 2002) was co-infiltrated with pGR-Pep-GP5e-GFP. Many studies have reported that the production of heterologous proteins could be enhanced by co-infiltration with a silencing suppressor (Lindbo 2007; Sainsbury et al. 2009; Saxena et al. 2011). Liu and Kearney showed that co-inoculation of p19, derived from *Tomato bushy stunt virus* (TBSV), and pSHEC/GFP could significantly increase the GFP expression in inoculated leaves (Liu and Kearney 2010). Consistently with these observations, GFP was detected in leaves co-infiltrated with p19 at 4 dpi. However, the level of GFP expression, which also reflected GP5e-GFP expression when using pGR-Pep-GP5e-GFP combined with p19, was much lower than using pGR-Pep-GFP alone (Figure 4.5A and B, upper panel). Furthermore, necrosis started in pGR-Pep-GFP infiltrated leaves at 7 dpi, which was 2 days later, when compared to pGR-Pep-GP5e-GFP-inoculated leaves. Also, the constructs containing GP5e-GFP showed a reduction in virus movement, as only a limited area of secondary upper leaves expressed GFP, when compared to the infiltrated leaves (Figure 4.5B, lower panel and Figure 4.5C). These results suggest that p19 can enhance the expression of GP5e-GFP by suppressing RNA silencing in infiltrated leaves. Nevertheless, without p19 the toxicity of GP5e probably caused cell death and silencing suppression in leaves and, as a result, viruses were not able to replicate and spread efficiently.

As GFP was remarkably observed in pGR-Pep-GFP infiltrated leaves at 4 dpi (Figure 4.3A), I conduct a time-course assay of protein expression for both pGR-Pep-GFP and pGR-Pep-GP5e-GFP from 3 to 9 dpi. Western blot analyses revealed that high levels of GFP or GP5e-

GFP could be acquired within 4-6 dpi. As mentioned above, GP5e-GFP infiltrated leaves exhibited necrosis at 7 dpi, while GFP infiltrated plants showed systemic infection throughout the plant at 8 dpi. This may explain the lower yield of proteins of interest in infiltrated leaves from 7-9 dpi. It was estimated that the yield of GP5e-GFP was 20 times lower than that of GFP as shown in protein quantification by immunoblotting. In addition, free GP5e-GFP exhibited increased instability, as evidenced by the increase in degraded proteins shown by western blotting, during time-course assays, when compared to GP5e-GFP fused with CP (Figure 4.7B). However, the expression of GP5e-GFP, in both free form and fusions, was approximately 0.036 mg/g fresh weight (Figure 4.7B), which is 100 times higher than previously documented levels of GP5 expressed in transgenic banana or tobacco (Chan et al. 2013; Chia et al. 2010). This demonstrates that this viral vector is able to express GP5e in the form of fusion with GFP within a short time frame (seven-day period). Fusion of GP5e with stabilizing protein domains, such as “fragment crystallizable chain” of immunoglobulins (Fc), hydrophobin I (HFBI) or elastin-like peptide (ELP) (Conley et al. 2009; Conley et al. 2011; Joensuu et al. 2010; Piron et al. 2014), may reduce its toxicity to host plants and enhance the stability of GP5e, which in turn enhance protein accumulation in plant systems. Moreover, the stabilizing proteins could also assist in maintaining the immunogenicity of GP5 and improving the delivery efficiency into animal systems for induction of immune response.

Despite the unexpectedly low levels of GP5e-GFP expression, the PepMV-based expression vector is a promising candidate for further refinements in order to acquire a high level of proteins of interest via virus replication. For instance, CP may be replaced by a heterologous protein, which will diminish the threat of unwanted dissemination of the virus, in terms of biosafety concerns. The CP may be provided *in trans* (i.e., via genetic transformation) to restore cell-to-cell movement (Sempere et al. 2011). This expression strategy has been proven to be highly successful for TMV-derived MagnICON vectors (Gleba et al. 2005; Marillonnet et al. 2004; Marillonnet et al. 2005). Vacuum infiltration may also be applied to deliver the viral vector to entire *N. benthamiana* plants, upgrading the system to large scale capacity. Moreover, *p19*, the silencing suppressor gene, may be introduced into this viral vector to help maximize the expression of heterologous proteins. To facilitate the cloning

process, pGR-Pep-F2A may be modified to include the entry sites for the Gateway cloning system. To enhance the cleavage efficiency of 2A, a short spacer such as glycine-serine-glycine (GSG), may be placed upstream of the F2A peptide (Wang et al. 2015). Through these optimizations, the PepMV vector will likely become an efficient, powerful expression system as a protein expression vector in *N. benthamiana*.

#### ***4.5 Conclusion***

In summary, I have described the construction of a versatile expression system, pGR-Pep-F2A, based on Ontario PepMV. This vector was used to express GFP and a potential subunit vaccine, e.g. GP5e, in *N. benthamiana* to control PRRSV. The yield of GFP has reached up to 2.03 mg/g fresh weight of leaf tissue at 5 dpi, whereas, the level of GP5e-GFP was much less at 0.036 mg/g fresh weight likely due to the specific toxicity of this protein in plant cells. The pGR-Pep-F2A vector has potential not only for protein expression, but also for pathogenic studies to expand the knowledge of PepMV. Further experimentation is required in order to optimize this viral vector and improve the expression of GP5 as a plant-made vaccine.

**Table 4.1** Primers used to construct the PepMV-based expression vectors

<b>Name</b>	<b>Sequence (5'-3')</b>
<b>SfoI-XmaI-CP36R</b>	TATAGGCGCCCTTCCCCCGGGCTCGAGAGTAC TGAACCAGAAGTAGCAGC
<b>BbvCI-TGB2-F</b>	TTTCCTCAGCAACAATCATAAC
<b>XmaI-F2A-CP-F</b>	TTCCCCCGGGGGAAGCGGAAATTTTGACCTTC TCAAGTTGGCGGGAGACGTGGAGTCCAACCCT GGGCCTGCTGACAATACCCAGTT
<b>SfoI-TDNA</b>	CAGGCGCCATTCGCCATTCAGGC
<b>ScaI-GFP-F</b>	CATTAAGTACTATGGTGAGCAAGGGCGAGGAGCT
<b>XmaI-cmyc-R</b>	CTGACCCGGGGAGGTCTTCCTCAGAGATCAA
<b>ScaI-GP5e-F</b>	CATTAAGTACTATGCTTGGCAAATGCTTAACAGC
<b>Pep6-R</b>	CACGTAGTTAACGCATGTACCACA
<b>Pep5-F</b>	CACACTACACTTCCTTCAGGACGC
<b>TGB3-F</b>	GTTCTAAAAGCAATCAACTT
<b>GFP-R</b>	CTTGTACAGCTCGTCCATGCCGAG
<b>Actin-F</b>	GGGATGTGAAGGAGAAGTTGGC
<b>Actin-R</b>	ATCAGCAATGCCCGGGAACA

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## Chapter 5: General discussion and conclusion

It has been over thirty years since the first biopharmaceutical, human growth hormone, was produced in sunflower (*Helianthus annuus*) and tobacco (*Nicotiana tabacum* var. petit Havana SR1) plants (Barta et al. 1986). Scientists, especially molecular biologists, have put an endless amount of dedication into molecular farming. The success story of ZMapp, an experimental drug produced in plants, is one of the fruits borne of this dedication. ZMapp is a combination of three monoclonal antibodies against Ebola virus and showed a significant 100% rescue rate when applied to infected Rhesus macaques (Qiu et al. 2014). With Ebola outbreaks in 2015, the United States Food and Drug Administration (FDA) approved ZMapp for use in new clinical drug trials in Liberia. ZMapp was produced in *N. benthamiana* by transient expression under the collaboration of Kentucky BioProcessing (Kentucky, USA), Mapp Biopharmaceutical Inc. and LeafBio (San Diego, USA), Defyrus Inc. (Toronto, Canada), the US government and the Public Health Agency of Canada (PHAC). Although ZMapp is still in clinical trials, it gives hope in the fight against the Ebola virus disease. The United States Department of Agriculture (USDA) gave approval to Dow Agro Sciences for the first plant-made vaccine, against *Newcastle disease virus* (NDV), in 2006. This vaccine was produced in tobacco suspension cells and is able to efficiently protect poultry from NDV virus (Vermij and Waltz 2006). These stories are not only milestones for molecular farming, but also demonstrate the competence of plants as bioreactors for plant-made vaccines against human and animal diseases.

PRRS is a devastating and recurring epidemic which affects the worldwide pork industry (Lunney et al. 2010; Topp et al. 2016). PRRS causes the death of pigs at all ages and reduces breeding herds by infection with porcine respiratory disease complex (PRDC) and/or reproductive failures (Lunney et al. 2016). Under farm conditions, PRRSV can co-infect with various bacteria and viruses, leading to severe and highly complex illnesses in pigs. Thus, antibiotics are often applied to infected pigs in PRRS outbreaks (Glass-Kaastra et al. 2013). In addition, this is an air-borne and very infectious disease, resulting in outbreaks within a short time frame (Cutler et al. 2011; Hermann et al. 2009; Tian et al. 2007). At present, no therapy exists. Vaccines, combined with other farming practices, play a critical role in the

fight against PRRS. Plant-made vaccines against PRRSV have become an attractive idea, as they provide the option of edible vaccines and lessen needle use and cold-chain requirements. Cold chain is the maintenance of optimal conditions during transport, storage and handling of vaccines. This not only reduces manufacturing and administration costs, but also eliminates the chance of PRRSV transmission via needles (Baker et al. 2012; Otake et al. 2002). As new health initiatives intend to decrease antibiotic use in livestock, in hopes of preventing antibiotic resistance in bacteria, the development of an effective plant-made vaccine will also help to fulfill this requirement (Topp et al. 2016).

Transient expression using viral vectors has been widely exploited to improve the levels of protein expression in molecular farming (Arntzen 2015; Lico et al. 2008; Lomonosoff and D'Aoust 2016). As opposed to stable transformation, this method is an alternative which can speed up the start-to-finish manufacture and optimization of vaccines produced in plants for quick responses to emerging infectious diseases. In this thesis, I investigated the potential of three viruses as viral vectors for the production of GP5-based subunit vaccines in plants. The three viruses are BPMV, CGMMV, and PepMV, which belong to the different genera, *Comovirus*, *Tobamovirus* and *Potexvirus*, respectively. The main purpose of this study is to develop a viral expression system for plant-made vaccines against PRRSV. These plant-made vaccines could be delivered to pigs by oral administration.

## **5.1 Discussion**

### **5.1.1 Can one viral system fit all desired requirements?**

In this thesis, I have worked with three different viruses which belong to distinct genera and families. It may be questioned as to whether one viral system among these virus-based expression systems can satisfy all the requirements, including protein yield, peptide presentation, oral administration, etc.

The protein yield of each virus-based expression system was affected by a variety of factors, such as plant virus type, host species, delivery method, and the characteristics of the protein of interest. As described in Chapter 2, an expression vector was developed, based on BPMV, which is distinguished from the other two vector candidate viruses, CGMMV and PepMV,

by its bipartite genome. BPMV also possesses a narrow host range within the legume family thus, soybean was chosen as a specific host for protein expression in this study. Soybean is a popular and widely grown crop in many countries, e.g. USA and Canada (Liu et al. 2016; Rao et al. 2014). The plant contains a high level of nutrients, beneficial isoflavonoids, and no nicotine, which, unlike tobacco species, is appropriate for direct feeding to pigs, without extra purification steps. Moreover, soybean plants also provide a high level of biomass, either in the field or in the greenhouse. However, soybean plants are recalcitrant to agroinfiltration. The only methods to deliver virus amplicons are mechanical inoculation using RNA transcripts or biolistic bombardment of DNA plasmids. The latter method was selected for this study, in order to facilitate the inoculation steps, without *in vitro* transcription. Soybean leaves were particle bombarded with viral DNA amplicons to produce BPMV, and these viruses subsequently spread throughout the plant via systemic virus movement. Infected plant materials were collected to inoculate the next passage of soybean plants, via mechanical inoculation. The results showed that GP5C-GFP was successfully expressed in soybean leaves at 30-45 dpi. The overall goal is to develop a low-cost BPMV-based expression vectors for oral vaccine production in soybean leaves.

As described in Chapter 3, the neutralizing epitope (NE) of GP5 was exposed on the outer surface of the viral particles of CGMMV in cucumber. To achieve this, a CGMMV peptide display vector was generated using a readthrough stop codon context. Viruses have been widely employed as nanocarriers for peptide display, which enhances the efficiency of subunit vaccines (Lico et al. 2008). A prerequisite using this strategy is the availability of knowledge of virus structure and topology. Since the CP of CGMMV is well characterized, the NE was inserted downstream of a readthrough context in the C-terminus of CP. This would display NE on the surface of chimeric virions, and maintain virus systemic movement in cucumber plants. Indeed, the results showed that the systemic leaves of CGMMV NE display vector-infiltrated cucumber contained NE-presenting virions.

In Chapter 4, a PepMV-based expression vector was established to produce GP5e-GFP in *N. benthamiana*, using the transient expression method. GP5e, the ectodomain of GP5, was codon optimized for expression in plants. In addition, co-agroinfiltration with p19, a

silencing suppressor, was also employed to enhance GP5e expression in plants. As a result, GP5e-GFP was successfully transiently expressed in *N. benthamiana* at 5 dpi.

As summarized above, three viruses were attempted for the development of a virus-based expression system to express a subunit vaccine in different plants. Each viral system is distinct to others in many ways. Virus type and host range are the first two main factors which must be carefully considered during viral vector development. These factors also have an influence on the method of infection. For example, soybean plants are a host of BPMV. The BPMV-based expression vectors could be delivered directly into plants by biolistic bombardment to initiate virus infection, and subsequent systemic virus movement is required to spread throughout infected plants. Therefore, construction of a BPMV-based expression vector should preserve correct virion assembly to ensure systemic virus movement. CGMMV and PepMV-based expression vectors are unable to infect soybean plants, as soybean is not a natural host. CGMMV was chosen to develop a peptide display system because its genomic organization and CP structure have been extensively studied and the viral vector is relatively easy to be modified for the insertion of short peptides (Ooi et al. 2006; Teoh et al. 2009; Zheng et al. 2015). In summary, there is no one viral system that can fit all purposes. Each system has its pros and cons which fit to specific requirements, such as the host plant, the peptide/protein of interest and the method of delivery. Therefore, we should select the right strategy and virus to maximize the beneficial characteristics of the virus for vaccine production in plants.

### 5.1.2 The viral vectors can compensate for the toxicity of GP5 to increase the yield of GP5-based production via transient expression *in planta*

Intensive research has been carried out on the expression of GP5 and other major structural proteins of PRRSV *in planta*. Other systems such as bacteria, insect cells, and mammalian cells have been also investigated for production of these proteins. The accumulation of PRRSV membrane protein (M) and nucleocapsid protein (N) in transgenic plants has been reported to reach as high as 5.1 µg/g of fresh corn callus and 2.62 mg/g of ground soybean seed (Hu et al. 2012; Vimolmangkang et al. 2012), respectively. In contrast, the expression of

PRRSV-GP5 in transgenic plants has usually been quite low (Piron et al. 2014). Accumulation of GP5 of PRRSV type 2 (NA) ranged from 110-267 ng/g of fresh weight leaf tissue in both transgenic tobacco and banana plants (Chan et al. 2013; Chia et al. 2010; Chia et al. 2011). There have been only two publications which showed higher accumulation levels of GP5. The first study demonstrated that GP5 (PRRSV type 2) was expressed at 2.5-4.7 µg/g fresh weight of leaf tissue and 0.8-1.2 µg/g fresh weight of tuber in transgenic potato plants (Chen and Liu 2011). The second study showed that expression of GP5 from PRRSV type 1 (EU) fused with stabilizing protein domain “Fc” was estimated to be 2.3 mg/g fresh weight in transgenic *Arabidopsis* seeds (Piron et al. 2014). As mentioned earlier in this thesis, transient expression has an advantage over stable transgenic plants given its flexibility and versatility in large scale-up operations and the quick manufacture of vaccines for emergency responses to infectious diseases. More recently, viral expression systems have been used for the transient expression of GP5 in plants. Uribe-Campero et al. reported the use of MagnICON vectors to transiently express GP5-M and GP5-T virus-like particles (VLPs) in *N. silvestris*, but did not disclose the expression yields (Uribe-Campero et al. 2015). The Lomonosoff group employed the pEAQ-HT vectors to express a codon-optimized GP5 ectodomain, without the transmembrane region and signal peptide, called GP5synt, in *N. benthamiana*. The product was insoluble at 5 mg/kg of infiltrated plant fresh weight (CORDIS 2015).

One of the bottlenecks that possibly impede GP5 expression *in planta* is the toxicity of GP5. PRRSV is known as a causal agent of apoptosis both *in vitro* and *in vivo* (Kim et al. 2002; Labarque et al. 2003; Miller and Fox 2004; Suárez et al. 1996). GP5 protein was shown to induce apoptosis when expressed in mammalian cells (Gagnon et al. 2003; Suárez et al. 1996). The N-terminal of GP5, which included the first 119 residues, has been mapped for apoptosis induction (Fernández et al. 2002). While the mechanism of GP5-inducing apoptosis remains unknown, this phenomenon has resulted in the low yield of GP5 expression in these cells (Gagnon et al. 2003). Similar results were also observed when GP5 was produced *in planta* (Peyret and Lomonosoff 2015; Thuenemann et al. 2013). Hydrophobicity of the GP5 transmembrane domain is believed to be the causal agent of the observed necrosis. Nevertheless, the expression of GP5oi, in which the signal peptide and

transmembrane region were deleted and only the ectodomain was kept, showed the expression at low levels when using pEAQ-HT (CORDIS 2015). Both GP5 and GP5-Tm (which has a deleted transmembrane region) were expressed at the same low level of 0.09% of TSP in transgenic *Arabidopsis* seeds (Piron et al. 2014). These data indicate that deletion of the transmembrane domain of GP5 did not affect expression levels of GP5 *in planta*. To overcome this challenge, many alternatives have been suggested, such as fusion to stabilizing proteins and compartment targeting (Piron et al. 2014), and/or plant codon optimization and silencing suppression (CORDIS 2015).

In Chapter 2 of this thesis, GP5 was halved into two main parts, the N- and C- termini, and expressed in a fusion with a stabilizing protein, such as GFP, in soybean leaf using BPMV-based vectors. Interestingly, only GP5C-GFP could be detected in the leaf extract, whereas, the GP5N-GFP-bombarded plants were not infected, although this experiment was repeated five times. I have speculated that the high toxicity of GP5N might interfere with BPMV replication and movement. Upon introduction of the modified BPMV vector into soybean leaf cells via biolistic bombardment, the recombinant virus must replicate in the bombarded cells, move intercellularly into neighboring cells and further spread systemically from the local leaf to the whole plant. GP5N toxicity causes plant cell death, i.e., necrosis and thus, prevents virus propagation throughout the whole plant, as was also reported in GP5-expressing mammalian cells (Gagnon 2003).

In Chapter 4, necrosis appeared at 5 dpi in infiltrated *N. benthamiana* when using pGR-Pep-GP5e-GFP alone. GP5e is a truncated GP5, which is codon optimized and includes the signal peptide and ectodomain only. No green fluorescent signals from GP5e-GFP were detectable in infiltrated leaves following infiltration, suggesting GP5e might also be toxic. PepMV infection was somehow rescued by co-infiltration of pGR-Pep-GP5e-GFP with p19, a silencing suppressor of CymRSV (Silhavy et al. 2002). As a result, green fluorescent signals from GP5e-GFP were visualized under a UV lamp or a confocal microscope, and the fusion protein was detected by western blot analysis. However, GP5e-GFP was mainly present in local agro-infiltrated leaves. GP5e-GFP PepMV could undergo systemic movement with p19 with a very weak expression of GP5e-GFP.



Taken together, these results indicate that the N-terminus of GP5 can cause toxicity in plant cells, leading to necrosis and silencing induction. Furthermore, viral vectors based on CGMMV or PepMV can overcome this obstacle to successfully increase the levels of peptide/protein of interest in plants by transient expression alone or with p19, respectively. Particularly, chimeric CGMMV presenting the GP5 NE accumulated to the level of 35.84 mg/kg fresh weight of leaf tissue in cucumber and GP5e-GFP was expressed at approximately 36.53 mg/kg fresh weight of leaf tissue in *N. benthamiana*. These expression levels are approximately 7 times higher than those previously reported using pEAQ-HT (CORDIS 2015).

### 5.1.3 pGR-Pep-F2A is a robust and versatile system which can function both for protein production and peptide/protein display

F2A-mediated cleavage allows for multiple proteins to be expressed in frame fused with viral CP as F2A can process the recombinant polyprotein into various products. In this study, free GFP was produced in *N. benthamiana* infiltrated with pGR-Pep-GFP (in which GFP-F2A was in frame fused with CP at the N-terminus in the pGR-Pep-F2A vector) at high levels of 2.03 g/kg fresh weight of leaf tissue, which was approximately 14% of TSP, at 5 dpi. This result suggests that pGR-Pep-F2A is a successful protein expression vector.

As mentioned in the introduction of Chapter 4, both PepMV and PVX belong to the genus *Potexvirus*. Although no crystallographic data for PepMV are available, the structure of PepMV is expected to be similar to PVX, where the N- and C- termini of CP are exposed on the surface of virions. As GFP was designed to insert into PepMV CP at the N-terminus, it was reasonable to expect that this protein would be displayed on the virus surface. As viral systemic movement was not affected and viral infection spread throughout the plants at 8 dpi, apparently GFP could be decorated on the surface of chimeric PepMV. However, I could not exclude the possibility that the free PepMV CP and the fused form of CP probably assembled together to form a chimeric PepMV, which displayed a protein of interest without steric hindrance, similar to the PVX "OVERCOAT" system (Santa Cruz et al. 1998).

In addition, a quantification of the expression levels of GP5e-GFP in the free form or its fusion with PepMV CP revealed that the latter form was more stable during a time course of infection. This also suggests that PepMV-CP can help to stabilize a protein of interest, especially GP5e, which is easily degraded in a cytoplasmic environment. In general, the PepMV-based vector provides a versatile system for multiple purposes of protein expression in *N. benthamiana*.

## 5.2 Conclusion

In conclusion, viral vectors have contributed immensely to the development of molecular pharming. In this study, I generated a variety of viral vectors based on BPMV, CGMMV and PepMV for the expression of GP5 or its truncated proteins and short peptides. Using these vectors, I have successfully produced these proteins/peptides in different plants using transient expression. GP5C-GFP, CGMMV-NEcmcy, and GP5e-GFP were expressed at 4.36, 35.84 and 36.53 mg/kg of leaf fresh weight in soybean, cucumber, and *N. benthamiana*, respectively, using BPMV, CGMMV, PepMV-based expression vectors, respectively. The CGMMV-NEcmcy and GP5e-GFP products are promising as plant-made, oral vaccines for pigs against PRRSV. Although the GP5C-GFP does not contain the ectodomain of GP5, it can still act as an adjuvant for vaccine delivery, as GP5C is known for its high immunogenicity. The construction of these virus-based expression vectors provides robust and versatile tools for the multi-purpose expression of recombinant proteins in plants, as oral vaccines or therapeutics. I believe that these viral vectors, especially the pGR-Pep-F2A, have the potential to serve as useful over-expression systems not only for research, but also for molecular farming industry, in the future.

## 5.3 Future prospects

The low expression of GP5 *in planta* has raised questions about its toxicity to plants. To answer this question, the potential mechanisms behind this phenomenon need to be investigated and elucidated. Also, the natural characteristics of GP5 domains, as well as its roles in PRRSV pathogenesis and host immunity, are unknown (Lunney et al. 2016; Prather

et al. 2013; Van Breedam et al. 2010). To accomplish high production levels of a plant-made vaccine based on GP5, it is necessary to understand all of these aspects.

Despite poor knowledge about PRRSV GP5 in plants, it is possible to improve the expression of GP5 *in planta* using multiple modifications (Piron et al. 2014). The GP5 of PRRSV type 1 had the transmembrane region removed and was fused with a stabilizing domain, pFc, to create GP5-Tm:pFc. The sequence of GP5-Tm:pFc was codon optimized as an Arabidopsis seed storage protein. The protein of interest was expressed and targeted to the ER in Arabidopsis seeds at up to 0.83% of TSP, when using a high speed expression vector for transgenic plants. The results demonstrated that GP5 could be engineered to increase the level of expression in plants. This study suggests that GP5e expression may be enhanced by fusion with other PRRSV structural proteins, such as the membrane protein (M), the nucleocapsid protein (N), minor glycoproteins (GP) as GP4 and GP3 proteins or stabilizing proteins, such as elastin-like polypeptides (ELP), hydrophobin I (HFBI), and pFc, and then targeted to the ER using a PepMV-based expression vector. This will allow multi-protein expression for PRRSV vaccines, as well as a reduction of GP5 degradation in plants, to achieve biobetter products.

The question of immunogenicity of these plant-made products as vaccines against PRRSV using BPMV, CGMMV or PepMV vectors must also be addressed. Thus, an immunization test should be conducted to assess whether these products are able to induce an immune responses in mouse or pig models. This test would also provide other necessary information, e.g. dosage, time, efficacy of oral administration, etc., for further applications. Since PRRSV pathogenesis is established through the mucosal surface in respiratory and reproductive tracts, it will be interesting to determine whether the plant-made vaccine can effectively trigger a strong immune response against PRRSV, when delivered by the oral route.

Peptide display is an attractive system which exploits viral vectors to produce nanocarriers for drug delivery and nanotechnology applications (Gleba et al. 2014; Lico et al. 2015; Steinmetz et al. 2009). Both the CGMMV-based and PepMV-based expression systems can be investigated for their competence in these novel research fields. More strikingly, CGMMV- and PepMV-based systems can also be combined to establish a hybrid system,

with benefits from both genera, *Tobamovirus* and *Potexvirus*, for synchronous expression of virus particles or specific recombinant proteins such as immunoglobulins. This will provide a powerful tool for both virus and interdisciplinary research, similar to the hybrid system of TMV and PVX (Dickmeis et al. 2015; Giritich et al. 2006).

Last but not least, biosafety concerns regarding the use of these viral vectors must be addressed. Since these viral vectors can replicate and release the virus into the environment, this may lead to the infection of healthy, non-target plants. There are two approaches to solve this issue: (1) physical barriers, and (2) biotechnology. In the first approach, containment can be maintained by the establishment of good manufacturing practices (GMPs) in molecular farming (Gleba et al. 2014; Holtz et al. 2015). Facilities would include a variety of containment level greenhouses and chambers which prevent the release of viruses into the natural environment. In the latter approach, virus vectors can be modified into deconstructed or inducible vectors, which eliminate some of the risk of contamination. It is practical to establish both methods as a part of a program to maintain biosafety.

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## *Curriculum Vitae*

**Hong Hanh Tran**

### **EDUCATION**

- |               |   |
|---------------|---|
| 2012- present | Ph.D. Biology<br><br>The University of Western Ontario<br><br>London, Ontario, Canada                     |
| 2010-2012     | M.Eng. Biomedical Engineering<br><br>Gachon University<br><br>Seongnam-si, Gyeonggi-do, Republic of Korea |
| 2005-2010     | B.Eng. Biotechnology<br><br>Hochiminh City University of Technology<br><br>Hochiminh City, Vietnam        |

### **HONOURS AND AWARDS**

- |           |  |
|-----------|--|
| 2016      | Plant Biotech Travel Award   |
| 2016      | Out - of Province Bursary (SOGS) Award                                   |
| 2012-2016 | Western Graduate Research Scholarship (WGRS)                             |
| 2014      | American Society for Virology (ASV) Graduate Student<br><br>Travel Award |
| 2010-2012 | Gachon Graduate Scholarship  |
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### **WORK EXPERIENCE**

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| 2012-2017 | Teaching Assistant, Department of Biology |
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The University of Western Ontario (Canada)

2012-2017 Research Assistant, Agriculture and Agri-Food Canada  
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2010-2012 Research Assistant, College of BioNano  
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### **REFEREED PUBLICATIONS**

**Tran HH**, Trinh KTL, Lee NY (2013) Pressure-driven one- step solid phase-based on- chip sample preparation on a microfabricated plastic device and integration with flow-through polymerase chain reaction (PCR). *Journal of Chromatography B* 936: 88-94.

Zhang H, **Tran HH**, Chung BH, Lee NY (2013) Solid-phase based on-chip DNA purification through a valve-free stepwise injection of multiple reagents employing centrifugal force combined with a hydrophobic capillary barrier pressure. *Analyst* 138(6):1750-1757.

**Tran HH**, Wu W, Lee NY (2012) Ethanol and UV-assisted instantaneous bonding of PMMA assemblies and tuning in bonding reversibility. *Sensors and Actuators B: Chemical* 181: 955-962.

**Tran HH**, Nguyen HH, Le TD, Nguyen HT (2010) Genetic transformation of *Lisianthus (Eustoma Grandiflorum Grise.)* with the CA-AFP (*Cicer Arietinum* Antifungal Protein) gene. *Journal of Biotechnology* (ISSN 1811-4989, in Vietnamese).

### **NON-REFEREED CONTRIBUTIONS**

**Tran HH**, Chen H, Menassa R, Huner N, Wang A (2016) Exploiting *Bean pod mottle virus* as a transient expression system to produce plant-made vaccines against porcine reproductive

and respiratory syndrome virus (PRRSV) in soybean leaves. Plant Biotech 2016, Queen's University, Kingston, Canada, June 2016.

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**Tran HH**, Chen H, Wang HY, Menassa R, Huner N, Wang A (2014) Developing a *Bean pod mottle virus*-based expression system for the expression of proteins of interest in plants. American Society for Virology 33<sup>rd</sup> Annual Meeting, Fort Collin, Colorado, USA, June 2014.

Trinh KTL, **Tran HH**, Zhang Y, Wu J, Lee NY (2013) One-step solid phase-based in-chip sample preparation and integration with flow-through polymerase chain. The 17<sup>th</sup> International Conference on Miniaturized Systems for Chemistry and Life Sciences, Freiburg, Germany, October 2013.

**Tran HH**, Lee NY (2011) A colorimetric detection of DNA for biosensor applications. The 5th International Conference on sensors (ASIASENSE 2011), Jeju, Republic of Korea, October 2011.

**Tran HH**, Trinh KTL, Lee NY (2011) Novel detection method of nucleic acid for nanobiosensor application. The Korean Society of Industrial and Engineering Chemistry Annual Conference, Gyeonggi-do, Republic of Korea, November 2011.

**Tran HH**, Lee NY (2011) Spatially- defined polymerization of a microsieve structure inside a channel for nucleic acid purification. The Polymer Society of Korea, Daejeon Convention Center, Daejeon, Republic of Korea, April 2011.