

Enhancement of plant expression vectors using replication and silencing suppressor elements

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" Seek knowledge from the cradle to the grave" – Prophet Muhammad (pbuh)

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Abstract

Molecular farming is gaining traction as a cost-effective platform to produce recombinant proteins. Further improvements can be made, however, to increase overall yield especially for difficult to express proteins. In this study virus-derived silencing suppressors and replication elements were used with the aim of increasing expression and yield of enhanced green fluorescent protein (EGFP) and the Zika PrME polyprotein in *Nicotiana benthamiana*.

A comparison of four viral silencing suppressor proteins was performed: these were tomato spotted wilt virus non-structural protein, NSs, tomato aspermy virus (TAV) 2b, tomato bushy stunt virus P19 and begomovirus alphasatellite Rep. Differences in EGFP expression in *N. benthamiana* due to the silencing suppression were determined using immunoblotting and fluorescence of EGFP. In addition, replication elements from three viruses (bean yellow dwarf virus [BeYDV], beak and feather disease virus [BFDV] and begomovirus alphasatellite) were assembled into novel plant expression vectors using GoldenBraid (GB) cloning technology and assessed using EGFP. Finally, the two approaches were combined in an attempt to express the Zika PrME polyprotein, which was assessed using immunoblotting.

EGFP expression was found to be greatest in the presence of the TAV 2b protein and no difference in fluorescence intensity between the original BeYDV replicating plant expression vector and that constructed using GB could be detected; however, the GB assembly of the BFDV and alphasatellite plant expression vectors was unsuccessful. The TAV 2b combined with the BeYDV replicating elements were used for the expression of Zika PrME. The gene was successfully cloned into the replicating BeYDV vector and a vector that does not replicate (negative control). The PrME was not detected using anti-His tag immunoblotting despite optimisation for *Agrobacterium* infiltration density, harvest day post infiltration, signal peptides and buffers during extraction.

In this study I demonstrated the following: that the TAV 2b protein out-performed all other silencing suppressors; that the GB cloning technology can be successfully applied in the development of novel plant expression vectors, although further optimisation is required for these and for Zika PrME expression. Further work in characterising the effect of silencing suppression on recombinant protein expression can be assessed using RT-qPCR to measure

the effect on mRNA levels. In summary, these improvements in plant recombinant protein expression can be readily applied to large scale production of novel therapeutics and vaccines.

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Chapter 1: Literature review

Introduction

The demand for pharmaceutical proteins has increased over the years due to emerging diseases. Vaccine production in turn is essential for preventing epidemics by providing protective immunity over long periods. It is important that vaccines are safe, easy to administer, cheap, stable and have minimum side effects (Shanmugaraj & Ramalingam 2014). Pharmaceutical proteins have traditionally been heterologously expressed in mammalian, bacterial and insect cells (Rybicki, 2010). However, these bioreactors present multiple problems such as scalability, safety concerns and high cost. On the other hand, molecular farming, the production of recombinant proteins in plants or plant cell cultures is a successful platform for protein production (Rybicki, 2014). Plants are advantageous in that they do not support human pathogens and unlike bacteria, do not produce endotoxins. In addition, they are also capable of post-translationally modifying recombinant proteins and can easily be scaled up to agricultural levels. Recombinant proteins can be produced using transient expression systems or stable transgenic lines. Transgenic plants are considered a traditional approach to the production of recombinant proteins; however, the approach requires long generation times and results in only moderate protein yields which contributes to the cost of downstream processing (Sabalza et al. 2014). Transient protein expression via agroinfiltration and viral based vectors is commonly used because higher expression can be achieved in less time and protein production can easily be scaled up since the transgene is not integrated into the genome to produce a transgenic plant (Daniell et al. 2009).

A preferred method of biopharming is the use of virus-based transient expression via plant tissue infiltration. This approach makes use of a shuttle vector-based *Agrobacterium tumefaciens* tumour-inducing (Ti) plasmid. In nature *Agrobacterium* causes tumour formation in plant roots known as crown-gall disease. Subsequently, a binary vector was designed and DNA could be inserted between the right and left borders and used to transform plant cells (Krenek et al. 2015). Furthermore, tissue infiltration using *A. tumefaciens* ensures that the vector is delivered and released into the host tissue (Fischer et al. 2004; Regnard et al. 2010). Agroinfiltration is very versatile, as several recombinant *Agrobacterium* - each one harbouring a different vector with a different gene - can be used to infiltrate the same plant. All that is

required is that the vector maintains a stable copy number in the *Agrobacterium*. (Pushko et al. 2010). *Nicotiana benthamiana*, a tobacco relative, has been shown to be useful for agroinfiltration and expression of recombinant proteins due to its ability to support replication of various viruses (Goodin et al. 2008).

The level of recombinant protein production is particularly important for commercial vaccine production. However, technical bottlenecks such as low yields and structural heterogeneity have hindered plant-based vaccine production. Protein yield depends on multiple factors such as genetic, epigenetic and biochemical environment. Manipulation of these different factors has been employed to increase protein yields in plants.

Generation of viral vectors

Virus-based vectors can be optimised for high protein expression or different plant host species. The viral genome is deconstructed or engineered in such a way that it eliminates non-essential viral genes but maintains genes of interest which allows for replication and expression of the recombinant protein (Gleba et al. 2007). In addition, viral plasmids are designed based on the replication of the viruses themselves. Earlier vectors used in agroinfiltration were not as robust as the current plant virus-based vectors (Lico et al. 2008). Each virus is unique and therefore has its own disadvantages and advantages such as its ability to express small or large proteins.

Vectors are categorised into first and second generation viral vectors. First generation vectors are capable of expressing all their genes including the gene of interest. This vector is essentially the wild-type virus that also allows for the expression of the recombinant protein under the control of a strong viral promoter. Plants can either be sprayed with the viral mixture or transfected with *Agrobacterium* as described by Azhakanandam et al., (2007), via 'agrospray' or 'wound and agrospray'. However, the researchers obtained a low yield of 0.04-0.3 % total soluble protein (TSP) with this technique, which needs further improvements especially for industrial use. On the other hand, there has been successful expression of viral coat protein fusions of different genetic epitopes. In addition their immunogenicity and certain levels of protection has been demonstrated in animal models (Gleba et al. 2007; Azhakanandam et al. 2007).

The limitations experienced with first generation vectors lead scientists to engineer deconstructed vectors known as second generation vectors. These vectors were rebuilt using only the viral elements that were essential for expression of the gene of interest in a plant host system. The vector was completed using non-viral components that allow for infectivity, movement, replication or amplification and viral particle assembly. However, the lack of systemic movement and low protein expression levels were still a problem. Removal of the coat protein was introduced to reduce metabolic resources devoted to synthesis of a large coat protein and the use of *Agrobacterium* for systemic movements in plants (Gleba et al. 2007). On the basis of these and many other findings a scalable protocol for protein expression and vector construction was designed.

RNA virus expression systems

RNA viruses were the first to be explored for use as viral vectors. Tobacco mosaic virus (TMV) is a single stranded (ss) RNA (+) virus that infects a wide range of plants especially tobacco. TMV is a rigid rod-shaped virus which belongs to the *Tobamovirus* genus and was the first virus investigated for viral vector development (Bos 1999). The well-known “deconstructed” MagniCON vector system from ICON Genetics relies on elements from TMV and turnip vein-clearing virus (TVCV). Magnifection, the technique for transfecting these deconstructed vectors, has been used for the expression of antibodies, interferons, growth hormones, antigens, adjuvants and enzymes (Gleba et al. 2005). TMV was also used to generate the broadly neutralizing antibody (bnMAb) known as VRCO1 against HIV-1 in *N. benthamiana* plants. The TMV replicon vector was able to produce \approx 150 mg of the bnMAb per kg of fresh leaf material 5-7 days post inoculation. These antibodies were biologically active in an HIV-1 neutralization assay and their activity was demonstrated in a topically applied microbicide against HIV infection (Hamorsky et al. 2013). Furthermore, a TMV RNA-based overexpression (TRBO) vector was used to express the allergen R8 from dust mites in tobacco plants. The TRBO vector lacks the TMV coat protein gene and is characterized by its high protein expression levels, efficient agroinfection and its inability to form viral particles in the host. The plant-derived R8 antigen performed identically to the parent allergen. This was shown by the binding capacity of R8 and the parent allergen to IgE and the ELISA results that showed that R8 activated the T_H1 and decreased the T_H2 cell growth. The R8 antigen could possibly be used as a candidate vaccine for allergen-specific immunotherapy of asthma (Li et al. 2013).

The cucumber mosaic virus (CMV) has a tripartite ssRNA genome and wide host range. Each genomic RNA is packaged in a separate icosahedral capsid which limits the size of any foreign transgene inserts (Hefferon, 2014b). A bipartite and tripartite CMV-based vector was designed to express GFP and *Acidothormus cellulolyticus* endo-1, 4- β -glucanase (E1) in *N. benthamiana*. The above CMV vector was based on a binary plasmid where the coat protein (CP) was replaced with a gene that encodes an α -1-antitrypsin [AAT]. Viral amplicons were induced upon the presence of an inducer (β -estradiol) which improved the efficiency of transgene expression. The researchers were able to show using GFP and E1 (a cellulose degrading enzyme) that the CMV-based vector was able to achieve transient recombinant protein expression at levels comparable to transgenic plants but with a reduced time and effort (Hwang et al. 2012). CMV particles have been successfully modified to display porcine circovirus type 2 (PCV-2) capsid protein epitopes on the surface of the particle (Gellért et al. 2012). These plant-produced CMV:PCV2 particles were able to induce PCV specific antibody responses and showed protection against PCV in pigs.

Cowpea mosaic virus (CPMV) has been extensively used as an expression system for the production of vaccines and other therapeutic proteins in plants. CPMV is an icosahedral, ssRNA (+) virus with a bipartite genome (Hefferon 2014b; Hefferon 2017). It was initially used for antigen presentation and was further developed by Medicago, Inc. (Durham, NC, USA) as a CPMV vector to generate virus like particle (VLP) carrying influenza virus HA antigens (Mardanov et al. 2017). The non-replicating expression vector, pEAQ, was also based on CPMV. The foreign gene is placed between the 5' leader sequence and 3' untranslated region (UTR) of RNA-2. A deletion of the in-frame initiation codon upstream of the main translation initiation site of RNA-2 contributed to a substantial increase in foreign protein expression (Sainsbury et al. 2009; Montague et al. 2011). These pEAQ vectors were used for the expression of anti-HIV antibodies and bluetongue virus-like particles (Thuenemann et al. 2013; Sainsbury et al. 2010; van Zyl et al. 2017). CPMV non-infectious VLPs also known as nanoparticles have also been used in immunotherapy studies against cancer. The empty CPMV (eCPMV) nanoparticles were shown to reduce lung melanomas and induce an anti-tumour response (Lizotte et al. 2016). In addition, they were able to demonstrate that the eCPMV nanoparticles cleared tumours in multiple anatomical locations such as the colon,

ovaries and breast. eCPMV was shown to be utilised as a direct cancer immunotherapy and not only as a drug delivery system to reduce tumours.

Positive sense RNA viruses have been predominantly used for the construction of plant expression vectors and also sparked special interest as delivery vehicles for immunotherapy (Hefferon 2014; Hefferon 2017). However, researchers have become much more interested in DNA viruses such as the *Geminivirus* because of their significant expression levels.

DNA virus expression systems

Although ssRNA viruses are predominant among plant viruses, ssDNA plant viruses are becoming popular for use as vectors and in plant molecular biology. Viruses such as tomato yellow leaf curl virus (TYLCV), African cassava mosaic virus (ACMV) and bean yellow dwarf virus (BeYDV) belonging to the *Geminiviridae* family are common ssDNA plant viruses that have a particular impact on the economy (Scholthof et al. 2011). They are also suitable for vector development and have been successfully engineered for recombinant protein expression.

Geminiviruses contain a small circular ssDNA genome ranging from 2.5–3.2 kb in length. The ambisense genome, monopartite and bipartite, is encapsidated in a twinned capsid. All geminiviruses contain a movement protein, a coat protein, a replication initiator protein (Rep) which is required for replication of the virus and an intergenic region, which holds a stem loop structure (Bisaro, 1996). These viruses replicate via rolling circle replication (RCR) with the help of the Rep protein which initiates replication at the stem-loop structures. Briefly, after the virus enters the nucleus the ssDNA is released which is then processed into a dsDNA intermediate by the host DNA polymerase. The Rep protein is then expressed and initiates RCR by nicking the dsDNA at a conserved nonanucleotide sequence located within the origin of replication (Bisaro, 1996; Hefferon, 2014a)

Geminiviruses have been of particular interest to molecular biologists because of their ability to accumulate to very high copy numbers which results in high levels of gene expression. One of the early geminiviral-based replicon systems made use of ACMV, which has a bipartite genome demonstrating that geminiviruses can be deconstructed (Ward et al. 1988). The researchers replaced the capsid protein with a chloramphenicol acetyltransferase (CAT) gene which resulted in 80 U/mg of CAT levels. Other earlier vectors used to express reporter genes in plant cells included maize streak virus and wheat dwarf virus (Timmermans et al. 1992;

Matzeit et al. 1991; Palmer & Rybicki 2001; Shen & Hohn 1995). A replicon based on the BeYDV genome was designed specifically for recombinant protein expression by the deletion of viral genes and addition of a CaMV expression cassette. A 40-fold enhanced expression of the β -glucuronidase (GUS) reporter gene in tobacco plants was reported. The researchers also confirmed that the Rep protein can be supplied in *trans* on a separate vector (Mor et al. 2003). Based on this, it was reported that a 20-fold increase in *Staphylococcus* enterotoxin B protein (SEB) was expressed using a BeYDV-derived replicon (Hefferon & Fan 2004). However, there were some limitations which included the difficulty of regenerating replicating geminivirus genomes in transgenic plants and transient expression was limited to a number of sites per plant (Rybicki & Martin 2014; Palmer et al. 1999; Gutierrez et al. 2004). In 2006, a transgenic plant expression system was developed using BeYDV elements to express GFP and the Norwalk norovirus (NV) CP from doubly-transformed tobacco NT-1 cells and in transgenic potato plants. The Rep protein was under the control of an alcohol-inducible promoter and a high copy number of replicons was achieved when triggered with ethanol (Zhang & Mason 2006). Furthermore, Huang *et al.* (2009) achieved maximum expression of hepatitis B core antigen (HBcAg) and NVCP after four days using a BeYDV replicon in leaves of *N. benthamiana*. To increase expression, the Rep/RepA was placed *in trans* and replication could be controlled by changing the strength of the promoter. The same group then constructed a single replicon system that contained the Rep/RepA genes *in cis* however, expression of HBcAg showed no substantial change when compared to the co-delivery of vectors. Regnard *et al.* (2010) designed a novel self-replicating expression shuttle vector, pRIC, based on a mild strain of the bean yellow dwarf virus (BeYDV-m) reported by Halley-Stott *et al.* (2007). The vector was designed to increase transgene expression through replicational increase in transgene copy number of the human papillomavirus type 16 (HPV-16) major CP and human immunodeficiency virus type 1 subtype C (HIV-1C) Pr⁵⁵ Gag-derived p17/p24 antigen. In addition, the enhanced green fluorescence protein (EGFP) was used as a reporter in *N. benthamiana*. The vector differs from other BeYDV-derived vectors by the presence of a *rep* gene *in cis* rather than *in trans*. This allows the vector to replicate independently increasing the gene copy number and in turn increasing protein expression. The BeYDV CP and movement protein (MP) genes were replaced with an antigen-encoding transgene under the control of a CaMV 35S constitutive promoter. The researchers were able to achieve a 2-3 orders of magnitude increase in amplicon copy number, 3-7 fold increase in HIV-1 and EGFP

protein expression and a 50 % increase for HPV-16 (Regnard et al. 2010). The Ebola glycoprotein (GP1) was also expressed using a geminiviral vector to produce an Ebola immune complex (EIC) which showed great potential as a plant-derived human vaccine (Seong et al. 2011). Hepatitis A virus (HAV) VP1 and an Fc antibody fragment have also been expressed in *N. benthamiana* using a beet curly top virus (BCTV) replicating vector. Another example is the use of the dual-module in-plant activation (INPACT) expression platform based on the replication machinery of tobacco yellow dwarf mastrevirus (TYDV). INPACT cassette encoding the GUS reporter accumulated up to 10% TSP within 3 days after ethanol treatment. The INPACT cassette is uniquely arranged to allow for tight regulation of Rep. The gene of interest is split and can only be expressed when Rep is induced to form circular replicons (Dugdale et al. 2013).

Although ssDNA viral vectors seem to be more advantageous than other viruses due to their wide host range, ability to replicate and vast amount of proteins produced during agroinfiltration, there is still room for improvement to increase recombinant protein expression.

Silencing suppressor proteins to increase protein expression

One of the advantages of plants is that they can easily be scaled up for industrial use. However, expression of recombinant proteins usually peaks three days after agroinfiltration and rapidly declines thereafter. Post-transcriptional gene silencing (PTGS) was the proposed reason for the rapid reduction in protein production (Voinnet et al. 2002; Johansen 2001). PTGS in plants involves the degradation of dsRNA, aberrant RNA and can also be triggered by replicating RNA and DNA viruses. During silencing these RNAs are processed by an RNase III type enzyme called Dicer into short, 21–24 nucleotide long, small interfering RNA (siRNA) duplexes. siRNAs activate the RNA-induced silencing complex (RISC) for target cleavage in a sequence-specific manner (Bernstein et al. 2001). The onset of PTGS triggers systemic silencing of the target RNA by sending silencing signals from one cell to another as well as to distant plant tissue (Olivier & Baulcombe 1997; Humber et al. 2003). Since viruses are inducers of RNA silencing in plants, viruses have had to evade or suppress RNA silencing. One-way viruses mitigate this defence is through the use of silencing suppressor proteins that can target different steps in the silencing pathway.

To date, many viral suppressor proteins have been identified including those from RNA and DNA viruses (Table 1). This group of proteins is diverse in sequence, functionality and evolutionary origin. This could be the reason that these silencing proteins target different steps of the silencing pathway (Palukaitis et al. 2008; Carrington et al. 2001).

Table 1: Summary of identified silencing suppressor proteins (Csorba et al. 2015; Silhavy & Burgyán 2004)

Family	Virus	Protein	RNA/DNA
<i>Carmovirus</i>	Turnip crinkle virus	P38	(+)ssRNA
<i>Cucumovirus</i>	Cucumber mosaic virus, tomato aspermy virus	2b	(+)ssRNA
<i>Closterovirus</i>	Beet yellows virus	P21	(+)ssRNA
	Citrus tristeza virus	P20, P23	
<i>Comovirus</i>	Cowpea mosaic virus	S protein	(+)ssRNA
<i>Hordeivirus</i>	Barley yellow mosaic virus	γb	(+)ssRNA
<i>Pecluvirus</i>	Peanut clump virus	P15	(+)ssRNA
<i>Polerovirus</i>	Beet western yellow virus, cucurbit aphid-born yellow virus	P0	(+)ssRNA
<i>Potexvirus</i>	Potato virus X	P25	(+)ssRNA
<i>Potyvirus</i>	Potato virus Y, tobacco etch virus, turnip yellow virus	Hc-Pro	(+)ssRNA
<i>Sobemovirus</i>	Rice yellow mottle virus	P1	(+)ssRNA
<i>Tombusvirus</i>	Tomato bushy stunt, cymbidium ringspot virus; carnation Italian ringspot virus	P19	(+)ssRNA
<i>Tobamovirus</i>	Tobacco mosaic virus; tomato mosaic virus	P30	(+)ssRNA
<i>Tymovirus</i>	Turnip yellow mosaic virus	P69	(+)ssRNA
<i>Tospovirus NSs</i>	Tomato spotted wilt virus	NSs	(-)ssRNA
<i>Tenuivirus</i>	Rice hoja blanca virus	NS3	(-)ssRNA
<i>Phytoreovirus</i>	Rice dwarf virus	Pns10	dsRNA
<i>Geminivirus</i>	African cassava mosaic virus	AC4, AC2	dsDNA
	Tomato yellow leaf curl virus	C2	

Although literature about the suppressor proteins is quite limited, the first viral suppressor protein identified was from the *Potyvirus* family in 1998 (Anandalakshmi et al. 1998). The potyvirus-encoded helper component proteinase (HC-Pro) not only suppresses transgene- and virus-induced silencing but also maintains genome replication, long-distance movement through plants and polyprotein processing (Ma et al. 2009). Different models have been proposed for the function of HC-Pro, one being that HC-Pro suppresses RISC activation by interacting with a protein/complex involved in siRNA unwinding (Chapman et al. 2004). Further studies clarified that HC-Pro impairs RNA silencing by inhibiting the assembly of RISC (Lakatos et al. 2006).

The silencing suppression activity of cucumber mosaic virus (CMV) 2b protein was discovered in the same silencing reversal assay used for HC-Pro (Brigneti et al. 1998). In the expression study, GFP was only expressed in newly emerged tissue but not in older tissue where RNA silencing already took place before the viral infection. This suppression activity is similar to that observed from either TAV 2b (encoded by tomato aspermy cucumovirus) or p19 (encoded by tomato bushy stunt tombusvirus). However, this is in contrast to that of HC-Pro and indicates that these proteins act at different stages of PTGS. Studies suggest that CMV 2b blocks silencing signals (Voinnet et al. 1998). Another study suggested that the localization signal and nuclear targeting signal encoded by CMV is also critical for the silencing activity of CMV 2b (Lucy et al. 2000)

Voinnet *et al.* (2000) for the first time, showed that a viral protein prevented systemic signalling of RNA silencing. The systemic silencing activity of p25 protein of the potato virus X (PVX) was determined by co-infiltrating transgenic GFP *N. benthamiana* with 35S-p25 and a replicating PVX-GFP recombinant virus. P25 was able to suppress the silencing in the infiltration zone induced by 35S-GFP but not the PVX-GFP. This suggests that p25 inhibits the signal production or something upstream thereof since both local and systemic RNA transgene signalling is inhibited (Li & Ding 2001).

Viral suppressor proteins regulate the complex defence arms race between plants and viral pathogens. Although studies on these proteins have been carried out for more than ten years, literature still lacks certain aspects of the molecular nature of viral suppressor proteins (Csorba et al. 2015). Further efforts should be directed at understanding these molecular

interactions and improving molecular tools for increasing production of foreign proteins in plants that may improve crop production.

As stated before biopharming requires tools to increase recombinant protein expression. One way is the use of silencing suppressors as well as replicating vectors to manipulate these components to improve protein expression.

Tools to improve construction of expression vectors

Plant synthetic biology has paved the way to ensure that plants and algae are engineered to be more useful by producing therapeutic compounds, addressing food insecurity and ecosystem contamination (Patron et al. 2015). Assembling different DNA components in high throughput manner is essential to the advancement of plant synthetic biology. The first introduction of standardisation was the development of BioBrick™, a DNA unit that is flanked by standardised sequences to ease the assembly with other BioBrick parts (Knight 2003). The parts contain certain sequences called a prefix and a suffix that determine which parts are compatible. BioBricks made it possible to store, share and assemble these modular biological parts in different combinations (Knight 2003; Ellis et al. 2011; Patron et al. 2015). However, the disadvantage of BioBricks is the 8 bp scar (nucleotides left after using other assembly methods) sequence which affects assembly of fusion proteins and ribosomal binding sites (RBS) (Ellis et al. 2011). Many other BioBrick assemblies have been developed to compensate for the limitations of the first BioBricks standards, including BglBricks which is described to reduce the amount of scar sequences to 6bp (Phillips & Silver 2006; Anderson et al. 2010). Furthermore, Gateway cloning (Hartley et al. 2000) and Clontech In-Fusion, a recombinase-based cloning technology is described as highly efficient but also leaves behind a 21 bp scar sequence. In addition, other cloning methods such as the Uracil-Specific Excision Reagent Fusion and especially Gibson Assembly were limited because plasmids became less efficient at larger sizes and a high error rate was observed during PCR amplification of 10 kb constructs (Geu-Flores et al. 2007; Gibson et al. 2009).

Assembly methods based on Type II restriction enzymes (RE) have been used to overcome the challenges of larger constructs. These enzymes cut outside their recognition sequence which generates overhangs that are different to that of the recognition sequence (Lippow et al. 2009). This is particularly useful when assembling other fragments with matching overhangs and results in a DNA product without any scar sequences. Golden Gate is a DNA

cloning method based on these Type II REs producing 4 bp overhangs that can be ligated with T4 ligase (Engler et al. 2008; Engler et al. 2009). This standardised modular cloning is efficiently performed in a single tube resulting in ‘scarless’ assemblies that are assembled together in a defined linear order. A limitation of this method is the removal of internal Type II enzymes sites from starting modules, which makes Golden Gate a non-reusable system (Engler et al. 2014). To overcome this, a “Golden Gate Modular Cloning (MoClo) Plant Parts Kit was developed. In addition, another strategy called GoldenBraid was introduced to allow reusability of these Golden Gate parts (Sarrion-Perdigones et al. 2011). Both methods allow for the construction of transcriptional units (TU) from basic parts which can further be assembled into specifically designed destination plasmids. GoldenBraid 2.0 was then developed to address key issues such as sharing common standard parts with other users, optimizing its speed and efficiency and making software and hardware more user friendly (Sarrion-Perdigones et al. 2013). Modular cloning methods are becoming popular among plant biologist since they allow for the construction of multigene constructs from pre-made or shared DNA parts. Recently, researchers were able to adapt the RNA-guided Cas9 system to GoldenBraid. The workflow and software tools were designed and optimized for the gRNA construction and can be accessed on the GB resource site. A transient expression assay was performed in *N. benthamiana* to determine the functionality and the efficiency of gRNA–Cas9 GB tools (Vazquez-Vilar et al. 2016).

Furthermore, these vectors and the different components described above can be used to address the threats of emerging infectious diseases such as Zika virus.

Significance of Zika virus expression in plants

The Zika virus (ZIKV) epidemic which started in 2015 has affected millions of people, especially those in Brazil and has had a major impact on global health and international travel. The virus has also been associated with microcephaly and other birth defects in new-borns and Guillain–Barré syndrome in adults (Powers et al. 2016). The World Health Organization (WHO) declared the outbreak as a Public Health Emergency of International Concern (Poland et al. 2018). Since then researchers across the world have been trying to understand and develop vaccines against ZIKV.

ZIKV is an arbovirus belonging to the *Flavivirus* genus which includes other clinically important arboviruses such as dengue (DENV), Japanese encephalitis virus (JEV) and West Nile virus

(WNV) (Lanciotti et al. 2008). The Zika virion is an enveloped icosahedron and contains a non-segmented, ssRNA (+) genome. The genome encodes three structural proteins, the capsid (C), pre-membrane and envelope protein (PrME), membrane protein (M) and seven non-structural proteins (Baronti et al. 2014). The virus is transmitted by *Aedes aegypti* mosquitoes and has spread across 85 countries (www.who.int).

One of the obstacles faced by researchers in the field of vaccine development is considerable cross reactivity that has been detected between ZIKV and other flaviviruses. This is particularly important for vaccine development in areas where other flaviviruses are also endemic (Priyamvada et al. 2016). Furthermore, antibody-dependent enhancement (ADE) of ZIKV antibodies is yet to be determined. ADE is a result of antibodies from a previous serotype facilitating the infection of a second serotype which results in a more detrimental disease. At present there are many ZIKV vaccine candidates in development which are based on various technologies ranging from live virus to protein-nanoparticle conjugates; however, a commercial vaccine will not be ready in the medium term (Lazear & Diamond 2016). It is therefore a priority to develop measures to control or limit the spread of ZIKV disease.

There is a crucial need for a highly specific diagnostic assay to ensure that case management, surveillance, control, and vaccine trials are carried out efficiently (Goncalves et al. 2018). Assays for ZIKV detection requires equipped laboratory facilities with diagnostic competent personnel (Waggoner & Pinsky 2016). Reverse transcription-PCR (RT-PCR)-based assays remains the gold standard for ZIKV RNA detection and distinguishing between other flaviviruses. In addition, viral RNA has a longer duration in body fluids (urine and saliva) than serum, increasing the rate of detection using RT-PCR (Musso et al. 2015; Gourinat et al. 2015; Waggoner & Pinsky 2016). However, current serological assays require more rigorous testing and increased specificity through decreasing cross reactivity.

Another method is the production of high affinity monoclonal antibodies (MAbs) to recognise specific ZIKV antigens which are required for an antigen detection assay. MAbs have the ability to bind to molecular structures with high affinities and are also available in different quantities as homogeneous reagents (Rose et al. 2016; Sørensen 2010). MAbs are generated by immunizing animals, usually mice, with a target antigen. These target antigens such as proteins are recombinantly expressed and purified in traditional systems such as *Escherichia coli*, yeast and mammalian expression systems (Sørensen 2010). ZIKV was primarily identified

via neutralizing antibodies in human sera. Due to high level of cross reactivity with other flaviviruses which gave a false positive result, the centre for disease control (CDC) warned that ZIKV infection cannot be confirmed with serological data only. Furthermore, though several Dengue NS1 antigen assays have been developed, antigen detection assays are not available yet (Waggoner & Pinsky 2016; Goncalves et al. 2018).

Assays to detect the Zika pathogen would rely on Nucleic-Acid Testing and high-affinity monoclonal antibodies that recognize specific epitopes on ZIKV antigens. Simpler RT-PCR methods such as cartridge based assays that can simultaneously detect ZIKV and other arboviruses from a single specimen in <2 hours have the ability to be used at or near the point-of-care (Goncalves et al. 2018). The development of more-portable molecular platforms for detection of ZIKV and other arboviruses includes recombinase polymerase amplification and loop-mediated isothermal amplification (Teoh et al. 2015). Other interesting technologies are also based on clustered regularly interspaced short palindromic repeats (CRISPR) and the use of paper-based strips for ZIKV detection (Niwa et al. 2014).

The cross-reactivity problem makes flavivirus antibody detection tools unreliable. Interestingly virus-specific neutralizing antibodies collected during the late convalescent phase (i.e. >2 months) had very little cross reactivity (Collins et al. 2017). This highlighted the importance of timing of collected samples for serological assays. Another method called ZIKV NS1 blockade-of-binding (BOB) ELISA showed high specificity and sensitivity and is used in laboratories in six different countries (Balmaseda et al. 2017). Nanotechnologies have also been developed for rapid ZIKV and DENV antibody or antigen detection.

ZIKV is a public health concern and the development of new diagnostic assays and platforms must be encouraged. It is essential that a product is available to use in the field, it must be robust, easy to use, cheap, and accurate and it must have demonstrable clinical impact. A platform such as biopharming would be invaluable to speed up ZIKV detection. Many other flavivirus proteins have been expressed and purified from plants. A plant-made WNV vaccine candidate, the domain III of the E protein produced in *N. benthamiana*, elicited a potent immune response in mice (He et al. 2014). Plant-produced WNV proteins and MAbs are able to identify and detect human IgM responses to WNV infection, in serological assays. Furthermore, Yang *et al.* (2017a) were able to generate plant-produced ZIKV E proteins that

bind to a range of MAbs that recognize various ZIKV E conformational epitopes and are highly immunogenic.

The need for a ZIKV detection system and a vaccine is still a high concern for public health. Funding and efficient platforms such as biopharming could speed up the process and prevent any further outbreaks.

Project aims and objectives

Biopharming is a promising platform for the production of recombinant proteins for use in the development of vaccine candidates and therapeutic agents. However, low protein yield is still an ongoing problem. The overall aim of this project was to increase protein expression by manipulating the DNA components to improve plant expression vectors.

Silencing suppressor proteins have been shown to help with the accumulation of recombinant proteins. The first objective was to determine which of the silencing suppressors that are currently being used in our lab generate the highest protein expression. This was determined by evaluating the level of the EGFP produced from pEAQ-*HT* and pRIC 3.0 with either P19, NSs, TAV 2b and the alphasatellite *rep* as the silencing genes.

The second objective was to assemble an optimized replicating plant expression vector with the addition of the best silencing suppressor. This was done by using replicating machinery, *rep*, from three different viruses, namely the bean yellow dwarf virus (BeYDV), beak and feather disease virus (BFDV) and begomovirus alphasatellite. The GoldenBraid cloning technique was used to assemble the replicating vectors and EGFP expression was used to determine the best expression vector.

Finally, to determine the efficacy of these replicating vectors, the attempt was made to express the ZIKV pre-membrane and envelope (PrME) protein in plants. Expression of the ZIKV PrME would contribute towards vaccine development and detection assays.

Chapter 2: Investigating the effect of silencing suppressor activity on

EGFP expression in plants

2.1. Introduction

Molecular farming is a cost-effective platform which makes use of plants for the expression of recombinant proteins. The practice of expressing recombinant proteins in plants is recognised worldwide. Recombinant proteins were originally produced in bacterial and yeast cells, mammalian cell lines and insect cells (Fischer et al. 2004; Twyman et al. 2003; Sabalza et al. 2014). Plants have many advantages since they are safe and inexpensive and can easily be scaled up to agricultural levels. They are also capable of post translational modification of the recombinant proteins required for their optimal activity. However, one of the shortfalls of molecular farming is the often lower yield of recombinant proteins in plants than is achieved in mammalian cells (Sabalza et al. 2014). The protein yield is affected by genetic, epigenetic, biochemical and environmental factors. Product yield can be influenced by genetic factors such as the efficiency of mRNA production, mRNA turnover and the efficiency of protein synthesis. Furthermore, different biochemical factors can affect protein accumulation such as subcellular localization by targeting the protein to the ER using signal- or retention sequences (Zimmermann et al. 1998). Another method to increase protein yield is the use of viral silencing suppressor proteins.

Viruses are one of the main causes of diseases in plants and animals. In plants, infection can cause major crop losses by stunting growth, yellowing, necrosis and other abnormalities (Wang et al. 2012). However, plants have developed a major antiviral defence mechanism called RNA silencing that targets and degrades foreign RNA molecules. This process is also known as post transcriptional gene silencing (PTGS) (Figure 1). RNA silencing involves an RNase III-like enzyme, DICER, that processes dsRNA into 21-25 nucleotides (nt) and produces small interfering RNAs (siRNAs) (Hammond et al. 2000). siRNAs activate the RNA-induced silencing complex (RISC) for target cleavage in a sequence-specific manner (Bernstein et al. 2001).

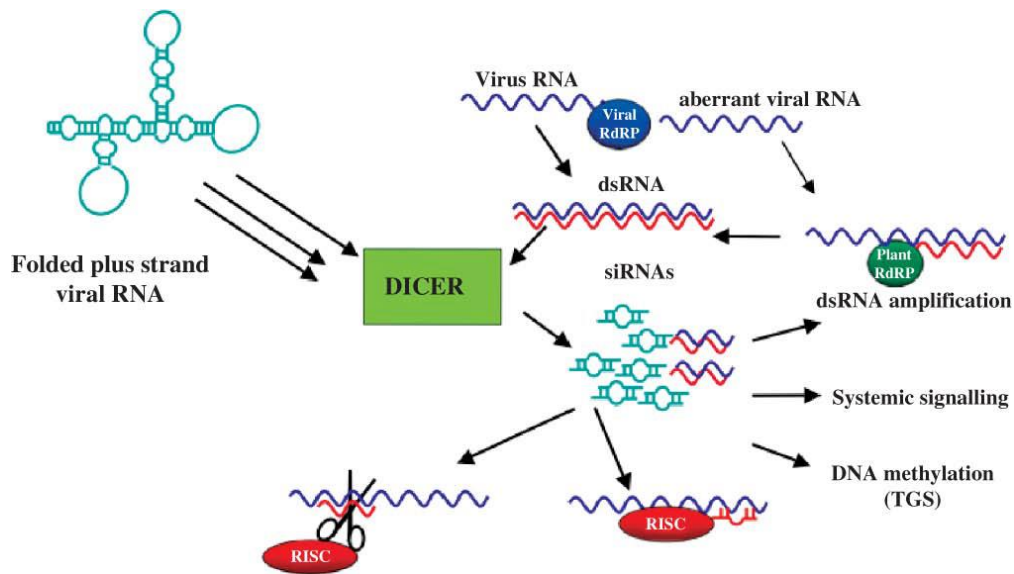


Figure 1: Virus induced RNA silencing. The figure represents a simplified model of the plant RNA silencing where double stranded (ds) RNA can be made by RdRP (RNA dependent RNA polymerase) and then diced up into 21–24 nt siRNAs. Viral siRNAs activate RISC complex for target cleavage or translational arrest (Palukaitis et al. 2008). Figure reproduced under the fair-use policy of the University of Cape Town.

These siRNAs are also responsible for systemic signalling and transcriptional silencing. Plant viruses are strong inducers and targets of virus induced gene silencing (VIGS) because the majority have RNA genomes and replicate via dsRNA intermediates (Palukaitis et al. 2008). This shows that an inducer can target degradation against itself and other high levels of foreign RNA (Johansen 2001). On the other hand, plant viruses have evolved strategies to counter this defence mechanism using viral proteins that inhibit PTGS (Silhavy & Burgyán 2004; Li & Ding 2001).

Viral suppressor proteins target step in the PTGS pathway suggesting that they evolved independently (Figure 2). Depending on the which step the proteins interfere with they can be placed into one of three groups. The first group inhibits PTGS in all the tissues of the plant which inhibits the accumulation of the processed 21-25 nucleotides RNAs (Mallory et al. 2001). The potyvirus-encoded helper component proteinase (HC-Pro) included in this group was one of the first viral proteins to be identified as a suppressor of PTGS (Palukaitis et al. 2008) (Figure 2). The second group, which includes cucumber mosaic virus protein 2b (Cmv2b), has no effect on tissues that have already activated PTGS but prevents the spread to emerging plant tissue. Studies suggest that Cmv2b protein blocks a nuclear step of PTGS that is critical for silencing suppression activity (Lucy et al. 2000; Chicas & Macino 2001). The third group includes the potato virus X p25 protein which has been suggested to block the

synthesis of the silencing signals, thereby preventing the spread of silencing (Voinnet et al. 2000; Chicas & Macino 2001). The literature about silencing suppression is still limited; however, a few viral suppressor proteins have been studied.

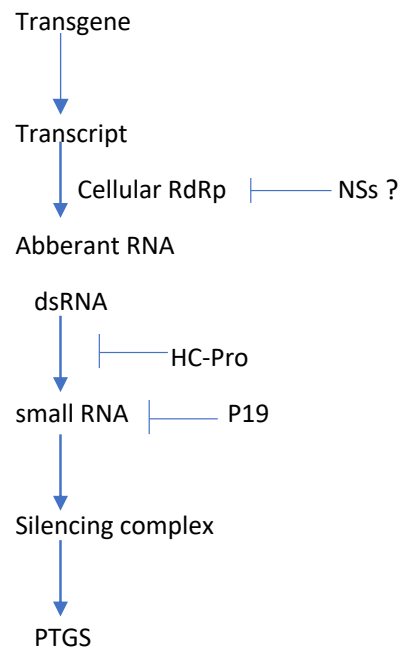


Figure 2: A model for transgene PTGS suppression. Transcription of a transgene results in production of dsRNA via cellular RdRp. The dsRNA is degraded by Dicer, an RNaseIII-like enzyme into 21- to 25-nucleotide RNAs (si RNAs). The silencing complex is induced for mRNA degradation. NSs could interfere with a step(s) for generating the dsRNA. HC-Pro suppression of silencing occurs at a step before accumulation of the small RNAs. TBSV p19 binds siRNAs.

Tomato spotted wilt virus (TSWV) species belongs to the *Tospovirus* genus within the *Bunyaviridae* family of multicomponent ssRNA viruses. The virus multiplies in insect cells and is transmitted by many thrips species (Adkins, 2000). The virus genome consists of three negative sense or ambisense ssRNA molecules. The negative sense L RNA (8.9 kb) encodes the L protein, a putative RNA dependent RNA polymerase (RdRP). The ambisense M RNA (4.8 kb) encodes the putative movement protein (NSm) and structural proteins. The ambisense S RNA (2.9 kb) encodes the nucleocapsid and NSs protein (Haan et al. 2016; Kormelink et al. 1993). The NSs protein is found mainly in the cytoplasm of plants infected with TSWV and the amount correlates with the severity of symptoms in infected plants. Takeda *et al.* (2002) showed that the TSWV NSs protein had RNA suppressor activity (Figure 2). They used a GFP

transient suppression assay that showed that the NSs protein suppressed sense transgene-induced PTGS. This suggests that NSs interferes with the step(s) before the plant's RdRP is able to synthesise dsRNA.

Tomato bushy stunt virus (TBSV), a unique RNA virus species, is a member of the *Tombusviridae* family. TBSV contains a 4.8 kb positive sense ssRNA genome that encodes five open reading frames (ORFs) (Oster et al. 1998; Qu & Morris 2002). RNA viruses generally require the expression of the coat protein (CP) or systemic movement protein in order to move long distances in plants; however, TBSV was reported to spread in the absence of CP expression (Scholthof et al. 1993). P19, derived from single guide RNA (sgRNA)-2, has been shown to be involved in suppression of PTGS of transgenes in *N. benthamiana* (Figure 2) (Voinnet et al. 1999; Carrington et al. 2001). The P19 protein was also shown to dramatically enhance transient expression of a range of proteins (Voinnet et al. 2002).

Tomato aspermy virus (TAV) is a species of the *Cucumovirus* genus that infects dicotyledonous and monocotyledonous plants. TAV has a segmented tripartite positive sense ssRNA genome. RNA-1 and RNA-2 encode proteins 1a and 2a which are involved in replication and internal transcription. RNA-3 and sgRNA-4 are translated into the movement and capsid protein. The viral suppressor of RNA silencing is encoded by ORF-2b (Salánki et al. 1997). Zhang *et al.* (2008) were able to show that the accumulation of 5' secondary siRNA and the RNA-dependent RNA polymerase (RdRP) was down-regulated by TAV 2b (Zhang et al. 2008).

Protein yield is an important factor in biopharming and determined by many other factors, one of these being the use of a silencing suppressor protein. In the experiments reported here three different silencing suppressors - NSs, P19 and TAV 2b - were compared to test their effects on recombinant protein expression (Appendix A).

2.2. Material and methods:

Construct preparation

The constructs pEAQ-P19-EGFP, pEAQ-TAV 2b-EGFP, pEAQ-NSs-EGFP pEAQ-No P19-EGFP, pRIC 3.0 and pRIC 3.0-EGFP were maintained in DH5- α chemically competent *E. coli* cells (*E. coli*™, Lucigen). Plasmids were obtained from the culture collection in the Biopharming Research Unit (MCB, UCT). The cells were streaked onto Luria-Bertani (LB) agar (0.5 % yeast, 1 % tryptone, 0.5 % sodium chloride, 1.5 % agar) plates containing 100 μ g/mL ampicillin and

left overnight (O/N) night at 37 °C. A single colony was picked and inoculated into 10 mL LB broth with 100 µg/ml ampicillin and incubated at 37 °C, for 16 to 18 hours (hrs) with agitation at 120 revolutions per minute (rpm). The plasmid DNA was extracted using the GeneJET Plasmid Mini-prep Kit (Thermo Scientific™, USA) as per manufacturer's instructions followed by quantification using a Nanodrop (Thermo Scientific™ USA).

2.2.1. Construction of vectors

Subcloning EGFP into pRIC 4.0

The EGFP gene was successfully cloned into the replicating plant expression vector, pRIC 4.0, which contain the TAV 2b silencing suppressor protein. The EGFP gene is a red-shifted variant of wild-type green fluorescent protein and has been previously shown to have a brighter fluorescence and higher expression in mammalian cells (BD Biosciences Clontech, CA, USA). PCR was carried out to amplify and add the restriction enzyme sites for cloning the EGFP gene into the pRIC 4.0. The sense primer contained a 5' *Nco*I site before the start codon which is compatible with the vectors *Afl*III site, and the antisense primer contained the 3' *Xho*I site downstream of the stop codon. The PCR mixture consisted of 1 x Phusion GC buffer, 200 µM dNTPs, 0.02 U/µL Phusion High-Fidelity DNA polymerase (Thermo Scientific), 0.5 µM GFP (*Nco*I) forward primer and 0.5 µM GFP (*Xho*I) reverse primer (Table 2) and 1 ng of pRIC 3.0-EGFP in a final volume of 50 µL. The primers amplified a 570 bp PCR product that was the EGFP gene with incorporated RE sites. The thermocycling was performed using a MyCycler™ thermal cycler (Bio-Rad) and the parameters were as follows: an initial denaturation cycle for 2 minutes at 95 °C was followed by 30 cycles of denaturation for 30 seconds at 95 °C, 30 seconds at an annealing temperature of 55 °C, extension for 90 seconds at 72 °C and a final cycle comprising of an extension at 72 °C for 2 minutes. The PCR product was resolved on a TBE agarose gel containing 2.5 mg/mL ethidium bromide and visualised under long-wavelength UV light (360 nm). The PCR product was excised under long-wavelength UV light and gel purified using the QIAquick® Gel Extraction kit (Qiagen, Netherlands) as per manufacturer's instructions. All enzymes used in this study were obtained from Thermo Scientific™ (USA) and New England Biolabs (NEB Massachusetts, USA).

Table 2: Primers used in this study as well as melting temperature (Tm) and PCR product size

Name	Forward	Reverse	Tm (°C)	Product size
EGFP	TCGAGCAC <u>CCATGGTG</u> AGC AA	CCGCTCGAGTTACTTGTACAGCTCGTC	55	738 bp
EGFP Int	CGTAAACGGCCACAAGTT	GTGCTCAGGTAGTGGTTG	53	585 bp
pTRA	CATTTCA <u>TTGGAGAGGACACG</u>	GAACTACTCACACATTATTCTGG	53	

*The restriction enzyme sites are represented by the underlined section in primer sequence

The DNA of pRIC 4.0 was retrieved from a glycerol stock in the BRU lab culture collection. The culture was streaked onto a luria agar (LA) plate with ampicillin (100 µg/ml) and incubated O/N at 37 °C. A single colony was subsequently inoculated in 10 ml LB with ampicillin (100 µg/ml) and incubated O/N with shaking. The DNA was extracted using the GeneJET Plasmid Mini-prep Kit (Thermo Scientific™, USA) as per manufacturer's instructions. The vector DNA, pRIC 4.0 was linearised with *AflIII* and *XhoI* for 1 hour and 30 minutes at 37 °C. The DNA product was purified as described above. The vector was then dephosphorylated with rAPID alkaline phosphatase as per manufacturer's instructions (Roche; Sigma-Aldrich).

The purified EGFP PCR product was digested with *NcoI* and *XhoI* for cloning into pRIC 4.0. After incubation for 1 hour at 37 °C, the DNA fragments were gel purified as described above. Ligation was performed using T4 DNA ligase from the Quick Ligation™ kit protocol (BioLabs® Inc.) with a 1:3 ratio of vector to insert in final volume of 20 µL. The mixture was incubated at room temperature for 5 minutes as per manufacturer's instructions.

Transformation

DH5-α chemically competent *E. coli* cells (*E. cloni*™, Lucigen) were transformed with a tenth of the ligation volume (2 µL) following the heat shock method (Sambrook and Russel 2001) and placed on ice for 15 minutes. The cells were heat shocked at 37 °C for 60 seconds and then placed on ice for 2 minutes. The cells were incubated with 500 µL of LB for 1 hour at 37 °C with shaking. Thereafter the pRIC 4.0-EGFP cells were plated onto LB agar plates with ampicillin (100 µg/mL) and incubated O/N at 37 °C.

Screening of positive clones

Colony PCR was used to screen for recombinant clones containing egfp. The PCR amplified a 546-bp region within egfp. A total volume of 40 μ L was used for the reaction and reagent concentrations were: 10 pmol/ μ L per EGFP Int primer (Table 2), 1.5 mM MgCl₂, 1 x GoTaq[®] Reaction Buffer, 0.2 mM dNTPs and 1.25 U GoTaq[®] DNA polymerase (Promega Madison, USA). The primers were designed to amplify a 546 bp DNA product internal to the EGFP gene. The thermocycling was performed using a MyCycler[™] thermal cycler (Bio-Rad) and the parameters were as follows: an initial denaturation cycle for 2 minutes at 95 °C was followed by 30 cycles of denaturation for 30 seconds at 95 °C, 30 seconds at the annealing temperature of 53 °C, extension for 35 seconds at 72 °C and 1 cycle comprising of a final extension at 72 °C for 5 minutes. The PCR products were visualized on a 1 % agarose gel to determine the positive colonies. The positive clones were picked from the agar plate and inoculated in 10 mL LB with appropriate antibiotics. The plasmid DNA was extracted using the GeneJET Plasmid Mini-prep Kit (Thermo Scientific[™], USA) as per manufacturer's instructions followed by quantification using a Nanodrop (Thermo Scientific[™] USA). RE digest with *EcoRV* and *XhoI* for 1 hour at 37 °C was carried out to confirm the size of the construct and fragments were resolved by gel electrophoresis on 1% TBE agarose gel. Clones were further confirmed by sequencing analysis (Macrogen Inc.) using pTRA sense and pTRA antisense primers (Table 2).

Agrobacterium tumefaciens

Agrobacterium tumefaciens strain GV3101, containing the helper plasmid pMP90RK, was used in the transformation of the pRIC 4.0-based construct. The cells were streaked out onto Luria-Bertani (LB) agar plates containing 50 μ g/mL carbenicillin, 50 μ g/mL rifampicin and 30 μ g/mL kanamycin for antibiotic selection and grown for 2-3 days at 27 °C. Thereafter, single colonies were grown in 10 mL LB broth with 50 μ g/mL carbenicillin, 50 μ g/mL rifampicin and 30 μ g/mL kanamycin at 27 °C, for 2-3 days with agitation at 120 rpm.

Electroporation and back transformation

Competent *A. tumefaciens* GV3101::p90RK cells were prepared as described by Wen-jun and Forde, (1989). Three hundred nanograms of the relevant construct was mixed with 100 μ L competent cells in a chilled 0.1 cm electroporation cuvettes (Bio-Rad) and left on ice for 5

minutes (Maclean et al. 2007). The cells were electroporated under 1.8 kV, 25 μ F and 200 Ω thereafter placed on ice and 900 μ L of LB added to the cuvette. The cells were pipetted into a sterile microcentrifuge tube and incubated at 27 °C for 2 hours. This was followed by plating the *Agrobacterium* on LA plates containing carbenicillin (50 μ g/mL), rifampicin (50 μ g/mL) and kanamycin (30 μ g/mL) and incubation at 27 °C for 3 days. The transformants were screened as described above (Wen-jun & Forde 1989).

Recombinant *Agrobacterium* was verified by back-transformation into *E. coli*. This was done by growing up 5 mL of the putative recombinant cells in LB containing the appropriate selection at 27 °C. The plasmid DNA was purified and transformed into competent *E. coli* as described above. Recombinant clones were confirmed by RE digest using *Xho*I and *Eco*RV.

2.2.2. Infiltration into *N. benthamiana*

Recombinant *Agrobacterium* cells were grown up in 10 mL LB with the appropriate antibiotics at 27 °C for 3 days to achieve an optical density at 600 nm (OD_{600}) of between 0.50-1.00. Five millilitres of the O/N culture was transferred to 50 mL LBB media (0.25 % tryptone, 1.25 % yeast extract, 0,50 % NaCl, 1 M-(N-morpholino) ethanesulfonic acid [MES] at pH 5.6 and 200 mM acetosyringone supplemented with carbenicillin (50 μ g/mL) and kanamycin (30 μ g/mL). The 50 mL cultures were incubated with agitation at 27 °C overnight. The O/N culture was diluted in infiltration media (10 mM MES [pH 5.6] and 20 mM $MgCl_2$ supplemented with 200 μ M acetosyringone) for a final OD_{600} = 0.50. The *Agrobacterium* solution was infiltrated into the abaxial air spaces of 4-6-week-old *N. benthamiana* leaves using a needleless syringe. The plants were grown at 22 °C with 16-hour light and 8-hour dark photoperiods.

2.2.3. Protein expression

Protein extraction

Total soluble protein was extracted for small scale expression studies. Briefly, three leaf discs were harvested using the inside of the top of a 2 mL microcentrifuge tube as a cutter. Two ceramic beads (Biogen) and 250 μ L per leaf disc of 1 X phosphate buffered saline (PBS) with the inclusion of Complete™, EDTA-free protease inhibitor (Roche Diagnostics, SA) as per manufacturer's instructions were added to the leaf discs. To remove particulate matter the samples were vortexed for 10 minutes, followed by centrifugation at 15 800 x g (\approx 13 000

rpm) for 5 minutes and this step was repeated. The final supernatant was then transferred to microcentrifuge tube. These clarified samples were stored at -20 °C prior to protein analysis. EGFP immunoblotting and fluorescence was standardised in that the same amount of fresh weight of plant material (same number of leaf discs) was used and that each sample was prepared identically.

EGFP fluorescence

EGFP fluorescence was quantified using a Glomax Multi+ Detection system (Promega, USA) with the blue optical kit as it excites the EGFP fluorophore with a maximum excitation wavelength of 460 nm and an emission wavelength of between 515-580 nm. A dilution series was made; 1:10 or 1:100, using sterile water and a 100 µL of each sample was loaded in triplicate into a black 384 well plate (Porvair, Whitehead Scientific).

Statistical analysis

Data were presented as mean and standard deviation (SD). ANOVA test or Student's t test was used for statistical analysis. $P < 0.05$ was considered statistically significant.

SDS-PAGE and immunoblot analysis

Samples were prepared by adding 5 x SDS sample application buffer (2 % SDS, 100 mM Tris-HCl, pH 7.5, 2 mM EDTA, 52 % glycerol, 4.3 % β -mercaptoethanol, 0.25 % bromophenol blue) to a final concentration of 1 x and heated at 90 °C for 3 minutes. Equal volumes of the samples were resolved on a 12.5 % polyacrylamide gel by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) at 20 mA for \approx 1 hour 30 minutes. The gel was transferred onto a nitrocellulose membrane by semi-dry blotting using a Bio-Rad Trans-Blot® Semi-dry transfer cell for approximately 90 minutes at 15 V. The membrane was then blocked with blocking buffer (10 x PBS, 2 % non-fat milk and 1 mL 10 % Tween-20) and incubated at room temperature for 30 minutes with gentle shaking. After removal of the blocking buffer, mouse monoclonal primary antibody (anti-GFP) (Sigma Aldrich®, Missouri, USA) diluted in blocking buffer (1:5000) was added to the membrane and probed for 2-4 hours at room temperature with shaking or O/N at 4 °C with shaking. The membrane was washed 4 x 15 minutes in

blocking buffer with shaking. It was then incubated in alkaline phosphatase-conjugated goat anti-mouse secondary IgG (SigmaAldrich, USA) at 1:5000 dilution for 2-4 hours at room temperature with shaking. The membrane was washed again 4 x 15 minutes in blocking buffer lacking milk, with shaking. The membrane was developed using 3-5 mL NBT/BCIP (nitroblue tetrazolium/5- Bromo-4-chloro-3-indoxyl-phosphate) phosphatase substrate (1-Component) (KPL, US) solution for 1 hour or until protein bands appeared after which the reaction was stopped using water.

2.3. Results

2.3.1. Vector construction

The EGFP gene was cloned into pRIC 4.0 by adding *NcoI* and *XhoI* restriction enzyme sites to the 5' and 3' ends of the gene respectively, using PCR. The PCR product and the linearised vector were ligated and transformed into *E. coli*. One colony was observed after incubation and the 546 bp band was confirmed using colony PCR (Figure 3a). The DNA was subsequently extracted and digested with *EcoRI* and *XhoI* RE which resulted in the expected banding pattern of 7201 and 874 bp (Figure 3b). The construct was further confirmed by sequencing (Macrogen) which agreed with predicted sequences on CLC Main Workbench 6. The pRIC 4-GFP was successfully electroporated into *A. tumefaciens* cells. A back transformation followed by colony PCR and RE digest was carried out to confirm that the correct DNA was electroporated into *A. tumefaciens*.

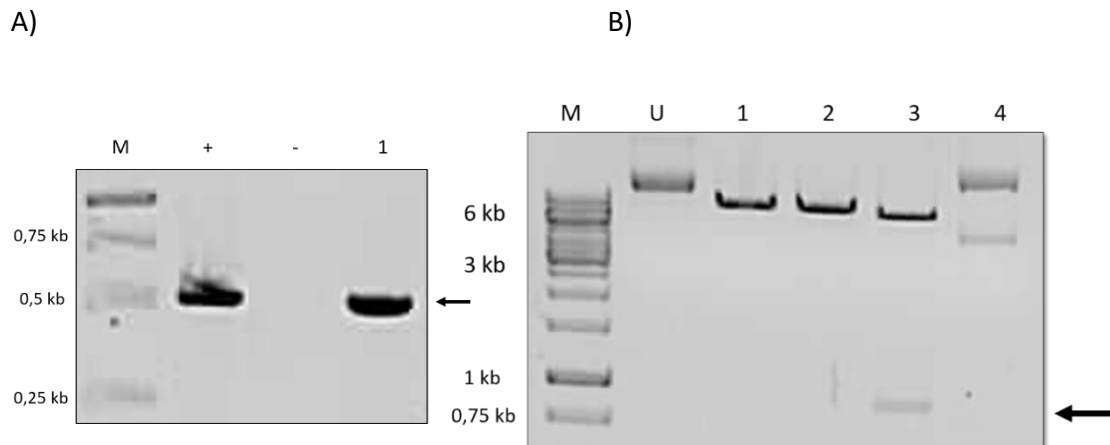


Figure 3: pRIC 4.0-EGFP vector confirmation. (A) Gel electrophoresis of colony PCR products to confirm the insertion of EGFP into the pRIC 4.0 vector. A PCR product of 546 bp (lane 1) corresponded with positive control (+). Molecular weight marker (M) and no template control (-). (B) Restriction enzyme mapping of pRIC 4.0-EGFP using *EcoRI* and *XhoI* produced the expected DNA fragments of 7201 and 874 bp (lane3). Undigested DNA control (U), *EcoRI* (lane 1), *XhoI* (lane 2), *EcoRI* and *XhoI* (lane 3), empty vector (lane 4)

2.3.2. Effect of RNA silencing suppressors on EGFP yield when transiently expressed from a non-replicating vector, pEAQ-HT

A small-scale expression time trial was carried out to determine the effect of the different silencing suppressors on EGFP yield. The accumulation of EGFP over time in plants infiltrated with *Agrobacterium* suspension was visualized using UV- light 3, 5 and 7 days post infiltration (dpi). The negative control containing no silencing suppressor, pEAQ-No P19-EGFP, had very low visible fluorescence throughout the time trial experiment (Figure 4).

Fluorescence was detectable for all the constructs although the intensity varied between the constructs. The least amount of green fluorescence was observed from the pEAQ-alphasatellite-EGFP vector whilst pEAQ-P19-EGFP, pEAQ-TAV 2b-EGFP and pEAQ-NSs-EGFP had higher levels of fluorescence in the infiltrated leaves (Figure 4).

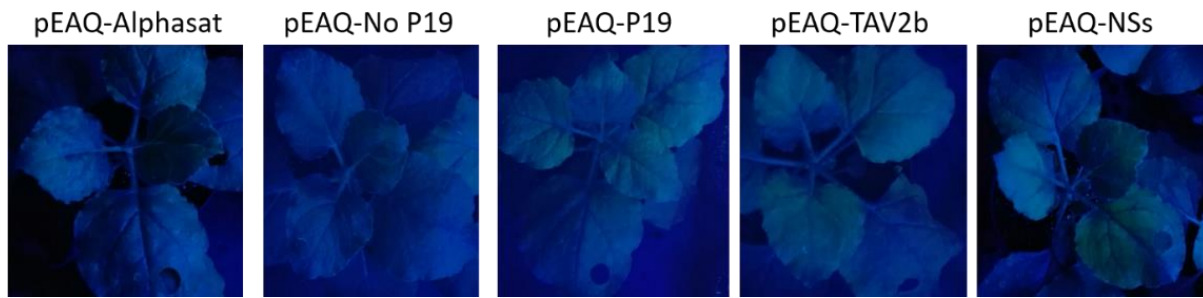


Figure 4: Fluorescence detection using UV from leaves infiltrated with different silencing suppressors 5 dpi. *Nicotiana benthamiana* leaves were infiltrated with pEAQ-alphasatellite-EGFP, pEAQ-No P19-EGFP, pEAQ-P19-EGFP, pEAQ-TAV 2b-EGFP and pEAQ-NSs-EGFP at OD₆₀₀ = 0.50. The plants were monitored for 7 days using UV light for green fluorescence detection.

The EGFP fluorescence was analysed and for days 5 and 7 there was no significant difference between the fluorescence of the three constructs, pEAQ-P19-EGFP, pEAQ-TAV 2b-EGFP and pEAQ-NSs-EGFP (Figure 5). This correlates with fluorescence observation seen in Figure 4. Although no significant difference was observed for the three vectors, fluorescence was significantly higher than the negative control and pEAQ-Alphasatellite-EGFP throughout the experiment. The negative control had little to no fluorescence and remained low for the entire experiment. The fluorescence detected from pEAQ-Alphasatellite-EGFP was comparable to the negative control with no significant difference observed between the two. There was a substantial decrease in fluorescence by day 7.

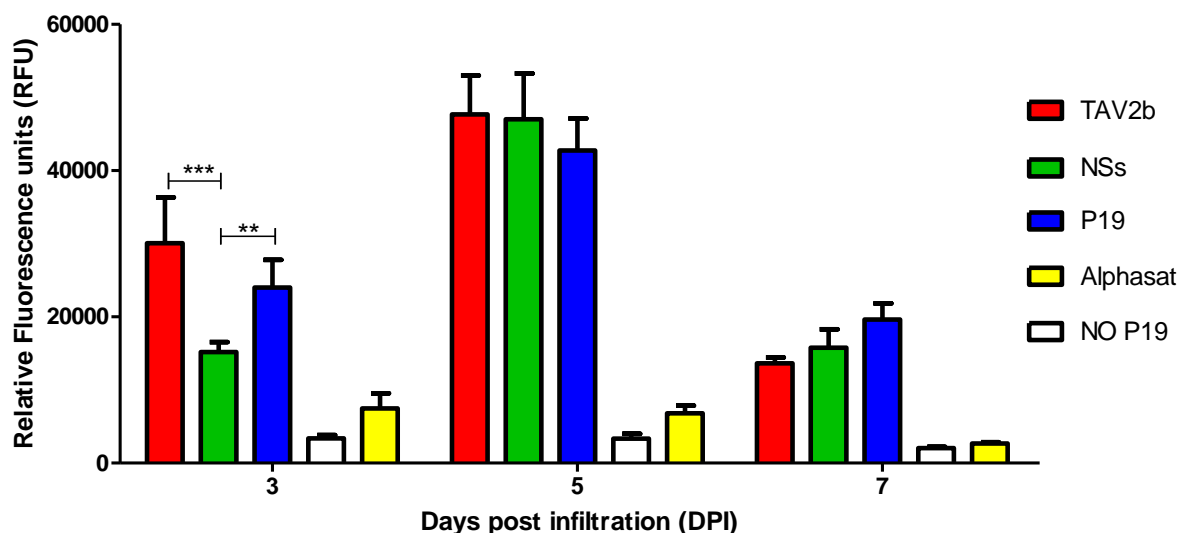


Figure 5: Fluorescence of plant-made EGFP in the presence of different silencing suppressors. Fluorescence from TSP extracts was measured 3, 5 and 7 days post infiltration with pEAQ-HT EGFP containing the silencing suppressors: P19, NSs, TAV 2b and alphasatellite Rep. A pEAQ-HT EGFP vector without a silencing suppressor served as a control. Each data point represents arithmetic mean of triplicates \pm SD. A 2Way ANOVA was used to evaluate statistical significance, P values represented as, *P<0.05.

2.3.3. Silencing suppressor activity in replicating systems

Three of the silencing suppressors showed the same enhancement in expression. Further investigations using a replicating vector focused on the smallest of the three proteins: TAV 2b. A small-scale syringe infiltration was carried out to determine the optimal optical density (OD) for the highest expression. The TAV 2b cassette was cloned into the BeYDV replicating vector pRIC 3.0, providing expression *in cis*, to create pRIC 4.0. The new vector was compared to pRIC 3.0 with and without NSs (routinely used in the lab) that supplied *in trans*. An empty pRIC 3.0 vector was used as a negative control (Regnard et al. 2010). EGFP expression and accumulation over time was determined on day 1 and 7 post infiltration via immunoblot analysis (Figure 6) from *N. benthamiana* leaves that were infiltrated with *A. tumefaciens* at an $OD_{600} = 0.50$. An EGFP specific antibody was used to detect the EGFP protein band (~ 27 kDa) which was observed for all the constructs except for the negative control pRIC 3.0 which lacked the GFP gene. Day 7 post infiltration produced a greater band intensity than day 1 which infers an increase in protein expression for all the vectors. Overall, pRIC 4.0-GFP produced a greater band intensity compared to all the other constructs. pEAQ-HT -EGFP band intensity is similar to the band intensity of pRIC 4.0-EGFP; however, according to the fluorescence results in Figure 5, the expression was still lower (Figure 6).

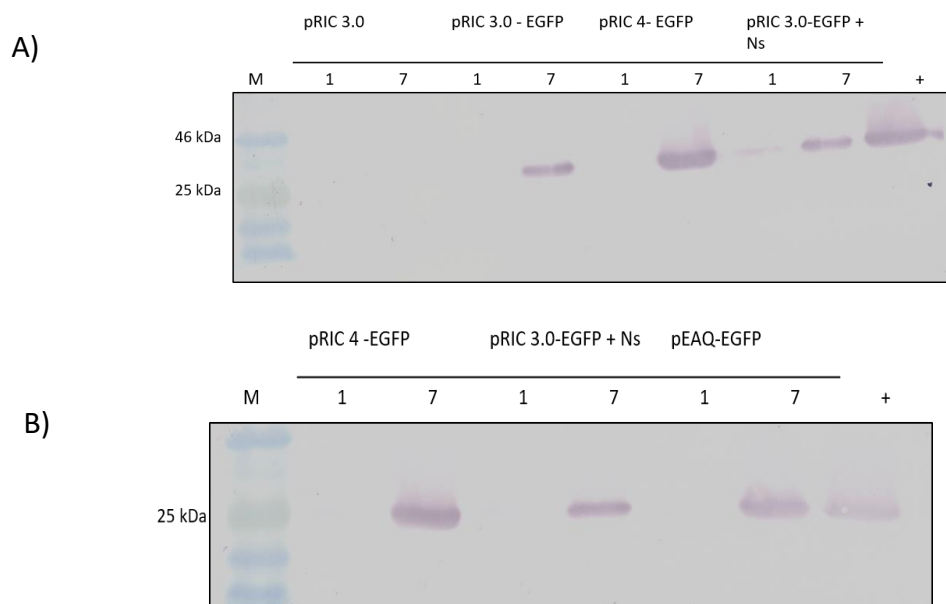


Figure 6: Immunoblots looking at the affect of two silencing suppressors, NSs *in trans* (routinely used in the lab) and TAV 2b *in cis*, on EGFP expression from replicating vectors. A) Expression on Day 1 and 7 of pRIC 4.0 EGFP incorporating TAV 2b *in cis* and pRIC 3.0 co-infiltrated with pBIN-NSs (a standard lab method). Experimental controls: pRIC 3.0 without EGFP, pRIC

3.0 EGFP lacking a silencing suppressor and EGFP positive control (+). Molecular weight marker (M). **B)** The replicating vectors with silencing suppressors compared with the non-replicating control pEAQ-HT- EGFP incorporating the TAV 2b *in cis*. Immunoblots were standardised using equal volumes of crude plant extract. EGFP was detected using mouse monoclonal primary antibody anti-GFP (1:5000).]

EGFP fluorescence was measured to determine the effect of silencing suppressor activity on EGFP protein expression levels (Figure 7). EGFP accumulation was the greatest when expressed from pRIC 4.0-EGFP containing the TAV 2b silencing suppressor with a 1.6-fold increase from day 3 to 7 post infiltration. Interestingly, there was no significant difference in EGFP expression levels between the control, pRIC 3.0 (empty vector) and pRIC 3.0-EGFP (Without any silencing suppressor). Furthermore, a slight increase is observed from day 3 to 7 for pRIC 3.0-EGFP with NSs *in trans* and was 3.6-fold lower than pRIC 4.0-EGFP (TAV 2b *in cis*) on day 7. A significantly higher EGFP expression was observed from pRIC 4.0-EGFP compared to all the other constructs, while the positive control, pEAQ-TAV 2b-EGFP (TAV 2b *in cis*) remained relatively stable from day 3 to 7.

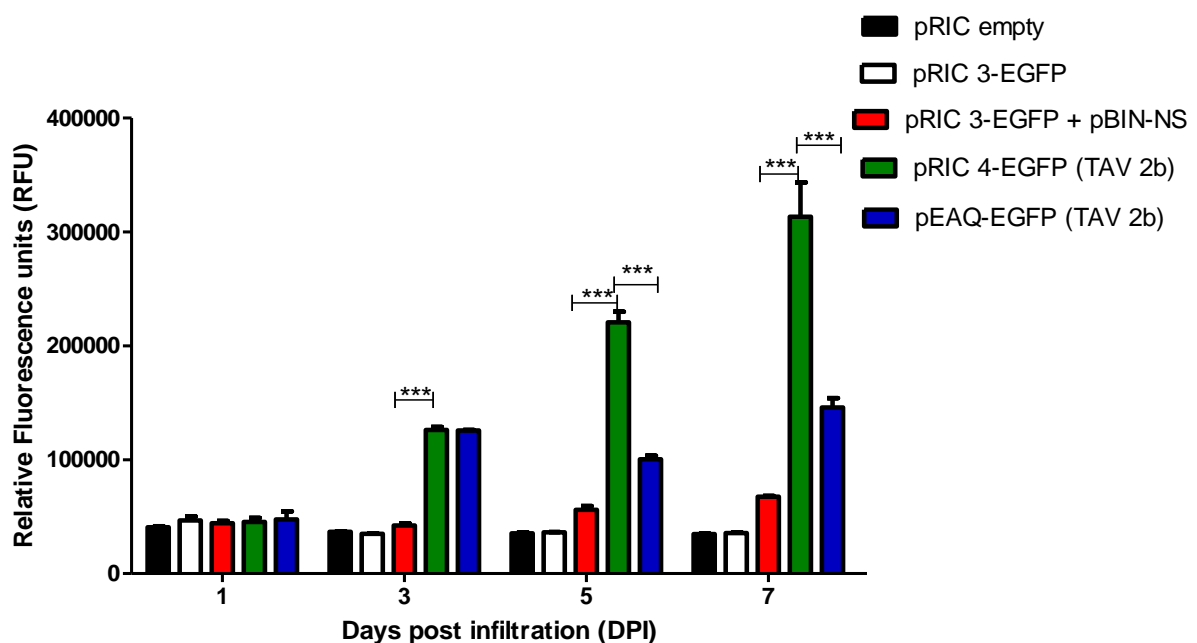


Figure 7: EGFP accumulation using replicating plant expression vectors and comparing silencing suppressors. Fluorescence from TSP extracts was measured on 1, 3, 5 and 7 days post infiltration with pRIC 3.0 EGFP and pRIC 4.0 EGFP containing TAV 2b. Controls include pRIC 3.0 without EGFP, pRIC 3.0 EGFP co-infiltrated with pBIN NSs and pEAQ-TAV 2b-EGFP. Each data point represents arithmetic mean of triplicates \pm SD. A 2Way ANOVA was used to evaluate statistical significance, P values represented as, *P<0.05.

2.4. Discussion

One way to potentially enhance expression of foreign genes in plants is the use of silencing suppressor proteins. RNA silencing in plants is an important antiviral defence mechanism; however, viruses have in turn developed proteins to counter this defence. This study looked at the effect of different silencing suppressors on GFP expression and accumulation in *N. benthamiana*.

Silencing suppressors have been used successfully for recombinant protein expression in plants. Different suppressor proteins, however, will have different effects on protein expression. This study demonstrated that the pEAQ-P19-EGFP (TBSV) and the pEAQ-NSs-EGFP (TSWV) systems (Figure 5) had similar expression levels even though the two silencing suppressor proteins (P19 and NSs) affect different stages of PTGS (Takeda et al. 2002; Silhavy & Burgyán 2004). Antibody expression has been shown to be enhanced by the presence of the TBSV P19 protein when compared to a P19 mutant (Saxena et al. 2011). Antibody production was increased between 3-5 fold when co-expressed with HCPro (757 mg/kg fresh weight) than those without (Vézina et al. 2009). P19 has also been used to enhance expression of several mAb as well as enzymes. Surprisingly the fluorescence from the negative control (i.e. pEAQ-NO P19-EGFP) was comparable to pEAQ-alphasatellite-EGFP. Alphasatellite Reps have been reported in the literature to have strong silencing suppressor activity (Nawaz-Ul-Rehman et al. 2010). The researchers infiltrated EGFP-silenced leaves with virus or satellite-encoded genes which were examined for reversal (suppression) of PTGS under UV illumination. Only the constructs expressing the alphasatellite Rep displayed a reversal of GFP silencing. The level of silencing suppression was comparable to that of P19 from TBSV. Although many begomoviruses and their associated satellites have been found to act as silencing suppressors, this was not evident in the current study. Not all satellite encoded Rep proteins have silencing activity, and a possible explanation for our results is that begomovirus-associated alphasatellite Reps are phylogenetically distinct from Reps of other begomovirus-associated alphasatellites. On the other hand, studies found that silencing suppression only takes place where there's active virus and alphasatellite replication, suggesting that the alpha-Rep protein might have a short half-life (Nawaz-Ul-Rehman et al. 2010)

The TAV 2b silencing suppressor greatly enhances EGFP expression at 5 dpi (Figure 5). The relative fluorescence of EGFP in the presence of TAV 2b was not significantly different than in

the presence of P19 and NSs, however P19 has been shown to increase symptoms of plant necrosis compared to TAV 2b (Yanez et al. 2017). The effect of TAV 2b on GFP expression could be explained by the fact that previous studies found that TAV 2b down-regulated the accumulation of RNA-dependent RNA polymerase (RdRP6) mRNA that is essential for the synthesis of secondary siRNA in plants during RNA silencing (Zhang et al. 2008; Moissiard et al. 2007). Moreover, the gene for TAV 2b is much smaller (≈ 287 bp) than the p19 (≈ 519 bp): this allows for the use of larger transgenes that won't affect infiltration or expression. The smaller size also improves efficiency of the vector when expressing the protein of interest (Huang et al. 2009; Sainsbury et al. 2009). pEAQ-TAV 2b-EGFP seems to have a similar expression as pRIC 4-EGFP based on immunoblot analysis (Figure 6) which is the opposite of what is observed in figure 7. This could be an over saturation of the blot giving the appearance that pEAQ-TAV 2b-EGFP and pRIC 4-GFP have a similar expression. Furthermore, the standardisation of EGFP was done by using the same volume which would mitigate against the effect of the replicating vectors on the plant. Since replicating vectors are shown to affect the plant leaves leading to necrosis. Normalising to TSP on these leaves (probably lower amount of host proteins due to necrosis) and healthy leaves (normal concentration of host proteins) resulting in a higher difference in EGFP. Normalising to TSP assumes that there is no difference in the levels of host proteins.

Another confusing result is the similar florescence between pRIC 3.0 (empty) and the pRIC 3-GFP (without silencing suppressor). No expression was expected from the empty pRIC 3.0 vector, but not for pRIC 3-GFP as it contains the EGFP gene. This could mean that EGFP was not expressed or that the absence of a silencing suppressor compromised the plant health which affected expression; however, immunoblot analysis indicated that expression of EGFP was apparent on Day 7. Potential markers for plant health could be identified to test the effect of vectors with and without silencing suppressors on the health of the plant. Silencing suppressors have been shown to delay the onset of plant necrosis and chlorosis. Further studies will need to be carried out to confirm this.

In conclusion, silencing suppressor proteins had a significant effect on EGFP expression level in this study, and an increase in gene copy number with a TAV 2b silencing suppressor can greatly enhance expression levels. The results confirm the increase in EGFP expression in the presence of a silencing suppressor protein. Expression is also greatly enhanced when a

replicating plant expression vector is used compared to a non-replicating vector. The results also provide evidence that the TAV 2b, P19 and NSs proteins are effective suppressors of GFP silencing.

Chapter 3: Viral replication and its effect on protein expression

3.1. Introduction

Deconstructed plant viral expression vectors have become a powerful molecular tool for the production of biopharmaceuticals. These viral vectors are capable of producing high levels of plant-produced proteins within a short period of time (Hefferon 2017).

The replication mechanisms of plant viruses have been exploited by molecular biologists to increase the expression of plant-produced biopharmaceuticals. Single-stranded RNA (ssRNA) viruses are predominant among plant viruses; however, ssDNA plant viruses are also becoming of greater importance in molecular biology and plant pathology. ssDNA viruses such as tomato yellow leaf curl virus (TYLCV), African cassava mosaic virus (ACMV) and bean yellow dwarf virus (BeYDV) belonging to family *Geminiviridae* have an important impact on the economy and are convenient for developing expression vectors (Hefferon 2014a; Scholthof et al. 2011). BeYDV belongs to the *Mastrevirus* genus and like other mastreviruses, has a single-component circular ssDNA genome (Figure 8) (Zhang et al. 2001). BeYDV has been utilized for production of monoclonal antibodies to Ebola, Zika and West Nile virus as well as for the production of novel encapsidated HPV pseudovirions (PsVs) in tobacco plants (Lamprecht et al. 2016; Huang et al. 2009). The BeYDV genome replicates via RCR with the aid of replication associated proteins (Rep/RepA). Its small simple genome can rapidly be amplified to very high copy numbers that can easily be manipulated, making it an attractive plant expression vector (Ward et al. 1988).

The viral genome contains a short intergenic region (SIR) which encodes the transcription termination signals and the DNA primer binding sites for complementary strand DNA synthesis (Figure 8). The replication associated gene (*rep*) is situated adjacent to the long intergenic region (LIR) that contains the bidirectional promoter and stemloop structure to which the Rep/RepA binds (Chen et al. 2011). The LIR and SIR are the two cis-acting elements that allow for successful BeYDV replication (Liu et al. 1998). Rep and RepA are the two

important regulatory proteins involved in replication. Rep is translated from spliced mRNA and is responsible for initiating RCR whereas RepA is translated from unspliced mRNA. Rep is the only protein required for replication but in the presence of RepA, replication is more efficient (Hefferon & Dugdale 2003; Zhang et al. 2001). Other proteins encoded include the movement protein (MP) and coat protein (CP) which are important for systemic spread and viral movement (Liu et al., 1998).

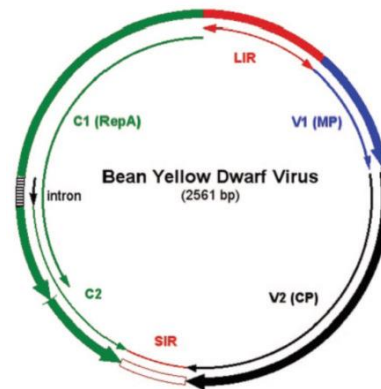


Figure 8: Bean yellow dwarf viral ssDNA circular genome structure. The BeYDV *rep* encodes the proteins Rep/RepA. BeYDV short intergenic region (SIR); long intergenic region (LIR) that contains the bidirectional promoter and stemloop structure. Movement (MP) and coat proteins (CP) (Chen et al. 2011). Figure reproduced under the fair-use policy of the University of Cape Town

Regnard et al. (2010) designed a novel self-replicating expression shuttle vector, pRIC (Figure 9), based on a mild strain of the BeYDV (BeYDV-m) (Halley-Stott et al. 2007). The vector was shown to increase transgene expression through replicational increase in transgene copy number of the human papillomavirus type 16 (HPV-16) major capsid protein (CP) and human immunodeficiency virus type 1 subtype C (HIV-1C) Pr55 Gag-derived p17/p24 antigen. In addition, the enhanced green fluorescence protein (EGFP) was used as an expression marker in *N. benthamiana*. The vector differs from other BeYDV-derived vectors by the presence of a *rep* gene *in cis* rather than *in trans*. This allows the vector to replicate independently, increasing the gene copy number and in turn increasing protein expression. The BeYDV coat protein (CP) and movement protein (MP) genes were replaced with an antigen-encoding transgene under the control of a CaMV 35S constitutive promoter (Regnard et al., 2010).

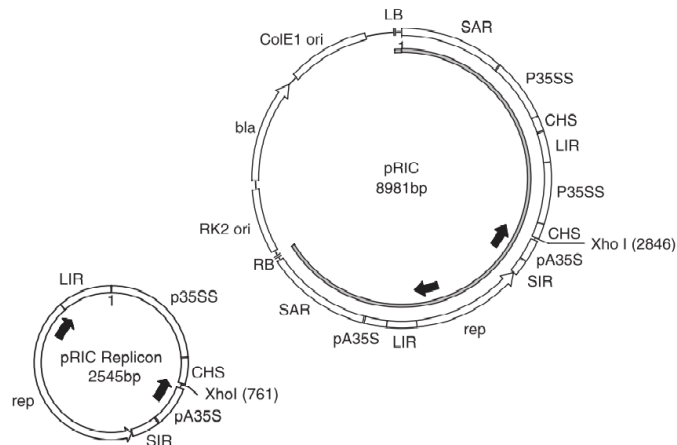


Figure 9: Schematic diagrams for the plant expression vector, pRIC. Circularized replicon after release from T-DNA and *Agrobacterium* vector, pRIC. ColE1 ori, origin of replication for *Escherichia coli*; RK2 ori, origin of replication for *Agrobacterium tumefaciens*; *bla*, ampicillin / carbenicillin-resistance *bla* gene; LBLeft (LB) and right (RB) borders for T-DNA integration. The BeYDV *rep* gene that encodes the proteins Rep/RepA is placed in *cis* rather than in *trans*. BeYDV short intergenic region (SIR); long intergenic regions (LIR) that contain the bidirectional promoter and stemloop structure. SAR, scaffold attachment region of the tobacco Rb7 gene; p35SS, CaMV 35S promoter with duplicated transcriptional enhancer; CHS, chalcone synthase 5'-untranslated region; pA35S, CaMV 35S polyadenylation signal. The grey bar represents the T-DNA transferred into the plant cell. Black arrows represents primer binding sites. (Regnard et al. 2010). Figure reproduced under the fair-use policy of the University of Cape Town

Other replicating DNA viruses: alphsatellites and beak and feather disease virus (BFDV)

Animal viruses such as the beak and feather disease virus (BFDV) and porcine circovirus (PCV) are part of the *Circoviridae* family and they also contain closed circular ssDNA. BFDV belongs to the *Circovirus* genus, which includes the smallest self-replicating mammalian viruses known (Delwart & Li 2012). BFDV infects wild and captive psittacine birds (Rahaus & Wolff 2003; Ortiz-Catedral et al. 2010). The disease ranges from the acute form seen in neonates to a chronic form that is mostly observed in adult birds (Fogell et al. 2016). BFDV has a relatively simple but compact circular ssDNA ambisense genome of approximately 2,000 nt's. The genome encodes the replication initiator protein (Rep) required for the initiation of replication and the capsid protein (CP). The intergenic region contains the origin of replication and the octanucleotide stem loop structure. The replication mechanism of circoviruses is similar to that of geminiviruses (Faurez et al. 2009; Delwart & Li 2012). It is postulated that it replicates via RCR through double stranded intermediates.

Alphasatellites are small circular DNAs that are capable of self-replication but which require a helper virus such as begomoviruses (family *Geminiviridae*) for dissemination, encapsidation and vector transmission (Nawaz-ul-Rehman & Fauquet 2009). The alphasatellite genome is

approximately 1380 bp in size and consists of one gene that encodes the Rep protein (alpha-rep) followed by an adenine rich region (A-rich) and a hairpin structure (Bridson et al. 2004). RCR is a common feature shared between alphasatellites and geminiviruses, as well as the stemloop structure and a nonanucleotide origin of replication (Nawaz-UI-Rehman & Fauquet 2009). In addition, Nawaz-UI-Rehman et al. (2010) have shown that Rep potentially acts as a suppressor of post transcriptional gene silencing (PTGS) (Nawaz-UI-Rehman et al. 2010).

Circoviruses such as BFDV share an evolutionary link with geminiviruses (Niagro et al. 1998). The non-coding regions and particularly the stem-loop structure are strikingly similar to those in the geminivirus genomes. Hexanucleotide repeats were found to flank the stemloop structure of BFDV and showed similarity with the geminivirus Rep binding site (Mankertz et al. 1998; Niagro et al. 1998). The high similarity of the Rep protein between the circoviruses BFDV and PCV with those of geminiviruses infers a phylogenetic relationship, and suggests that the Rep protein from these viruses originated from a common ancestor (Timmermans et al. 1992). Geminiviruses evolved along with their satellite circular ssDNA (Nawaz-ul-Rehman & Fauquet 2009). Their transcription regulation has been widely studied and some promoters have shown constitutive expression while others are more regulated (Zhang et al. 2012).

This study aimed to improve expression in plants based on the replication machinery of BeYDV and other replicating ssDNA genomes (BFDV and alphasatellites). To ease the designing and production of the replicating vectors, the GoldenBraid (GB) cloning technology was used to standardise the pRIC 3.0 replicating parts. The assembly of large DNA constructs from smaller fragments has seen significant developments in synthetic biology over the years. In addition, speed and efficiency is a crucial feature for any cloning strategy including the ability to reuse DNA parts (Ellis et al. 2011; Sarrion-Perdigones et al. 2013; Gibson et al. 2009; Hartley et al. 2000). GB cloning allows for exchange of standard DNA parts to facilitate the construction of complex multigene structures using specific type IIS restriction enzymes, *BsaI* and *BsmBI* (Engler et al. 2008; Sarrion-Perdigones et al. 2011). Previous assemblies such as the Golden Gate technique allowed for standardisation however, these standard parts were not able to be used for subsequent assemblies (Engler et al. 2008; Engler et al. 2014). To solve this GB makes use of a first level destination plasmid, α , that becomes the entry plasmid for the second level destination plasmid, Ω . The type IIS RE cut a few base pairs away from the recognition site resulting in a 4 bp overhang flanking each DNA part. The flanked DNA parts

define each part's relative position in the multipartite assembly and can efficiently be joined together via the compatible overhangs. The 4 bp overhang determines the order of each part in the final assembly (Figure 10) (Sarrion-Perdigones et al. 2013). The GB restriction-ligation cycles and type IIS RE enzymes ensure the stability of the desired assembly, thus making these cycles more efficient than the classical two steps of restriction and ligation (Alonso and Stepanova 2015). The technique is based on same enzymatic reaction as MoClo, a modular cloning system that differs by the addition of an extra level (level 0) for cloning of 'subparts' (Weber et al. 2011).

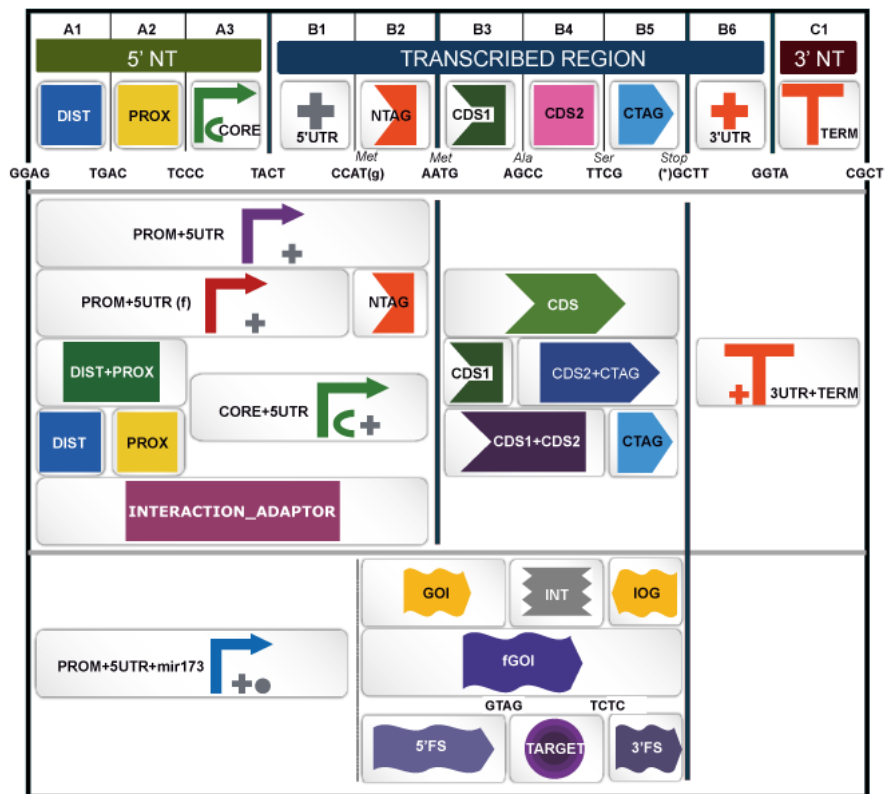


Figure 10: The GB schematic of the 4 nt code for each part in the assembly. The nt code determine the position of each GB part to ensure correct assembly. Thorough planning needs to be done to decide where each part is placed and multiple parts can be made based on this diagram. E.g. defined functional regions: complete promoter region or full coding region. Image from GB cloning website - <https://gbcloning.upv.es/search/>
Figure reproduced under the fair-use policy of the University of Cape Town

In order to further improve protein expression from pRIC 3.0, it is postulated that regulation of replication of the BeYDV-derived vector may be required to increase protein expression. Previous studies have shown that pRIC 3.0 can amplify the gene of interest via a 100x to 1000x increase in transgene copy number (Figure 9). The problem with this is, that although the

vectors strongly enhance gene expression at a transcriptional level, protein accumulation only increases 1.5- to 7-fold (Regnard et al. 2010). Based on this, the aim was to construct replicating vectors using the replication machinery from BeYDV (plant virus), alphasatellites (plant) and BFDV (avian virus) to divert resources to protein translation. The vectors consist of the viral stem loop structure that contains a nucleotide motif recognised by Rep to initiate replication. The idea is that when Rep from the plant virus binds to the stemloop structure of the avian virus, difference in the stemloop nucleotide sequence may result in fewer DNA replicons and potentially higher protein expression as a result. The EGFP gene was used as a control and to test vector efficacy between the different vectors.

3.2. Material and methods:

3.2.1. Construction of vectors

GoldenBraid cloning method

GB parts can be classified as:

- 1) **GB patches:** DNA fragments obtained from PCR amplification to remove any internal type IIS RE sites and add the appropriate 4-nucleotide overhang (Figure 10) that defines their category within the transcriptional unit (TU) (Process known as Domestication). The primers are obtained from the GB Domesticator tool (www.gbcloning.org/do/domestication).
- 2) **GB parts:** The GB patches stored as inserts within a specially designed entry vector, the universal part domesticator (pUPD).
- 3) **GB destination vectors (pDGBs):** can be categorise into two levels according to the enzyme that releases the transcriptional unit/ module and to their backbone:
 - a. **Alpha, α , level plasmids** have kanamycin resistance and release the insert upon *BsaI* digestion used for multipart assembly. To streamline the process, five alpha level plasmids (pDB alpha 11, 12, 13, 14 and 2R) were designed by Dr Tomas Moravec (Institute of Experimental Botany, Czech Republic). All alpha level plasmids will generate identical 4 bp overhangs after cutting with *BsaI* (GGAG/CGCT). However, the difference comes in when removing assembled gene cassettes from the alpha level plasmids using *BsmBI*. Here, Dr

Moravec added 4 nucleotides in each alpha level plasmid to ensure that each assembled gene cassette is ligated in the correct order. The overhang at the end of 1st gene cassette will be the same as the overhang at the start of the next gene cassette (Table 3). In addition, the first 4 bp code of pDB- α 11 corresponds to the 1st code of A1 (Figure 10) while the last 4 bp code of pDB- α 14 corresponds to 2nd code of C1 (Figure 10), maintaining the original GB code. The pDB- α 2R is the only plasmid that allows the cassette to be in a reverse orientation (Table 3).

- b. **Omega, Ω , level plasmids** contain spectinomycin resistance and *BsmBI* sites used for the final assembly.

All the destination plasmids contain 2 restriction/recognition site for the type IIS RE. The RE site and the recognition site are carefully placed in opposite directions on the destination plasmid and differ by the antibiotic resistant marker (Sarrion-Perdigones et al. 2011).

Table 3: Table representing the 4 bp overhangs within the alpha level plasmids. The overhangs at the end allow for ease of the assembly order of the gene cassettes when cloning from alpha level to omega level. The overhang at the end of 1st gene cassette will be the same as the overhang at the start of the next gene cassette. pDB3- Ω 2 is the plasmid used in the final assembly of GB cloning.

	BsmBI cut Fwd	BsmBI cut Rev
pDB-α11	GGAG	CCAT
pDB-α12	CCAT	GCTT
pDB-α13	GCTT	GGTA
pDB-α14	GGTA	GTCA
pDB-α2R	GTCA	CGCT
pDB3-Ω2	GGAG	CGCT

Domestication of GB patches

The glycerol stocks containing the template sequence were selected from the Biopharming Research Unit (BRU) culture collection database, inoculated in 10 mL LB with appropriate antibiotics and DNA extracted using the QIAquick® DNA Extraction Kit (Qiagen®) as per manufacturer's instructions. The appropriate 4 bp overhangs were selected and cloned as follows: The rep/RepA was cloned as a coding sequence (CDS) according to the convention, B3-B5 i.e. AATG-GCTT. The BeYDV LIR 1, BFDV IR and alphasatellite IR were cloned as A1-C1 part, i.e. GGAG-CGCT. BeYDV LIR 2 as a promotor sequence according to the convention, i.e. A1-B2, GGAG-AATG, to fool the primer design tool, the internal stop codon was changed and

one base deleted so that the sequence length would be a multiple of 3. The SIR was cloned as conventional terminator, B6-C1, GCTT-CGCT (Figure 10). Primers were designed using the GBCLONING.org website. Each sequence was submitted to the GBCLONING.org domesticator program as a FASTA file. The program checks for the presence of internal type IIS RE sites and automatically designs mutagenic primers however, if there is a type IIS RE present two sets of primers will be produced (Table 4). The advantage of GB2.0 mutagenesis is that typically only one round of PCR is necessary and no gel purification step is necessary. The PCR mixture consisted of 10 pmol/ μ L of forward and reverse primers (Table 4), 1.5 mM MgCl₂, 1x Phusion HF buffer, 3 % DMSO, 10 mM dNTP and 0.02 U/ μ L Phusion DNA polymerase (Thermo Scientific) and 1 ng of template DNA. In the case where two patches were amplified, 1 ng of each patch was added to the reaction mix. The thermocycling parameters using the MyCycler™ thermal cycler (Bio-Rad): an initial denaturation for 30 s at 98 °C, followed by 5 cycles of denaturation for 10 s at 98 °C, anneal for 30 s at 58 °C and 20 s at 72 °C for extension. Another 25 cycles comprising of denaturation for 10 s at 98 °C, anneal for 30 s at 60 °C and 20 s at 72 °C for extension and 1 cycle at 72 °C for 2 minutes. Two microlitres of the PCR mix were used for verification on a 1% agarose gel. Once the amplicons were verified, the remaining 40 μ L was column purified (QIAGEN).

Table 4: Goldenbraid primers used to amplify GB patches. RE sites (yellow), 4bp code for domestication only (grey) and 4bp convention for multipart assembly (purple)

4 bp overhang	GB PATCH	FORWARD	REVERSE	Used for
AATG-GCTT	BeYDV rep/repA	GCGC CGTCTC GCTCGAATG CCTTCTGCTAGTAAGAAC	GCGC CGTCTC GATACCTTA TGATCTCGGAAATCTC	To amplify from pRIC 3.0 (Regnard et al. 2010)
	BeYDV rep/repA p2	GCGC CGTCTC GGTAT CTCCTCGCAAATCTGAC	GCGC CGTCTC GCTCAAAGC TCAGTGACTCGACGATTCCC	
GGAG-CGCT	BeYDV LIR 1	GCGC CGTCTC GCTCGGGAG GTGACTCCGAGGGGTTGCCT	GCGC CGTCTC GCTCAAGCG AGCAAATACCATCACATCGTATAT	
GGAG-AATG	BeYDV LIR 2	GCGC CGTCTC GCTCGGGAG AAACTCTAGTCAATACCATCACAT	GCGC CGTCTC GCTCAATT TTGTTGTGACTCCGAGGGGT	
GCTT-CGCT	BeYDV SIR	GCGC CGTCTC GCTCGGCTT AACGTGCCTCTCCTCATACG	GCGC CGTCTC GCTCAAGCG AATGATTATTTTATGAATATA TTTCATTGTGC	
AATG-GCTT	BFDV rep	GCGC CGTCTC GCTCGAATG CCGTCCAAGGAGGGCTC	GCGC CGTCTC GATGT CTCGGGGACTTATTG	To amplify from pMA BFDV 84A 1.1mer (GQ165756)
	BFDV rep p2	GCGC CGTCTC GACAT GGTACAAGGAGGACTG	GCGC CGTCTC GCTCAAAGC TCAAAAATTGATGGGGTGGGC	
GGAG-CGCT	BFDV IR	GCGC CGTCTC GCTCGGGAG CTGCAGAGGTGCCACAGG	GCGC CGTCTC GCTCAAGCG TGTTCCCGGCGACTGTGAT	
GGAG-CGCT	alphasatellite IR	GCGC CGTCTC GCTCGGGAG GCTGCAGCTTGCGTGCTCGC	GCGC CGTCTC GCTCAAGCG CCCCGGGCTTAAGCGAGAGA	To amplify from pUC57 alphasatellite
AATG-GCTT	alphasatellite Rep	GCGC CGTCTC GCTCGAATG ATCGATATGCCTCCGCACA	GCGC CGTCTC GCTCAAAGC CAATTGTTAATCCAAGTAATC TTCAC	To amplify from pUC57 alphasatellite rep CaMV cassette
GGAG-CGCT	TAV 2b	GCGC CGTCTC GCTCGGGAG TTTAAACGAATTCGAGCTCGG	GCGC CGTCTC GGCCT CGTGTCTGGTTTATATT	To amplify from pRIC 4.0 vector
	TAV 2b p2	GCGC CGTCTC GAGGC GACTAAACCTGGAGCC	GCGC CGTCTC GCTCAAGCG TTTAAACATCTTTTATCTTTA GAGTTAAG	
GGAG-CGCT	Alphasat IR Bsal	GCGC GGTCTC GCTCGGGAG GCTGCAGCTTGCGTGCTCGC	GCGC GGTCTC GCTCAAGCG CCCCGGGCTTAAGCGAGAGA	To amplify from pUC57 alphasatellite
	pUPD2	CCCATCAACTCGAGTGCCA	GAGGAAGCCTGCATAACG	Colony PCR and sequencing
	pJET	CGACTCACTATAGGGAGAGCGGC	AAGAACATCGATTTTCCATGGCAG	

The table represents all the primer sequences used to amplify the specific genes. Primers were obtained from the gbcloning.upv.es website after submission of the template sequence.

After the DNA extraction, the PCR products were cloned into the domesticator plasmid pUPD2. Reaction mix consisted of 80 ng of column-purified PCR product and 40 ng of pUPD2, 1 µL of *BsmBI* (Thermo Scientific), 1 U T4 ligase, 1X T4 ligase buffer (Thermo Scientific) in a

final volume of 10 μ L. All the RE used in this study were purchased from Thermo Scientific. The thermocycling parameters using the MyCycler™ thermal cycler (Bio-Rad): initial digestion cycle for 20 minutes at 37 °C, followed by 45 cycles of restriction-ligation reaction for 2 minutes at 37 °C and 5 minutes at 16°C. Final digestion of excess destination plasmid for 30 minutes at 37 °C and deactivation of the enzymes for 20 minutes at 80 °C. Two microlitres of the restriction-ligation reaction mix were used to transform DH5- α chemically competent *E. coli* cells (*E. cloni*™, Lucigen) and plated onto Luria broth (LB) agar plates with chloramphenicol (CAM) (20 μ g/mL). DH5 α *E. coli* do not require IPTG and therefore only 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (40 μ g/mL X-gal) was used for blue-white selection. White colonies were screened via PCR using pUPD forward and reverse primer (Table 4). The positive clones were confirmed by RE digest and sequencing.

Alpha level assembly

Once the domesticated constructs were successfully confirmed the DNA was mixed with appropriate alpha receiver plasmid (Table 5). Reaction mix consisted of 80 ng of the domesticated GB patches and 40 ng of the alpha level receiver plasmid, 1 U T4 ligase, 1X T4 ligase buffer (Thermo Scientific) and water to 10 μ L. The cycling parameters for the restriction-ligation reaction remained the same as described above. Two microlitres of the restriction-ligation reaction mix were used to transform chemically competent *E. coli* on LB agar plates with kanamycin (50 μ g/mL) and 40 μ g/mL X-gal for blue-white selection. The white colonies were confirmed by RE digest using *EcoRI* and sequencing.

Omega (Final) assembly

Reaction mix consisted of 80 ng of each assembled gene cassette in their respective alpha level vectors and 40 ng of the pDB3- Ω 2 level receiver plasmid, 1 μ L of *BsmBI*, 1 μ L of T4 ligase, 1.5 μ L of 10x T4 ligase buffer (Thermo Scientific) in final volume of 15 μ L. The cycling parameters for the restriction-ligation reaction remained the same as described above. Two microlitres of the restriction-ligation reaction mix were used to transform chemically competent *E. coli* that were plated on LB agar plates with spectinomycin (50 μ g/mL) and 40 μ g/mL X-gal for blue-white selection. White colonies selected were confirmed by RE digest using *EcoRI* and sequencing.

Table 5: Layout of the final assembly

	Alpha level receiver plasmid				
	pDB α 11	pDB α 12	pDB α 13	pDB α 14	pDB α 2R
GB-pRIC 4-GFP (BeYDV)	TAV 2b	stuffer	LIR	<u>CaMV</u> -GFP cassette	LIR-Rep-SIR
Alphasatellite - GFP	α - IR	<u>CaMV</u> -GFP cassette	α - sat-Rep Cassette	α - IR	TAV 2b
Alphasatellite - GFP (BFDV rep)	α - IR	<u>CaMV</u> -GFP cassette	BFDV-Rep Cassette	α - IR	TAV 2b
BFDV -GFP	BFDV-IR	<u>CaMV</u> -GFP cassette	BFDV-Rep Cassette	BFDV - IR	TAV 2b
BFDV -GFP (alphasatellite)	BFDV-IR	<u>CaMV</u> -GFP cassette	α - sat-Rep Cassette	BFDV - IR	TAV 2b

Agrobacterium transformation

Competent *A. tumefaciens* EHA105 cells were thawed on ice and 1 μ L plasmid DNA was added to 40 μ L of competent EHA105 cells in a 1.5 mL microcentrifuge tube. The tube was frozen in liquid nitrogen for about 5 min and thawed at 37 °C. The freeze/thaw cycle was repeated, 0.5 mL LB was added and incubated at 28°C with shaking for 30-50 min. The cells were pelleted in a microcentrifuge for 1 min at maximum speed. Four hundred microlitres of the supernatant were removed and the pellet was resuspended in the remaining 100 μ L LB. The cells were plated onto spectinomycin (50 μ g/mL) and rifampicin (50 μ g/mL) LB agar plates and incubated at 27°C for 2 days.

3.2.2. Gene copy number and replicon formation

DNA extraction and DNA detection

Total DNA was extracted from plant tissue using Extract-N-Amp Plant PCR kit (Sigma Aldrich USA) as per manufacturer's instructions. Briefly, one leaf disc was harvested using a 2 mL microcentrifuge tube, resuspended in 100 μ L extraction buffer and heated for 10 minutes at 95 °C. After heating, 100 μ L of the dilution buffer was added and stored at 4 °C.

Rolling circle amplification (RCA)

To confirm the presence of the replicon, rolling circle amplification (RCA) was performed on total DNA using a TempliPhi DNA Amplification kit (Amersham Biosciences) as per manufacturer's instructions. RCA utilises the bacteriophage Φ 29 DNA polymerase and randomly adds hexamers that bind and exponentially amplify ss or ds circular DNA. The RCA products were digested with *Eco*RI and the positive control, pUC19 vector was digested with *Bam*HI at 37 °C for 1 h 30 min. The DNA samples were resolved on 1 % agarose gel at 120 V.

Real time quantitative PCR (qPCR)

DNA was quantified using qPCR performed using the Rotor-Gene RG3000A (Corbett Research, Australia) real time PCR machine together with the LuminoCt SYBR Green qPCR ReadyMix (Sigma-Aldrich, USA). The reaction was performed in triplicate using 0.1 mL tubes and extracted total DNA samples were diluted by a factor of 10. The reaction mixture contained 1 μ L of diluted DNA as a template in a 20 μ L reaction, 0.4 μ L of 10 μ M EGFP internal Fwd and Rvs primer (Table 2) and 10 μ L LuminoCt SYBR Green qPCR ReadyMix (Sigma-Aldrich, USA). pJET 1.2- p35SS-EGFP-pA35SS expression cassette prepared from *E.coli* cells was used as control to construct the standard curve for analysis. Cycling parameters: an initial denaturation for 2 minutes at 95 °C was followed by 40 cycles of denaturation for 3 s at 95 °C, annealing and extension for 30 s at 60 °C and hold for 5 s at 72 °C. Gene copy number was normalized to total plant DNA.

3.2.3. Protein expression

Protein extraction, assay of EGFP fluorescence and SDS-PAGE and immunoblot analysis were done as described in Chapter 2 (See Chapter 2.2.3).

3.3. Results

3.3.1. GoldenBraid assembly proof of concept

In order to become familiar with the GB cloning technique, the BeYDV plant expression vectors pRIC 3.0-GFP (Regnard et al. 2010) and pRIC 4.0-GFP (contains the TAV 2b silencing suppressor) were disassembled into standardised GB2.0 parts (Table 6).

Table 6: layout of the BeYDV expression vectors for the GB assembly

	pDBα11	pDBα12	pDBα13	pDBα14	pDBα2R
GB-pRIC 3-GFP (BeYDV)	stuffer	stuffer	LIR	<u>CaMV</u> -GFP cassette	LIR-Rep-SIR
GB-pRIC 4-GFP (BeYDV)	TAV 2b	stuffer	LIR	<u>CaMV</u> -GFP cassette	LIR-Rep-SIR

All the domesticated parts and alpha level assembly were confirmed by RE digest and sequencing (Data not shown). The final assembly was confirmed using RE digest with *EcoRV* and the correct banding pattern (6674 bp, 2676 bp, 1270 bp) was observed. GB-pRIC 4-GFP was digested with *EcoRI* and only one out of the two selected colonies resulted in the expected band size 7533 bp, 3767 bp, 750 bp represented by the red arrows (Figure 11A).

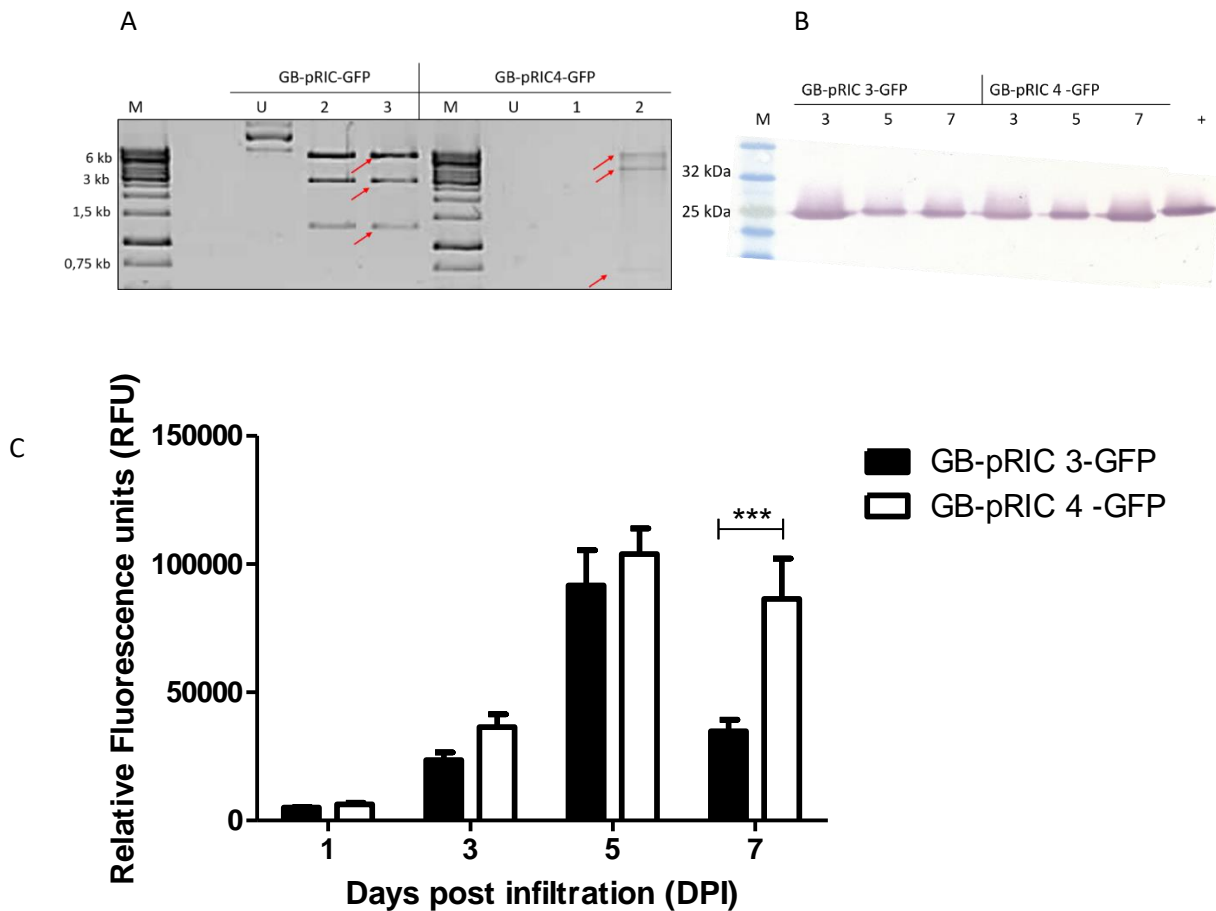


Figure 11: Goldenbraid GB-pRIC 3-GFP and GB-pRIC 4-GFP expression. (A) vector construction confirmation. GB-pRIC 3-GFP with *EcoRV* and the expected band sizes 6674 bp, 2676 bp, 1270 bp. GB-pRIC 4-GFP was digested with *EcoRI* resulting in the expected band size 7533 bp, 3767 bp, 750 bp; (U) Undigested DNA (B) Western blot analysis. *N. benthamiana* leaves were infiltrated with *A. tumefaciens* at an OD = 0.5. Leaf disc were harvested on day 3, 5, and 7, (+) positive control. Equal volumes of the crude extracts were loaded and detected using a rabbit anti-GFP antibody (1:2000). (C) Relative fluorescence units (RFU) of the Green fluorescent protein (GFP). Fluorescence reading were observed using the Glomax Multidetector system (Promega) at a maximum excitation wavelength of 460 nm and emission wavelength of between 515-580 nm. Each data point represents arithmetic mean of triplicates \pm SD. A 2Way ANOVA was used to evaluate statistical significance, P values represented as, *P<0.05.

GFP expression was determined via immunoblot analysis (Figure 11B) from *N. benthamiana* leaves. Our in-house pRIC 4-GFP was used as a positive control. Detection of the GFP protein band (~ 27 kDa) was observed for both constructs with day 3 having a greater band intensity. The intensity of the bands increases by day 7 for GB-pRIC 4-GFP construct only, while GB-pRIC 3-GFP band intensity remained fairly stable. The expression level differences between the two constructs were measured using relative fluorescence units (RFU). A higher RFU value was achieved when GFP was expressed from GB-pRIC 4-GFP with a 1.9-fold change over days 3 to 5 (Figure 11C). Furthermore, there was no significant difference in the RFU between the two

constructs on day 5 but GB-pRIC 3.0-GFP was significantly reduced by day 7 compared to GB-pRIC 4.0-GFP.

3.3.2. Effects of the BeYDV rep gene on EGFP expression

It was previously shown that protein expression from the pRIC 3.0-GFP plant vector does not correlate with the increase in gene copy number (Regnard et al. 2010). Using the GB technique for initial cloning the affect of BeYDV Rep/RepA on protein expression and gene copy number of the constructs was determined. The aim was to reduce the gene copy number and sequester resources to increase protein expression. BeYDV Rep/RepA protein binds to the LIR and initiates replication, resulting in replicon formation from the larger parent plasmid (Table 7, highlighted section). *N. benthamiana* leaves were infiltrated with **(1)** GB-pRIC 3.0-GFP (no rep): the vector does not contain the Rep/RepA gene to initiate replication; **(2)** GB-pRIC 3.0-GFP, contains all the necessary parts required for replication and **(3)** GB-pRIC 3.0-GFP (Rep outside: the Rep/RepA gene is placed on the outside of the replicon) (Table 7).

Table 7: layout of the re-assembled pRIC 3.0 vectors using GB cloning. The rows represent 3 constructs with different positions of the BeYDV rep gene. The highlighted section represents the parts required for replicon formation.

	pDB α 11	pDB α 12	pDB α 13	pDB α 14	pDB α 2R		
GB-pRIC 3.0-GFP (no rep)	Stuffer	Stuffer	LIR	eGFP	SIR	Stuffer	LIR
GB-pRIC 3.0-GFP	Stuffer	Stuffer	LIR	eGFP	SIR	Rep/RepA	LIR
GB-pRIC 3.0-GFP (Rep outside)	Rep/RepA	Stuffer	LIR	eGFP	SIR	Stuffer	LIR

The plant health was observed for 7 dpi for all the constructs (Figure 12). The plants infiltrated with GB-pRIC 3.0-GFP and GB-pRIC 3.0-GFP (Rep outside) displayed mild necrosis 5 dpi and deteriorated by day 7. Symptoms of infection in plants infiltrated with GB-pRIC 3.0-GFP (no rep) were delayed and mild chlorosis was observed by 7 (Figure 12). Due to the poor health of the plants, leaf discs were only harvested up until day 5 for optimum expression results.

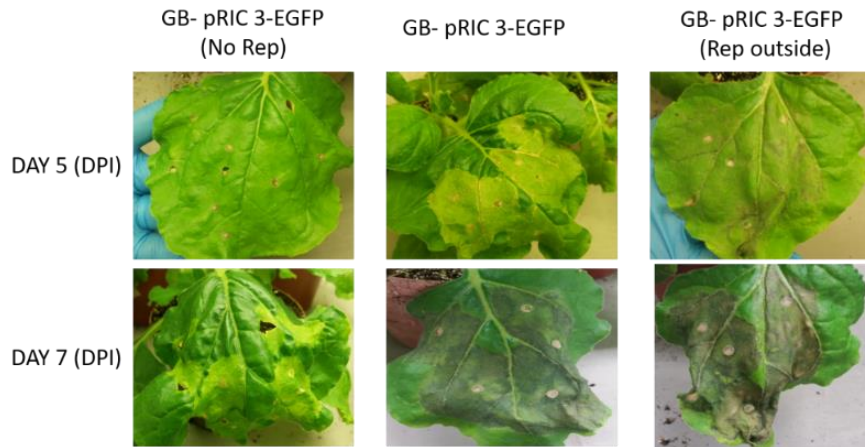


Figure 12: Effect of Rep gene on leaf morphology. Leaf morphology was monitored from 1 dpi up until 7 dpi and the figure shows a comparison between 5 and 7 dpi.

GFP RFU were measured to compare expression levels between the three constructs (Figure 13). GFP expression was the greatest when expressed from GB-pRIC 3.0-GFP (Rep outside) peaking on day 3. Surprisingly GB-pRIC 3.0-GFP expression levels were similar to GB-pRIC 3.0-GFP (no rep). By day 5 a decrease in expression levels was observed for all three constructs.

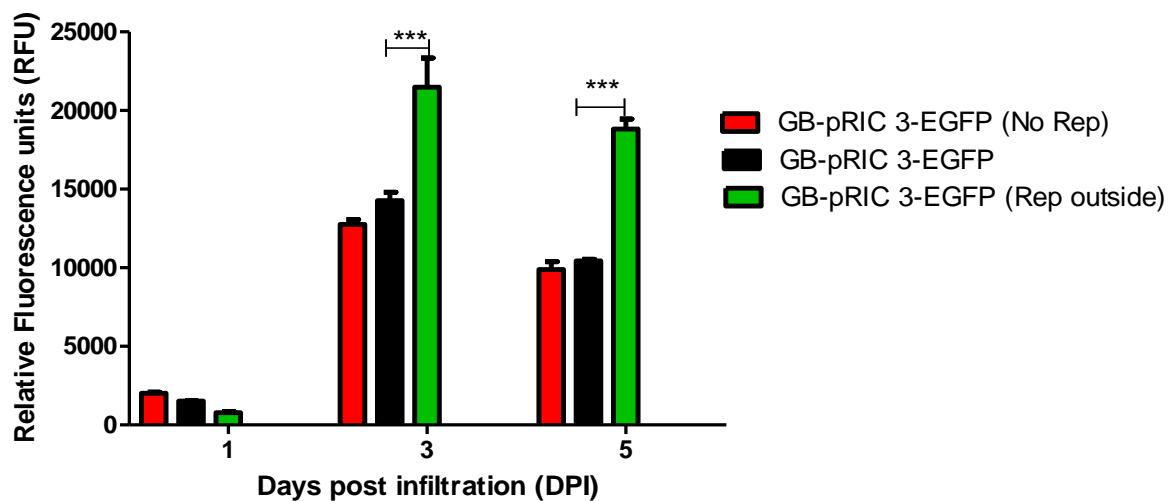


Figure 13: Time trial expression of EGFP. Relative fluorescence units (RFU) of EGFP produced in *N. benthamiana*; GB pRIC 3-EGFP (no rep), GB pRIC 3-EGFP and GB pRIC 3-EGFP (Rep outside) expression vectors were infiltrated at an $OD_{600} = 0.5$. Leaves were harvested 1, 3 and 5 days post infiltration followed by protein extraction. Fluorescence readings were observed using the Glomax Multidetection system (Promega) at a maximum excitation = 460 nm and emission wavelength = 515-580nm. Each data point represents arithmetic mean of triplicates \pm SD. A 2Way ANOVA was used to evaluate statistical significance, P values represented as, * $P < 0.05$.

The BeYDV Rep/RepA protein is responsible for replication, circularization of the replicon. DNA copies of GFP using qPCR from all three constructs were compared to determine the effect of the Rep/RepA on gene copy number. In general, an increase from 10^5 to 10^9 gene copy number was observed up until day 3 with a slight decrease by day 5 (Figure 14A). GB-pRIC 3.0-GFP peaks by day 3 which is comparable with the GFP expression levels observed for the same construct in figure 13. The same result was observed for GB-pRIC 3.0-GFP (Rep outside) although the expression level was lower compared to GB-pRIC 3.0-GFP (Figure 14A). GB-pRIC 3.0-GFP (no rep) copy number remains relatively constant with a decrease by day 5. In addition, the presence and formation of the amplicon was confirmed using RCA (Figure 14B). Absence of any bands for GB-pRIC 3.0-GFP (no rep) confirms that replication did not take place and correlates with the fluorescence and gene copy number observed above. The two bands observed (1100 bp and 2846 bp) for GB-pRIC 3.0-GFP adds up to the actual size of a replicon which is 3946 bp. This coincides with the high gene copy number but does not correlate with the low GFP RFU expression. Once again, the opposite is observed for GB-pRIC 3.0-GFP (Rep outside) where a faint band of ~2700 bp proves the replicon formation took place and a relatively intermediate copy number of replicons correlates with high GFP expression.

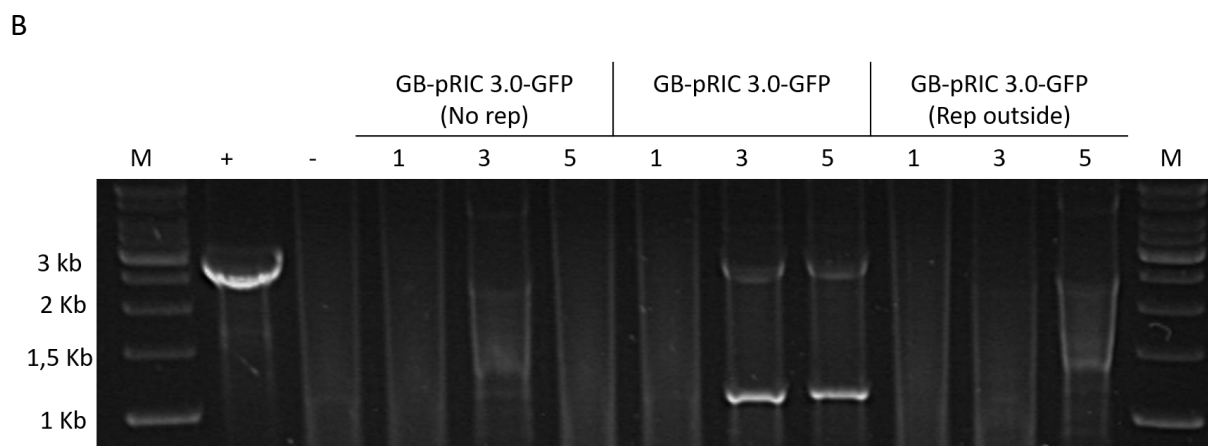
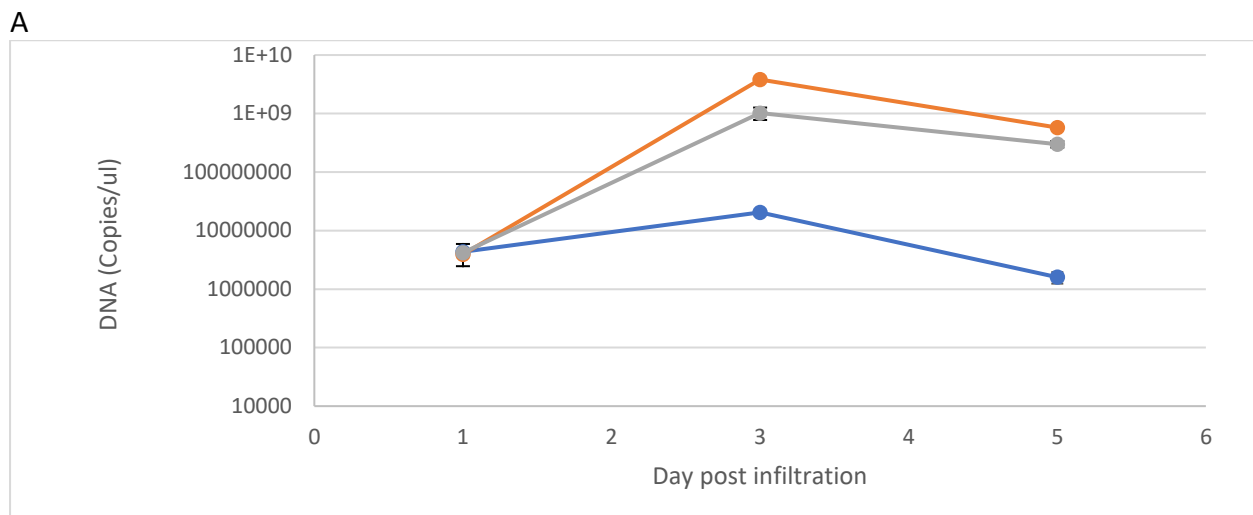


Figure 14: Effect of Rep/RepA on gene copy number and replicon formation. (A) Gene copy number was determined from plant extracted DNA using qPCR of GB pRIC 3-EGFP (no rep) (Blue), GB pRIC 3-EGFP (orange) and GB pRIC 3-EGFP (Rep outside) (Grey) expression vectors which were infiltrated at an $OD_{600}=0.5$. Leaves were harvested 1, 3- and 5-days post infiltration followed by protein extraction. Error bars represent standard deviation. **(B)** Electrophoresis gel of RE digested RCA products from total plant extracted DNA. The bands indicate the size of the expected BeYDV amplicon over 5 days, (+) pUC19 positive control (-) no template control and (M) DNA ladder.

3.3.3. Effect of protein expression using the replication machinery from BFDV and alphasatellites.

Having demonstrated the ease of use and efficiency of the GB technique as well as the effect of the BeYDV Rep/RepA protein on protein expression, I wanted to determine whether Reps from different viruses would increase protein expression. The objective of this study was to design replicating vectors using the replication machinery from BFDV, an avian virus, and plant-derived alphasatellites. Using the GB cloning technique, I aimed to determine how the

replication machinery from the above viruses would affect GFP expression in plants and how it compares to GB-pRIC 4-GFP.

Goldenbraid construct confirmation

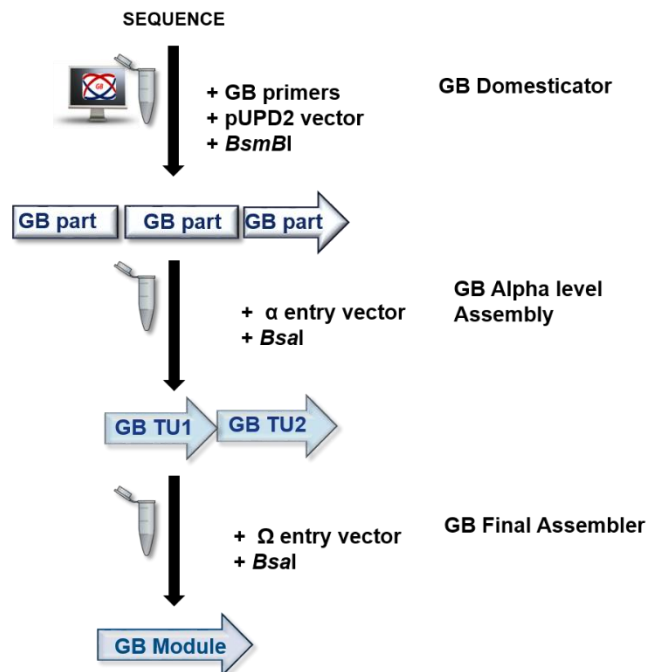


Figure 15: Basic scheme of the GB procedure. Computer screens indicate software-assisted steps. GB parts and TUs can be built and stored in a data base. Figure reproduced under the fair-use policy of the University of Cape Town

The first step of the GB process was the domestication of all the GB DNA patches to remove any internal type IIS RE (*Bsal* and *BsmBI*) sites (Figure 15). Successful PCR amplification was observed by the presence of the expected fragment size (Figure 16A); alphasatellite Rep 998 bp, alphasatellite IR 266 bp (Figure 16B), BFDV Rep patch1 775 bp, BFDV Rep patch2 153 bp, BFDV IR 202 bp, TAV 2b cassette patch1 1036 and TAV 2b cassette patch2 462 bp. After the restriction-ligation reaction using the *BsmBI* RE all the clones screened for pUPD_ alphasatellite rep and pUPD_ BFDV rep were positive with an amplicon size of 1259 and 993 bp respectively. Three out of the 4 white colonies screened for pUPD_ BFDV IR (463 bp) and pUPD_ TAV 2b (1760 bp) were positive (Figure 16C). Unfortunately, no colonies were observed for the alphasatellite IR and was it therefore not possible to clone it into the pUPD vector.

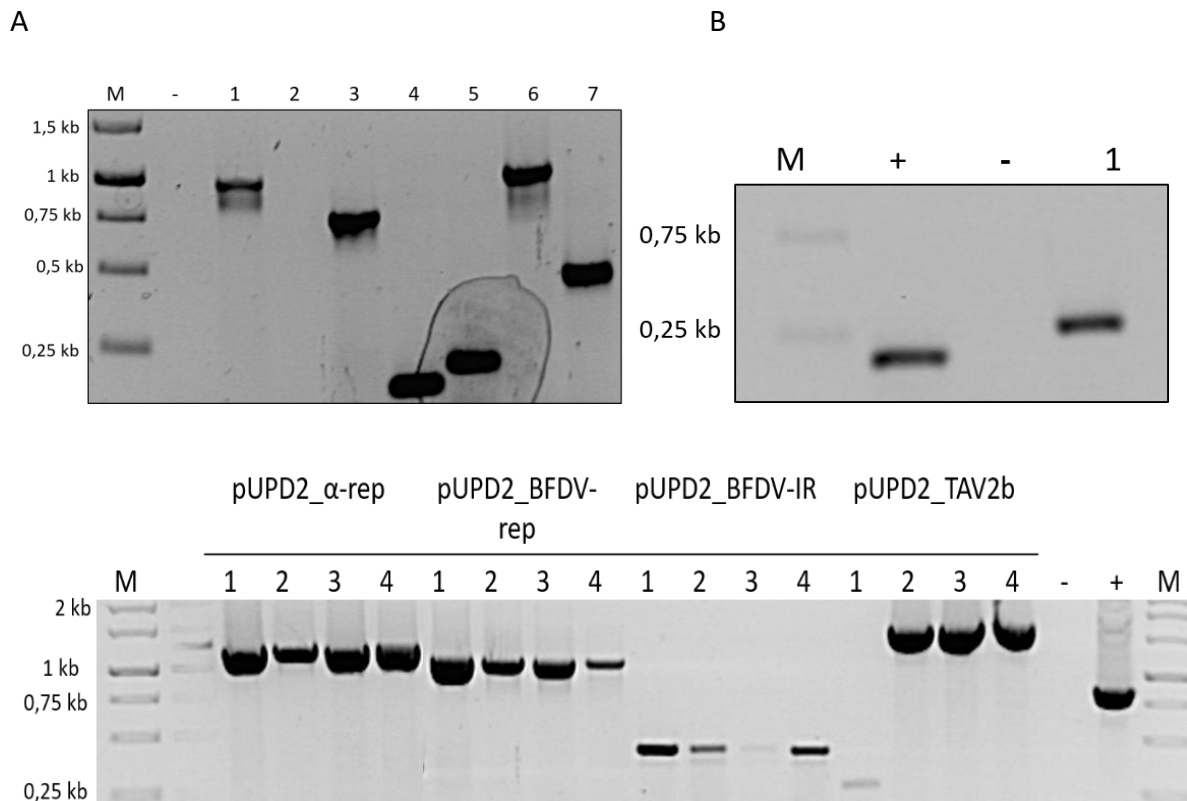


Figure 16: Domestication of the BFDV, alphasatellite DNA parts and the TAV 2b. (A) PCR amplification; (M) Marker; (-): negative, no template control; lane 1 alphasatellite rep, lane 2 alphasatellite IR, lane 3 BFDV Rep patch 1, lane 4 BFDV Rep patch 2, lane 5 BFDV IR, lane 6 TAV 2b cassette patch 1 and lane 7 TAV 2b cassette patch 2. **(B)** Successful PCR amplification of the alphasatellite IR. (-): negative, no template control and (+) positive control and lane 1) alphasatellite IR. **(C)** Colony PCR screening of white colonies using pUPD primers. (-) negative (No DNA), (+) empty pUPD vector. The expected product size was observed for pUPD_alphasatellite rep (1259 bp), pUPD_BFDV Rep (993 bp), pUPD_BFDV IR (463 bp) and pUPD_TAV 2b (1760 bp). The PCR amplicons were separated on a 1% agarose gel at 120 V for 1 hr.

The DNA was extracted for two positive clones from each construct and was further confirmed using a RE digest with *BsaI* (Figure 17). The *BsaI* RE should cut out the insert from the pUPD backbone. The expected banding pattern was observed for all 4 constructs represented by the red arrows except for colony 1 of pUPD2_alphasatellite-rep. The undigested bands which were not subjected to RE digest had the correct band size. The constructs were finally confirmed by sequencing and the results agreed with predicted sequences on Benchling (<https://benchling.com>). The DNA concentration ranged from 78.2 ng/μL to 120 ng/μL (data not shown), this could be the reason for the very faint bands observed especially 168 bp BFDV IR.

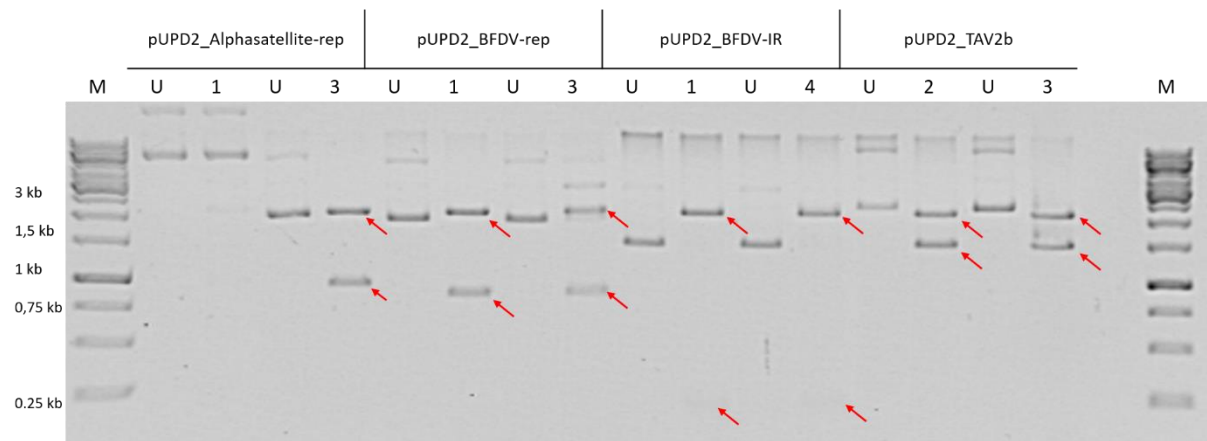


Figure 17: Domestication confirmation. Electrophoresis gel of the Restriction enzyme digest to confirm the domesticated constructs using *BsaI*. The red arrows show the correct band sizes for all the constructs. (M) DNA marker; (U) Undigested DNA; pUPD2_alphasatellite-rep= 2105 bp and 964 bp; pUPD2_BFDV-rep = 2105 bp and 868 bp; pUPD2_BFDV-IR = 2105 and 168 bp; pUPD2_TAV 2b = 2105 bp and 1465 bp.

Alpha level assembly confirmation

The domesticated GB patches were now ready for the alpha level assembly as described in table 7. After the restriction-ligation reaction with *BsaI*, a RE digest was carried out using *EcoRI* RE and the expected banding pattern was observed for all the alpha level constructs (Figure 18).

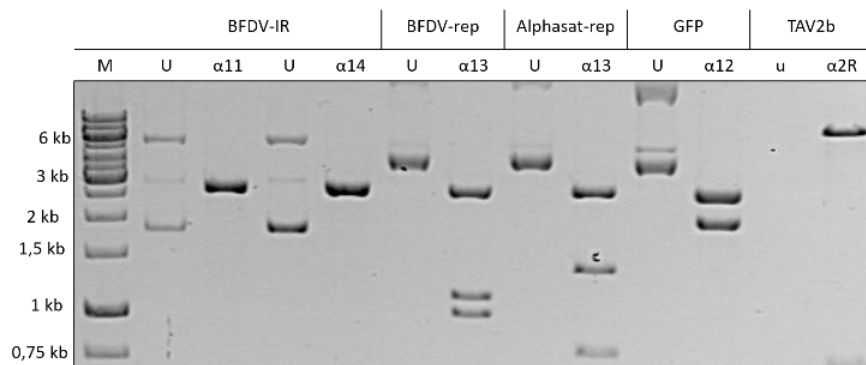


Figure 18: Restriction enzyme digest confirmation of the Alpha level constructs. Digest was carried out with *EcoRI* RE. All the constructs had the correct band sizes with the following fragment sizes; pDB α 11-BFDV_IR: 2574 & 206 bp, pDB α 14-BFDV_IR: 2574 & 198 bp, pDB α 13-BFDV_rep: 2574, 1120, 994 & 27 bp, pDB α 13-alphasatellite_rep: 2574, 1363 & 874 bp, pDB α 12-GFP_cassette: 2574 & 1994 bp, pDB α 2R-TAV 2b_cassette: 7823 & 750 bp. (M): marker and (U): undigested

Since no growth was observed, for the domestication step, the alphasatellite IR was then amplified using a new set of primers (Table 4) that contained the *BsaI* RE site for cloning directly into the alpha level vector (pDB α 11 and pDB α 14) and bypassing the domestication step. The alphasatellite IR amplicon was blunt end cloned into pJET 1.2 vector using the CloneJET PCR Cloning Kit (Thermo Scientific). Ten positive colonies were screened using pJET

specific primers and a 398 bp product was observed for 7 colonies (Figure 19A). Colonies 2, 9 and 10 were selected and digested with *Bsa*I which successfully cuts out the 237 bp IR, represented by the black arrow (Figure 19B). The pJET-*alphasatellite* IR was added to the restriction-ligation reaction with *Bsa*I to clone it into the alpha level vectors, pDB α 11 and pDB α 14. A few white colonies grew on the pDB α 14 plate and 5 were selected for RE digest with *Bsm*BI (Figure 19C). Unfortunately, the expected banding pattern was not observed and thus the *alphasatellite* IR was yet to be cloned into pUPD.

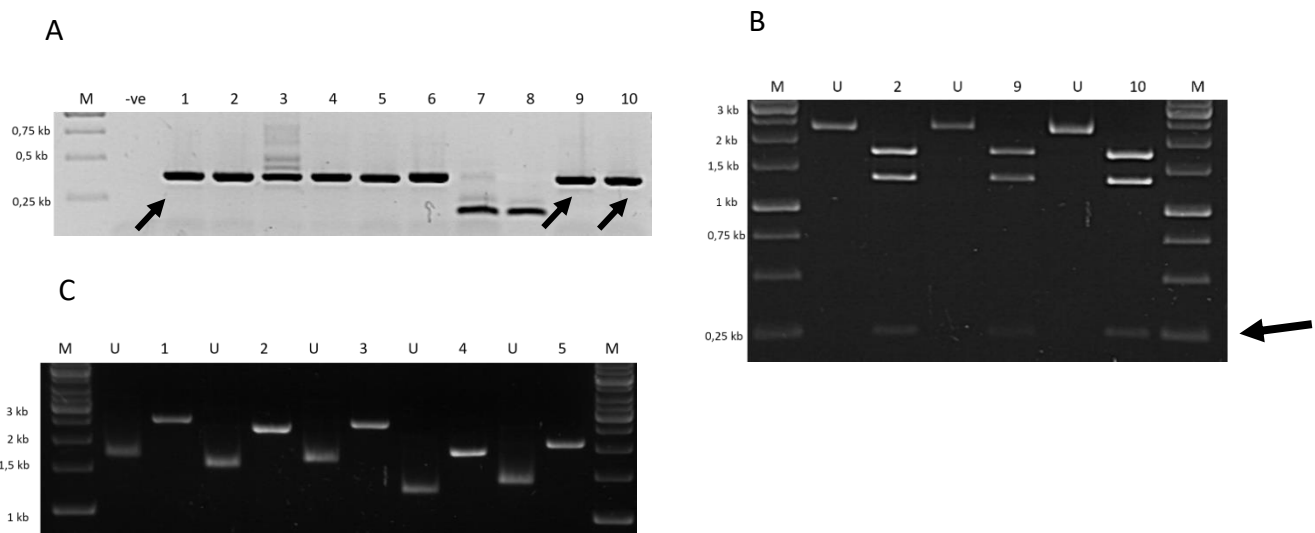


Figure 19: Confirmation of pJET 1.2_ *alphasatellite* IR and *alphasatellite* IR in pDB α 14. (A) Colony PCR of the positive clones using pJET primers, (M): marker, (-ve): No DNA template control. (B) Restriction enzyme digest confirmation of pJET 1.2_ *alphasatellite* IR colonies using *Bsa*I. The expected banding sizes 2625, 380 & 237 bp (U): undigested DNA. (C) RE digest of the pDB α 14_ *alphasatellite* IR, unfortunately the expected band size, 2596 & 241 bp, was not observed

Final assembly confirmation

The final step was to place all the alpha level assemblies with the final entry vector pDB3- Ω 2 for the last restriction-ligation reaction. Since the *alphasatellite* IR was not domesticated nor cloned into the alpha level vectors, I was unable to construct the *alphasatellite* vectors as described in table 5. I attempted to construct the BFDV vectors with GFP expression cassette, GB-BFDV-GFP, as well as the BFDV vector with the *alphasatellite* rep gene, GB-BFDV-GFP (*alphasatellite*) (Table 5). For the assembly of GB-BFDV-GFP vector, 17 colonies were observed after growth on spectinomycin plates. Unfortunately, after multiple attempts no growth took place after inoculation into LB media. A different version of the final entry vector was then used, pDB1- Ω 1, based on the pGREEN expression vector which has a lower transformation

efficiency than the original pDB3-Ω2. After transformation with the pDB1-Ω1-BFDV-GFP construct, only 16 white colonies and a ~120 blue colonies were observed. Two of the white colonies were digested with *EcoRI* (Figure 20). The bands observed were not of the expected sizes and seems to be the size of the empty pDB1-Ω1 (~3000 bp). The same results were observed for the GB-BFDV-GFP (alphasatellite rep) vector. However, the pDB3-Ω2 entry vector was used in this case and 24 white colonies had grown with only 2 blue colonies. After RE digestion the bands represented by the white arrows adds up to the size of an empty pDB3-Ω2 vector, 7295 bp (Figure 20).

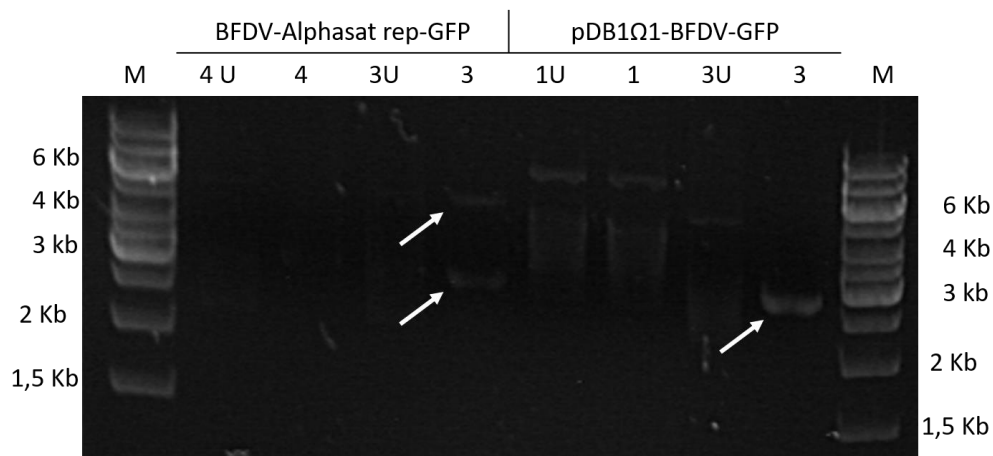


Figure 20: Gel electrophoresis of the RE digest of GB-BFDV-GFP (alphasatellite) and GB-BFDV-GFP vectors. (M) Marker; (U) undigested; BFDV-Alphasat-rep-GFP \approx 3500 bp and 2500 bp; pDB1-Ω1 \approx 3000 bp

3.4 Discussion

Expression systems based on plant viruses have become one of the most useful tools in molecular biology (Hefferon & Fan 2004; Zhang & Mason 2006; Huang et al. 2009; Regnard et al. 2010). Geminiviruses are among the viral vectors tested as an expression platform. Their replication features makes them particularly useful to increase the gene copy number and expression of foreign proteins (Regnard et al. 2010; Palmer & Rybicki 1997; Hayes et al. 1989). Regnard et al. (2010) showed high level of protein expression from the replicating vector, pRIC 3.0 compared to its non-replicating counterpart. However, gene copy number was still 100x-1000x greater than the protein expression levels that only increased 1.5-7 fold even in the presence of a pBIN-NSs silencing suppressor protein (Regnard et al. 2010). In this study I aimed to improve expression based on the replication machinery of the BeYDV and other replicating viruses (BFDV and alphasatellites). To ease the designing and production of the replicating vectors, the GB technology was used to standardise the pRIC 3.0 replicating parts. The GB assembly technology incorporates these features through the standardisation of DNA sequences or parts to form multi complex constructs (Sarrion-Perdigones et al. 2013; Sarrion-Perdigones et al. 2011). To prove the efficiency and ease of this technique, the pRIC 3.0 vector was disassembled into standard DNA parts and re-constructed into GB-pRIC 3.0-GFP and GB-pRIC 4.0 (TAV 2b). The constructs were successfully confirmed (Figure 11A) and a high level of GFP expression was observed 5 dpi (Figure 11C). This entire expression system took approximately 8-9 days to assemble and verify. This might seem time consuming but once all the parts are set up in the databases, multiple constructs can be assembled and verified within one week. The assembly, in particular, is faster than BioBricks (Knight 2003). The binary assembly of BioBricks allows only two DNA pieces to be flanked by a set of restriction sites and assembled together to form one part. This slows down the process when trying to assemble multiple parts (Knight 2003; Sarrion-Perdigones et al. 2011). Overall, I showed that DNA parts can be standardized and efficiently assembled into replicating plant expression vectors.

Plant virus expression systems have been used for efficient expression of heterologous proteins (Gleba et al. 2007). Specific interest has been given to replicating vectors based on geminiviruses. Here I tried to explore the use of the replication machinery from alphasatellites, and the avian virus BFDV. Both viruses replicate with aid of the Rep protein,

via RCR (Cheung 2015; Nawaz-ul-Rehman & Fauquet 2009). To achieve this the GB technique was used to try and assemble these replicating vectors. The *rep* genes as well as their IR were successfully amplified and cloned into the domesticator pUPD plasmid (Figure 17). These parts have now been standardised and placed into a data base for future use. Due to the close similarities of the BFDV and alphasatellites replication component, I anticipated that I would be able to observe significant effect on protein expression. The alphasatellite IR could not be domesticated and it was suggested to directly clone it into the alpha level entry vectors, however this was also unsuccessful. The 266 bp DNA fragment was blunt end cloned into pJET 1.2 and was confirmed using PCR and RE digest and therefore cannot be toxic, since it was maintained in *E. coli*. The problem appears to emerge when cloning into the GB entry vectors. This will need further investigation since all the nt overhangs were confirmed to be correct. The rest of the parts were assembled in their relative alpha level vector and were successfully confirmed. However, the final assemblies were not successful (Figure 20). Based on observation, after transformation one can tell by the number of colonies whether the reaction was successful. Many white colonies with few blue colonies indicate a successful clone. Equal amounts of white and blue colonies suggest that too much of destination plasmid was present and/or that the RE did not work well. No or only a handful of colonies means that either one of the parts were missing in the assembly reaction or was considerably under-represented. In this case very few white colonies were observed compared to the blue colonies. On plates where white colonies were observed, it would be worthwhile to screen more or all of the white colonies or design primers that would amplify different parts of the final construct. This would ensure that all the parts have been ligated. Furthermore, *BsmBI* enzymes have an optimal temperature of 37 °C (Alonso and Stepanova 2015). Enzymes from different suppliers have different optimal incubation temperatures affecting the efficiency of the restriction–ligation reactions. An increase above 37 °C would affect T4 ligase activity and stability. Example the *BsmBI* enzyme used in this study was from Fermentas (Thermo Scientific) and has temperature optimum of 37 °C but needs high dithiothreitol (DTT) concentration in the buffer. On the other hand, *BsmBI* from New England Biolabs (NEB) has a temperature optimum 55 °C but does not need the DTT. In addition, frequent freezing and thawing cycles reduces the efficiency of *BsmBI* reactions. In future work, increasing the DTT concentration would be an important step to ensure the efficiency of the enzyme as well as making 10 µL aliquots of the RE. Since DNA stock of each part are stored at -20 °C in a database, it would

be best to first confirm that the concentration of the DNA is correct by quantifying it or viewing it on an agarose gel. Perhaps it would be best to extract DNA from fresh colonies that were streaked from a glycerol stock.

Furthermore, I exploited the use of the multifunctional Rep/RepA protein from the BeYDV based vector, pRIC 3.0, to try and increase protein expression. The Rep/RepA protein aids replication which increases the gene copy number and enhances protein expression within the vector based replicons (Regnard et al. 2010). To further improve protein expression from pRIC, it was suspected that regulating replication by placing the *rep* gene on the outside of the replicon, may be required to increase protein expression. BeYDV replication has been shown to be highly efficient and may divert resources away from protein translation (Palmer & Rybicki 1997; Regnard et al. 2017). DNA replicons are particularly useful because they replicate in the nucleus using host factors to aid replication and can house large foreign inserts. Here I show that the Rep/RepA protein had a significant effect on the plant morphology and GFP expression (Figure 13). GB-pRIC 3.0-GFP (no rep) had a similar fluorescence value as GB-pRIC 3.0-GFP (Figure 13) but GB-pRIC 3.0-GFP achieved the highest gene copy of 10^9 while GB-pRIC 3.0-GFP (no rep) was significantly lower (Figure 14A). The GFP fluorescence results are contrary to what was demonstrated by Regnard et al. (2010) where the pRIC 3.0-GFP vector had a significant increase in protein expression compared to its non-replicating counterpart, pTRAc. In addition, the EGFP copy number increased from 10^6 to 10^9 copies 3 days post inoculation when expressed from pRIC 3.0-GFP which corresponds with the results obtained for the GB-pRIC 3.0-GFP. Interestingly, a noticeable improvement of GFP expression was achieved when using the GB-pRIC 3.0-GFP (Rep outside) vector which had the greatest RFU even though the *rep/repA* was placed on the outside of the replicon. The gene copy number was also significantly high even though the RCA results were less conclusive (Figure 14B). This is in line with other findings where the Rep/repA was placed *in trans*. Mor et al. (2003) demonstrated that BeYDV-derived vector co-delivered by bombardment with a Rep/RepA-supplying vector greatly enhanced GFP expression and resulted in replicon formation. Moreover, a previous study demonstrated that co-infiltration of the Rep-supplying vector and a replicon vector resulted in enhanced EGFP expression with the addition of p19 post translational silencing suppressor (Huang et al. 2009). The increase in GFP expression from the GB-pRIC 3.0-GFP (no rep) could be due to the cowpea mosaic virus

(CPMV) expression cassette. The pRIC vectors contain a cauliflower mosaic virus (CaMV) expression cassette. A previous study found increased protein accumulation as well as self-assembled virus like particles (VLPs) in the absence of the replication function of a disabled version of the Cowpea mosaic virus RNA-2 (Sainsbury & Lomonossoff 2008). Geminiviral promoters appear to be tightly regulated depending on the stage of viral life cycle (Palmer & Rybicki 1997). It would be worthwhile to investigate RNA levels to determine which step, replication or translation, leads to the decrease in protein expression. These possibilities are the subject of ongoing research.

Plants have an innate ability to induce a hypersensitive response (HR) in the presence of a pathogen attack (Greenberg, 1997; Van Wezel et al. 2002). The HR restricts multiplication and systemic spread of the pathogen. The decrease in gene copy number and GFP fluorescence also coincides with onset of leaf necrosis and chlorosis. A significant amount of necrosis and chlorosis of the leaves was observed in the presence of the Rep/RepA protein whereas in its absence the symptoms were significantly delayed (Figure 12). In addition, Van Wezel et al., (2002) were able to demonstrate that the presence of the Rep from two begomoviruses, the African cassava mosaic virus (ACMV) and the Tomato yellow leaf curl virus-China, triggered an HR. This infers that the replication machinery of the BeYDV based vectors can affect the leaf morphology which seems to have affected the protein expression levels (Figure 13). Pathogens have a counter defence which makes use of certain proteins that act as suppressors of post transcriptional gene silencing (PTGS) (Voinnet et al. 1999). The addition or co-expression with a silencing suppressor such as the P19 protein of TBSV has been shown to reduce PTGS and improve protein yields (Peyret & Lomonossoff 2015).

In conclusion, I was able to show that the GB cloning technology could be useful for disassembling and reassembling DNA parts; however, this was not the case when using DNA parts that were not in the data base. In addition, we were able to show that BeYDV Rep protein had a significant effect on protein expression depending on where it was placed within the vector.

Chapter 4: Testing of Zika virus PrME-His expression by the optimized expression vectors

4.1. Introduction

Zika virus (ZIKV) is a vector borne pathogen that was first isolated in 1947 from rhesus macaque monkeys in the Zika forest of Uganda. Recent outbreaks of Zika fever in Latin America, however, made it an emerging infectious disease (Calvet et al. 2016). Prior to this the virus had remained relatively dormant with only a few cases reported before 2007. Transmission of the virus is via the *Aedes aegypti* mosquito and the first major outbreak occurred in Yap (Federated states of Micronesia) in 2007, when approximately 75 % of the population was infected (Duffy et al. 2009). Since then ZIKV has spread sporadically to French Polynesia (2013-2014) (Hancock et al. 2014), the Cook Islands, Easter Islands, New Caledonia, and most recently the Latin Americas (Plourde & Bloch 2016). By June 2017, the World Health Organization reported that 85 countries have reported mosquito-borne Zika virus transmissions. This created a challenge particularly in areas where those infections co-circulate with dengue and other febrile-illnesses and has resulted in a heavy burden on health systems.

Virology and epidemiology

The virus belongs to *Flaviviridae* family and falls under the *Flavivirus* genus which includes other clinically important arboviruses such as dengue (DENV), Japanese encephalitis virus (JEV) and West Nile virus (WNV) (Lanciotti et al. 2008). In addition, the virus originated from two distinct African and Asian lineages as determined by phylogenetics, and is closely related to the only other member of its clade, Spondweni virus (Plourde & Bloch 2016). The Zika virion is an enveloped icosahedron and contains a non-segmented, single-stranded, positive sense RNA genome. The genome encodes for three structural proteins, the capsid (C), pre-membrane (Pr), membrane (M) and an envelope (E) protein and seven non-structural proteins (Baronti et al. 2014). The E protein consists of 3 domains (I, II and III), is responsible for virus entry and attachment, and is a major target for neutralizing antibodies (Dai et al. 2016). The virus enters the host cell via endocytosis which results in particle disassembly due to the acidification of the endosomal vesicle (Figure 21).

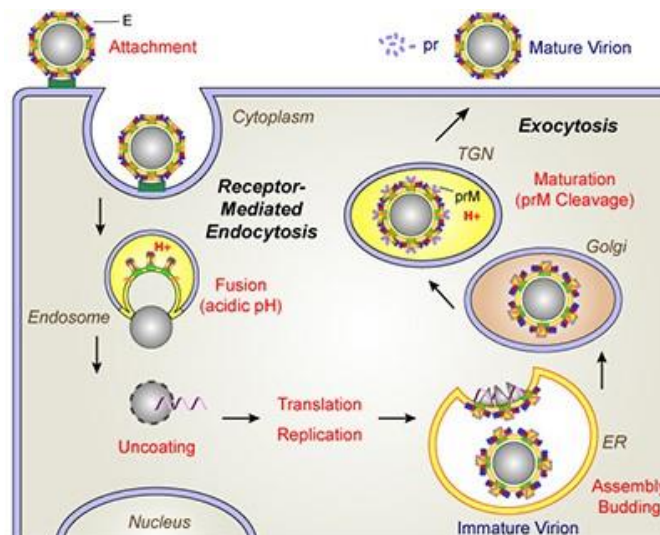


Figure 21: Flavivirus life cycle (virologie.meduniwien.ac.at). Figure reproduced under the fair-use policy of the University of Cape Town

This causes the E protein to undergo an irreversible conformational change from a dimer to a trimer due to the acidic endosomal environment (Modis et al. 2004). The genome is released into the cytoplasm and the positive sense RNA is translated into a single polyprotein. The polyprotein is then processed by viral and host proteases (Allison et al. 1995; Lindenbach & Rice 2007). Viral assembly takes place in the endoplasmic reticulum (ER), forming immature virions that contain 60 spiky heterotrimeric protrusions of E and PrM arranged in icosahedral formation (Figure 22) (Zhang et al. 2003; Dai et al. 2016; Mukherjee et al. 2016). At this stage the immature virions are considered non-infectious since the E protein cannot undergo conformational change required for viral fusion (Zybert et al. 2008). Virus maturation occurs in a low pH environment as the immature virions travel through the *trans*-Golgi network (TGN). The low pH induces a structural change of the PrME heterodimer into homodimer which also exposes the furin protease recognition site. The host cell furin protease cleaves the PrM, releasing the M protein and a soluble Pr protein. Cleavage of PrM is an important event in flavivirus maturation and essential step in the infectious life cycle (Elshuber et al. 2003). The PrM protein acts as a chaperone for correct folding of E protein (Lorenz et al. 2002). The M protein consists of a short extracellular domain attached to the membrane via two extracellular transmembrane α -helices and remains associated with the virion even after cleavage. M gets buried by the ectodomain of E protein, resulting in rare antibody responses against the M protein (Setoh et al. 2011; Davis et al. 2001; Darwish & Khor 2016; Chavez et

al. 2010). The E protein is the focus for the development of neutralizing antibodies, which have been found to mainly target domain III. The type-specific neutralizing epitopes are located in the upper lateral surface of domain III and the peptides are considered a potential candidate for vaccine development. Studies also show that fewer cross-reactive epitopes are found in domain III compared to I and II of the E protein (Beltramello et al. 2010; Chavez et al. 2010).

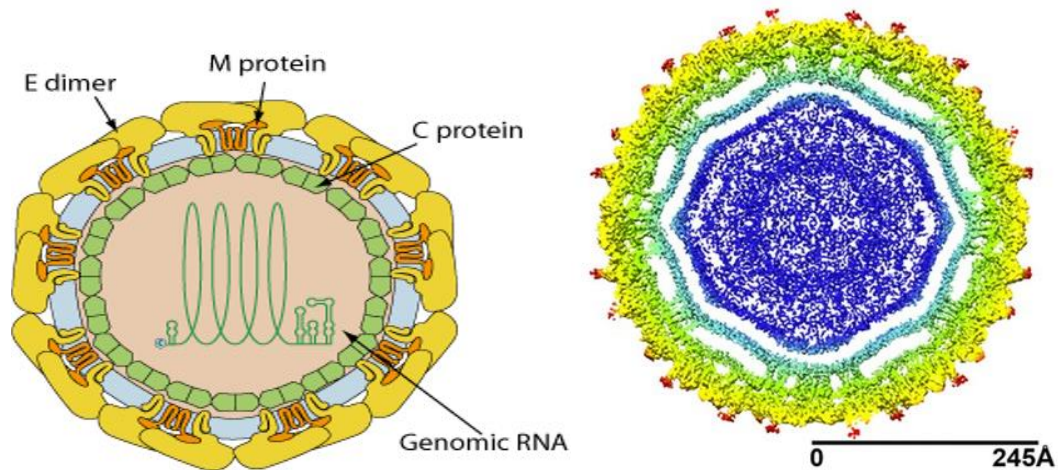


Figure 22: Structure of Flavivirus. (A) The virus consists of the envelope (E) protein (yellow), membrane (M) protein (orange) and capsid (C) protein (green). (B) The cryo-EM structure of the mature ZIKV at near atomic resolution (3.8Å). A cross-section of ZIKV showing the radial density distribution. The protein and RNA core (dark blue) contacts the inner layer of the viral membrane (aqua) and the surface proteins (red, yellow and green). Adapted from viralzone.expasy.org and (Sirohi et al. 2016) . Figure reproduced under the fair-use policy of the University of Cape Town

Diagnoses, Treatment and control

The incubation period of ZIKV in humans is approximately one week; however, infection in many cases is asymptomatic (loos et al. 2014). Symptoms occur about two to seven days after the mosquito bite, which is similar to dengue fever. Symptoms consist of mild fever, rash and headaches, myalgia, arthralgia and conjunctivitis (Duffy et al. 2009). Zika virus is also possibly associated with congenital microcephaly and Guillain–Barré syndrome. This has been confirmed with the detection of virus RNA in the amniotic fluid and brain tissue of foetuses with microcephaly (Powers et al. 2016).

During the acute phase of the illness viral nucleic acids can be detected using reverse transcription PCR (RT-PCR) (Plourde & Bloch 2016). Immunoglobulin M (IgM) antibodies are detected by IgM-capture enzyme-linked immunosorbent assay (MAC-ELISA). Unfortunately, anti-Zika virus IgM and IgG are often cross-reactive with other flaviviruses (and particularly DENV) thus, the specificity of serologic tests is limited.

There are currently no vaccines available against ZIKV infection. However, there are a few ZIKV vaccine platforms that are currently being developed as vaccine candidates: DNA based vaccines (NIAID), purified inactivated virus, plasmid-based DNA vaccine and a recombinant rhesus adenovirus serotype 52 vector that expresses the ZIKV PrME. The vaccines elicited neutralizing antibodies and completely protected monkeys against ZIKV challenge (Abbink et al. 2017). In addition, messenger RNA (mRNA) based vaccines that were tested in animals include a nucleoside-modified mRNA vaccine that is formulated with lipid nanoparticles (Pardi et al. 2017). Although these candidates look promising, the safety and cost associated with the vaccines still needs to be addressed before they can become viable. A recent study successfully demonstrated the production of plant-produced virus-like particles (VLPs) based on the hepatitis B virus core antigen (HBcAg) that displays ZIKV Domain III (DIII). The HBcAg-zDIII VLPs provided a protective immunity in mice against other Zika strains and did not enhance dengue virus infection (Yang et al. 2017a; Yang et al. 2017b).

In this study, I attempted to express the pre-membrane (PrM) and enveloped (E) protein with a His tag, using two plant expression vectors. The aim was to express the ZIKV-PrME gene in *N. benthamiana* from different plant expression vectors to determine vector efficacy and produce plant-made ZIKV VLPs.

4.2. Material and methods:

4.2.1. Construction of vectors

Gene synthesis

The PrME gene sequence was assembled as a consensus through multiple sequence alignment and then synthesized by GenScript® (China) so as to include restriction sites for cloning into the plant expression vectors (Figure 23A). The gene was also codon-optimized for expression in tobacco plants.

Cloning into pEAQ-HT and pRIC 4.0

The gene was successfully cloned into the two plant expression vectors pRIC 4.0 (a geminivirus-derived replicating vector) and pEAQ-HT (Sainsbury et al. 2009), which contains a hyper-translatable element (Figure 23B). A 6x histidine tag sequence was added to the C-terminal end of the E gene using high-fidelity PCR. The PrME sense primer contained the *AgeI* and *BspHI* for cloning into pEAQ-HT and pRIC 4.0 respectively (Table 8). The 6x histidine tag and *XhoI* site were added using two antisense primers, PrME 3 his R and PrME 3 his *XhoI* R. The first high-fidelity PCR 50 μ L had final concentrations of 1x Phusion GC buffer, 200 μ M dNTP's, 0.02 U/ μ L Phusion DNA polymerase (Thermo Scientific™ (USA)), 0.5 μ M PrME sense primer and 0.5 μ M PrME 3 his antisense primer (Table 8) and 1 ng/ μ L of pEAQ-PrME template DNA. The primers amplified a 2024 bp DNA product encompassing the PrME gene and incorporating 3 histidine codons. The thermocycling was performed using a MyCycler™ thermal cycler (Bio-Rad) and the parameters were as follows: an initial denaturation cycle for 30 s at 98 °C was followed by 30 cycles of denaturation for 10 s at 98 °C, 30 s at an annealing temperature of 56 °C, extension for 60 s at 72 °C and a final cycle comprising of an extension at 72 °C for 300 s. The PCR product was resolved on TBE agarose gel containing 2.5 mg/mL ethidium bromide and visualised under long-wavelength UV light (360 nm). The PCR product was excised under long-wavelength UV light and gel purified using the QIAquick® Gel Extraction kit (Qiagen, Netherlands) as per manufacturer's instructions. The purified PCR product was used as a template for a PCR to include the final histidine codons and restriction enzyme site (Table 8). The reaction conditions were kept the same as described above except 0.5 μ M PrME 3 His *XhoI* reverse primer (Table 8) was used. The PCR product was purified as described above. All restriction enzymes used in this study were obtained from Thermo Scientific and New England Biolabs (NEB). The vector DNA, pEAQ-HT and pRIC 4.0, was extracted using the GeneJET Plasmid Mini-prep Kit (Thermo Scientific) as per manufacturer's instructions. The vector DNA was linearised with *AflIII/XhoI* (pRIC 4.0) and with *AgeI/XhoI* (pEAQ-HT) for 1 hour at 37 °C. The DNA fragments were purified as described above.

The purified PrME-6x His PCR product was digested with either *AgeI/XhoI* or *BspHI/XhoI* for cloning into pEAQ-HT and pRIC 4.0 respectively. After incubation for 1 hour at 37 °C, the DNA fragments were gel purified as described above. DNA ligation was performed using T4 DNA ligase from the Quick Ligation™ kit protocol (BioLabs® Inc.) with a 1:3 ratio of vector to insert

in final volume of 20 μ L. The mixture was incubated at room temperature for 5 minutes as per manufacturer's instructions.

Table 8: Primers used for PCR and qPCR

Name	Sequence	Tm (°C)	Additional notes
PrME Fwd	5' GCGACCGTTCATGACAAG 3'	61	Cloning into the plant expression vectors
PrME 3 His R	5' atggtgatgAGCTGAAACAGCAGTAGAAAGAAA 3'	60	
PrME 3 His XhoI R	5' CCGCTCGAGctaataatgatggtgatggtgatgAGCTGAAACAGCC 3'	62	
PrME internal F	5' ATGCCCAATGCTTGATGAAGG 3'	56	Used for colony PCR
PrME internal R	5' CCAAAGTAGCCTCAGCCCTAGG 3'	58	
pTRA-FP	5' CATTTCATTTGGAGAGGACACG 3'	64	Used for sequencing
pTRA-RVS	5' GAACTACTCACACATTATTCTGG 3'	64	
pEAQ-HT F	5' TTCTTCTTCTTGCTGATT GG 3'	56	
pEAQ-HT R	5' CACAGAAAACCGCTCACC 3'	56	

Primers were designed using the CLC Main Workbench 6 (QIAGEN Bioinformatics) and synthesised by the DNA Synthesis Unit (MCB, UCT).

***Escherichia coli* preparation and transformation**

Escherichia coli preparation and transformation of DNA were done as described in Chapter 2 (See Chapter 2.2.1).

Screening of positive clones

Putative positive clones were screened using colony PCR in a final volume of 40 μ L. The final reaction concentrations were 10 μ M of PrME Int Fwd primer and the PrME Int Rvs primer (Table 8), 1.5 mM MgCl₂, 1x GoTaq® Reaction Buffer, 0.2 mM dNTP and 1.25 U GoTaq® DNA polymerase (Promega, USA). The primers were designed to amplify an 890 bp DNA product encompassing the PrME gene. The thermocycling parameters using the MyCycler™ thermal cycler (Bio-Rad): an initial denaturation cycle for 2 minutes at 95 °C was followed by 30 cycles of denaturation for 30 s at 95 °C, 30 s at an annealing temperature of 51 °C, extension for 54 s at 72 °C and a final cycle

comprising of an extension at 72 °C for 2 minutes. The PCR products were visualized on a 1 % agarose gel to determine the positive colonies. The positive colonies were picked from the agar plate and inoculated in 10 mL LB with appropriate antibiotics. The plasmid DNA was extracted using the GeneJET Plasmid Mini-prep Kit (Thermo Scientific) as per manufacturer’s instructions followed by quantification using a Nanodrop (Thermo Scientific™). Restriction enzyme digest with *EcoRV/XhoI*, *BspHI*, *AgeI/XhoI* and *PstI* for 1 hour at 37 °C was carried out to confirm the fragment sizes of the construct and resolved by gel electrophoresis on 1% TBE agarose gel. Clones were further confirmed by sequencing analysis (Macrogen Inc.) using pTRA fwd and pTRA Rvs primers (Table 8) for pRIC 4.0-PrME- His and pEAQ-HT Fwd and pEAQ-HT Rvs for pEAQ-PrME- His. The sequencing result was also aligned to other sequences in the NCBI Genbank database using BLAST.

Agrobacterium tumefaciens

Agrobacterium tumefaciens preparation and transformation and back transformation were done as described in Chapter 2 (see Chapter 2.2.1).

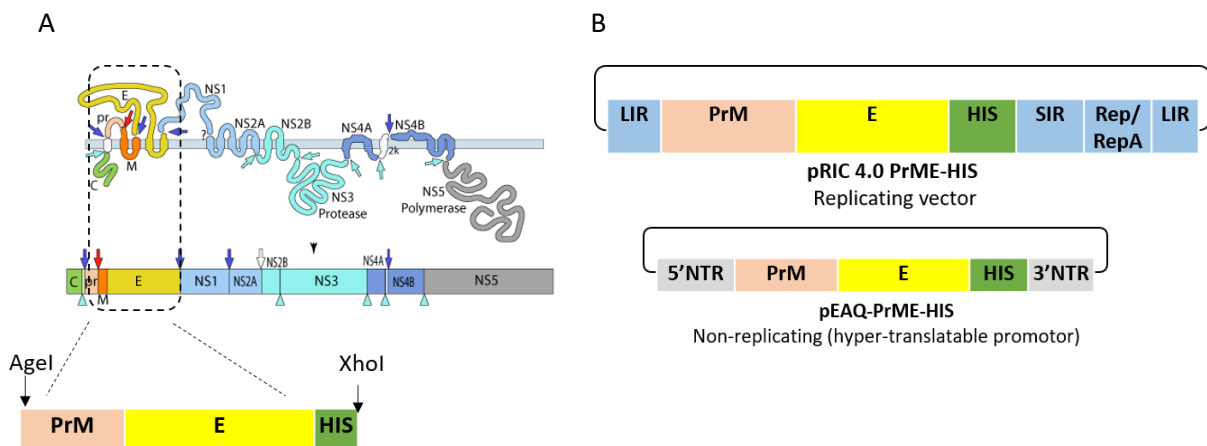


Figure 23: The region of the ZIKV polypeptide and final constructs used for the expression in *N. benthamiana*. (A) A consensus sequence of the PrME region was codon-optimised and synthesized by GenScript. A 6x His tag sequence was incorporated at the C-terminal end of the E gene. (B) The PrME 6x His gene was cloned into pEAQ-HT and pRIC 4.0 using *AgeI* and *BspHI* respectively on the 5’ end and *XhoI* on the 3’ end. LIR, BeYDV long intergenic region; SIR, BeYDV short intergenic region; Rep/RepA, BeYDV replication associated gene; 3’NTR, non-translated region. (Regnard et al. 2010; Sainsbury et al. 2009; viralzone.expasy.org)

4.2.2. Infiltration into *N. benthamiana*

Infiltration into *N. benthamiana* was done as described in Chapter 2 (See Chapter 2.2.2).

4.2.3. Protein expression

Total protein was extracted for small scale expression studies. Briefly, three leaf discs were harvested using the top of a 2 mL microcentrifuge tube. Two ceramic beads (Biogen) and 250 μ L per leaf disc of one of the following buffers: 1X phosphate buffered saline (PBS), General lysis buffer, 8M urea extraction buffer, acidic extraction buffer (Yang et al. 2017a) or 100 mM Tris-HCl (Table 9). Some of the buffers included the CompleteTM, EDTA-free protease inhibitor (Roche Diagnostics) as per manufacturer's instructions. The samples were vortexed at maximum speed for 10 min and centrifuged at 15871 x g (13000 rpm) for 5 minutes. The supernatant was transferred to a 2 mL microcentrifuge tube and centrifuged at 15871 x g (13000 rpm) for 5 minutes. The previous step repeated and the clarified samples were stored at -20 °C prior to protein analysis.

Table 9: Different protein extraction buffers used for PrME extraction from *N. benthamiana* leaves

Name	Ingredients	Protease inhibitor
PBS extraction buffer	1X phosphate buffered saline (PBS)	Complete TM , EDTA-free protease inhibitor
General lysis buffer	50 mM Tris-HCl pH =7.5, 100 mM NaCl, 1 mM DTT, 5% glycerol	Complete TM , EDTA-free protease inhibitor
Urea extraction buffer	300 mM NaCl, 50 mM Tris (pH 7.6), 1 M DTT and 8 M Urea	
Acidic buffer	1 mM EDTA & 1 x PBS pH 5.2	
Tris-HCl extraction buffer	100 mM Tris-HCl (pH 7.6), 1% TritonX-100	

SDS-PAGE and immunoblot analysis

Samples were prepared by adding 5x SDS sample application buffer (2% SDS, 100 mM Tris-HCl, pH 7.5, 2 mM EDTA, 52% glycerol, 4.3% β -mercaptoethanol, 0.25% bromophenol blue) to a final concentration of 1x and heated at 90 °C for 3 minutes. Equal volumes of the sample were resolved on a 12.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) at 20 mA for approximately 90 minutes. The SDS gel was transferred onto a nitrocellulose membrane by semi-dry blotting using a Bio-Rad Trans-Blot[®] Semi-dry transfer cell for 1 hour at 15 V. The membrane was then blocked with blocking buffer (1 x PBS, 2%

Bovine serum albumin (BSA), 1% tween-20) and incubated at room temperature for 30 min with gentle shaking. The membrane was then incubated with 1:2000 dilution of anti-His-tag mouse IgG antibody (Bio-Rad) diluted in blocking buffer, was added to the membrane and probed for 2-4 hours at room temperature at 4 °C with gentle shaking. Thereafter the membrane underwent four 15 minute washes in blocking buffer with shaking followed by incubation with a 1:2000 dilution of anti-mouse secondary antibody conjugated to alkaline phosphatase (Sigma-Aldrich®) for 2-4 hrs at room temperature. All the above steps were performed with gentle shaking in blocking buffer without milk. The 6× His-labeled protein were visualised with 3-5 mL NBT/BCIP (Roche, Switzerland) solution for 1 hour or until protein bands appeared after which the reaction was stopped using water.

4.3. Results

4.3.1. Vector construction of pRIC 4-PrME- His and pEAQ-PrME- His

The successful cloning of the pRIC 4-PrME-His and pEAQ-PrME-His final constructs was confirmed using colony PCR. Positive clones would result in a PCR product of 890 bp and this band size was observed for the positive control as well (Figure 24A). As expected, there were no bands observed for the negative (no template) control. Colonies that contained empty plasmid would not produce any bands because internal PrME primers were used. All clones tested positive with one positive clone selected for each construct and confirmed by restriction enzyme digest. The correct banding pattern was observed for both constructs; undigested pRIC 4.0-PrME- His had a band of approximately 9382 bp and when digested with *Bsp*HI resulted in three bands, 1012 bp, 3179 bp and 5203 (Figure 24B). pEAQ-PrME- His when digested with *Xho*I/*Age*I resulted in 9953 bp and 2043 bp bands, the latter being the PrME- His gene. The constructs were then further confirmed by sequencing. The sequencing results agreed with predicted sequences on CLC Main Workbench 6. The vectors were successfully electroporated into *A. tumefaciens* cells. A back transformation was performed to confirm that the correct DNA was electroporated into *A. tumefaciens* (data not shown).

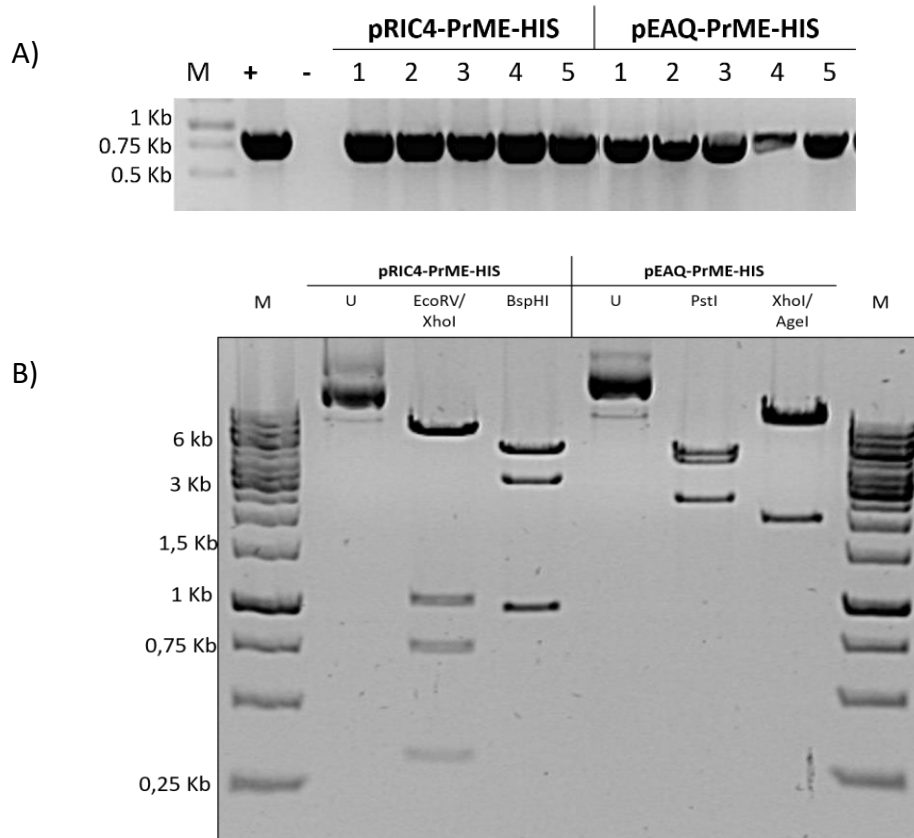


Figure 24: Confirmation of construction. (A) Colony PCR of the recombinant clones using internal PrME primers with an expected product size of 890 bp. Positive control (+) and negative control (-). **(B)** Restriction enzyme digest confirmation of the pRIC 4-PrME- His and pEAQ-PrME- His. The Following DNA sizes was observed: **pRIC 4.0-PrME- His EcoRV/XhoI** 339 bp, 773 bp, 1073 bp and 7201 bp; **BspHI** 1012 bp, 3179 bp and 5203 bp. **pEAQ-PrME- His, PstI** 2515 bp, 4407 bp, 5078 bp, **XhoI/AgeI** 2043 bp and 9953 bp.

4.3.2. Transient expression in *N. benthamiana* and comparison of extraction buffers

A small-scale expression time trial was carried out to determine the optimal optical density (OD₆₀₀) for the highest expression, and the plant health was observed. The positive control was a Rift Valley fever virus (RVFV) nucleocapsid (N) protein that has been shown to express well in plants: these became necrotic on day 9, and leaves infiltrated with pRIC -PrME-HIS also turned yellow on day 9 (Figure 25). Plants infiltrated with pEAQ-PrME-His remained healthy throughout time trial.

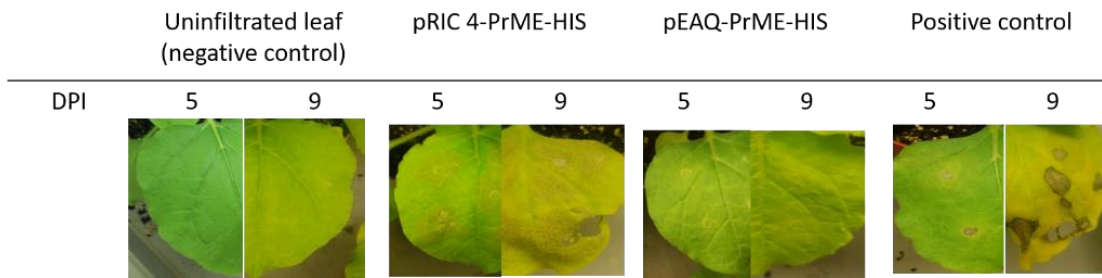


Figure 25: Effect of the ZIKV-PrME-HIS expression on leaf morphology of *N. benthamiana*. Leaf morphology was monitored from 1 dpi up until 9 dpi and the figure shows a comparison between 5 and 9 dpi. The positive control was a RVFV nucleocapsid (N) protein.

In order to obtain the correct protein size, one needs to determine the best extraction buffer that will produce the desired protein. Since PrME was still novel to plant expression, different protein extraction buffers were tested to obtain the predicted protein size of approximately 75 kDa (Table 9). Initially TSP was extracted using 1x PBS but no specific proteins were detected during immunoblotting. This was repeated using a Tris-HCL extraction buffer and only the positive control of 25 kDa band was detected (Figure 26A). Since the protein was still undetectable an acidic extraction buffer was then used to remove RuBisCo (a plant contaminating protein). Unfortunately, the 74 kDa PrME-HIS protein was not detected from any of the above extractions buffer. It was then decided to try a general lysis buffer and to test the solubility of the protein with the addition of 8 M urea. Both the pellet and supernatant were loaded onto the gel but no band was observed (Figure 26 B).

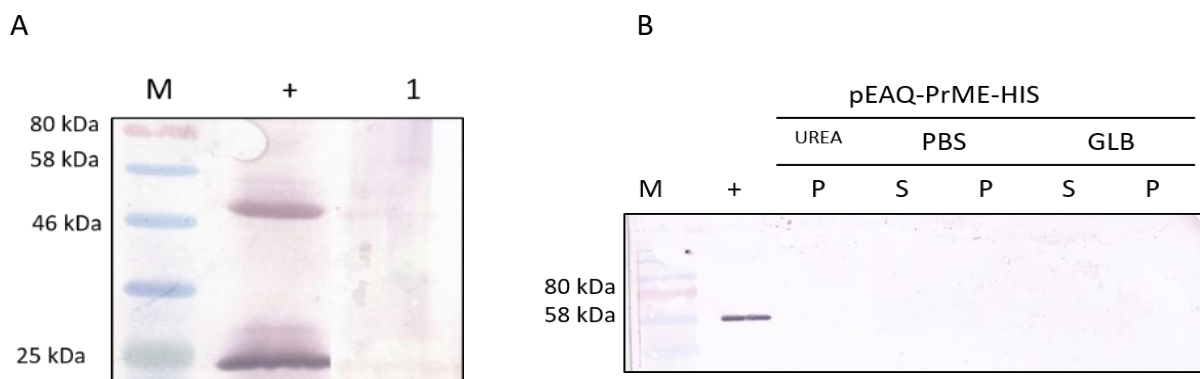


Figure 26: Western blot analysis for expression buffer comparison. Small scale expression of pEAQ-PrME followed by protein extraction. **(A)** Tris-HCL + 1 % TritonX-100. 1: pEAQ-PrME-HIS, +: positive control (pEAQ-N-His) **(B)** Urea, PBS and general lysis buffer, P: pellet, S: supernatant, +: positive control.

4.4. Discussion

Different platforms have been used to make ZIKV vaccine candidates. These platforms include plasmid DNA vaccines, inactivated viruses, modified mRNA encapsulated by nanoparticles and adenovirus vaccines (Pardi et al. 2017; Abbink et al. 2017). The above candidates also show promising protection in mice and Rhesus macaque monkeys. However, these platforms present multiple problems such as scalability, high downstream cost and risk of not inactivating live viruses. Yang et al. (2017a) recently developed a subunit vaccine based on the E protein that was successfully expressed in plants and conferred immunogenicity in animals. A 6x His tag was added to both the N- terminus and C-terminus of the ZIKV E protein DNA coding sequence and then cloned into the TMV-based expression vector of the MagniCON system. A range of monoclonal antibodies that recognize various E conformational epitopes were able to bind to the plant produced ZIKV E. In addition, ELISA results showed that ZIKV E accumulated to >160 µg per gram of leaf fresh weight (LFW) 6 days post agroinfiltration (DPI). Furthermore, the group also designed a virus-like particle (VLP) carrier based on the HBcAg that displays the domain III of ZIKV E protein. The results showed that it could be easily and quickly produced and purified in large quantities (Yang et al. 2017a).

In order to test the efficacy of our in-house vector pRIC 4.0, I aimed to clone the PrME gene with a His tag into this vector and to optimize PrME expression. In addition, the PrME- His gene was cloned into the pEAQ-HT to compare which vector had the higher protein expression levels. The PrME-His gene was successfully cloned into pRIC 4.0 and pEAQ-HT and was further confirmed by sequencing (Macrogen). Unfortunately, the PrME-His protein was undetectable on a western blot using anti-His antibody. The same result was observed for all the different protein extraction buffers. Furthermore, the correct use of controls confirmed that the lack of expression observed for the ZIKV protein in plants was protein related.

The lack of protein expression could be explained by the fact that virus assembly takes place in the ER to form immature, non-infectious virions (Zybert et al. 2008; Mukherjee et al. 2016). Targeting proteins to the ER improves protein yields, allows for glycosylation if need be and prevents proteolytic degradation by retaining the protein in the ER (Schouten et al. 1996; Stoger et al. 2014). It has been shown that adding a retention sequence to C-terminal end of the E protein will allow for translocation to the ER and in turn viral assembly. On the contrary, the PrME gene used in this study does not include the native signal peptide which is found on

the C terminal end of flavivirus C protein. The hydrophobic signal sequence anchors the C terminal end to the ER membrane which allows the Pr and M protein to be translocated into the ER (Qi et al. 2008). Martinez et al., (2011) successfully demonstrated expression of dengue virus E protein with an ER retention sequence in *N. tabacum* and *Morinda citrifolia*. The expression cassette consisted of a signal peptide at the N-terminus and an ER retention/retrieval sequence, KDEL (Lys-Asp-Glu-Leu) was added to the C-terminus. The CPrME (capsid, premembrane and envelope) expressed with a KDEL sequence had higher accumulation levels than the constructs without (Martínez et al. 2011). This would be useful strategy to increase expression levels of the ZIKV PrME-His used in this study.

Another alternative would be to co-express the PrME protein with furin protease. Furin is a human protease responsible for proteolytic cleavage of PrM into soluble Pr and M proteins which is a crucial step for maturation and infection. Elshuber et al., (2003) showed that furin protease cleavage of PrM is important for tick-borne encephalitis (TBE) virus. Mutation of the cleavage site resulted in immature, non-infectious TBE virions in BHK-21 cells. Moreover, co-expression of the human furin enhanced the accumulation of active human transforming growth factor beta (TGF- β) in *N. benthamiana*. The researchers also observed proper processing of extracted LAP- TGF- β and concluded that plants are a suitable platform for producing complex proteins that require accurate post-translational processing to be biologically active (Wilbers et al. 2016).

Plant morphology after infiltration plays an important role in protein expression and accumulation. Infection-like symptoms were observed in plants for the replicating vector pRIC 4.0 and more so for the positive control, pEAQ-N-His (RVFV). Previous studies have shown that Rep can induce a hypersensitive response (HR) in *N. benthamiana*. The HR is associated with local necrosis and production of hydrogen peroxide (Van Wezel et al. 2002). However, the symptoms observed in this study were not as severe as reported in previous studies. This reduction in necrosis may be due to the addition of the TAV 2b silencing suppressor gene on the pRIC 4.0 vector. Silencing suppressors have been reported to prolong expression of the transgene and reduce infection like symptoms (Takeda et al. 2002).

Determining an optimal extraction protocol is an important step to obtaining the desired protein. Different extraction buffers were used to determine whether changing buffer conditions would result in PrME extraction. The extraction buffers were unsuccessful in

extracting any of the desired protein, since no ZIKV PrME-His proteins were detected on an immunoblot. This may be due to the lack of protein translation taking place for PrME-HIS in the plant cell, since the positive control was detected using the same buffers.

Since no ZIKV PrME was detected other alternatives need to be considered to express the protein in plants. The flavivirus E protein contains neutralizing epitopes located within the domain III. These peptides are considered important for vaccine development. Studies also show that these epitopes are less cross reactive compared to domain I and II of the E protein (Beltramello et al. 2010; Chavez et al. 2010). Expression of the PrM with the E protein assists with obtaining the genetically correct conformation of E (Konishi & Mason 1993). Yang et al., (2017a) recently transiently expressed the ZIKV E protein in *N. benthamiana* which was highly immunogenic in mice. The ZIKV E coding sequence with His tags was cloned into a TMV-based expression vector and the researchers were able to achieve substantial protein yields and >90 % purity. They were also able to show that the E protein was correctly folded since it was recognised by a range of mAb that bind to various epitopes of the E protein (Yang, Sun, et al. 2017). This research confirms that the ZIKV E can be expressed on its own and should be considered for eliciting neutralizing antibodies.

In conclusion, I successfully cloned the ZIKV-PrME-His into two plant expression vectors pRIC 4-PrME-His and pEAQ-PrME-His which was confirmed by sequencing. However, I was unable to achieve PrME-His expression in *N. benthamiana*. I would assume that either its transcription or translation within the plant cell that is not taking place. Further studies will need to be carried out to determine whether mRNA is made and to obtain PrME-His expression in plants.

Chapter 5: General conclusion

The level of recombinant protein expression and yield is particularly important for commercial vaccine production. However, low protein yields and structural heterogeneity have hindered plant-based vaccine production. Thus, different strategies have been developed to increase protein yields in plants. Plant viruses have been used to introduce foreign genes into plants since the early 1980s. Virus-based vectors are useful because of their rapid replication and because they can be optimised for high protein expression or use in different plant host species. In this study we focused on improving our current expression vectors by examining silencing suppressor activity and the application of replication elements taken from different viruses.

RNA silencing is a major antiviral plant defence mechanism that targets the viral genome for degradation. Plant viruses use RNA silencing suppressor proteins to counter the plant host antiviral response by inhibiting various aspects of the silencing pathway. This prevents viral DNA degradation and the inhibition of foreign gene expression. Silencing suppressor genes are useful to increase expression of recombinant genes in plant. In this study we explored the use of different silencing suppressor proteins to determine which one would best improve protein expression. TAV 2b had the greatest impact on recombinant protein expression compared to other more commonly used proteins (P19 and NSs). Since the TAV 2b gene is much smaller than p19, it will reduce the vector size and increase the efficiency of the expression vectors. Further studies that investigate mRNA levels should be carried out using reverse transcriptase PCR (RT-qPCR) and compared to other constructs containing silencing suppressors.

Furthermore, one of the main aims of this project was designing and creating replicating plant expression vectors using the replication elements from BeYDV, BFDV and alphasatellites. The GB cloning technique was used for the first time to ease the cloning process. We were able to deconstruct and re-assemble our in-house replicating vectors pRIC 3.0 and pRIC 4.0 and confirm that the technique was efficient and useful. However, the same was not achieved when trying to assemble the BFDV and alphasatellite expression vectors. Although domestication and the multipart assembly of all the DNA parts were confirmed, the final assembly was not successful. More time and research are needed to troubleshoot this problem. An alternative would be to use a different destination plasmid such as the

commonly used plasmids, pRIC 4 or pEAQ-*HT*. Once all the parts are in the same vector, one could digest with the appropriate RE and clone it into the common expression vectors. Although, this would take longer and defeat purpose of the whole GB efficient technique, due to designing primers and finding the appropriate RE, it would help with generating the new vectors in due time. Overall the aim is to design the replicating expression vectors and interchange the rep genes between the vectors to improve protein expression in plants.

Finally, the expression efficiency in *N. benthamiana* of the above-mentioned vector, incorporating the TAV 2b gene and BeYDV replication elements, was tested with ZIKV PrME-HIS protein. The PrME-HIS was first cloned into the plant expression vectors, pRIC 4.0 and pEAQ-*HT* to see if there was any expression of the protein in plants. Unfortunately, no expression was achieved despite optimising different protein extraction buffers, day of harvest post infiltration and *Agrobacterium* infiltration density. Experiments that we could consider include targeting the ZIKV PrME proteins to the ER which has been shown to improve protein yields, allowing for glycosylation if need be and preventing proteolytic degradation. In addition, co-expression with a furin protease could enhance accumulation and aid proper processing of the proteins. Expression of the ZIKV E protein alone should also be considered as it contains less cross-reactive epitopes

In conclusion, I was able to determine that the TAV 2b silencing suppressor protein was better at improving EGFP expression. The GB technique was shown to ease the difficult cloning processes. However, further optimisation is required for the expression of ZIKV PrME, as discussed.

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Appendix A

	Size (bp)	BeYDV replication elements	Promoter (Enhancer)	Reporter	(Enhancer) Terminator	BeYDV replication elements		Promoter	Silencing suppressor	Terminator	
pRIC 3.0 (no EGFP)	9881	LIR	p35SS	-	pA35SS	SIR	<i>rep/repA</i>	LIR	-	-	-
pRIC 3.0 EGFP	9699	LIR	p35SS	<i>egfp</i>	pA35SS	SIR	<i>rep/repA</i>	LIR	-	-	-
pRIC 4.0 EGFP	8048	LIR	p35SS	<i>egfp</i>	pA35SS	SIR	<i>rep/repA</i>	LIR	p35S	TAV 2b	pA35S
pPEAQ-HT EGFP (TAV 2b)	11404	-	p35S (CPMV 5' UTR)	<i>egfp</i>	(CPMV 5' UTR) NosT	-	-	-	p35S	TAV 2b	pA35S
pPEAQ-HT EGFP (NSs)	11560	-	p35S (CPMV 5' UTR)	<i>egfp</i>	(CPMV 5' UTR) NosT	-	-	-	p35S	NSs	pA35S
pPEAQ-HT EGFP (α -sat rep)	11101	-	p35S (CPMV 5' UTR)	<i>egfp</i>	(CPMV 5' UTR) NosT	-	-	-	p35S	α -sat rep	pA35S
pPEAQ-HT EGFP (P19)	10675	-	p35S (CPMV 5' UTR)	<i>egfp</i>	(CPMV 5' UTR) NosT	-	-	-	p35S	P19	pA35S
pPEAQ-HT EGFP (no P19)	8755	-	p35S (CPMV 5' UTR)	<i>egfp</i>	(CPMV 5' UTR) NosT	-	-	-	-	-	-

Figure 1: Table presenting pRIC 3.0 and pEAQ-HT constructs with the different silencing suppressor gene. The major features are labelled, and total size indicated. CaMV, cauliflower mosaic virus 35S promoter and terminator; Nos, nopaline synthase terminator (blue); CpMV UTR (blue), silencing inhibitor (red), BeYDV replication elements (yellow) and *egfp* (green).