Optimization of a Viral System to Produce Vaccines and other Biopharmaceuticals in Plants

by

Andy Diamos

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Graduate Supervisory Committee:

Hugh Mason, Chair
Tsafrir Mor
Brenda Hogue
Valerie Stout

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ABSTRACT

Plants are a promising upcoming platform for production of vaccine components and other desirable pharmaceutical proteins that can only, at present, be made in living systems. The unique soil microbe *Agrobacterium tumefaciens* can transfer DNA to plants very efficiently, essentially turning plants into factories capable of producing virtually any gene. While genetically modified bacteria have historically been used for producing useful biopharmaceuticals like human insulin, plants can assemble much more complicated proteins, like human antibodies, that bacterial systems cannot. As plants do not harbor human pathogens, they are also safer alternatives than animal cell cultures. Additionally, plants can be grown very cheaply, in massive quantities.

In my research, I have studied the genetic mechanisms that underlie gene expression, in order to improve plant-based biopharmaceutical production. To do this, inspiration was drawn from naturally-occurring gene regulatory mechanisms, especially those from plant viruses, which have evolved mechanisms to co-opt the plant cellular machinery to produce high levels of viral proteins. By testing, modifying, and combining genetic elements from diverse sources, an optimized expression system has been developed that allows very rapid production of vaccine components, monoclonal antibodies, and other biopharmaceuticals. To improve target gene expression while maintaining the health and function of the plants, I identified, studied, and modified 5' untranslated regions, combined gene terminators, and a nuclear matrix attachment region. The replication mechanisms of a plant geminivirus were also studied, which lead to additional strategies to produce more toxic biopharmaceutical proteins. Finally, the

mechanisms employed by a geminivirus to spread between cells were investigated. It was demonstrated that these movement mechanisms can be functionally transplanted into a separate genus of geminivirus, allowing modified virus-based gene expression vectors to be spread between neighboring plant cells. Additionally, my work helps shed light on the basic genetic mechanisms employed by all living organisms to control gene expression.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 History of Plant-Made Biopharmaceuticals

The production of desirable biological molecules has greatly increased in the past two decades. This includes a wide range of products such as monoclonal antibodies, enzymes, and growth factors. With 120 billion US dollars in global sales per year, these biologics now account for the largest and fastest growing sector of pharmaceuticals worldwide (Butler and Meneses-Acosta 2012).

Insulin, the first recombinant protein approved for human use (Johnson 1983), was produced in a prokaryotic expression system using E. coli. However, in recent years, eukaryotic expression systems have come to dominate, largely due to their ability to produce complex proteins with correct post-translational modifications that are currently beyond the capabilities of prokaryotic cells (Almo and Love 2014). Chinese Hamster Ovary cells are the platform of choice for the majority of currently available biopharmaceuticals, due to their extensive molecular characterization and proven regulatory history (Zhu 2012).

Since the 1980s, plants have been explored as alternative platforms to produce vaccine antigens, recombinant proteins such as antibodies, and other biologics. Plant-based systems have the capacity to rapidly produce high levels of recombinant protein in a manner that is highly scalable, potentially using current infrastructure to be grown on an agricultural scale (Streatfield et al. 2001). Additionally, unlike mammalian systems,

plants do not harbor animal pathogens, reducing the risk of transmitting disease (Thanavala et al. 2006). Finally, unlike prokaryotic systems, plants are capable of making post-translational modifications necessary for producing complex recombinant proteins (Vitale 2005).

In 1989, the first monoclonal antibody was synthesized in transgenic tobacco plants (Hiatt et al. 1989) and three years later the first vaccine component, the hepatitis B surface antigen, was produced (Mason et al. 1992). Despite two decades of research since these developments, today only three plant-made biologics have been approved for commercial sale. The most notable of these is glucocerebrosidase by Protalix Biotherapeutics, which was the first intravenously injectable pharmaceutical made in plants (Pastores et al. 2014).

Significant progress has been made towards modifying plants for biopharmaceutical production. By silencing or mutating β1,2-xylosyltransferase and core α1,3-fucosyltransferase, the genes responsible for the production of plant-specific glycans, the production of biopharmaceuticals with more human-like glycans has been achieved (Strasser et al. 2008; Castilho and Steinkellner 2012). These systems have been used to produce antibodies against respiratory syncytial virus and ebolavirus with highly homogenous glycan profiles that provide enhanced potency even compared to antibodies produced in mammalian cell cultures, due to the effects of glycosylation on effector function (Zeitlin et al. 2011; Hiatt et al. 2014).

1.2 Viral Transient Expression Vectors

Initial plant-based production methods, including all three commercially available biopharmaceuticals as of 2016, have relied on the use of stable nuclear genetic transformation. These methods have suffered from the long lengths of time necessary to generate the transgenic lines, and overall low yields approaching at most approximately 1% total soluble protein (Lico et al. 2008). To solve these issues, recent research has instead focused on the use of agrobacterium to deliver virus-based vectors to transiently express foreign proteins, producing much higher yield in a matter of days instead of months. The vast majority of ongoing clinical trials use these methods, rather than stable transgenic plants (Gleba et al. 2014).

1.2.1 Vector Delivery Using Agroinfiltration

To transfer a viral vector to the majority of a plant's cells, the soil microbe *Agrobacterium tumefaciens* is typically employed. Certain *Agrobacterium* strains harbor a tumor-inducing plasmid which contains a specific T-DNA (transfer DNA) region delineated by 23 base pair border repeats. The native T-DNA can be removed and replaced by an expression cassette containing the T-DNA border regions, allowing the delivery of virtually any DNA of interest to the plant nucleus (McCullen and Binns 2006). *Agrobacterium*-mediated DNA transfer is extremely efficient, able to infect >96% of leaf cells (Komarova et al. 2010). Additionally, this process can be scaled up to allow the infection of large amounts of plants simultaneously using vaccine infiltration (Simmons et al. 2009).

The ability of *Agrobacterium* to perform T-DNA transfer revolves around both a set of plant cell attachment genes found on the bacterial chromosome, as well as a set of *vir* genes contained on the tumor-inducing plasmid that are directly involved in the T-DNA transfer process. With the exceptions of *virA* and *virG*, the many other *vir* genes are not expressed except in the presence of certain plant compounds often produced during plant wounding (Stachel and Nester 1986). In the presence of phenols, aldose monosaccharides, low pH, and low PO_4, the membrane-bound sensor VirA phosphorylates the cytoplasmic VirG, which then binds to specific DNA regions on the tumor-inducing plasmid to activate the many *vir* genes (Brencic et al. 2005). The chromosomally-encoded protein ChvE is responsible for sensing monosaccharide components of plant cell walls, leading to binding and activation of the periplasmic region of VirA, and also plays roles in chemotaxis (Shimoda et al. 1993; He et al. 2009).

To initiate T-DNA transfer, VirD2 nicks the T-DNA border repeats and becomes covalently attached to the 5' end to protect against exonucleolytic degradation (Gelvin 2003). An additional sequence adjacent to the right border, known as the overdrive sequence, facilitates T-DNA generation (Peraltal et al. 1986). VirE2 coats the single-stranded T-DNA and plays important roles in nuclear localization and chromosomal integration once inside the plant cell (Citovsky et al. 1992). A type IV secretion system is constructed out of VirB1-11 proteins and VirD4 to perform T-DNA transfer. Besides VirD2 and VirE2 bound T-DNA, VirE3, VirF, and VirD5 are known to also be transferred to the plant cell. The precise function of many of the proteins involved in T-

DNA transfer is not fully known, and it is likely that many unidentified components of the transfer process still exist (McCullen and Binns 2006; Nester 2015).

1.2.2 MAGNIcon Expression System

The use of viral vectors to improve plant-based biopharmaceutical production generally relies on the amplification of either DNA or RNA of a protein of interest using viral replication elements. As such, many of the unnecessary viral components have been removed, and the remaining components have been optimized, resulting in "deconstructed" vectors (Giritch et al. 2006). In most virus vectors, the capsid protein is generally removed to free up genome space and to prevent the production of infectious virions, allowing the insertion of other desirable genes.

Of the many plant expression platforms developed, the MagnICON system developed by Icon Genetics is the most successful system to date. It is based around the RNA virus tobacco mosaic virus (TMV), with modifications for delivery as a T-DNA by *Agrobacterium*. In this system, the 126kDa and 183kDa replication proteins act together to amplify the viral genome in the plant host cell cytoplasm. RNA-dependent RNA polymerase activity produces a full-length negative-sense genomic strand. From this, large quantities of mRNA for a gene of interest, which was used to replace the TMV coat protein, are made from the coat protein subgenomic promoter. Additionally, full-length positive-sense recombinant genomes are produced, which spread cell-to-cell through plasmodesmata due to the action of the 30kDa TMV movement protein (Liu and Nelson 2013).

Depending on the individual protein being produced, yields between 0.2 to 5.0 grams of recombinant protein per kilogram of leaf material have been reported in 5-12 days (Marillonnet et al. 2004; Klimyuk et al. 2014). Due to the ability of TMV to move cell-to-cell without its coat protein, very low concentrations of agrobacterium (OD_{600} = ~0.002) are needed to efficiently infect the vast majority of plant cells. The MagnICON system has been successfully employed to produce numerous biopharmaceutical proteins, including individualized vaccines for Non-Hodgekin's lymphoma, which recently completed clinical trials. Many other clinical trials with biopharmaceuticals made in both small- and large-scale production facilities are currently underway using the MagnICON system (Klimyuk et al. 2014).

Despite its success, the MagnICON system has several drawbacks. It is unable to express more than one protein using the same virus, due to inherent limitations in the lifecycle and genome size of RNA viruses. To circumvent this, it was necessary to identify other noncompeting viruses capable of replicating in the same cell as tobacco mosaic virus. Potato virus X serves this purpose for the production of two proteins simultaneously in the same cell; however, at present it is not possible to express three or more proteins using this system, which prohibits the production of complex biopharmaceuticals such as IgAs or heteromultimeric vaccine antigens such as bluetongue, influenza, or rotavirus virus-like particles (Thuenemann et al. 2013). TMV vectors have also failed to produce high levels of certain larger proteins such as HPV L1, possibly due to genetic instability of larger inserts (Matić et al. 2012). The host range of tobacco mosaic virus is very narrow, making use of TMV-based vectors limited outside

of the *Nicotiana* genus. Additionally, significant plant tissue necrosis has been observed when TMV-based vectors are used to produce proteins that are less hospitable to plants (Mathew et al. 2014a)

1.2.3 Bean Yellow Dwarf Virus Expression System

To circumvent these issues, a transient expression system based on the geminivirus bean yellow dwarf virus (BeYDV) has been explored. In this system, the BeYDV replication proteins are exploited to amplify a gene of interest to high copy number in the plant cell nucleus. These replicons hijack and saturate the plant transcription machinery, leading to the production of large amounts of recombinant protein. Due to the noncompeting nature of BeYDV replicons, multiple proteins can be produced in the same cell from the same vector. There seems to be no known limit to the size or number of proteins that are able to be efficiently produced (Chen et al. 2011). Additionally, the host range of BeYDV allows the use of these vectors in many dicot plant species, such as tobacco and lettuce (Lai et al. 2012b).

Initial efforts to use BeYDV for recombinant protein production replaced the CP and MP with an expression cassette driven by the strong 35S promoter from cauliflower mosaic virus (Mor et al. 2003b). When Rep/RepA were produced either on the same vector as the gene of interest or supplied by a separate vector, high levels of replication and a corresponding increase in protein accumulation was shown for a variety of biopharmaceuticals. The virus-like particles hepatitis B core antigen and norovirus capsid protein were produced at a level of 0.8 and 0.34 mg per gram leaf fresh weight (LFW) respectively (Huang et al. 2009). Additionally, it was shown that two separate proteins

could be produce in the same cell by either codelivering two vectors, or placing two BeYDV replicons in tandem on the same T-DNA vector, allowing production of multimeric proteins such as monoclonal antibodies. The anti-ebolavirus GP1 monoclonal antibody 6D8 was produced at a level of 0.5 mg/g leaf fresh weight (Huang et al. 2010).

1.3 Mastrevirus Replication

The geminiviruses comprise a family of small single-stranded DNA viruses which replicate in the nucleus of host cells, associating with host histones to form chromatin structures known as viral minichromosomes (Pilartz and Jeske 2003). BeYDV was isolated from the common bean *Phaseolus vulgaris* and has been shown to infect many other dicots. BeYDV and other mastreviruses produce only four proteins: a coat protein (CP) and movement protein (MP), which are produced by the virion sense DNA strand, and two replication proteins produced on the complementary sense DNA strand: Rep and RepA. Rep and RepA are produced from a single intron-containing transcript: RepA is the predominant product from the unspliced transcript, while a relatively uncommon excision of an intron alters the reading frame to produce Rep. Production of all viral proteins is driven by a bidirectional promoter in the long intergenic region (LIR) which also contains the viral origin of replication. A short intergenic region (SIR) is also present which has transcription terminator signals and is the origin of complementary strand synthesis (Liu et al. 1998).

The mastrevirus Rep protein, which is produced early in infection, is a multifunctional protein which initiates rolling circle replication by nicking a conserved stem-loop sequence in the LIR. The majority of replication then occurs using cellular

machinery to extend the free 3’ end of the nicked viral replicon, though it is likely that Rep recruits many of the involved cellular factors. Rep also plays a role in ligating newly synthesized DNA to create circular viral genomes and is likely a helicase (Gutierrez 1999; Choudhury et al. 2006). In related geminiviruses, Rep has been shown to form homo-oligomers, or possibly hetero-oligomers with RepA or other proteins, which may play a role in replication (Horváth et al. 1998; Krenz et al. 2011).

RepA is thought to function in creating a cellular environment suitable for replication. Some evidence suggests this occurs by binding retinoblastoma-related proteins, which are involved in cell cycle regulation. With RepA bound, previously sequestered transcription factors are able to initiate S-phase gene production, creating the cellular machinery necessary for viral replication (Gutierrez et al. 2004). An LxCxE motif is thought to contribute to this by facilitating retinoblastoma-related protein binding. However, other functions of RepA, many of which are as of yet unidentified, have also been shown to enhance viral replication (Ruschhaupt et al. 2013). A set of proteins known as GRAB proteins, which are involved in leaf development and senescence, have been shown to interact with RepA (Lozano-Durán et al. 2011).

Both Rep and RepA repress their own transcription, and RepA transactivates the CP and MP, allowing their expression only late in infection (Muñoz-Martín et al. 2003). Additionally, both Rep and RepA are thought to be suppressors of post transcriptional gene silencing, though this activity is weak compared to other viral silencing suppressors (Wang et al. 2014). The Rep protein from the related begomoviruses is also a suppressor of transcriptional gene silencing, which is accomplished by downregulating cellular DNA

methylases (Rodríguez-Negrete et al. 2009). It is unknown if either Rep or RepA from BeYDV fulfills this role.

The end result of BeYDV replication is the amplification of high levels of viral replicons in the nucleus of infected plant cells. These replicons serve as both double-stranded transcription templates to drive expression of viral genes, and as single-stranded genome copies, which are packaged into virions for cell-to-cell and systemic spread.

1.4 Geminivirus Movement

As geminiviruses replicate in plant nuclei, they must cross two barriers in order to transit between cells: the pores of the nuclear envelope to exit to the cytoplasm, and the intercellular channels, known as plasmodesmata, to spread cell-to-cell (Lucas 2006). Though these general barriers must be overcome by each geminivirus, the specific mechanisms of doing so differ between the different genera.

1.4.1 Bipartite Begomovirus Movement

The begomoviruses are the largest, most well-studied geminivirus genus and are responsible for devastating crop damage worldwide (Scholthof et al. 2011). They are divided into the monopartite begomoviruses, which contain only one singles-stranded circular DNA component, and the bipartite begomoviruses, which contain both a DNA A and DNA B component and will be the focus of this review. Both the DNA A and DNA B components are small, strictly falling between 2.5 and 2.8 kilobases. The DNA A component produces the replication protein Rep, the coat protein, and auxiliary proteins that regulate transcription, suppress gene silencing, and enhance replication. The DNA B

component is responsible for virus movement, producing only two proteins: a nuclear shuttle protein (NSP) and a movement protein (MP) (Jeske 2009).

The NSP is responsible for nuclear import and export of replicated viral genomes (Krichevsky et al. 2006). Both the MP and NSP have been shown to bind DNA in a sequence non-specific manner that instead depends on form and size, though the specifics differ between species. In general, genome-sized DNA molecules (2.5kb to 2.8kb) are efficiently transported, likely evolutionarily constrained by the size that can pass through the plant plasmodesmata (Rojas et al. 1998; Gilbertson et al. 2003; Hehnle et al. 2004). Interestingly, the NSP has been shown to not be required for virus infectivity with several begomoviruses, likely due to overlapping function with the coat protein (Zhou et al. 2007b). Conversely, coat protein mutants are still able to spread cell-to-cell, but lack systemic movement (Sudarshana et al. 1998). The DNA-A component of the bipartite begomoviruses is still capable of systemic spreading in the absence of DNA-B, though in a very limited capacity (Hou et al. 1998). NSP has been implicated as an inducer of the hypersensitive response, though the viral TrAP protein counteracts this response in some species (Hussain et al. 2007). The NSP also has virulence functions, acting to disable translation suppression pathways by interacting with NIK-1 (Zorzatto et al. 2015).

Inside the nucleus, viral genomes have been shown to interact with NSP and histone H3 (Zhou et al. 2011). The current model is that a complex of histone H3, NSP, and viral genomic DNA is exported out of the nucleus, where it interacts with the MP. The MP-NSP-genome-histone H3 complex is then trafficked to the cell periphery and through the plasmodesmata to neighboring cells (Krenz et al. 2010). Though the exact

mechanism of transport is not fully understood, heat shock cognate 70kDa protein has been implicated in the movement of viral replicons through a network of stromules which are formed upon infection and connect the nucleus, plastids, and plasmodesmata (Krenz et al. 2012).

1.4.2 Mastrevirus Movement

The mastreviruses are much less studied than the begomoviruses, though they result in significant crop damage, especially in sub-Saharan Africa (Shepherd et al. 2007). The mastreviruses have only one small circular single-stranded DNA genome between 2.6 to 2.8 kb in length and comprised of only four genes: two replication association proteins, the coat protein, and a movement protein. The coat protein replaces the function of the NSP, shuttling viral replicons out of the nucleus (Liu et al. 1999a). Additionally, the coat protein is required for ssDNA production (Azzam et al. 1994). After nuclear export, the MP localizes the NSP-DNA complex to the cell periphery for cell-to-cell transport (Jeffrey et al. 1996).

The MP from mastreviruses does not seem to bind to DNA directly, instead interacting with the CP-DNA complex. Thus, mastreviruses require the coat protein for all forms of movement. Interestingly, though the CP has been shown to bind DNA in a sequence non-specific manner *in vitro*, attempts to achieve movement with recombinant mastrevirus genomes have failed (Shen and Hohn 1995; Liu et al. 2001). These studies suggest the possibility that some other feature is necessary for viral movement, such as DNA secondary structure, or binding sites in the viral genome that interacts with either

the MP, NSP, or other cellular proteins. Mastrevirus movement has also been shown to be restricted to nearly genome-sized DNA molecules (Shen and Hohn 1995).

1.4.3 Geminivirus Recombination and Complementation

Genetic recombination is a major driving force of virus evolution and has been very well documented for geminiviruses (Varsani et al. 2008). Unlike the RNA viruses, which contain error-prone polymerases, geminiviruses replicating using host machinery which has much higher fidelity. Despite this, they maintain error rates similar to those of RNA viruses (Duffy et al. 2008). It seems likely that the high mutation rate coupled with the high propensity for recombination has resulted in the emergence of many devastating agricultural diseases caused by geminivirus infection (Lefeuvre et al. 2009; Lefeuvre and Moriones 2015). As would be expected of viruses that arose through inter-species and inter-strain recombination, there are many examples of the capacity for the genetic components of related viruses to functionally replace each other.

Exchange of genetic components can alter the host range or tissue specificity of a virus. The virus susceptibility of a given plant species differs drastically between even closely related plant viruses, likely due to the ongoing arms race between plant defenses and virus counter-defenses (Rojas et al. 2005). Cassava mosaic geminiviruses, formerly relatively benign pathogens, underwent recombination to produce a much more severe strain that has severely constrained cassava production in Africa (Legg and Fauquet 2004). In Spain, a tomato-infecting geminivirus isolated in a wild reservoir appeared to be a recent genetic recombinant between Tomato yellow leaf curl Sardinia virus and

Tomato yellow leaf curl virus, and had a broader host range than either of the two parent viruses (García-Andrés et al. 2006).

Researchers have studied the ability for geminivirus genetic components to complement each other by creating chimeric viruses. In one fascinating example, Briddon et al. exchanged the coat protein from African cassava mosaic begomovirus (ACMV), which is transmitted by white fly, with the coat protein of beet curly top curtovirus (BCTV), which is transmitted by leafhopper. The chimeric ACMV acquired the ability to be successfully transmitted by the BCTV leafhopper vector, illustrating the powerful complementary potential of geminivirus recombination (Briddon et al. 1990).

Abutilon mosaic virus (AbMV) is restricted to the phloem, whereas the related bean dwarf mosaic virus (BDMV) can spread through epidermal, cortical and phloem cells in *Phaseolus vulgaris* (Wang et al. 1996; Wege et al. 2000). Complementing the DNA-A from AbMV with the DNA-B of BDMV conferred enhanced tissue invasion to the AbMV DNA-A, successfully moving AbMV out of the phloem. Interestingly, the DNA-B from AbMV did not limit the movement capacity of BDMV DNA-A, despite its phloem limitation when paired with its own DNA-A (Levy and Czosnek 2003). Additionally, a begomovirus lacking DNA B entirely was complemented in movement function by both a curtovirus and a topocuvirus mixed infection (Briddon and Markham 2001). These interactions demonstrate the complex synergy that exists between geminiviral genetic components.

There is substantial evidence for intra- and inter-species recombination between mastreviruses that rivals that of the begomoviruses (Kraberger et al. 2013). Nevertheless,

in contrast to the many examples of successful complementation among members of the begomoviruses that extend cross-genus to the curtoviruses and topocuviruses, there are almost no examples of successful complementation among the mastreviruses. Multiple chimeras created between BeYDV and maize streak virus were unable to replicate or infect either host (Liu et al. 1999b). The only report of viable genetic complementation with a mastrevirus was carried out by swapping the coat and movement protein from similar strains of maize streak virus, which shared high levels of sequence homology (van der Walt et al. 2008).

1.5 The Plant Immune Response to Viral Infection

1.5.1 Plant innate immunity

A cornerstone of both the plant and animal immune systems is the ability to undergo cell death in response to infection. This serves to limit the replication and spread of the invading pathogen. In one arm of the plant immune system, these pathogens are recognized by certain unique markers that the hosts have evolved to detect, called pathogen-associated molecular patterns (PAMPs) (Segonzac and Zipfel 2011). PAMPs are recognized by transmembrane receptors, including receptor-like kinases and other receptor-like proteins, found throughout cellular compartments, known together as pattern recognition receptors (PRRs) (Bonardi et al. 2012). Two of the most well-studied examples of plant PRRs involve the recognition of bacterial flagellin-derived peptide flg22 and bacterial translation elongation factor (Boller and Felix 2009). When detected by the plant cell, PAMPs trigger a signaling cascade which rapidly results in the

production of reactive oxygen species and antimicrobial compounds (Dodds and Rathjen 2010).

To counteract this pattern-triggered immunity (PTI), plant pathogens have developed numerous and diverse counter-defense proteins known as effectors or virulence factors (Jones and Dangl 2006). As a counter-counter-defense, plants have evolved an array of resistance proteins and other cellular sensors to recognize pathogen effector proteins. A leading hypothesis, known as the guard hypothesis, postulates that host proteins, most of which are in the nucleotide binding site-leucine rich repeat (NB-LRR) family, "guard" critical cellular components from manipulation by pathogens. In this manner, a relatively small set of plant proteins can protect against a multitude of subversive effector proteins (Dangl and Jones 2001). Plant recognition of pathogen effectors results in effector-triggered immunity (ETI), mediated by signaling cascades that invoke the hypersensitive response (HR), release of reaction oxygen species, autophagy, host translation shutoff, and programmed cell death (Nishimura and Dangl 2010; Zhou et al. 2014b; Zorzatto et al. 2015).

1.5.2 Geminiviruses and the Hypersensitive Response

Numerous virus proteins have been identified as virulence factors which either trigger the HR, or work to counteract it. In begomoviruses, the bean dwarf mosaic virus nuclear shuttle protein (NSP) was shown to activate the HR in bean plants (Garrido-Ramirez et al. 2000), and this activity was mapped to its N-terminus of the NSP (Zhou et al. 2007b). Interestingly, the TrAP protein from tomato leaf curl New Delhi virus prevents the activation of the HR generated by its NSP (Hussain et al. 2007).

Additionally, the NSP is known to interact with a host immune NB-LRR receptor-like kinase to enhance virus pathogenicity, and recently has shown to be involved in preventing translation shutoff in response to virus infection (Sakamoto et al. 2012; Zhou et al. 2014b).

The V2 protein from tomato yellow leaf curl virus is also a suppressor of the HR. It was shown to inhibit the enzymatic activity of CYP1, a papain-like cysteine protease involved in immunity and cell death (Bar-Ziv et al. 2015). The Rep protein from African cassava mosaic virus also elicited the HR in *Nicotiana benthamiana* (Van Wezel et al. 2002), and it was further reported that altering a single amino acid reversed HR induction without affecting protein function (Jin et al. 2008). A set of small subviral elements known as beta-satellites that associate with begomoviruses have also been shown to enhance disease severity and induce the HR through the action of the βC1 protein (Tahir and Mansoor 2011). While there are many studies on the role of the HR in begomovirus infection, there are virtually no reports of the role of the HR during mastrevirus infection.

1.5.3 RNA Silencing and Viral Defense

RNA silencing is a ubiquitous mechanism among eukaryotes for targeted degradation of mRNAs, though it particularly crucial for plant defense against viral pathogens. RNA silencing functions at both the DNA level, in transcriptional gene silencing, and at the RNA level, in post-transcriptional gene silencing. RNA silencing is initiated by dsRNA, which arises through RNA virus replication intermediates, aberrant or improperly processed transcripts, or RNA secondary structure. dsRNA serves as a substrate for Dicer-like proteins (DCL), which cleave dsRNA into 21-24nt small

interfering RNAs (siRNA). These small RNAs are loaded into argonaute (AGO) proteins, forming a RNA-induced silencing complex (RISC) which cleaves or translational represses identical or high similar RNA sequences (Weiberg and Jin 2015).

In RNA viruses, it is thought that viral replication, which requires synthesis of antisense RNA strands, as well as native RNA secondary structure, create dsRNA which then activates the RNA silencing pathways (Szittya et al. 2010). DCL4, with DCL2 serving redundant function, are the primary dicers involved in anti-viral activity, producing 21-nt siRNAs. Mutants in these genes are associated with increased virus infectivity (Andika et al. 2015). By contrast, many AGO proteins function in viral defense: 10 separate AGO genes have been identified in *Arabidopsis*, with AGOs 1, 2, 5, 7, and 10 having suggested antiviral roles (Carbonell and Carrington 2015).

Once activated, siRNAs derived from initial dsRNAs can be used by cellular RNA-dependent RNA polymerases (RDRs) to synthesize dsRNA from any ssRNA targeted by the RNA silencing machinery, thus amplifying and expanding the initial signal (Devert et al. 2015). High levels of virus-derived siRNAs are generated during infection, which can result in plant recovery. As a counter-defense, virtually every plant virus produces viral suppressors of RNA silencing, which interfere with many different levels of the RNA silencing machinery (Csorba et al. 2015).

Plant DNA viruses, such as the geminiviruses and pararetroviruses, do not contain genomic RNA, and thus viral transcripts must serve as the source of dsRNA for activation of RNA silencing. As these viruses contain circular genomes with bidirectional promoters, it has been suggested that overlapping mRNAs serve as the source for dsRNA

production (Chellappan et al. 2004), however support for this hypothesis has been conflicting (Paprotka et al. 2015). Highly expressed genes have been shown to be targets for RDR6-mediated dsRNA synthesis (Que et al. 1997; Béclin et al. 2002; Schubert et al. 2004; Luo and Chen 2007b). Geminiviruses have been shown to produce large quantities of viral siRNAs, and encode various suppressors of RNA silencing (Raja et al. 2010; Aregger et al. 2012).

Interestingly, unlike RNA viruses, DNA viruses are also targeted by transcriptional gene silencing through RNA-directed DNA methylation. Geminiviruses have been shown to associate with cellular histones, forming viral minichromosomes (Pilartz and Jeske 2003; Paprotka et al. 2011). These minichromosomes are thought to be susceptible to the transcriptional gene silencing pathway mediated by DCL3, which produces 24-nt siRNAs that interact with AGO4 to enable DNA methylation. Cytosine methylation of the viral genome is associated with plant recovery from infection, and many geminiviruses encode suppressors of the transcriptional gene silencing machinery (Raja et al. 2014). Therefore geminiviruses must evade plant defenses at both the transcriptional and post-transcriptional level.

1.6 Control of Gene Expression and Translation by 5’ and 3’ Untranslated Regions

The genetic region upstream from the coding sequence of a gene, known as the 5’ untranslated region (UTR), plays a critical role in gene translation (Kawaguchi and Bailey-Serres 2002). The sequence context directly surrounding the initiation codon ATG, known as the kozak consensus, can drastically effect translation efficiency (Kozak 1986). In monocotyledonous and dicotyledonous plants, it has been shown that the -3 and

+4 positions (where the ATG is defined as +1 to +3) have the greatest impact on translation initiation. The presence of a purine at these positions has been shown to favor maximal translation. For dicots, a sequence context of TAAA(A/C)AATGGC was found to be optimal, though some studies have found slightly different results (Agarwal et al. 2009; Sugio et al. 2010).

Viruses are obligate parasites of translational machinery, and thus much of the work studying optimal 5' UTRs has been done on viral sequences, which are often very efficiently translated. While most endogenous 5' UTRs require a 7-methylguanosine cap, many RNA viruses lack such a structure. Instead, these viruses rely on either an internal ribosome entry site (IRES) in the 5' UTR, or a 3' cap-independent translation element (CITE) (Kneller et al. 2006). These elements allow direct recruitment of translation initiation factors, such as eIF4F in plants, or of ribosomal subunits, in the absence of a 5' cap. They also serve other functions, such as preventing viral mRNA from being degraded (Nicholson and White 2011; Walsh and Mohr 2011).

1.6.1 Virus and Plant 5' UTRs

The 5' UTR from the genomic RNA of tobacco mosaic virus, known as the omega leader, is one of the most well-studied enhancers of gene translation (Gallie and Walbot 1992). It contains a poly(CAA) region which is thought to be involved in enhancing translation initiation by recruiting eIF4F (Gallie 2002). It has been proposed than the 5' UTR folds into a unique tertiary structure with non-canonical base pairing (Agalarov et al. 2011). The TMV UTR contains an IRES capable of cap-independent translation (Dorokhov et al. 2002). Additionally, the 3' UTR contains several

pseudoknots and a t-RNA like structure thought to play a role in both mRNA stabilization and translational enhancement (Chujo et al. 2015).

Several other viral 5' UTRs have been found to greatly enhance translation, such those from alfalfa mosaic virus (Gehrke et al. 1983), tobacco etch virus (Gallie et al. 1995), and pea seadborne mosaic virus (Nicolaisen et al. 1992). Two native plant 5' UTRs were also identified that provided translational enhancement comparable to viral 5' UTRs (138). Additionally, a synthetic 5' UTR was created which was also reported to enhance translation at a level similar to the TMV 5' UTR (Kanoria and Burma 2012).

1.6.2 3' Cap-independent Translation Enhancers

Many viruses utilize RNA structures in the 3' UTR, known as 3' CITEs, that play a critical role in translation. One of the most well-studied of the 3' CITEs is the barley yellow dwarf virus (BYDV)-like translation element (BTE). The BTE contains a conserved 17-nucleotide sequence, and forms a radiating loop structure which binds the eIF4G subunit of eIF4F with high affinity (Truniger et al. 2008). In addition to the BTE, the BYDV 3' UTR contains a hairpin with an apical loop which engages in a long-distance RNA-RNA interaction with its 5' UTR. This interaction is thought to mediate transfer of translation elements to the 5' end of the viral mRNA, which results in ribosome scanning (Rakotondrafara et al. 2006).

In addition to the BTE, there are many other variations of 3' CITEs including the PTE from panicum mosaic virus, which instead recruits eIF4E (Wang et al. 2009), the I-shaped and Y-shaped CITEs which recruit eIF4F (Fabian and White 2006; Nicholson et al. 2010), and the T-shaped CITE discovered in turnip crinkle virus that directly binds

60S and 80S ribosomes (Stupina and Simon 2013). Some viruses contain more than one 3’ CITE. The RNA2 of pea enation mosaic virus contains 3 independent CITEs which mediate interaction with both translation initiation factors and 40S, 60S, and 80S ribosomal subunits (Fan et al. 2012). These diverse plant and viral elements demonstrate the important role of both the 5’ and 3’ UTRs in the efficient translation of mRNA.

1.7 Improving the BeYDV Expression System

BeYDV, a very small virus, performs very few functions itself, instead relying almost entirely on host machinery. Thus BeYDV must compete for replication, transcription, and translation components with the plant cell. In order to maximally produce recombinant proteins, BeYDV-based vectors can be altered to more optimally compete for cellular resources, and limit activation of gene silencing and cell death pathways. Additionally, different strategies are explored to ensure efficient delivery of vectors to plant cells.

1.7.1 Optimizing Genetic Elements of BeYDV Vectors

To maximize protein production, BeYDV vectors have made various modifications to the wildtype genetic elements. The strong 35S promoter from cauliflower mosaic virus (CaMV) has been used to drive transgene expression, rather than the native bidirectional LIR promoter. Additionally, 5’ UTRs from several viruses have been tested to enhance transgene translation, and various 3’ UTRs have been employed to enhance transcript stability and 3’ end processing. The coding sequences of genes of interest have also had their codons optimized and detrimental sequences removed. Lastly, the P19 protein from tomato bushy stunt virus has been coexpressed to

inhibit RNA silencing (Chen et al. 2011; Kim et al. 2015). Each of these modifications has only been investigated with preliminary studies and it is thus likely that there is great potential to further enhance recombinant protein production by optimizing BeYDV vectors.

The 35S promoter from CaMV is one of the most well studied and widely used plant promoters due to its high activity in a wide range of plants and plant tissues (Odell et al. 1985; Bhullar et al. 2007). It was found that the expression from the 35S promoter could be improved by duplicating the enhancer region (Kay et al. 1987). Several promoters that exceed the transcription activity of the 35S promoter have been reported. In one study, a synthetic hybrid created by combining elements of the mannopine synthase promoter with elements from the 35S promoter was found to further improve transcription (Comai et al. 1990). In another study, a promoter region derived from Cestrum yellow leaf curling virus was found to enhance expression up to 28-fold higher than the 35S promoter using β-glucuronidase reporter in stable and transient assays (Sahoo et al. 2014). However, BeYDV vectors generate very high levels of replicon DNA which are thought to saturate the transcription machinery, suggesting that a stronger promoter may fail to increase transcript production (Mor et al. 2003a). It instead may be possible to increase utilization of each transcript by optimizing its genetic components.

Original work with BeYDV vectors employed the 5' UTR from tobacco etch virus (TEV), which contains an IRES and is an efficient enhancer of translation (Carrington and Freed 1990; Gallie et al. 1995). However, in native TEV infection, VPg protein is linked to the 5' end of the viral mRNA, and further improves translation (Khan

et al. 2008). This indicates that the TEV 5' UTR alone without VPg may not be providing maximal translational enhancement. A transient expression system based in large part on translational enhancement was developed using the 5' and 3' UTRs from cowpea mosaic virus, suggesting translational enhancement is a vital component of transgene production (Peyret and Lomonossoff 2013). Many other viral and plant UTRs have been identified which have been reported to exceed the translational enhancement of the TEV 5' UTR, however these studies were generally carried out using *in vitro* translation systems or transgenic plants and may exhibit tissue-specific and species-specific differences (Fan et al. 2012). Further 5' UTR testing is needed using transient expression assays in *Nicotiana benthamiana* to optimize translational enhancement of BeYDV vectors.

As BeYDV replicates in the nucleus and utilizes the plant host transcription machinery, an efficient terminator plays a key role in maximizing transgene protein production. Transcript termination and polyadenylation are necessary for nuclear export, mRNA stability, efficient translation, and the prevention of gene silencing (Luo and Chen 2007a; Moore and Proudfoot 2009). Many terminators have been investigated for their potential to enhance protein production. The 3' UTR from the potato pinII gene was found to enhance hepatitis B virus surface antigen 10-50 fold in transgenic potato compared to the agrobacterium-derived nopaline synthase terminator (Richter et al. 2000). Combining the nopaline synthase terminator with the 35S terminator from cauliflower mosaic virus resulted in a 5-65 fold enhancement of yellow fluorescent protein production compared to the 35S terminator alone (Beyene et al. 2010). The tobacco extensin terminator was found to enhance expression of the GI norovirus capsid

protein up to 6-fold compared to the vspB terminator from soybean using transient expression assays with BeYDV vectors in *Nicotiana benthamiana* (Rosenthal, 2012). While it was shown that the extensin terminator was efficient at preventing most readthrough transcription, further optimization may be possible by combining the extensin terminator with other strong terminators. In addition, BeYDV vectors contain a bidirectional promoter which drives transcription of the viral replication proteins, which may also benefit from improved transcript termination, preventing potential read-through transcription and dsRNA generation, leading to RNA silencing.

Scaffold/Matrix attachment regions (MARs) have been explored as genetic elements capable of enhancing transgene expression in plant systems. MARs are AT-rich regions thought to be involved in higher-order chromatin structure and preferentially associate with nuclear matrix, a complex cellular structure with many proposed roles (Liebich et al. 2002; Calikowski et al. 2003). Experiments in whole plants and plant cell cultures have shown that the presence of MARs can enhance transcription of flanking genes. Studies on the tobacco Rb7 MAR found that it can also increase the proportion of transformants expressing a transgene (Halweg 2005). Additionally, MARs have been implicated in the reduction of transgene silencing (Mlynarova 2003). The tobacco TM6 MAR was shown to reduce repressive DNA methylation in flanking promoter regions (Ji et al. 2013). While MARs have traditionally been used only in transgenic systems, a transient expression system based on geminivirus replication may benefit from the inclusion of MARs. Furthermore, agrobacterium T-DNA has been shown to associate with histones (Lacroix et al. 2008; van Heusden et al. 2015)

1.7.2 Efficient Vector Delivery to Plant Cells

BeYDV vectors have replaced the native MP and CP with the desired gene of interest. This has had the consequence of abolishing cell-to-cell movement. Rather than relying on cell-to-cell movement to distribute viral replicons to neighboring cells, a high density of *Agrobacterium* must instead be used to individually transfer T-DNA to every plant cell. This is undesirable for industrial-level production of pharmaceutical proteins, as growth of large amounts of *Agrobacterium* requires more infrastructure and is more costly (Gleba et al. 2014).

Previous attempts to use altered mastrevirus replicons with the native CP and MP have failed. Inserting an herbicide resistance expression cassette into the wildtype sequence of maize streak virus did not hinder replication, but abolished systemic movement, presumably due to increased genome size (Shen and Hohn 1995). Further studies retained the wildtype genome size by using two separate replicons with movement functions on one, and replication functions on the other, each of proper size. Yet, recombination quickly produced wildtype virus, which predominated. As an alternate strategy, wildtype virus was coinfected with a replication-competent vector of proper size. These efforts still lead to preferentially spreading of only the wildtype genome (Palmer and Rybicki 2001). These studies suggest that, despite the prevailing notion that the CP and MP interact in a sequence-nonspecific manner (Elmer et al. 1988), some selectivity exists beyond genome size, at least for MSV.

Of the eight recognized genera of geminiviruses, only one group from one genus does not require the CP for movement function: the bipartite begomoviruses. These

viruses encode a separate NSP, which performs the role of DNA export from the nucleus. This role, which is typically carried out by the CP in other viruses, allows interaction of replicated viral DNA with the cytoplasmic MP and subsequent spread cell-to-cell through plasmodesmata, though the exact details of this interaction have not been fully elucidated (Krenz et al. 2012).

The MP and NSP from Bean dwarf mosaic virus (BDMV) bind DNA in a sequence-nonspecific manner that instead depends on size and form. The MP and NSP preferentially interact with open-circular double-standed DNA in the 2kb to 10kb range in vitro. This interaction was shown for BDMV DNA but also DNA ladder, sheared plant genomic DNA, and with various plasmids (Rojas et al. 1998). Using microinjection experiments with plants, the MP was then shown to preferentially transport genome-sized DNA in the 2.5kb-3kb range. Interestingly, these results were also observed for non-viral DNAs, indicating that movement capacity *in planta* was indeed not dependent on a specific viral sequence (Gilbertson et al. 2003). These experiments suggest that the MP and NSP from BDMV would retain movement functionality with similarly sized replicons from other DNA viruses, such as BeYDV.

Besides viral movement, other methods could be employed to increase the efficiency of expression vectors to plant cells. The ability of different *Agrobacterium* strains to efficiently transfer T-DNA varies, and has been modified to some extent. A hypervirulent strain, EHA105, was found to have enhanced virulence related to its unique *virB* and *virG* genes. Many studies have found that mutations in *virG* result in constitutive activation of all *vir* genes in the absence of plant inducers like phenols, low

pH, and sugars, especially an asparagine to aspartic acid mutation at amino acid 54 (Scheeren-Groot et al. 1994). Additionally, the extracellular sensor VirA appears to contain repressive domains that only become activated in the presence of plant inducers. Removing these domains can lead to constitutive *vir* gene activation (Lin et al. 2014). A plant caspase-resistant mutant of VirD2 was also found to improve T-DNA delivery in plants (Reavy et al. 2007).

1.7.3 Modifying BeYDV Replication

Previous studies with BeYDV vectors have determined that further increasing the replicon copy number is not correlated with a corresponding increase in mRNA or protein production, possibly indicating that such a large number of replicons are produced that the plant transcriptional machinery becomes saturated. In native BeYDV infection, the presence of the CP results in the accumulation of single-stranded viral DNA. The CP sequesters replicon DNA to form virions, shuttles replicon DNA out of the nucleus, and, in concert with the MP, facilitates cell-to-cell movement and systemic spread of viral DNA (Liu et al. 2001). These interactions reduce the amount of double-stranded viral DNA capable of being used for transcription. As modified BeYDV expression vectors do not contain the CP and MP, the amount of double-stranded DNA available in the nucleus to serve as a transcription template may exceed wildtype levels.

Plants employ the hypersensitive response (HR) as a mechanism to combat viral infection. The HR is characterized by a burst of reactive oxygen species and the formation of necrotic lesions resulting from programmed cell death, which occur as a means to destroy infected cells and limit virus spread (de Ronde et al. 2014). The

BeYDV expression system often results in the onset of plant leaf necrosis in as little as 3-4 days for certain proteins, which can limit their production (Phoolcharoen et al. 2011). Many viral proteins are known activators of the HR, including the geminivirus Rep proteins (Van Wezel et al. 2002).

Taken together, these studies suggest that more viral replicons are produced than are needed in the BeYDV expression system, and that viral replication proteins may induce the plant HR. Therefore, reducing rep and/or repA expression in BeYDV vectors may reduce the HR without negatively impacting protein expression, as saturating levels of mRNA could still be produced from fewer viral replicons. In fact, as RNA silencing and the HR are interrelated pathways that act in concert against invading viruses, reducing the onset of HR may also prevent premature silencing of BeYDV vectors, in addition to early cell death (Zvereva and Pooggin 2012).

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CHAPTER 2

5' AND 3' UNTRANSLATED REGIONS STRONGLY ENHANCE PERFORMANCE OF GEMINIVIRAL REPLICONS IN NICOTIANA BENTHAMIANA LEAVES.

2.1 Abstract

We previously reported a recombinant protein production system based on a geminivirus replicon that yields high levels of vaccine antigens and monoclonal antibodies in plants. The bean yellow dwarf virus (BeYDV) replicon generates massive amounts of DNA copies, which engage the plant transcription machinery. However, we noticed a disparity between transcript level and protein production, suggesting that mRNAs could be more efficiently utilized. In this study, we systematically evaluated genetic elements from human, viral, and plant sources for their potential to improve the BeYDV system. The tobacco extensin terminator enhanced transcript accumulation and protein production compared to other commonly used terminators, indicating that efficient transcript processing plays an important role in recombinant protein production. Evaluation of human-derived 5' untranslated regions (UTRs) indicated that many provided high levels of protein production, supporting their cross-kingdom function. Among the viral 5' UTRs tested, we found the greatest enhancement with the tobacco mosaic virus omega leader. An analysis of the 5' UTRs from the Arabidopsis thaliana and Nicotinana benthamiana photosystem I K genes found that they were highly active when truncated to include only the near upstream region, providing a dramatic enhancement of transgene production that exceeded that of the tobacco mosaic virus omega leader. The tobacco Rb7 matrix attachment region inserted downstream from the gene of interest

provided significant enhancement, which was correlated with a reduction in plant cell death. Evaluation of Agrobacterium strains found that EHA105 enhanced protein production and reduced cell death compared to LBA4301 and GV3101. We used these improvements to produce Norwalk virus capsid protein at >20% total soluble protein, corresponding to 1.8 mg/g leaf fresh weight, more than twice the highest level ever reported in a plant system. We also produced the monoclonal antibody rituximab at 1 mg/g leaf fresh weight.

2.2 Introduction

Recombinant protein production systems have become an integral part of medicine, industry, and research. Biopharmaceutical proteins, including monoclonal antibodies, enzymes, growth factors, and other biologics, are the largest and fastest growing sector of all pharmaceuticals (Butler and Meneses-Acosta, 2012). Nearly all of these recombinant proteins are made with traditional bioreactors using mammalian, insect, or microbe cell cultures. In recent years, plant systems have been extensively explored as alternative expression systems that offer safety, cost-effectiveness, scalability (Huang et al., 2009, Thuenemann et al., 2013, Klimyuk et al., 2014, Mortimer et al., 2015). The potential of plant-based systems has been demonstrated by the approval of the first plant-derived therapeutic for Gaucher's disease and by the advancement of many plant-made biologics to late-stage clinical development (Gleba et al., 2014). However, the economic feasibility of plant-based systems is strongly yield dependent, and thus, methods of increasing transgene expression are crucial for the success of plants as a recombinant protein production platform.

We and others have previously reported the potential for viral vectors based on bean yellow dwarf virus (BeYDV) to be used for biopharmaceutical production. In this system, the BeYDV replication elements are used to amplify the genes of interest to high copy number in the plant cell nucleus in the form of circular DNA replicons. These replicons utilize the nuclear transcription machinery, leading to the production of large amounts of recombinant protein that is dependent on replication (Huang et al., 2009, Huang et al., 2010, Regnard et al., 2010). Due to the noncompeting nature of BeYDV replicons, multiple proteins can be produced in the same cell from the same vector. This contrasts with many RNA virus systems, where the coinfiltration of two different vectors based on the same virus backbone results in one vector being preferentially amplified in a single cell, thus inhibiting the coproduction of multiple proteins in the same cell. This problem has been partially addressed by the identification of TMV and PVX as noncompeting viruses for the production of proteins with two heterosubunits (Giritch et al., 2006), however this system is incapable of producing proteins with more than two heterosubunits. In the BeYDV system, there is presently no known limit to the size or number of proteins that are capable of being efficiently produced (Chen et al., 2011). Additionally, the host range of BeYDV allows the use of these vectors in many dicot plant species, such as tobacco and lettuce (Lai et al., 2012). Nicotiana species are the most widely used plant for recombinant protein production due to susceptibility to virus infection, ease of vacuum infiltration, and high biomass (Gleba et al., 2014).

While BeYDV vectors strongly enhance gene expression at the level of transcription, replicon amplification greatly exceeds the enhancement of protein

accumulation (Huang et al., 2009, Huang et al., 2010, Regnard et al., 2010). Moreover, for mRNA transcripts to be efficiently utilized, the interplay of multiple post-transcriptional cellular processes is required, many of which are controlled by the regions upstream and downstream of the gene coding sequence. The 5' untranslated regions (UTR) plays an important role in optimizing transgene production by competing with cellular transcripts for translation initiation factors and ribosomes, increasing mRNA half-life by minimizing mRNA decay or post-transcriptional gene silencing, and avoiding deleterious interactions with regulatory proteins or inhibitory RNA secondary structures (Chiba and Green, 2009, Moore and Proudfoot, 2009, Jackson et al., 2010).

The 5' UTR from the genomic RNA of tobacco mosaic virus, known as the omega leader, is one of the most well-studied enhancers of translation (Gallie and Walbot, 1992). Several other viral 5' UTRs have been found to greatly enhance transgene production in many plant systems, including those from alfalfa mosaic virus (Gehrke et al., 1983), tobacco etch virus (Carrington and Freed, 1990), and pea seadborne mosaic virus (Nicolaisen et al., 1992). Many RNA viruses, such as barley yellow dwarf virus, also have 3' UTRs that contain 3' cap-independent translation enhancers, which enhance reporter production in tobacco and oat protoplasts (Fan et al., 2012). The combination of the 5' and 3' UTRs from cowpea mosaic virus improved protein production in transient expression assays using Nicotiana benthamiana, likely due to translational enhancement (Sainsbury et al., 2009). Two native plant 5' UTRs were identified that improved transgene production at levels comparable to viral 5' UTRs in transgenic cotton and tobacco (Agarwal et al., 2014). Additionally, a synthetic 5' UTR was also reported to

enhance protein production at a level similar to the TMV 5' UTR in transgenic tobacco and cotton (Kanoria and Burma, 2012).

Genetic elements downstream from the gene of interest also play a crucial role in optimizing protein production. Proper transcript termination and polyadenylation are necessary for nuclear export, mRNA stability, efficient translation, and prevention of gene silencing (Luo and Chen, 2007, Moore and Proudfoot, 2009). Several terminators have been investigated for their potential to enhance protein production in plants. The 3' UTR from the potato pinII gene was found to enhance hepatitis B virus surface antigen 10-50 fold in transgenic potato compared to the Agrobacterium-derived nopaline synthase terminator (Richter et al., 2000). Combining the nopaline synthase terminator with the 35S terminator from cauliflower mosaic virus resulted in a 5-65 fold enhancement of yellow fluorescent protein production compared to the 35S terminator alone (Beyene et al., 2011).

Additionally, chromatin scaffold/matrix attachment regions (MARs) have been explored as genetic elements capable of enhancing transgene production in plant systems. MARs are AT-rich regions thought to be involved in higher-order chromatin structure and that preferentially associate with nuclear matrix, a complex cellular structure with many proposed roles (Liebich et al., 2002, Calikowski et al., 2003, Halweg et al., 2005). Experiments in whole plants and plant cell cultures have shown that the presence of MARs can enhance transcription of flanking genes. The tobacco Rb7 MAR also increased the proportion of plant transformants expressing a transgene (Halweg et al., 2005). Furthermore, MARs have been implicated in the reduction of transgene silencing

(Mlynarova et al., 2003). The tobacco TM6 MAR was shown to reduce repressive DNA methylation in flanking promoter regions and enhance recombinant protein production in transgenic tobacco (Ji et al., 2013). An expression vector based on a mild strain of BeYDV that contained the Rb7 MAR has been previously reported, though a comparison to a vector without the MAR was not made (Regnard et al., 2010). The ability for MARs to enhance protein production in transient expression systems has not been thoroughly investigated.

Agrobacterium-mediated T-DNA transfer (reviewed in (McCullen and Binns, 2006) is the preferred method of gene delivery in plant transient expression systems (Chen and Lai, 2015). However, Agrobacterium is a plant pathogen that has complex effects on infiltrated leaf tissues and often elicits a cell death response (Ditt et al., 2001, Veena et al., 2003). Many studies have found variable effects of different Agrobacterium strains, depending on the plant species and system used. One study found that strain GV3101 provided higher transgene expression in *Nicotiana benthamiana* and *Nicotiana excelsiana* than strains LBA4404, C58C1, at6, at10, at77 and A4 (Shamloul et al., 2014). Additionally, many Agrobacterium strains vary greatly in their T-DNA transfer efficiency. Super virulent strains based on strain A281, such as EHA105, were shown to overexpress virG, a transcriptional activator which regulates vir gene expression (Jin et al., 1987). Constitutively activated virG mutants (Gao et al., 2006) have been used to increase T-DNA transfer efficiency, even when supplied on a separate plasmid (van der Fits et al., 2000). A mutant form of virD2 was found to enhance gene delivery to tobacco

cells (Reavy et al., 2007). These studies suggest there is potential to improve Agrobacterium T-DNA transfer and minimize deleterious plant cell interactions.

In the present study, we investigated the potential for diverse genetic elements to enhance protein production using BeYDV vectors. We show that optimizing the 5' UTR and 3' transcription terminator region substantially enhances the production of GFP, Norwalk virus capsid protein (NVCP), and the monoclonal antibody rituximab. Further, we demonstrate the potential for a MAR to reduce cell death and enhance protein production in a transient expression system. We also show that the choice of Agrobacterium strain can play an important role in plant cell death and recombinant protein yield. Using these optimizations, we have achieved yields of vaccine antigens and monoclonal antibodies equal to or greater than the highest levels ever reported in plant systems.

2.3 Materials and Methods

2.3.1 Vector construction

Geminiviral replicon with colE1 origin of replication

We constructed a T-DNA backbone vector containing the colE1 origin to enable high-copy replication of plasmids in E. coli. The T-DNA vector pGPTV-Kan (Becker et al., 1992) was digested with BglII and the vector fragment ligated to produce pGPTVKbb containing the pRK2 oriV, trfA, and nptIII (kanamycin resistance) genes. The colE1 origin from pUC19 was amplified by PCR with primers oriE-Pst-F and oriE-Mlu-R (Table 1), digested with PstI-MluI and ligated with pGPTVKbb digested likewise, to yield pVEKtrf, which was digested with BspEI and religated to produce pEKtrf (thus

lacking oriV). The oriV segment was amplified by PCR from pGPTV-Kan with primers oriV-Bgl-F and oriV-R1-R, digested with BglII-EcoRI and ligated with pEKtrf digested BglII-MfeI to give pEKtrfV. A DNA segment containing the A. tumefaciens T-DNA left border was inserted by ligation of the 2631bp BglII-BspEI fragment from pHB114 (Richter et al., 2000) with pEKtrfV digested likewise, yielding pEKtrfVa. The backbone from pEKtrfVa was incorporated into a geminiviral replicon T-DNA vector by a 3-fragment ligation: pEKtrfVa digested PvuI-BspEI, pBYR2p19 (Chen et al., 2011) digested PvuI-XbaI (2747 bp), and pBYR2p19-GFP digested XbaI-BspEI (3677 bp), yielding pBYR2e-GFP. The GFP cds in pBYR2e-GFP was replaced with the pUC19 polylinker (XbaI to SacI) by digestion/ligation of both plasmids with XbaI-SacI, to make pBYR2eFa.

3' terminator constructs

We constructed geminiviral replicons with different 3' terminator regions downstream of reporter genes. pBYGFP.R (Huang et al., 2009) contains the tobacco etch virus (TEV) 5'UTR and the soybean vspB 3' region flanking the GFP cds. The tobacco (Nicotiana tabacum) extensin gene 3' flanking region, 732 bp including an intron of 226 bp, was amplified by PCR using primers Ext1 and Ext2, which introduced a SacI site at the 5' end and EcoRI site at the 3' end. After digestion with SacI and EcoRI, the extensin 3' region was substituted for the vspB 3' region in pBYGFP.R to make pBYGFP.REF. Constructs pBYNVCP.R and pBYNVCP.REF were generated by replacing the GFP coding sequence of the pBYGFP.R and pBYGFP.REF with the Norwalk virus capsid protein (NVCP) cds from psNV210 (Zhang and Mason, 2005) using XhoI and SacI sites.

5'UTR constructs

We constructed expression vectors having different 5'UTRs linked to reporter genes. The shuttle cloning vector pBY-GFP212 was constructed by 4-fragment ligation: pBY027 (Mor et al., 2003) digested PstI-EcoRI (vector), pBTI210.3 (Judge et al., 2004) digested PstI-NcoI (820 bp 35S promoter + TMV 5'UTR), pGFPi210 (Huang et al., 2009) digested NcoI-SacI (726 bp GFP cds), and pBYR2p19 digested SacI-EcoRI (482 bp tobacco extension 3' region). Oligonucleotides (Table 1) encoding different 5'UTR segments were designed to anneal with 5' ends compatible with a cut XhoI site (5' protruding TCGA) and 3' ends compatible with a cut NcoI site (5' protruding CATG). The annealed oligonucleotides were phosphorylated with polynucleotide kinase + ATP, and ligated with pBY-GFP212 digested XhoI-NcoI to produce the various 5'UTR constructs, pBY-GFP212-XX. The constructs were ligated into pBYR2eFa on MfeI-SacI fragments, to give various P35S-5'UTR-GFP-Ext3' constructs named pBYR2eXX-GFP (Fig. 1).

Selected constructs were converted to nonreplicating vectors by deletion of the BeYDV Rep genes and the downstream LIR, accomplished by digesting with BamHI-AvrII, filling the recessed 3' ends with Klenow fragment DNA polymerase, and ligating the vector fragment, to give plasmids named pBYL2eXX-GFP. A nonreplicating construct with TMV 5'UTR was constructed by ligation of pBYL2e20-GFP digested MfeI-SacI (vector) and pBYR2e-GFP digested MfeI-SacI (1150 bp) to yield pBYL2eFc-GFP. Truncations of the A. thaliana psaK (PSI) 5'UTR in nonreplicating vectors were made. PCR amplification of pBYL2ePSIa-GFP with primers PSI3'-Xho-F and Ext3i-R,

digestion of the product with XhoI-SacI, and insertion into pBYL2eFc-GFP digested XhoI-SacI yielded pBYL2ePSI3'-GFP, containing the 3' 41 nt of the 5'UTR. A similar deletion of the 3' end was produced by PCR amplification of pBYL2ePSIa-GFP with primers 35S-Bsa-F and PSI5'-Xba-R, digestion of the product with MfeI-XbaI, and insertion into pBYL2eFc-GFP digested MfeI-XbaI to make pBYL2ePSI5'-GFP. Replicating vectors containing the 3' 41nt of the AtPsaK 5' UTR were generated by digesting pBYL2ePSI3'-GFP with XbaI-FseI (vector fragment) and inserting the XbaI-FseI fragment from either pBYR2e-GFP or pBYR2eFa-sNV to generate pBYR2eP3-GFP and pBYR2eP3-sNV respectively.

Homologs of A. thaliana psaK were identified using the Sol Genomics *Nicotiana benthamiana* draft genome (https://solgenomics.net/organism/Nicotiana_benthamiana/genome). pBYR2e-GFP was digested XhoI-SacI and the GFP fragment was inserted into psNV120e (a nonreplicating T-DNA vector; details available upon request) digested XhoI-SacI yielding pGFPe-TMV. The upstream region from the first psaK homolog, referred to as NbPsaK1, was PCR amplified from *Nicotiana benthamiana* genomic DNA using primers PsaK1-Xho-F and PsaK1-Xba-R, and the second homolog, referred to as NbPsaK2, was amplified similarly using primers PsaK2-Xho-F and PsaK2-Xba-R. The PCR fragments were digested with XhoI-XbaI and ligated into pGFPe-TMV digested XhoI-XbaI yielding pGFPe-NbPsaK1 and pGFPe-NbPsaK2. A truncation of the NbPsaK1 5' UTR was generated by PCR amplifying pGFPe-NbPsaK1 with primers PsaK1T-Xho-F and Ext3i-R, digestion of the product with XhoI-SacI, and insertion into pGFPe-TMV digested XhoI-SacI to yield

pGFPe-NbPsaK1T. pGFPe-NbPsaK2T was created similarly using PCR primers PsaK2T-Xho-F and Ext3i-R, followed by XhoI-SacI digestion and insertion into pGFPe-TMV digested XhoI-SacI.

Selected 5'UTRs were modified to contain XbaI sites at the 3' end for fusion with the Norwalk virus capsid protein (NVCP) cds. The shuttle vectors (pBY-GFP212-XX) were amplified by PCR with reverse primers containing an XbaI site and M13-R, and the resulting products digested PstI-XbaI and ligated with pBYR2eFa-sNV digested likewise, thus yielding the various vectors names pBYR2eXX-sNV.

The rituximab heavy chain was obtained by PCR amplifying pMAP-RitX-G1-B (a kind gift from Mapp Biopharmaceuticals, San Diego CA) with primers BAA-Xba-F and RituxG-Sac-R. The resulting PCR fragment was digested with XbaI-SacI and ligated into a derivative of pBY027 (Mor et al., 2003), digested likewise, yielding pBYR0-LRtxGT. The rituximab light chain was similarly cloned by amplifying pMap-RitX-K-b (Mapp Biopharmaceuticals) with BAA-Xba-F and RituxK-Sac-R, digested XbaI-SacI, and ligated into a derivative of pBY027 digested likewise to yield pBYR0-LRtxKF. To generate T-DNA vectors, the rituximab heavy chain was obtained by XhoI-SacI digestion of pBYR0-LRtxGT and inserted into pBYR2e-GFP (vector) digested XhoI-SacI to yield pBYR2e-MRtxG. The rituximab light chain was obtained by XhoI-SacI digestion of pBYR0-LRtxKF and inserted into pBYR2e-GFP digested XhoI-SacI to yield pBYR2e-MRtxK. The AtPsaK 5' UTR fused to the rituximab heavy and light chains was obtained by digesting pBYR0-LRtxGT XbaI-SacI (heavy chain) or pBYR0-LRtxKF XbaI-SacI

(light chain) and ligating into pBYR2ePSI-GFP (vector) digested XbaI-SacI to yield pBYR2ePSI-MRtxG and pBYR2ePSI-MRtxK respectively.

MAR constructs

The tobacco Rb7 MAR was PCR amplified from genomic DNA using primers Mar-1 and Mar-2 designed to create EcoRI sites on either end. The amplified fragment was digested with EcoRI and ligated into pBY027 digested likewise to yield pBY027-MAR. pBY027-MAR was PCR amplified with primers Mar-1 and Mar-Kpn-2 to create a KpnI site on the 3' end. To generate a KpnI in the BeYDV vector, primers LIR-R and Kpn-F-SIR were used to amplify the LIR-C1/C2-SIR segment of pBYGFP.REF. A 3-fragment ligation consisting of pBYGFP.REF (vector) digested HindIII-EcoRI, the HindIII-KpnI digested segment of the LIR-C1/C2-SIR PCR product, and the KpnI-EcoRI digested MAR fragment was used to make pBYR-GEM. To create the 5' MAR, pBY027-MAR was PCR amplified with primers Mar-Pst1 and Mar-Pst2. The product was digested with PstI and ligated into pBYR-GEM digested with SbfI to make pBYR-MGEM. The SacI-FseI fragment containing the 3' MAR from pBYR-MGEM was ligated into vectors containing the rituximab heavy chain (pBYR2e-MrtxG) or light chain (pBYR2e-MRtxK) to yield pBYR2e-MRtxGM and pBYR2e-MRtxKM respectively. The 5' + 3' MAR rituximab construct was created by digesting pBYR-MGEM with XhoI-AscI to obtain the 5' MAR fragment, and ligating it into pBYR2e-MRtxG or pBYR2e-MRtxK, yielding pBYR2e-MMGM and pBYR2e-MMKM respectively.

2.3.2 Agroinfiltration of Nicotiana benthamiana leaves

Binary vectors were separately introduced into Agrobacterium tumefaciens LBA4404, LBA4301, GV3101, or EHA105 by electroporation. The resulting strains were verified by restriction digestion or PCR of plasmid DNA, grown overnight at 30°C, and used to infiltrate leaves of 5- to 6-week-old *Nicotiana benthamiana* maintained at 23-25°C. Briefly, the bacteria were pelleted by centrifugation for 5 min at 5,000g and then resuspended in infiltration buffer (10 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.5 and 10 mM MgSO4) to OD600=0.2. The resulting bacterial suspensions were injected by using a syringe without needle into leaves through a small puncture (Huang and Mason, 2004). For antibody coinfiltrations, Agrobacterium suspensions were mixed such that the final concentration of each corresponded to OD600=0.2. Plant tissue was harvested at 4 DPI unless otherwise noted.

2.3.3 Protein extraction

Total protein extract was obtained by homogenizing agroinfiltrated leaf samples with 1:5 (w:v) ice cold extraction buffer (25mM sodium phosphate, pH 7.4, 100mM NaCl, 1mM EDTA, 0.2% Triton X-100, 10 mg/mLsodium ascorbate, 10 mg/mL leupeptin, 0.3 mg/mL phenylmethylsulfonyl fluoride) using a Bullet Blender machine (Next Advance, Averill Park, NY) following the manufacturer's instruction. To enhance solubility, homogenized tissue was rotated at room temperature for 30 minutes. The crude plant extract was clarified by centrifugation at 10,000g for 10 min at 4°C. Protein concentration of clarified leaf extracts was measured using a Bradford protein assay kit (Bio-Rad) with bovine serum albumin as standard.

2.3.4 SDS-PAGE

For SDS-PAGE, clarified plant protein extract was mixed with sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 200 mM dithiothreitol, 0.02 % bromophenol blue), boiled for 10 minutes, and separated on 4-15% polyacrylamide gels (Bio-Rad). For GFP fluorescence, PAGE gels were visualized under UV illumination (365nm). PAGE gels were stained with PageBlue protein staining solution (Thermo Fisher) or Coomassie stain (Bio-Rad) following the manufacturer's instructions. Following protein staining, the 26kDa band corresponding to GFP or the 58kDa band corresponding to NVCP were analyzed using ImageJ software to quantify the band intensity.

2.3.5 ELISA

NVCP concentration was analyzed by sandwich ELISA as described (Mason et al., 1996). Briefly, a rabbit polyclonal anti-NVCP antibody was bound to 96-well high-binding polystyrene plates (Corning), and the plates were blocked with 5% nonfat dry milk in PBS. After washing the wells with PBST (PBS with 0.05 % Tween 20), the plant extracts were added and incubated. The bound NVCP were detected by incubation with guinea pig polyclonal anti-NVCP antibody followed by goat anti-guinea pig IgG-horseradish peroxidase conjugate. The plate was developed with TMB substrate (Pierce) and the absorbance was read at 450 nm. Plant-produced NVCP was used as the reference standard (Kentucky BioProcessing).

For rituximab quantification, plant protein extracts were analyzed by ELISA designed to detect the assembled form of mAb (with both light and heavy chains) as described previously (Giritch et al., 2006). Briefly, plates were coated with a goat anti-human IgG

specific to gamma heavy chain (Southern Biotech, AL). After incubation with plant protein extract, the plate was blocked with 5% nonfat dry milk in PBS, then incubated with a HRP-conjugated anti-human-kappa chain antibody (Southern Biotech) as the detection antibody. Human IgG was used as a reference standard (Southern Biotech).

2.3.6 GFP fluorescence

Leaves producing GFP were photographed under UV illumination generated by a B-100AP lamp (UVP, Upland, CA).

2.3.7 Statistical analysis

For each experiment, plants of the same age were used to minimize developmental differences. Additionally, experiments were designed to compare each construct directly on the same leaf to minimize leaf-to-leaf variation. Comparisons between leaves were made using leaves of similar developmental stage. Data are presented as mean ± S.E. Three or more independent infiltrations were made for each experiment and compared using Student's t-test (two-tailed). $P < 0.05$ was represented with two stars (**) and $P < 0.01$ was represented with three stars (***).

2.4 Results

2.4.1 Diverse 5' UTRs greatly impact transgene production

Previously, we reported BeYDV vectors that contained the 5' UTR from either tobacco etch virus (TEV) or tobacco mosaic virus (TMV). In order to systematically evaluate the role of the 5' UTR on transgene production, we created a series of BeYDV transient expression vectors containing diverse 5' UTRs from viral, plant, and human sources upstream from the green fluorescent protein (GFP) gene (Fig. 1). As the

nucleotides directly surrounding the start codon are known to play a role in translation initiation, we standardized all vectors to contain the nucleotides ACC (to accommodate the NcoI site) or ACA preceding the ATG, which has been reported to be optimal for dicot plants (Sugio et al., 2010). We found no difference in the performance of vectors with ACC or ACA. These vectors were delivered to *Nicotiana benthamiana* leaves by agroinfiltration and monitored for green fluorescence. To minimize leaf-to-leaf variation, each leaf was infiltrated with a vector containing the TMV 5' UTR as an internal control alongside vectors containing the 5' UTRs to be tested.

First, we compared a set of 11 human-derived sequences found to provide cap-independent translational enhancement (Wellensiek et al., 2013). There is evidence that some 5' UTR elements function cross-kingdom, especially A-rich polypurine sequences (Dorokhov et al., 2002, Terenin et al., 2005). We found that many of the human 5' UTRs, as well as a polypurine sequence, produced bright green fluorescence under UV illumination (Fig. 3A). To further analyze GFP production, protein extracts from agroinfiltrated leaves were separated by SDS-PAGE followed by Coomassie staining or visualization under UV light, and the GFP band intensity was quantified by densitometry. Gel quantification showed many of the human-derived 5' UTRs, as well as the polypurine 5' UTR, produced GFP at a level comparable to the commonly used plant viral 5' UTRs from TEV and TMV (Fig. 3B). These data indicate that 5' UTRs from sources outside of the plant kingdom can support high levels of translation in *Nicotiana benthamiana*.

Next, we tested the 5' UTRs from RNA plant viruses. Among the 5' UTRs tested, the TMV 5' UTR appeared to provide the brightest fluorescence, followed by TEV (Fig. 3A). Using gel quantification, the TMV 5' UTR provided a >40% increase in GFP yield over the TEV 5' UTR (Fig. 3B). A truncation containing only nucleotides 1-22 of the TMV 5' UTR performed as well as the full length sequence, indicating that the poly(CAA) region of the TMV 5' UTR is not necessary for high levels of translation in *Nicotiana benthamiana*, at least in a replicating system (Fig. 3B). Constructs containing the 5' UTR from alfalfa mosaic virus (AMV), as well as constructs containing the 5' and 3' UTRs from barley yellow dwarf virus (BYDV) or pea enation mosaic virus (PEMV), showed poor GFP production and were not studied further (Fig. 3A).

We also wished to test the activity of plant-derived 5' UTRs in the BeYDV system. It has been reported that a 5' UTR derived from 63 nucleotides upstream from the start codon of the Arabidopsis thaliana photosystem K subunit (AtPsaK) enhanced transgene expression in transgenic tobacco leaves (Agarwal,et al., 2014). Using our system, we found that the 63nt AtPsaK 5' UTR produced intense green fluorescence, and gel quantification data indicate that GFP production was increased by >20% compared to the TMV 5' UTR (Fig. 3A, Fig. 3B). The AtPsaK 5' UTR (accession NM_102775) appears to be a truncation of the full-length 129nt 5' UTR. To further delineate the active region of the AtPsaK 5' UTR, we created deletions at its 5' and 3' ends and tested their potential to enhance GFP production in nonreplicating transient expression vectors. Using gel quantification, we found that a truncation removing nucleotides -1 to -23 upstream from the start codon resulted in a ~14% decrease in GFP production, while a truncation

removing nucleotides -42 to -63 resulted in a ~13% increase in GFP production (Fig. 4). A similar ~12% enhancement was observed in replicating GFP vectors (Fig. 3B).

To determine whether the 5' UTRs from related psaK genes have potential for high levels of transgene production, two psaK homologs were identified from *Nicotiana benthamiana* (referred to as NbPsaK1 and NbPsaK2) and their 5' upstream regions were cloned into nonreplicating GFP expression vectors. These vectors were agroinfiltrated alongside the TMV and AtPsaK 5' UTRs for comparison. By gel quantification, the 163nt upstream region from NbPsaK1 was found to have very minimal activity, whereas the 170nt upstream region from NbPsaK2 produced GFP at ~50% of the level of the TMV 5' UTR (Fig. 4). Inspection of the nucleotide sequence revealed the presence of upstream ATGs in both NbPsaK1 and NbPsaK2. As the 3' end was the most active region of the AtPsaK 5' UTR, similar truncations were made for the NbPsaK upstream regions (referred to as NbPsaK1 3' and NbPsaK2 3'). These new constructs were agroinfiltrated alongside the full-length version and tested by gel quantification. The NbPsaK1 truncation enhanced GFP production by >20-fold compared to the original vector (Fig. 4). The NbPsaK2 truncation enhanced GFP production by 2.4-fold compared to the original vector, corresponding to a >40% improvement compared to the TMV 5' UTR (Fig. 4). These results indicate that the regions 40-60nt upstream from the A. thaliana and *Nicotiana benthamiana* psaK genes are highly active in *Nicotiana benthamiana* leaves, and are capable of enhancing protein production at a level greater than the widely used TMV 5' UTR.

To further assess the potential of the 5' UTR to improve transgene production, several promising 5' UTRs were tested in BeYDV vectors producing NVCP. Protein extracts from agroinfiltrated leaf samples were normalized for TSP and analyzed by NVCP ELISA. In general agreement with the results found for GFP, several of the human 5' UTRs performed as well as the TMV 5' UTR, and the TMV 5' UTR resulted in a ~30% increase in NVCP production compared to the TEV 5' UTR (Fig. 5). Additionally, vectors containing the AtPsaK 5' UTR produced NVCP at 15.9 ± 1.5% TSP compared to 11.3 ± 1.0% TSP for the TMV 5' UTR (Fig. 5). Further, the truncated form of the AtPsaK 5' UTR (AtPsaK 3') produced as much or more NVCP as the unmodified 5' UTR. These data further demonstrate the capacity of the 5' UTR to enhance recombinant protein production, and show that the enhancing activity of the unmodified or truncated AtPsaK 5' UTR is not gene-specific.

2.4.2 Matrix attachment regions enhance transgene production and reduce plant cell death

The presence of MARs has been reported to enhance transgene production using transgenic systems (Halweg et al., 2005, Xue et al., 2005, Ji et al., 2013). Many of the postulated mechanisms by which MARs enhance transgene production, such as by preventing repressive chromatin modifications or by the interaction of chromatin with the nuclear matrix, require the gene of interest to be organized into chromatin. Thus it is unclear whether MARs would function in transient expression systems that do not involve stable chromosomal integration. However, replicated geminivirus DNA has been shown to associate with cellular histones, forming viral minichromosomes (Pilartz and

Jeske, 1992, Pilartz and Jeske, 2003). Therefore, we investigated the potential for MARs to improve BeYDV vectors.

The tobacco Rb7 MAR was inserted into BeYDV vectors (Fig. 1) either with two copies flanking the expression cassette (5' + 3' MAR), or one copy in the 3' position (3' MAR). Placing the MAR only in the 5' position was not found to be as effective as the other two configurations in preliminary studies and was not pursued further (data not shown). Leaves of *Nicotiana benthamiana* were co-infiltrated with BeYDV vectors containing the rituximab heavy and light chains both either with or without the Rb7 MAR at either the 5' or 5' + 3' positions. Protein extracts from infiltrated leaf spots were normalized for TSP and assayed for rituximab production by IgG ELISA. Remarkably, it was found that while both MAR-containing vectors enhanced IgG production, the vector containing only the 3' MAR resulted in a 3.4-fold increase in IgG production, representing 14.3 ± 1.6 % TSP for the 3' MAR vector compared to 4.2 ± 1 % TSP for the control with no MAR elements (Fig. 6A). Inspection of the infiltrated leaves revealed a substantial reduction in leaf tissue necrosis with the MAR-containing vectors (Fig. 6B). These data indicate that MARs have potential to enhance protein production using geminiviral transient expression vectors, and this enhancement is correlated with a reduction in plant cell death.

2.4.3 Effects of Agrobacterium strain on transgene production and cell death

The choice of Agrobacterium strain has been shown to play an important role in many aspects of transient protein production, including T-DNA transfer efficiency, plant health, and overall yield (Gleba et al., 2014, Shamloul et al., 2014, Sheikh et al., 2014).

To investigate the effects of Agrobacterium strain on recombinant protein production, we introduced BeYDV GFP vectors to strains LBA4301, GV3101, and EHA105. Leaves of *Nicotiana benthamiana* were agroinfiltrated with each strain at OD600 of 0.2 and monitored for plant health and GFP production. At 4 DPI, spots infiltrated with GV3101 developed faint leaf browning, whereas the other two constructs had no detectable changes from uninfiltrated leaf tissue (data not shown). By 7 DPI, leaf spots infiltrated with GV3101 had become severely necrotic, while EHA105 or LBA4301 only had just begun to develop necrotic tissue (Fig. 7B). Inspection of leaves under UV light revealed that fluorescing leaf regions infiltrated with EHA105 were substantially brighter than areas infiltrated with either of the other strains (Fig. 7A).

To further compare the effects of EHA105 and GV3101 more quantitatively, BeYDV rituximab vectors were introduced to each strain and agroinfiltrated into *Nicotiana benthamiana* leaves. Leaf extracts were normalized for TSP and analyzed by IgG ELISA. In agreement with the data obtained using GFP vectors, Agrobacterium strain EHA105 substantially improved rituximab production: 10.9 ± 1.6 % TSP for EHA105 compared to 5.3 ± 1.1 % TSP for GV3101 (Fig. 7C). Additionally, the increase in rituximab production was correlated with a reduction in plant cell death. These results demonstrate the importance of Agrobacterium strain on improving recombinant protein production.

2.4.4 Optimized genetic elements function synergistically to further enhance transgene production

We determined the potential for the enhancing effects of the genetic elements identified in the present study to function synergistically with one another.

Agrobacterium strain EHA105 was observed to perform better with all tested constructs, and was used for the remainder of studies (data not shown). BeYDV NVCP vectors were created which contained the AtPsaK 5' UTR and extensin terminator with or without the 3' Rb7 MAR, and were agroinfiltrated using strain EHA105 into *Nicotiana benthamiana* leaves. NVCP ELISA showed that insertion of the 3' Rb7 MAR paired with the AtPsaK 5' UTR and extensin terminator significantly enhanced NVCP production, yielding 20.3 ± 1.5% TSP compared to 15.7 ± 1.3% TSP for the construct lacking the MAR (Fig. 8A, last two columns). This yield corresponds to 1.8mg NVCP per gram leaf fresh weight. These results, compared with previous data, indicate that the optimizations identified in this study provide synergistic enhancement of transgene production, enabling very high levels of recombinant protein production (Fig. 8A, Fig. 8B).

2.5 Discussion

In recent years, transient expression systems have become the method of choice for plant-based recombinant protein production. In addition to their high yields, the rapid speed of these systems (4-5 days for BeYDV vectors) offers many unique advantages over stable transgenic systems, such as the ability to produce personalized therapeutics as reported for non-Hodgkin's lymphoma (Bendandi et al., 2010, Tuse et al., 2015), and to rapidly respond to virus outbreaks or bioterrorism events (D'Aoust et al., 2010). Additionally, transient expression systems circumvent the regulatory issues associated with the creation of genetically modified organisms.

The most widely used transient expression system, magnICON, uses viral vectors derived from TMV and PVX (Giritch et al., 2006). Due to the competing nature of many RNA viruses, this system cannot produce recombinant proteins with more than two heterosubunits, excluding the efficient production of secretory IgAs, IgMs, and heteromultimeric virus-like particles, among other desirable biopharmaceuticals (Chen and Lai, 2013). A nonreplicating system based on cowpea mosaic virus was has been used to produce bluetongue virus-like particles, allowing proper assembly of four heterosubunits (Thuenemann et al., 2013). However, this system lacks the high yields associated with the other replicating systems (Gleba et al., 2014).

To circumvent these issues, we developed a transient expression system based on BeYDV which generates noncompeting DNA replicons to drive high-level production of heteromultimeric proteins (Huang et al., 2010). While this system generates massive amounts of DNA copies of the target gene(s) which are thought to result in a saturation of the plant transcription machinery, the disparity between gene copy number, transcript accumulation, and protein production suggested that each transcript was not being efficiently utilized by the plant cell (Huang et al., 2009, Huang et al., 2010). Therefore, we hypothesized that optimizing the genetic elements involved in efficient transcript processing, stability, and utilization could further improve the BeYDV system.

In the current study, we present a comprehensive comparison of diverse genetic elements and assess their potential to enhance plant-based recombinant protein production. We compared a large set of 5’ UTRs derived from human, plant, and viral sequences. In agreement with previous studies demonstrating cross-kingdom translational

enhancement of certain 5' UTRs (Dorokhov et al., 2002, Terenin et al., 2005), we found that many of the human sequences, as well as the polypurine 5' UTR described by Dhorokhov et al., provided high levels of GFP production in leaves of *Nicotiana benthamiana*, in some cases out-performing the routinely used viral 5' UTRs from tobacco etch virus or alfalfa mosaic virus (Fig. 3A, Fig. 3B). Among the virus-derived 5' UTRs tested, we found the TMV 5' UTR provided the highest level of transgene expression. Some of the viral elements tested, especially those containing long 3' UTRs, performed very poorly. As RNA viruses are not typically adapted for the plant nucleus, many of these sequences may contain cryptic splice sites or other detrimental elements. We suspect that rigorous optimization of these sequences, such as through the insertion of introns and removal of sequences known to destabilize mRNA, could significantly improve the performance of genetic elements derived from these viruses.

Interestingly, despite the historic success of viral elements in driving high levels of protein production, we also found that the plant-derived 5' UTRs from the psaK homologs of both A. thaliana and *Nicotiana benthamiana* were capable of enhancing recombinant production by as much as 40% more than the widely used TMV 5' UTR (Fig. 4). In particular, the first 40-60nt directly upstream from the ATG seemed the most potent, possibly due to the removal of inhibitory regulatory sequences further upstream (Fig. 4). Furthermore, we investigated the potential of the tobacco extensin terminator to enhance transgene production. It was found to prevent read-through transcription, enhance mRNA accumulation, and enhance protein production at a level greater than the 35S or nopaline synthase terminators, among other commonly used gene terminators

(data to be presented elsewhere, Fig. 2). We anticipate that further investigation of other native genetic elements from highly expressed plant genes has great potential to improve recombinant protein production systems.

MARs have a well-supported history of enhancing transgene production in transgenic plants (Halweg et al., 2005, Xue et al., 2005, Wang et al., 2007, Zhang et al., 2009, Ji et al., 2013), though their features seem variable or, in many cases, poorly understood. In the present study, we show that a MAR increases transgene production and reduces cell death in a plant transient expression system. Replicated geminivirus DNA has been shown to be organized into chromatin (Pilartz and Jeske, 1992, Pilartz and Jeske, 2003), and subject to repressive DNA methylation (Raja et al., 2008), indicating that MARs could be functionally active in BeYDV replicons. We found that insertion of the Rb7 MAR had a substantial enhancing effect on rituximab production, improving yield by 3.4-fold (Fig. 6A). The MAR was most active when placed 3' of the expression cassette, in contrast to other studies which found optimal placement upstream from the promoter, or in both positions (Zhang et al., 2009). Inspection of the tobacco Rb7 sequence reveals the presence of many polyadenylation and transcription termination signals, suggesting the alternative hypothesis that the 3' MAR is acting as a second gene terminator or otherwise stabilizing the mRNA. Double terminators have been found to have a dramatic enhancing effect on transgene production (Beyene et al., 2011). Unexpectedly, we also found a dramatic decrease in cell death associated with the insertion of the tobacco Rb7 MAR (Fig. 6B). Further studies are underway to

characterize the function of the Rb7 MAR and other MARs in enhancing transgene production and reducing cell death in the BeYDV system.

One of the drawbacks of transient expression systems compared to stable transgenics is the requirement for Agrobacterium to deliver the gene of interest to the plants. An ideal Agrobacterium strain should minimize deleterious plant cell interactions while providing efficient T-DNA transfer to reduce the concentration of Agrobacterium required for complete gene delivery to all plant cells. We wished to evaluate different strains of Agrobacterium using the BeYDV system. EHA105 has been reported to overexpress virG, a transcriptional activator that regulates T-DNA transfer through induction of vir gene expression (Jin et al., 1987). Additionally, GV3101 and LBA4404 have been reported to have differing effects on the activation of plant immune response genes through the production of cytokinins (Sheikh et al., 2014). Previously, we found that strain GV3101 enhanced transgene production compared to strain LBA4404 (data not shown). In this study, we compared strains GV3101, LBA4301, and EHA105, and found that EHA105 both enhanced transgene production, and reduced plant cell death. Our results demonstrate that Agrobacterium strain can have a dramatic effect on recombinant protein production systems (Fig. 7). A strain CryX is reported to provide 100-1000 times the gene delivery efficiency compared to commonly used Agrobacterium strains (Gleba et al., 2014). These studies indicate there is great potential to reduce plant toxicity and improve T-DNA transfer efficiency by optimizing the Agrobacterium strain.

In conclusion, by optimizing the gene terminator, 5’ UTR, and Agrobacterium strain, and by targeted insertion of MAR elements, we have dramatically improved the

BeYDV transient expression system. We have used this system to produce NVCP at up to 20% TSP, corresponding to 1.8mg per gram leaf fresh weight, a >4-fold improvement over the original vector (Huang et al., 2009) and more than twice the highest level ever reported in a plant-based system (Santi et al., 2008). Furthermore, we have also produced the monoclonal antibody rituximab at up to 1 mg per gram leaf fresh weight, which is twice the highest level previously reported for a monoclonal antibody using BeYDV vectors (Huang et al., 2010). We expect these improvements to be broadly applicable to other DNA expression systems. Additionally, these modifications could be used to fine-tune expression in cases where multiple proteins need to be produced at different levels.

2.6 Figures

2.6.1 Table 1 Oligonucleotides used in this study

PsaK1-Xho-F	TACTCGAGCTGAAACAGTCCATTCTGAGGC
PsaK1-Xba-R	GGTCTAGATTTAATTTGCAGCAACTCAACTTTTTTTTCTC
PsaK1T-Xho-F	CCCTCGAGAAAAGCCAATTAAACTAAAAAAAGAAGAG
PsaK2-Xho-F	ATCTCGAGACAAGTATCTTAGTGTATCCAGAATAGCC
PsaK2-Xba-R	GCTCTAGATGTTGCAGAAATTTCAAAGAATTGGAAATGC
PsaK2T-Xho-F	AACTCGAGAAACAAACAAAATCAACAAATATAGAAAATAACG
Ext1	GTGAGCTCGAAGTGACATCACAAAGTTGAAG
Ext2	CAGAATTCGTCATAACTGTAGAAATGATTCC
GFP-f	GTCCAGGAGCGCACCATCTTCT
GFP-r	GATGCCCTTCAGCTCGATGCGGTT
Mar-1	GCGAATTCTCGATTAAAAATCCCAATTATATTTGG
Mar-2	GCGAATTCACTATTTTCAGAAGAAGTTCCC
Mar-Pst1	GCCTGCAGTCGATTAAAAATCCCAATTATATTTGG
Mar-Pst2	GCCTGCAGACTATTTTCAGAAGAAGTTCCC
5D5-F	TCGACATATTGAAGAGACAGAGTGATATATAAAACTGCTAAC
5D5-R	CATGGTTAGCAGTTTTATATATCACTCTGTCTCTTCAATATG
10-F	TCGAAGAATTTTTAGTCAAGAAGTGAC

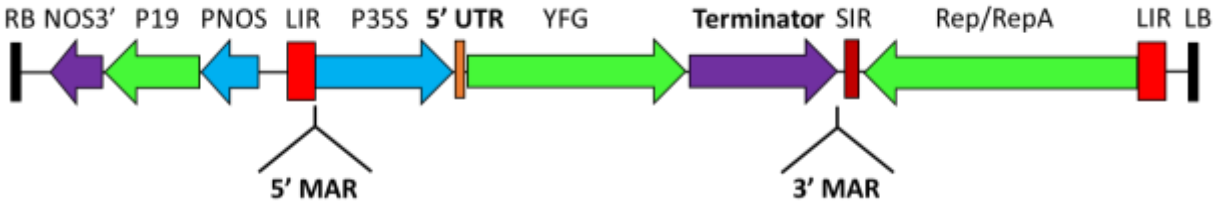
10-R	CATGGTCACTTCTTGACTAAAAATTCT
12-F	TCGAAGTGGACGTCAATACTTACGCAC
12-R	CATGGTGCGTAAGTATTGACGTCCACT
13-F	TCGAAGATTTAAGTGACGATAAAGTTAC
13-R	CATGGTAACTTTATCGTCACTTAAATCT
19-F	TCGAAGTTGTTTTGGATTTAGTCAAGC
19-R	CATGGCTTGACTAAATCCAAAACAACT
20-F	TCGAGGATATGAATGTTGAACAGCTTAC
20-R	CATGGTAAGCTGTTCAACATTCATATCC
23-F	TCGAACGAATGCAATCTTGGACGTTAC
23-R	CATGGTAACGTCCAAGATTGCATTCGT
26-F	TCGATGTGAGAATGAATGTTAGCAAAC
26-R	CATGGTTTGCTAACATTCATTCTCACA
43-F	TCGATTATTGCTGAAGTTTTGAGTTAC
43-R	CATGGTAACTCAAAACTTCAGCAATAA
43-Bsa-F	GCGGTCTCCCTAGTTATTGCTGAAGTTTTGAGTTA
48-F	TCGATGAAGAGAAAGTTGAAATTGTAC
48-R	CATGGTACAATTTCAACTTTCTCTTCA
54-F	TCGAAGTGAACTGCAAACGGATTACAC
54-R	CATGGTGTAATCCGTTTGCAGTTCACT
PPT-F	TCGAAAAAGAAGGAAAAAGAAGGGAAGAAAAGGAC
PPT-R	CATGGTCCTTTTCTTCCCTTCTTTTTCCTTCTTTT
PSI-F1	TCGAAAAAACAAAAATAAAAAAAACATCGCACAAGAAA
PSI-F2	ATAAAAGATTTGTAGAATCAACTAAGAAACCATG
PSI-R1	CATGGTTTCTTAGTTGATTCTACAAATCTTTTATTTTC
PSI-R2	TTGTGCGATGTTTTTTTTATTTTTGTTTTT
TMV-F	TCGAACAATTACTATTTACAATTACAC
TMV-R	CATGGTGTAATTGTAAATAGTAATTGT
BYDV5-Xho-F	TTCTCGAGTGAAGATTGACCATCTCACAAAAGC
BYDV5-Nco-R	TTCCATGGTGGCGGTGGGGATAGAAGGG
BYDV3-Kpn-F	AAGGTACCAGTGAAGACAACACC
BYDV3-Sac-R	ATGAGCTCGGGTTGCCGAACTGC
PEMV-F1	TCGAGGGTATTTATAGAGATCAGTATGAACTGTGTCGCTAGGATCAAGCGG
PEMV-F2	TGGTTCACACCTGACTTCACCCCTGGCGAGGGCGTGAAGTCTAC
PEMV-R1	CATGGTAGACTTCACGCCCTCGCCAGGGGTGAAGTCAGGTGTGAACCACCGC
PEMV-R2	TTGATCCTAGCGACACAGTTCATACTGATCTCTATAAATACCC

PEMV3-Bsr-F	ATTGTACAAGTAAGGCTTCGCTTCCCGCC
Xho-AMV5-F	TCGAGTTTTTATTTTTAATTTTCTTTCAAATACTTCCAACAT
PSI3'-Xho-F	CGCTCGAGTCGCACAAGAAAATAAAAGATTTG
PSI5'-Xba-R	CCTCTAGATTTTATTTTCTTGTGCGATGTTTT
PSI5'-Nco-R	CCCCATGGTTTTATTTTCTTGTGCGATGTTTT
PSI-Xba-R	GGTCTAGATTTCTTAGTTGATTCTACAAATCTTTTA
5D5-Xba-R	GGTCTAGATTAGCAGTTTTATATATCACTCTGTC
10.10-Xba-R	CGTCTAGATCACTTCTTGACTAAAAATTCTTC
10.43-Xba-R	CGTCTAGATAACTCAAAACTTCAGCAATAATC
10.20-Xba-R	CGTCTAGATAAGCTGTTCAACATTCATATCC
oriE-Pst-F	CCCTGCAGACCAAGTTTACTCATATATAC
oriE-Mlu-R	CCACGCGTAAAAAGGCCGCGTT
oriV-Bgl-F	GCAGATCTCGACGAGCAAGGCAAGA
oriV-R1-R	GGGGAATTCAATGGCAAGGACTGCC
Ext3i-R	CAATTTGCTTTGCATTCTTGAC
35S-Bsa-F	GCGGTCTCGGCATGGTGGAGCACGA
MAR-Kpn-2	GCGGTACCACTATTTTCAGAAGAAGTTCCC
kpn-f-SIR	GTGGTACCGAGTGTACTTCAAGTCAGTTGG
BAA-Xba-F	CCTCTAGAACAATGGCTAACAAACATCTTTCTTTG
RituxG-Sac-R	CCGAGCTCTTACTTACCAGGTGAAAGAGAC
RituxK-Sac-R	CCGAGCTCTTAGCACTCTCCCCTATTAAAAG

2.6.2 Table 2. List of the 5’ UTR DNA sequences used in this study

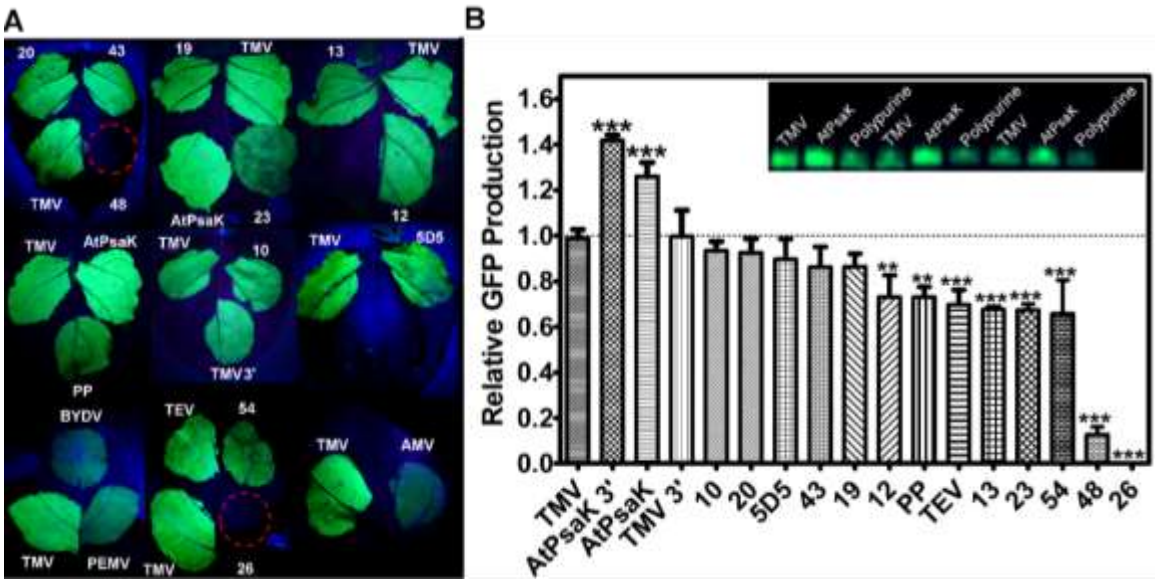
Name	Description	Sequence
TMV	Tobacco mosaic virus	GTATTTTTACAACAATTACCAACAACAACA AACAACAAACAACATTACAATTACTATTTA CAA
TMV 3’	Tobacco mosaic virus	ACAATTACTATTTACAATTACA
AMV	Alfalfa mosaic virus	TTTTTATTTTTAATTTTCTTTCAAATACTTCC A
TEV	Tobacco etch virus	GAATTAATTCTCAACACAACATATACAAAA CAAACGAATCTCAAGCAATCAAGCATTCTA CTTCTATTGCAGCAATTTAAATCATTTCTTT TAAAGCAAAAGCAATTTTCTGAAAATTTTC ACCATTTACGAACGATAG
5D5	Human-derived	CATATTGAAGAGACAGAGTGATATATAAAA CTGCTAA
10	Human-derived	AGAATTTTTAGTCAAGAAGTGA
12	Human-derived	AGTGGACGTCAATACTTACGCA
13	Human-derived	AGATTTAAGTGACGATAAAGTT
19	Human-derived	AGTTGTTTTGGATTTAGTCAAG
48	Human-derived	TGAAGAGAAAGTTGAAATTGTA
54	Human-derived	AGTGAACTGCAAACGGATTACA
20	Human-derived	GGATATGAATGTTGAACAGCTT
23	Human-derived	ACGAATGCAATCTTGGACGTTA
26	Human-derived	TGTGAGAATGAATGTTAGCAAA
43	Human-derived	TTATTGCTGAAGTTTTGAGTTA
PP	Synthetic polypurine	AAAAGAAGGAAAAAGAAGGGAAGAAAAG GG
AtPsaK	*A. thaliana* psaK	AAAAACAAAAATAAAAAAAACATCGCACA AGAAAATAAAAGATTTGTAGAATCAACTAA GAAA
AtPsaK 5’	5’ end of AtPsaK (deletion of nucleotides 1-23)	AAAAACAAAAATAAAAAAAACATCGCACA AGAAAATAAAA
AtPsaK 3’	3’ end of AtPsaK (deletion of nucleotides 42-63)	TCGCACAAGAAAATAAAAGATTTGTAGAAT CAACTAAGAAA
NbPsaK1	*Nicotiana benthamiana* psaK	CTGAAACAGTCCATTCTGAGGCCACAAACT CCTTGCTTTGGGTAATGGGCCTATGTCACA GAAACTTGTTTGGAACCCCAGTAGATTTAT ACAAACAATTTTGTCAAAAGCCAATTAAAC

		TAAAAAAAGAAGAGAAAAAAAAGTTGAGT TGCTGCAAATTAAA
NbPsa K1 3’	3’ end of NbPsaK1 (deletion of nucleotides 59-163)	AAAAGCCAATTAAACTAAAAAAAGAAGAG AAAAAAAAGTTGAGTTGCTGCAAATTAAA
NbPsa K2	*Nicotiana benthamiana* psaK	ACAAGTATCTTAGTGTATCCAGAATAGCCC CTTCTGTGGCCACAAACTCTTCAAGTGGCC ATGCCACAGAAACTTCTTTCCACCAGAAAA GGGTTTATAACAATTTAAACAAACAAAATC AACAAATATAGAAAATAACGCATTTCCAAT TCTTTGAAATTTCTGCAACA
NbPsa K2 3’	3’ end of NbPsaK2 (deletion of nucleotides 75-170	ATAACAATTTAAACAAACAAAATCAACAAA TATAGAAAATAACGCATTTCCAATTCTTTG AAATTTCTGCAACA



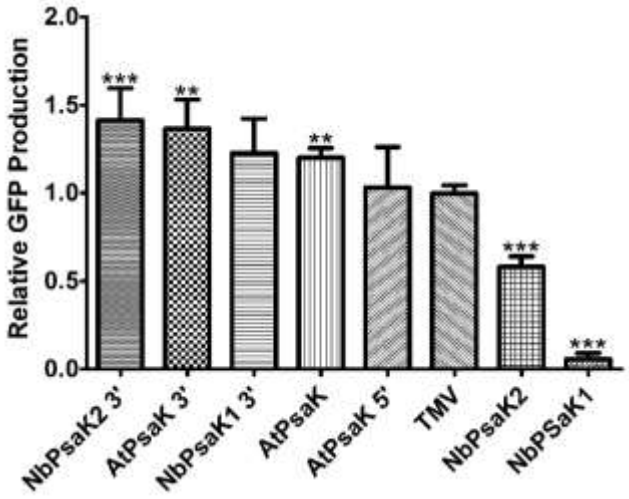
2.6.3 Figure 1. Vector Map

Generalized schematic representation of the T-DNA region of the BeYDV vectors used in this study. RB and LB, the right and left borders of the T-DNA region; NOS3', *Agrobacterium* nopaline synthase 3' element; P19, tomato bushy stunt virus P19 silencing suppressor; PNOS, *Agrobacterium* nopaline synthase promoter; LIR, long intergenic region of the BeYDV genome; 5'/3' MAR, tobacco Rb7 matrix attachment region; P35S, 35S promoter from cauliflower mosaic virus; Rep/RepA, C1/C2 ORFs from BeYDV encoding the viral replication proteins. The 5' UTR, terminator, and 5'/3' MAR elements are as described in each subsequent section.

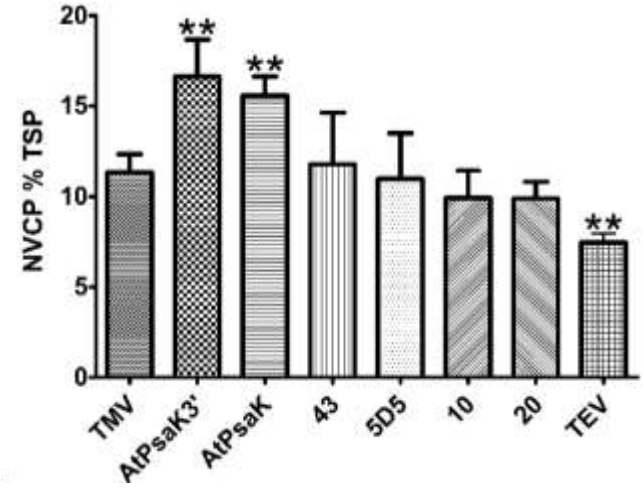


2.6.4 Figure 3. Evaluation of Diverse 5' UTRs on GFP Production
Leaves of *Nicotiana benthamiana* were agroinfiltrated with BeYDV containing different 5' UTRs upstream from the GFP gene vectors (vector pBYR2eXX-GFP, where XX denotes the individual 5' UTRs). (A) Leaves were photographed at 4 DPI under UV illumination (365nm). Images are representative of 3-4 independently infiltrated leaves. (B) Agroinfiltrated leaves were harvested between 4-5 DPI and extracts were analyzed by SDS-PAGE followed by observation under UV illumination (365nm) and Coomassie staining. GFP band intensity was quantified using ImageJ software, using native plant proteins as a loading control. Columns represent means ± standard error of three or more independently infiltrated leaves. All leaves were infiltrated with the TMV 5' UTR vector in addition to the other vectors as an internal control for leaf and plant variability. Two stars (**) indicate $p < 0.05$ and three stars (***) indicate $p < 0.01$ as compared to TMV by student's *t*-test. 5' UTR key (position -1 taken as first nucleotide upstream from ATG): TMV, tobacco mosaic virus full length 5' UTR; AtPsaK 3', nucleotides -1 to -41 of AtPsaK gene; AtPsaK, nucleotides -1 to -63 of AtPsaK gene; TMV 3', nucleotides -1 to -21 of TMV; TEV, tobacco etch virus full length 5' UTR; PP, synthetic polypurine sequence; AMV, full length alfalfa mosaic virus 5' UTR; BYDV, full length barley yellow dwarf virus 5' and 3' UTRs; PEMV, full length pea enation mosaic virus RNA 2

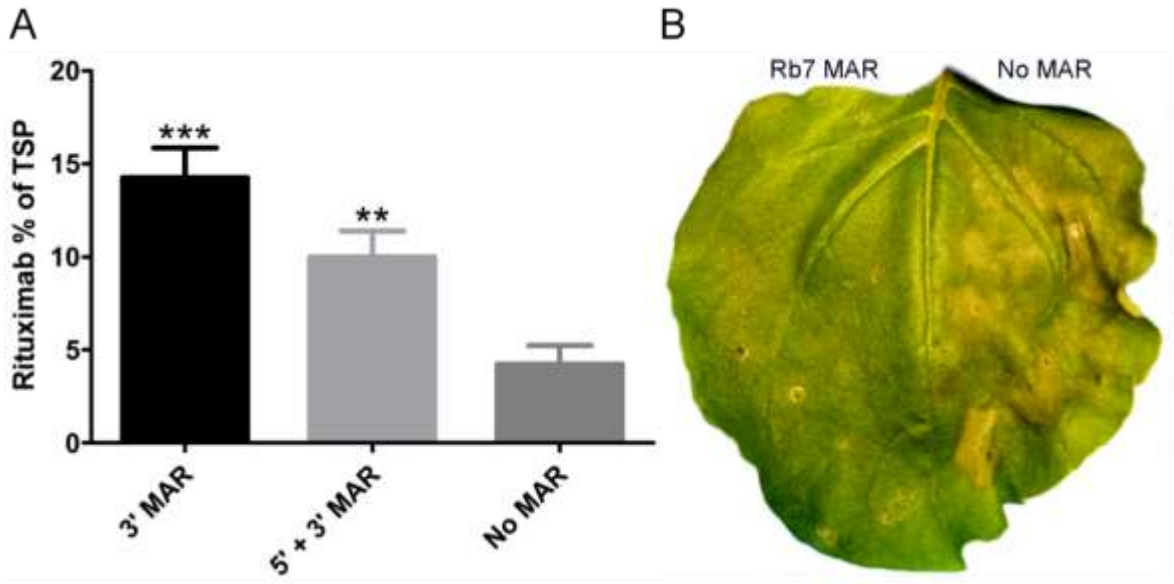
5’ and 3’ UTRs; 10; 20; 5D5; 43; 19; 12; 13; 23; 54; 48; 26, human-derived 5’ UTR sequences.



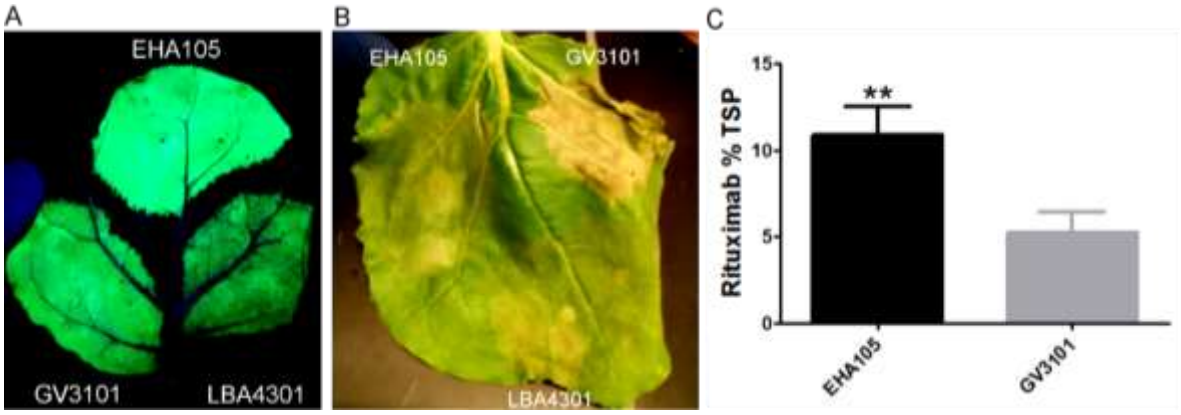
2.6.5 Figure 4. Truncated psaK 5’ UTRs provide high levels of transgene production Leaves of *Nicotiana benthamiana* were agroinfiltrated with nonreplicating vectors containing different psaK 5’ UTRs, or the TMV 5’ UTR, upstream from the GFP gene (pGFPe-XX, where XX denotes the 5’ UTR). Agroinfiltrated leaf tissue was harvested at 5 DPI and leaf extracts were analyzed by SDS-PAGE followed by coomassie staining. GFP band intensity was quantified using ImageJ software, using native plant protein bands as a loading control. Columns represent means ± standard error from four independently infiltrated samples. Two stars (**) indicate $p < 0.05$ and three stars (***) indicate $p < 0.01$ as compared to TMV by student’s *t*-test.



2.6.6 Figure 5. Evaluation of 5' UTRs on NVCP production
Agroinfiltrated leaves of *Nicotiana benthamiana* were harvested between 4-5 DPI and protein extracts were analyzed for NVCP production (vector pBYR2eXX-sNV) by ELISA. NVCP concentration in leaf extracts was normalized by total soluble protein. Columns represent results from four independently infiltrated leaf samples ± standard error. Two stars (**) indicate $p < 0.5$ as compared to TMV by student's *t*-test.

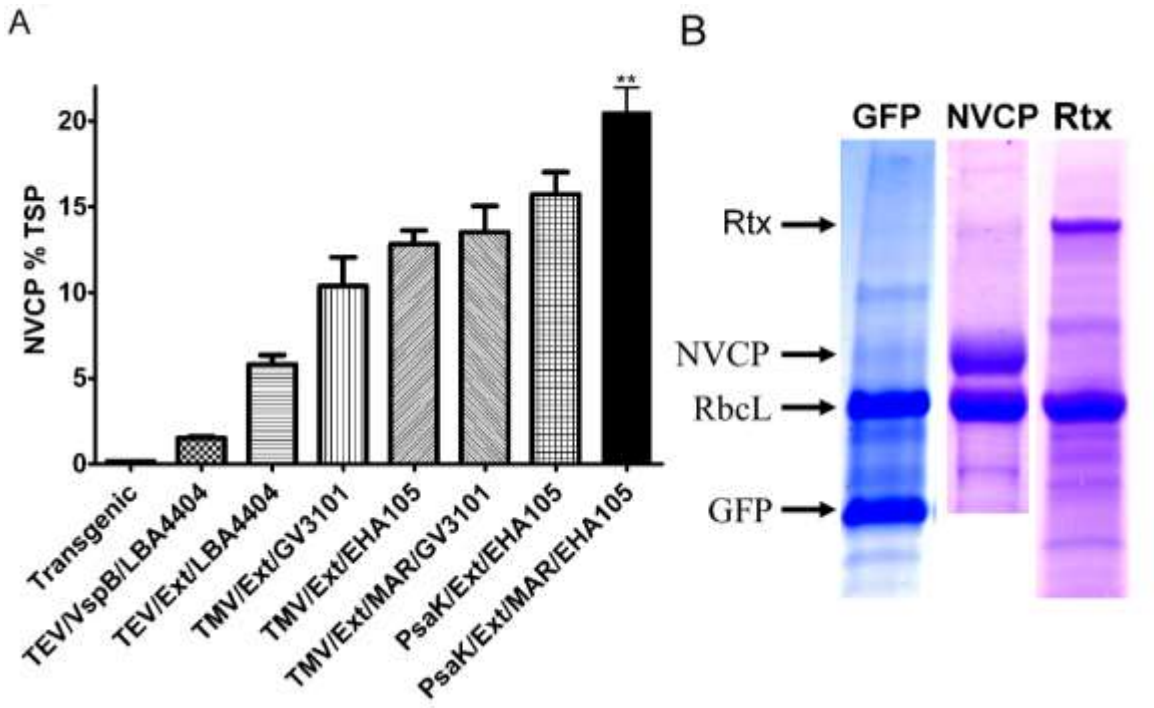


2.6.7 Figure 6. Rb7 MAR enhances transgene production and reduces cell death
(A) Protein extracts from leaves of *Nicotiana benthamiana* were infiltrated with BeYDV vectors containing the rituximab heavy chain and light chain were analyzed by ELISA. Constructs contained either the Rb7 MAR both upstream from the promoter and downstream from the terminator (5' + 3' MAR, vectors pBYR2e-MMGM and pBYR2e-MMKM); the downstream Rb7 MAR only (3' MAR, vectors pBYR2e-MrtxGM and pBYR2e-MrtxKM); or no modifications (No MAR, vectors pBYR2e-MRtxG and pBYR2e-MRtxK). Rituximab concentrations were normalized by total soluble protein. Columns represent results from four independently infiltrated leaf samples ± standard error. Two stars (**) indicate $p < 0.5$ and three stars (***) indicate $p < 0.01$ as compared to construct No MAR by student's *t*-test. (B) *Nicotiana benthamiana* leaves were agroinfiltrated with BeYDV rituximab vectors either containing 5' + 3' MAR (left half of leaf) or no MAR (right half of leaf). A representative leaf was photographed under visible light at 4 DPI.



2.6.8 Figure 7. *Agrobacterium* strain EHA105 increases transgene production and reduces cell death

(A,B) Leaves of *Nicotiana benthamiana* were infiltrated with *Agrobacterium* strains EHA105, GV3101, or LBA4301, each harboring a BeYDV GFP vector (pBYR2eFa-GFP). Representative images of four independently infiltrated leaves were photographed at 4 DPI under UV illumination (A) or 7 DPI under visible light (B). (C) Leaves of *Nicotiana benthamiana* were infiltrated with *Agrobacterium* strains EHA105 or GV3101 harboring BeYDV rituximab vectors (pBYR2e-MRtxG and pBYR2e-MRtxK). Leaf extracts were analyzed for rituximab production by sandwich ELISA and data was normalized by total soluble protein. Columns represent data from four independently infiltrated samples ± standard error. Two stars (**) indicates $p < 0.05$ by student's t-test.



2.6.9 Figure 8. BeYDV Vector Improvements Enhance Transgene Production Synergistically

(A) Summary of improvements in NVCP production. Extracts from leaves of *Nicotiana benthamiana* agroinfiltrated with BeYDV NVCP vectors were analyzed by sandwich ELISA. Data were normalized for total soluble protein. Columns represent results from four or more independently infiltrated leaf samples ± standard error. Two stars (**) indicates $p < 0.05$ as compared to AtPsaK/Ext/EHA105 by student's *t*-test. Transgenic, estimated yield (Santi et al., 2006); TEV, tobacco etch virus 5' UTR; VspB, 3' UTR from soybean *vspB* gene; LBA4404, *Agrobacterium* strain LBA4404; Ext, tobacco extensin terminator; TMV, tobacco mosaic virus 5' UTR; GV3101, *Agrobacterium* strain GV3101; EHA105, *Agrobacterium* strain EHA105; MAR, tobacco Rb7 matrix attachment region inserted 3' of the gene terminator; psaK, 5' UTR from *A. thaliana* psaK gene. (B) Extracts from leaves of *Nicotiana benthamiana* agroinfiltrated using strain EHA105 with BeYDV GFP (pBYR2eP3-GFPM) or NVCP vectors (pBYR2eP3-sNVM) were separated on SDS-PAGE gels and stained with Coomassie dye. The bands corresponding to GFP (26kDa), NVCP (58kDa), and Rituximab (~150kDa) are indicated, along with the rubisco large subunit rbcL.

CHAPTER 3

CHIMERIC 3' FLANKING REGIONS STRONGLY ENHANCE GENE EXPRESSION IN PLANTS

3.1 Abstract

Plants represent a promising platform for the highly-scalable production of recombinant proteins. Previously, we identified the tobacco extensin terminator lacking its intron as an element that reduced transcript readthrough and improved recombinant protein production in a plant-based system, compared to other commonly used terminators. In this study, we systematically compared nonreplicating plant expression vectors containing over 20 commonly used or newly identified terminators from diverse plant and plant viral sources using agrobacterium-mediated transient expression in plant leaves. We found that 8 gene terminators enhance reporter gene expression significantly more than the commonly used 35S and NOS terminators, with the intronless extensin terminator providing the highest level of GFP production of any single terminator tested, increasing GFP production by 13.6-fold compared to the NOS terminator. Combining terminators in tandem produced large synergistic effects, with many combinations providing a >25-fold increase gene expression compared to NOS alone. Addition of the tobacco Rb7 or TM6 matrix attachment regions (MAR) strongly enhanced protein production when added to most terminators, with the Rb7 MAR providing the greatest enhancement. Using deletion analysis, we found that the full activity of the 1193bp Rb7 MAR required only a 463bp region at its 3' end. Addition of the Rb7 MAR to combined terminators further enhanced protein production in a terminator-dependent manner. The best combinations provided a >60-fold increase compared to the NOS terminator alone.

These combinations were then placed in a replicating geminiviral vector, providing a total of >150-fold enhancement over the original NOS vector. These results demonstrate the importance of the 3' flanking region in optimizing gene expression, and show the potential for combined 3' flanking regions to improve DNA-based recombinant protein production systems.

3.2 Introduction

Plant-based recombinant protein production systems are increasingly emerging as a promising alternative to traditional mammalian and microbial cell culture systems due to unique advantages of lower costs, high scalability, and improved safety (Chen and Davis 2016; Kamarova *et al.,* 2010). Case studies have shown the potential for large cost reductions in capital investment and the cost of goods for plant-made therapeutics compared to conventional methods (Tusé *et al.,* 2014; Nande *et al.,* 2016). The capacity for these systems to rapidly and safely produce therapeutics has been demonstrated by two success stories: the FDA approval of an enzyme replacement therapy for Gaucher's disease, which became the first plant-made therapeutic (Zimran *et al.,* 2011; Fox 2012); and the monoclonal antibody therapy ZMapp given during the 2014 Ebola outbreak, which was shown to protect against lethal virus challenge (Lyon *et al.,* 2014; Qui *et al.,* 2014). Many strategies for improving protein production in plants have been explored, such as viral expression systems, subcellular targeting, agrobacterium strain, expression host, promoters, introns, and 5' untranslated regions (UTR). However, another key component in many of these systems is the 3' UTR, which has not been systematically optimized.

The 3’ UTR determines the fate of each transcript through a complex interplay of nuclear and cytoplasmic processes. Polyadenylation is a well-studied process involving cleavage of the nascent mRNA strand and the addition of a tract of 100-200 adenosine residues, forming the poly(A) tail. Plant polyadenylation requires the coordinated action of three main genetic signals: the UA-rich cleavage element (CE), which contains the cleavage and polyadenylation site; an A-rich near upstream element (NUE); and a UG-rich far upstream element (FUE). Alteration of any of these three signals can drastically impact gene expression (Rothnie 1996; Loke *et al.,* 2005; Hunt 2008; Mathew *et al.,* 2011). Polyadenylation also plays a pivotal role in transcription and translation. The 3’ end of the mRNA and accompanying processing machinery are in close contact with the 5’ end of the gene, a phenomenon known as gene looping. By this mechanism, efficient polyadenylation has been linked to enhanced transcription through RNAPII recycling (Tan-Wong *et al.,* 2009; Mapendano *et al.,* 2010; Andersen *et al.,* 2012; Tan-Wong *et al.,* 2012). Quality control of mRNA is also highly dependent on 3’-end processing, as improperly polyadenylated transcripts are targeted for nuclear degradation. Furthermore, the 3’-end processing machinery participates in coordinating the assembly of an export-competent mRNP (Libri *et al.,* 2002; Moore and Proudfoot, 2009; Qu *et al.,* 2009; Singh *et al.,* 2015). Poly(A) binding proteins, along with numerous other proteins which interact with the 3’ UTR, play an essential role in translation by participating in translation initiation factor recruitment, ribosome recycling, and mRNA decay (Wiginton *et al.,* 2014).

The 3’ UTR can profoundly affect gene silencing. RNA-dependent RNA polymerase 6 (RDR6) plays a key role in synthesizing dsRNA from overexpressed viral or transgene transcripts to initiate or amplify the silencing cascade leading to transcript degradation (Allen and Howell 2010, Qin *et al.,* 2012). Improperly processed transcripts producing high levels of read-through transcription were targeted for silencing by this pathway (Luo and Chen 2007). Additionally, a study that combined two terminators in tandem found a large increase in protein production compared to either terminator alone, possibly as a result of reduced read-through transcription leading to reduced gene silencing (Beyene *et al.,* 2011). RDR6 also has affinity for transcripts lacking proper polyadenylation, further underscoring the importance of the 3’ UTR in mitigating gene silencing (Baeg et al. 2017).

Besides the gene terminator, other 3’ genetic elements contribute to gene expression in plants. Chromatin scaffold/matrix attachment regions (MAR) are AT-rich regions that associate with the nuclear matrix and are thought to affect higher-order chromatin structure (Liebich *et al.,* 2002, Calikowski *et al.,* 2003, Halweg *et al.,* 2005). The tobacco Rb7 MAR, when placed at both the 5’ and 3’ ends of the expression cassette, increased the likelihood and magnitude of GFP production in tobacco cells (Halweg *et al.,* 2005). Additionally, MAR have been implicated in the reduction of transgene silencing (Mlynarova *et al.,* 2003). The tobacco TM6 MAR reduced repressive DNA methylation in flanking promoter regions and enhanced recombinant protein production in transgenic tobacco (Ji *et al.,* 2013). We found that the Rb7 MAR reduced leaf cell death and substantially increased protein production in a replicating geminiviral transient

expression system, but the effect required the MAR to be positioned at the 3' end of the gene terminator (Diamos *et al.,* 2016). The potential of MAR to enhance protein production in transient expression systems has not been thoroughly investigated.

Despite the well-established critical role of the 3' UTR in facilitating high levels of gene expression, few studies have focused on the gene terminator in the context of recombinant protein production. For nearly 30 years, the most widely used terminators have been the nopaline synthase (NOS) and octopine synthase (OCS) terminators from *Agrobacterium tumefaciens,* and the 35S terminator from cauliflower mosaic virus (MacFarlane *et al.,*1992; Ellis *et al.,* 1987; Pietrzak *et al.,* 1986). In transgenic potato plants, the 3' UTR from the potato *pinII* gene provided 10-50 times greater accumulation of hepatitis B surface antigen compared to the NOS terminator (Richter *et al.,* 2000). More recently, the terminator from a heat shock protein from *Arabidopsis* (AtHSP) was found to provide a 2-fold increase in GUS production in transient or stable transgenic *Arabidopsis* plants compared to NOS (Nagaya *et al.,* 2010). Previously, we identified the tobacco extensin terminator, which reduced transcript readthrough and improved transgene production substantially compared to the NOS or 35S terminators, an effect which was amplified when its native intron was removed (data to be presented elsewhere). In this study, we systematically investigate the potential of the gene terminator and 3' flanking region to improve plant-based expression comparing expression vectors containing diverse terminators from plant and viral sources.

3.3 Materials and Methods

3.3.1 Vector construction

An agrobacterium T-DNA binary vector containing the 35S promoter, tobacco mosaic virus 5' UTR, GFP gene, and full-length tobacco extensin terminator was constructed by 3-fragment ligation: the vector backbone from pPS1 (Huang and Mason, 2004) was obtained by XhoI-EcoRI digestion; the TMV 5' UTR-GFP fragment was excised from pBYR2e-GFP (Diamos et al., 2016) by XhoI-SacI digestion; and the intronless extensin terminator was excised from pBY-GFP212 (Diamos et al., 2016). The resulting vector, pPS-OGFP-EU, was used to construct single and double terminator constructs. The DsRed gene was amplified from pBYDsRed (Huang et al., 2010) with primers DsR-Xba-F and VspHT, digested XbaI-SacI, and inserted into pPS-OGFP-EU to create pPS-ODsR-EU. Terminators were amplified by PCR using primers (Table 1) designed to insert SacI and EcoRI restriction sites at its 5' and 3' ends, respectively, digested SacI-EcoRI, and ligated into pPS-OGFP digested likewise. The pinII, NOS, and rbcS 3' regions were obtained from pHB114 (Richter et al. 2000), pHB103 (Richter et al. 2000), and pRTL2-GUS (Carrington et al. 1991) respectively by SacI-EcoRI digestion and cloned into pPS-OGFP digested likewise.

For double terminator constructs, the upstream segment was amplified by PCR using primers (Table 1) designed to insert a SacI site at the 5' end, and BsaI site at the 3' end. The downstream segment was amplified with a BsaI site at the 5' end designed to generate compatible overhang with the upstream BsaI site, and an EcoRI site at the 3' end. The final construct was assembled by 3-fragment ligation: pPS-OGFP-EU digested

SacI-EcoRI, the upstream segment digested SacI-BsaI, and the downstream segment digested BsaI-EcoRI.

For MAR constructs, the tobacco Rb7 or TM6 MAR was inserted downstream from the terminator. First, the Rb7 MAR was inserted into pPS-OGFP-EU by three fragment ligation: pPS-OGFP-EU was digested PvuI-SphI to obtain the vector fragment; pPS-OGFP-EU was digested PvuI-EcoRI to obtain the GFP cassette; and pBYR2e-MRtxGM (Diamos et al., 2016) was digested EcoRI-SphI to obtain the Rb7 MAR fragment. The resulting vector was digested KpnI-AgeI, the ends were blunted with Klenow fragment DNA polymerase, and the vector fragment was self-ligated to yield pPS-OGFPM-EU. The TM6 MAR (genbank accession KC555564) was PCR amplified from tobacco genomic DNA using primers TM6-EcoRI-F and TM6-KpnI-R (Ji et al. 2013), digested EcoRI-KpnI, and inserted into pUC19. The EcoRI-AvrII fragment containing the TM6 MAR was then excised and inserted into pPS-OGFPM-EU digested likewise to yield pPS-OGFPT-EU. Single or double terminators were inserted into pPS-OGFPM-EU or pPS-OGFPT-EU by SacI-EcoRI digestion as described for pPS-OGFP-EU. For Rb7 deletion mutants, native restriction sites were used as shown in Fig. 6. After digestion with each respective enzyme, the ends were blunted with Klenow fragment DNA polymerase, and self-ligated.

3.3.2 Agroinfiltration of plant leaves

Binary vectors were separately introduced into *Agrobacterium tumefaciens* GV3101by electroporation. The resulting strains were verified by restriction digestion or PCR, grown overnight at 30°C, and used to infiltrate leaves of 5- to 6-week-old

Nicotiana benthamiana, tobacco, or lettuce maintained at 23-25°C. Briefly, the bacteria were pelleted by centrifugation for 5 min at 5,000*g* and then resuspended in infiltration buffer (10 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.5 and 10 mM MgSO4) to OD600 = 0.2. The resulting bacterial suspensions were infiltrated using a syringe without needle into leaves through a small puncture (Huang *et al.* 2004). Plant tissue was harvested 5 days post infiltration (DPI). Leaves producing GFP were photographed under UV illumination generated by a B-100AP lamp (UVP, Upland, CA).

3.3.3 Protein extraction and analysis

Total protein extract was obtained by homogenizing agroinfiltrated leaf samples with 1:5 (*w:v*) ice cold extraction buffer (25mM sodium phosphate, pH 7.4, 100mM NaCl, 1mM EDTA, 0.1% Triton X-100, 10 mg/mL sodium ascorbate, 0.3 mg/mL PMSF) using a Bullet Blender machine (Next Advance, Averill Park, NY) following the manufacturer's instruction. To enhance solubility, the homogenized samples were end-over-end mixed at room temperature for 30 min. The crude plant extract was clarified by centrifugation at 13,000*g* for 10 min at 4°C. Protein concentration of clarified leaf extracts was measured using a Bradford protein assay kit (Bio-Rad) with bovine serum albumin as standard. For SDS-PAGE, clarified plant proteins extract were mixed with sample buffer containing a final concentration of 50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.02 % bromophenol blue, and separated on 4-15% polyacrylamide gels (Bio-Rad). For GFP or DsRed fluorescence, PAGE gels were visualized under UV illumination (365nm) and stained with Coomassie stain (Bio-Rad) following the manufacturer's instructions. The fluorescent band corresponding to GFP was analyzed

using ImageJ software to quantify the band intensity using native plant protein bands as an internal loading control.

3.4 Results

3.4.1 Evaluation of Diverse Terminators on GFP Production

To systematically evaluate diverse terminators, we constructed expression vectors using 20 different terminators from plant and viral sources, placed 3' of a GFP reporter gene, which was driven by the strong 35S promoter and tobacco mosaic virus 5' UTR (Fig. 1). These constructs were delivered to *Nicotiana benthamiana* leaves by agroinfiltration and evaluated for GFP production. To minimize leaf-to-leaf variation, each leaf was also infiltrated with a GFP vector containing the intronless tobacco extensin terminator (EU) as an internal control, which we previously found to be a potent enhancer of gene expression (Diamos *et al.*, 2016).

While much previous work used the NOS and 35S terminators, the *A. thaliana* 18.2 kDa heat shock protein terminator (AtHSP) was reported to enhance transgene production compared to the NOS terminator (Nagaya *et al.*, 2010). In agreement with these results, we found that the AtHSP terminator provided a 2.5-fold increase in GFP production compared to the NOS terminator (Fig. 2). Also consistent with previous work in transgenic potato (Richter *et al.*, 2000), the potato *pinII* 3' UTR provided a very strong 8.5-fold increase compared to the NOS terminator (Fig. 2). The rubisco small subunit (rbcS) 3' UTR from pea showed a 5.4-fold enhancement. These data demonstrate that many terminators from diverse plant species have high activity in *Nicotiana benthamiana*.

To identify new candidates, a genome-wide study of mRNA stability levels in *A. thaliana* (Narsai *et al.,* 2007) was used to locate genes with potentially stability-enhancing 3' UTRs. We identified a *Nicotiana benthamiana* homolog of the *A. thaliana* 17.6kDa class II heat shock protein (At5g12020). The 3' flanking region from this homolog (referred to as NbHSP) was highly active, increasing reporter gene expression by 6.3-fold compared to the NOS terminator, more than doubling the enhancement provided by the AtHSP terminator (Fig. 2). We also identified an *Nicotiana benthamiana* homolog of *A. thaliana* actin 7 (At5g09810). While the downstream 617-nt region of this gene (NbACT) enhanced expression by 3.9-fold compared to NOS, extending the 3' UTR to include more downstream sequence (1044 nt) resulted in a large 8.9-fold enhancement (NbAct3, Fig. 2).

Many of the most highly active genetic elements in recombinant protein production systems are derived from viral sources. Therefore, we investigated the potential of viral terminators to enhance gene expression. The downstream short intergenic region (SIR) from the coat protein gene of bean yellow dwarf virus (BeYDV) showed no intrinsic terminator function by itself, or when additional downstream viral sequence (SIR 3') was included (Fig. 2). However, when an upstream region (SIR 5'/3') from the bean yellow dwarf virus coat protein coding sequence was also included, it was highly functional, providing GFP production 3-fold greater than the NOS terminator (Fig. 2). These data suggest that upstream elements present in the BeYDV coat protein gene are required for proper 3' end processing. The downstream sequences from the BeYDV rep and repA genes were also found to be highly active, providing a 5.6-fold and 2-fold

respective enhancement. To test sequences from other geminiviruses, the terminators of bean dwarf mosaic virus (BDMV) genes were also investigated. A 282 nt sequence including the 3' end of the nuclear shuttle protein, the intergenic region, as well as the 3' end of the movement protein (BDB282) performed similarly to the NOS terminator. However, when an additional 200nt of the downstream movement protein sequence was included (BDB501), it provided a 6.4-fold enhancement. A construct containing the BDMV coat protein downstream sequence alone was not functional, again suggesting that necessary signals may also reside in the gene coding sequence upstream from the terminator.

Taken together, these results show that many 3' UTRs from diverse sources exceed the enhancement provided by the commonly used NOS or 35S terminators, at least in a transient expression system in *Nicotiana benthamiana* leaves. Consistent with our previous work, the EU terminator outperformed the other 19 3' UTRs tested, providing a 13.6-fold increase compared to the NOS terminator, indicating that it is a uniquely potent enhancer of gene expression.

3.4.2 Combined Gene Terminators Strongly Enhance GFP Production

A double terminator consisting of the 35S terminator fused to the NOS terminator greatly enhanced protein production in various plant species compared to either terminator alone (Beyene *et al.*, 2011). To investigate the potential for tandem terminators to synergistically enhance recombinant protein production, we tested combinations of those previously tested in Fig. 2. We found an 18.4-fold enhancement by the 35S-NOS double terminator compared to the NOS terminator alone (Fig. 3),

exceeding the highest production by the best single terminator. Interestingly, reversing the position of the two terminators (NOS-35S) provided a much lower 11.2-fold enhancement, but still greatly exceeded the GFP production obtained with either terminator alone.

Fusion of 35S with pinII, NbHSP, and BDB501 3' regions all substantially enhanced protein production compared to either terminator alone. However, despite the individual superiority of each of these terminators compare to NOS, when paired with 35S, none exceeded the GFP production of 35S-NOS (Fig. 3, compare 35S-Pin2, 35S-NbHSP, 35S-BDB501, and 35S-NOS). In contrast, fusion of the individually strong NbACT terminator to the 3' end of either the 35S or NbHSP terminators resulted in a potent enhancement of GFP production, exceeding the production of 35S-NOS (Fig. 3).

As the extensin EU terminator was the best individual terminator identified, we evaluated its potential combined with other terminators. Addition of either the NbHSP, NOS, or 35S terminators to the 3' end of the EU terminator nearly doubled the GFP production provided by EU alone, exceeding the gene expression provided by 35S-NOS. The two best individual terminators, EU and NbACT, when combined, exceeded all other combinations, providing a remarkable 37.7-fold increase compared to NOS alone (Fig. 3, EU + NbACT). Interestingly, although the 35S terminator performed best when placed 5' of the NOS terminator, the opposite was found when paired with EU: the enhancement provided by 35S-EU was significantly lower than EU-35S (Fig. 3). Furthermore, addition of BDB501 to EU resulted in a slight decrease in expression (Fig. 3). Therefore, these

results indicate that terminators placed in tandem interact either synergistically or antagonistically, in a context-dependent manner.

Previously, we found that the 5' and 3' UTRs from the RNA viruses barley yellow dwarf virus (BYDV) and pea enation mosaic virus (PEMV) severely inhibited expression in *Nicotiana benthamiana* leaves using a replicating system containing the extensin terminator (Diamos *et al.,* 2016). A non-replicating expression system based on the 5' and 3' UTRs from cowpea mosaic virus (CMPV) was reported to enhance gene expression largely due to incorporation of the viral 3' UTR after the NOS terminator (Sainsbury and Lomonossoff 2008). In this study, we evaluated virus-derived 3' UTRs in nonreplicating vectors. Similar to our results with replicating vectors, we found that the 3' UTRs from PEMV, BYDV, and tobacco necrosis virus D strongly inhibited gene expression when inserted downstream from the EU terminator, and the TMV 3' UTR had a negligible effect on gene expression (Fig. 3). In agreement with the results of Sainsbury et al., pEAQ-HT-GFP, which contains the cowpea mosaic virus 5' and 3' UTRs, provided a 17.1-fold increase compared to NOS alone (Fig 3). However, this vector also contains the P19 suppressor of RNA silencing. Coinfiltration of EU with P19 provided a 29.9-fold increase compared to NOS (Fig. 3, compare EU and EU + P19). The 3' UTRs derived from DNA viruses also performed very well. While the BeYDV SIR showed no terminator function by itself, addition of the SIR to the 3' end of EU nearly doubled its GFP production. Interestingly, although the intron-containing extensin terminator (IEU) performed very poorly on its own compared to the intronless version (EU), addition of the BeYDV SIR completely negated the detrimental effect of the intron

(Fig. 3, compare IEU + SIR and EU + SIR). Addition of the 35S terminator to IEU also greatly enhanced expression, however in this case the total yield was lower than the comparable vector with the intron removed (Fig. 3, compare IEU + 35S and EU + 35S). These results indicate that viral 3' flanking regions, have potential to strongly increase gene expression when inserted downstream from the gene terminator.

3.4.3 Matrix Attachment Regions Are Potent Enhancers of Transient Expression

While MAR have been widely used in transgenic expression systems, there are few reports of their use in transient expression systems. We found that the tobacco Rb7 MAR strongly enhanced transient expression in a replicating geminiviral transient expression system when placed downstream from the gene terminator (Diamos *et al.*, 2016). To more fully characterize the potential for MAR to function in transient expression systems, the tobacco Rb7 and TM6 MAR were inserted into nonreplicating GFP expression vectors in combination with 8 different gene terminators.

Insertion of the Rb7 MAR downstream from the EU terminator resulted in a striking 3-fold enhancement of GFP production (40-fold compared to NOS alone), exceeding the best double terminator configuration (Fig. 4, compare EU and EU + Rb7). Interestingly, similar to the SIR, the Rb7 MAR also dramatically improved production of the otherwise weak IEU terminator, increasing expression 19-fold, bringing it nearly on par with the EU-Rb7 vector (Fig. 4, compare IEU, IEU + Rb7, and EU + Rb7 and Fig. 5). To verify that the observed enhancement was unique to the Rb7 MAR, a control DNA sequence of similar size, derived from a synthetic norovirus capsid protein coding sequence, was instead inserted downstream of GFP, and found to provide no significant

difference in GFP production (Fig. 4, compare EU and EU + Control). Inspection of the AT-rich Rb7 MAR sequence reveals many terminator-like elements, however in the absence of a terminator no detectable GFP activity was produced using the MAR as the sole 3' region, indicating that it does not act as a fully functional terminator (Fig. 5). Consistent with our findings in replicating systems, positioning of the Rb7 MAR 5' of the promoter had no effect on gene expression (Fig. 5c).

We further found that Rb7 MAR provided a large enhancement when used in conjunction with the 35S (13.8-fold), AtHSP (13.6-fold), NOS (12-fold), BDB501 (3.6-fold), or NbHSP (2-fold) terminators (Fig. 4). In the absence of the Rb7 MAR, the NbHSP terminator provided nearly 3-fold more expression than the 35S terminator. However, upon addition of the MAR, the 35S/Rb7 combination provided double the expression of NbHSP/Rb7 (Fig. 4). Interestingly, while the Rb7 MAR substantially enhanced 7 of the 8 terminators tested, the NbACT terminator was unaffected by addition of the Rb7 MAR.

The tobacco TM6 MAR reportedly exceeded the enhancing effect of the Rb7 MAR in transgenic tobacco (Ji *et al.* 2013). To test the TM6 MAR in our transient expression system, the full sequence was cloned from tobacco plants and inserted in place of the Rb7 MAR. The TM6 MAR enhanced GFP production when paired with the 35S, NOS, or IEU terminators, but not with the NbACT, similar to our findings for the Rb7 MAR (Fig. 4, purple bars). However, the magnitude of the enhancement provided by the TM6 MAR was significantly less than that of the Rb7 MAR in all combinations tested (Fig. 4, Fig. 5).

Using deletion studies, we determined which regions of the 1193-bp Rb7 MAR were responsible for the observed enhancement. Deletion of nucleotides 144-1193 or 437-1193 eliminated the enhancing effect of the Rb7 MAR, however deletion of nucleotides 1-144, 144-437, 1-437, 421-730, or 1-730 did not impair MAR activity (Fig. 6). In fact, a small but repeatable increase in the enhancement provided by the Rb7 MAR was observed upon deletion of nucleotides 1-730 (Fig. 6). These data indicate that a relatively short region at the 3' end of the Rb7 MAR is responsible for all of the observed enhancement in this system.

3.4.4 Synergistic Enhancement of Combined 3' Flanking Regions

We investigated the potential for double terminators and the Rb7 or TM6 MAR to further increase gene expression when used in combination. Addition of the Rb7 to the EU-35S double terminator significantly increased the expression provided by the double terminator alone (2.4-fold), and by either individual terminator with or without Rb7 MAR (Fig. 7, compare EU + Rb7, 35S + Rb7, EU + 35S + Rb7 and Fig. 4 EU + 35S). This represents a 56.7-fold total increase compared to the NOS terminator. However, the fold-increase provided by addition of the Rb7 MAR to EU-35S was smaller than the increase provided by addition of Rb7 to either EU or 35S alone (Fig. 5). These results indicate that double terminators and the Rb7 MAR can synergistically enhance gene expression when used in combination.

Interestingly, despite the failure of the Rb7 MAR to enhance expression of the NbACT terminator by itself, the 35S-NbACT double terminator was further enhanced by 2.4-fold upon addition of the Rb7 MAR, more than doubling the expression provided by

35S-Rb7 (Fig. 7). EU-NbACT, the highest expressing double terminator, was also improved by 1.5-fold upon addition of the Rb7 MAR. Since the enhancement provided by these combinations was very similar to EU-35S, these results indicate that both terminators alone need not have strong synergy with the Rb7 MAR in order for it to provide a large enhancing effect to a double terminator.

While the 35S and NOS terminators were substantially improved when combined in either orientation (Fig. 4), and while addition of the Rb7 MAR to the 35S-NOS double terminator provided a further 1.9-fold increase in expression (compare Fig. 7 35S + NOS + Rb7 to Fig. 4 35S + NOS), the total yield of 35S-NOS-Rb7 was no better than the single terminator construct 35S-Rb7 (Fig. 7, compare 35S + NOS + Rb7 and 35S + Rb7). Similarly, although we observed a large synergy between the Rb7 MAR and the individual terminators AtHSP, EU, BDB501, and 35S, and although the expression provided by the double terminators AtHSP-EU, 35S-EU, and EU-BDB501 was enhanced by addition of the Rb7, all performed worse than EU-Rb7. Furthermore, one double terminator combination was not improved at all by the presence of a MAR. Addition of the Rb7 MAR to NbHSP-Pin2 resulted in a small 1.1-fold increase, which was not statistically significant (compare Fig. 7 NbHSP + Pin2 + Rb7 to Fig. 5 NbHSP + Pin2). These data suggest a complex set of interactions between both terminators and the Rb7 MAR controls gene expression.

3.4.5 Evaluation of Combined 3’ Flanking Regions in a Replicating System

Previously, we reported a plant transient expression system based on the geminivirus bean yellow dwarf virus, which enhances gene expression by increasing

accumulation of DNA copies of the gene of interest (Huang *et al.* 2009). We found that expression was substantially increased by insertion of the extensin terminator and the Rb7 MAR, among other modifications (Diamos *et al*., 2016). To evaluate the potential of combined 3' UTRs to function in this system, several of the best performing 3' UTR combinations were cloned into geminiviral vectors expressing GFP. The geminiviral vector containing EU-Rb7 enhanced expression 3.1-fold more than the nonreplicating vector (Fig. 8). Similarly, the two best nonreplicating vectors containing EU-35S-Rb7 and 35S-NbACT-Rb7 were further enhanced 2.7-fold and 2.5-fold, respectively, when placed in geminiviral vectors (Fig. 8). Similar to their non-replicating counterparts, in geminiviral vectors the 35S-EU-Rb7 and 35S-NbACT-Rb7 combinations increased GFP production by up to 20% compared to a replicating vector containing only EU-Rb7 (Fig. 8).

3.4.6 Gene-Specific and Plant-Specific Activity of Single and Combined 3' Flanking Regions

To determine whether the identified terminators performed similarly with a reporter gene other than GFP, vectors containing a variety of individual or combined terminators were constructed with the DsRed gene replacing the GFP gene. DsRed shares no sequence homology with GFP. For single terminators, the extensin terminator still provided the highest level of gene expression (Fig. 9), in agreement with our previous data expressing GUS or Norwalk virus capsid protein (data to be presented elsewhere). Other terminators performed similarly as when paired with GFP, however some small differences were observed, such as the increased activity of NbHSP and 35S with DsRed

compared to NOS (Fig. 9). While the EU-35S double terminator substantially exceeded the DsRed production with EU alone, the 35S-NOS double terminator performed substantially worse with DsRed than with GFP, failing to exceed the enhancement provided by EU alone (Fig. 9a). Use of the Rb7 MAR strongly enhanced DsRed expression with most terminators, and the enhancement provided by Rb7 exceeded that of TM6 in the one case tested (Fig. 9). Interestingly, while 35S-Rb7 performed very well with GFP, it did not with DsRed. These results suggest that some terminator combinations exhibit gene-specific activity, but most results were consistent with both genes.

The functionality of genetic elements often varies among species. To assess the generality of these results in other plant systems, a subset of 3' UTRs were tested in tobacco and lettuce. Similar to our results in *Nicotiana benthamiana*, in both tobacco and lettuce plants GFP gene expression with EU was >10-fold higher than with NOS, and EU exceeded all other single terminators tested (Fig.10). While EU performed substantially better than all other single terminators in its native tobacco, the AtHSP, NbHSP, and NbACT performed nearly as well as EU in lettuce (Fig.10). Addition of the Rb7 MAR strongly enhanced GFP expression when added to EU, and the 35S-NbACT-Rb7 combination, which performed very strongly in *Nicotiana benthamiana*, also performed very strongly in tobacco and lettuce (Fig. 10). Despite its strong performance in *Nicotiana benthamiana*, IEU-35S-Rb7, which contains the extensin intron, had much lower expression in tobacco (Fig. 10). Similarly, NbHSP-NbACT with or without Rb7 MAR performed substantially worse in tobacco than in *Nicotiana benthamiana* (Fig. 10).

3.5 Discussion

Previously, we identified the tobacco extensin terminator as a potent enhancer of gene expression in replicating or nonreplicating systems (Diamos *et al.* 2016, and data to be presented elsewhere). In this study, to more broadly assess the potential of 3' flanking regions to enhance gene expression in plant systems, we systematically compared a diverse set of terminators from various plant and viral sources. Narsai *et al.* (2007) reported a genome-wide analysis of mRNA stability in *A. thaliana*, showing that characteristic 3' UTR motifs are enriched in long-lived or short-lived transcripts. To rationally derive putative terminator candidates with potential to enhance gene expression, we identified *Nicotiana benthamiana* homologs of two highly stable *A. thaliana* transcripts: an 18.8 kDa class II heat shock protein gene, and an actin-like gene. Both terminators outperformed all of those frequently used previously (Fig. 2). Since we tested only two terminators identified in this manner, we suspect there is great potential to uncover other highly active terminators. Overall, we found that 12 terminators exceeded the performance of 35S or NOS.

Highly expressed genes from strong promoters are targets for RNA silencing (Que *et al.* 1997; Schubert *et al.* 2004), mediated by RDR6 (Béclin *et al.* 2002). Luo and Chen (2007) demonstrated that improperly terminated mRNAs result in RDR6-mediated silencing of the transgene, and that the use of a 35S-NOS double terminator reduced this effect while enhancing GUS expression is transgenic *A. thaliana*. Beyene *et al.* (2007) also found a large enhancing effect of a 35S-NOS double terminator in several plant species. Therefore, we wished to investigate the potential for other terminator

combinations to function synergistically in tandem. While the intronless EU terminator by itself was significantly better than all 19 other individual terminators tested, we identified 8 double terminators that significantly exceeded the performance of EU alone, 7 of which significantly outperformed the 35S-NOS double terminator (Fig. 3). We found that nearly every combination outperformed either individual terminator alone, showing that tandem-linked terminators have excellent potential to enhance gene expression. Interestingly, in both tested cases, reversal of the position of the two terminators resulted in a substantial difference in expression, indicating that the observed enhancement does not arise entirely from the individual action of the two terminators, but rather on a synergistic interaction between the two terminators, which depends in part on their relative positions. Furthermore, a 35S-NOS terminator enhanced expression strongly; however, the opposite was observed with 35S-EU: expression was enhanced by 50% when 35S was positioned downstream from EU. These results indicate that the optimum results depend on specific terminator pairings as well as order.

The 3' flanking regions from RNA viruses contain many mechanisms to enhance mRNA stability or increase translation (Fan *et al.,* 2012; Simon and Miller 2013). However, when expressed in the plant nucleus, these 3' regions may contain cryptic splice sites and other detrimental sequences. Most of the RNA virus-derived 3' flanking regions we tested were poorly functional when transiently expressed in *Nicotiana benthamiana* leaves, except for those derived from cowpea mosaic virus. The 5' and 3' UTRs from cowpea mosaic virus were reported to be potent enhancers of protein expression (Sainsbury and Lomonossoff 2008). In general agreement, we found pEAQ-

HT-GFP, which contains the NOS terminator and the cowpea mosaic virus UTRs, enhanced GFP expression 17.1-fold compared to NOS alone. However, the cowpea mosaic virus vector pEAQ-HT-GFP also contains the P19 suppressor of RNA silencing which likely enhances RNA stability (Sainsbury et al. 2009), making direct comparisons to other 3' UTRs difficult. While pEAQ-HT-GFP provided 20% more GFP than the extensin terminator alone, it provided 40% less when extensin was also supplemented with P19 (Fig. 3). We also found other highly expressing terminator combinations identified here were also strongly enhanced by addition of P19 (data not shown). As pEAQ-HT-GFP contains the relatively weak NOS terminator, we suspect the cowpea mosaic virus flanking regions may perform better on a strong terminator, unless some particular synergy exists with NOS.

Flanking regions derived from nucleus-adapted DNA viruses, such as the geminiviruses, were found to be potent enhancers of gene expression, especially when used in conjunction with a functional terminator. The short 200 bp SIR from bean yellow dwarf virus showed no terminator activity by itself, but was found to strongly increase gene expression when used in conjunction with the extensin terminator, on par with the best double terminator combinations tested. However, extending the SIR to include upstream and downstream coding sequence from the BeYDV coat and rep proteins showed that it also has strong terminator function on its own. Similar results were obtained with 3' UTRs obtained from bean dwarf mosaic virus. These results highlight the influence of the upstream gene coding sequence on 3' UTR function. Further work is

needed to better characterize the enhancing potential of geminiviral 3' UTRs, and to determine whether the observed enhancing effect of the SIR is terminator-specific.

Though MAR were previously used in transgenic systems, we found the tobacco Rb7 MAR substantially improved gene expression using a geminiviral transient expression system (Diamos *et al.* 2016). Only a small percentage of T-DNA delivered by agrobacterium undergoes chromosomal integration, while the majority is transiently transcribed in the nucleus. It has been shown that the agrobacterium proteins VirE2, which coats the T-DNA, and VirD2, which attaches to 5' end of the T-DNA and mediates nuclear entry, both associate with cellular histones (Lacroix *et al.* 2008; van Heusden *et al.* 2015). As MAR are thought to influence chromatin structure, the association of T-DNA with histones suggests a possible mechanism by which MAR function in vectors delivered by agroinfiltration. Here, we find that both the tobacco Rb7 and TM6 elements greatly enhance transient gene expression in agroinfiltrated leaf tissue (Fig. 4). The effect of the Rb7 MAR varies in a promoter-dependent manner (Mankin *et al.* 2003). Similarly, we find that the effect of the MAR also varies in a terminator-dependent manner. EU was the strongest individual terminator and EU-Rb7 was the strongest MAR combination, exceeding the best double terminator by over 50%. However, while NbACT was the second strongest individual terminator, NbACT3-Rb7 was the lowest expressing MAR combination. Interestingly, we saw a similar effect with NbACT-TM6, suggesting that some enhancing activity present in both MAR is not active when paired with the NbACT 3' region. All other terminators greatly benefited from addition of the Rb7 or TM6

MARs, although the magnitude of the enhancement varied in a manner that was not correlated to the individual expression level mediated by each terminator alone.

Ji *et al.* (2013) found that the TM6 MAR enhanced GUS expression at a level greater than the Rb7 MAR in transgenic tobacco. However, we consistently found that the Rb7 MAR increased transient expression more than the TM6 MAR. This observed discrepancy could be due to different expression systems, or different reporter genes. We found that the entire enhancing activity of the Rb7 MAR resides in a 463 bp region at its 3' end. Although a detailed characterization of the functional regions of the Rb7 MAR has not been reported, the region we found to be dispensable includes several AT-rich regions, a matrix attachment recognition sequence motif, and a topoisomerase II binding site, all of which were previously suspected to play a role in MAR function (Allen *et al.* 1996). Ji *et al.* (2013) found that deletion of similar MAR elements did reduce the enhancing effect of TM6. Additionally, it has been reported that the TM2 MAR functions best when placed 5' of the gene of interest (Zhang *et al.* 2009), whereas we found no effect of 5' insertion of the Rb7 MAR . As MAR are thought to contain numerous different active regions, there may be differences in the key functional regions of the Rb7, TM2, and TM6 MARs. Alternatively, while Rb7 and TM6 are both clearly active in transient expression systems, the mechanisms by which expression is enhanced may differ between transient and transgenic systems. Further studies are needed to resolve these discrepancies.

Previously, we found that combining optimized 5' UTRs and the Rb7 MAR resulted in a synergistic enhancement of gene expression (Diamos *et al.* 2016). Other

studies obtained favorable results by duplicating or combining highly functional genetic elements, such by tandem-linking TM2 MAR (Zhang *et al.* 2002) or combining the 5' UTR from alcohol hydrogenase and the AtHSP terminator (Limkul 2015). Here, we find that combining double terminators with the Rb7 MAR enhanced gene expression more than either component by itself in some, but not most, cases (Fig. 7). Particularly high synergy was observed between the Rb7 MAR and the EU-35S, 35S-NbACT, and EU-NbACT double terminators, reaching very high expression levels of up to 60-fold more than the NOS terminator alone. All three combinations were highly effective double terminators in the absence of MAR. However, while both EU and 35S also individually displayed high synergy with Rb7, the NbACT terminator alone gained no benefit from the Rb7 MAR, suggesting that both terminators need not function well with the Rb7 MAR for the combination to be beneficial. More studies are needed to understand the mechanism of this synergistic enhancing activity.

Other variable effects were also observed when combining double terminators with MARs. While 35S-NOS was a relatively strong double terminator, it had little synergy when combined with Rb7. Similarly, while the AtHSP terminator had high synergy with the Rb7 MAR, double terminators containing AtHSP did not improve expression compared to AtHSP-Rb7 alone (Fig. 7). As all AtHSP double terminators tested in this study had AtHSP positioned as the upstream terminator, it is possible reversing terminator positions may result in better performance. Notably, EU-35S-Rb7 was one of the best combinations tested, but the reversed 35S-EU-Rb7 had a 50% reduction in expression (Fig. 7).

We have created a replicating transient expression system based on the geminivirus bean yellow dwarf virus, which amplifies the gene of interest to high copy number in the plant nucleus (Huang *et al.* 2009, Huang *et al.* 2010). By incorporating optimized 5' and 3' UTRs with other modifications, we have used this system to produce vaccine antigens and pharmaceutical proteins at levels greater than or similar to the highest levels reported in plant-based systems (Diamos *et al.* 2016). Here, we find that gene expression with the double terminator and MAR constructs 35S-NbACT-Rb7 and EU-35S-Rb7 is improved by ~2.5-fold when placed in a replicating vector, a 20% increase compared to the best replicating construct containing only a single terminator and MAR (Fig. 8). This represents a more than 150-fold increase compared to the original NOS vector alone, providing an estimate yield of >50% total soluble protein or >4-5mg recombinant protein per kg of leaf tissue, which appears to approach the theoretical limit achievable in a plant-based system.

The upstream gene coding sequence has been shown to interact with the 3' UTR. The NOS terminator contains a cryptic polyadenylation site that requires an upstream element to be present for its function (Sanfaçon and Hohn 1990; Sanfaçon et al. 1991). We found that the intergenic regions of bean yellow dwarf virus and bean dwarf mosaic virus both require upstream coat protein coding sequence for terminator function (Fig. 2). These results indicate that 3' UTRs may perform differently in the context of different genes of interest. Using DsRed as an alternative reporter gene to GFP with no shared homology, we found that most single or combined 3' UTRs performed similarly relative to one another, with a few notable exceptions. The 35S-NOS and 35S-Rb7 3' UTRs both

performed substantially worse with DsRed than they did with GFP, however both still performed better than 35S alone. Despite these combinations performing worse, the 35S terminator alone performed better with DsRed than with GFP, and combinations with the 35S terminator positioned as the second terminator were still highly functional (Fig. 9).

Lettuce has been shown to be a promising plant system capable of rapidly producing recombinant proteins (Lai et al. 2012b; Chen et al. 2016). To further investigate the generality of our results, we also tested a variety of 3' UTRs in tobacco and lettuce. While minor differences were observed among the single terminators, such as better performance of AtHSP, NbHSP, and NbACT compared to EU, EU was still the best individual terminator. Further, combined terminators containing the Rb7 MAR substantially outperformed any individual terminator tested (Fig 10). However, a few of the terminator combinations that performed very well in *Nicotiana benthamiana* performed much more poorly in either lettuce or tobacco. As we did not test every identified combination with multiple genes or other plant systems, it is possible that other gene-specific or plant-specific effects exist that we did not discover here. Therefore, our data suggests the optimal terminator for a given system must be determined empirically. However, the potent enhancing effect of the intronless EU terminator has been demonstrated with GFP, DsRed, GUS, and Norwalk virus capsid protein, in *Nicotiana benthamiana*, lettuce, and tobacco (Fig. 2, Fig. 9, Fig. 10, and data to be presented elsewhere), indicating that the terminator is intrinsically highly active in many gene contexts. Additionally, our results clearly demonstrate that combining terminators is a highly effective strategy to improve gene expression in a variety of systems.

In conclusion, we have identified a diverse set of gene terminator regions that greatly exceed the gene expression provided by the most commonly used terminators in *Nicotiana benthamiana*, tobacco, and lettuce leaves. The intronless tobacco extensin terminator is a uniquely potent enhancer of gene expression. In nearly every case tested, double terminators outperformed either individual terminator alone, often exceeding the gene expression of the best individual terminators by more than 2-fold. We find that MAR, especially the 3' end of the Rb7 MAR, are also strong enhancers of gene expression in transient systems, and when combined with double terminators, synergistically enhance expression. Incorporating these combined terminators into a replicating geminiviral expression system has allowed us to produce recombinant proteins comparable to the highest levels ever reported in a plant-based system. We anticipate that the 3' UTR combinations identified here to have broad potential to improve other DNA-based plant expression systems.

Acknowledgements

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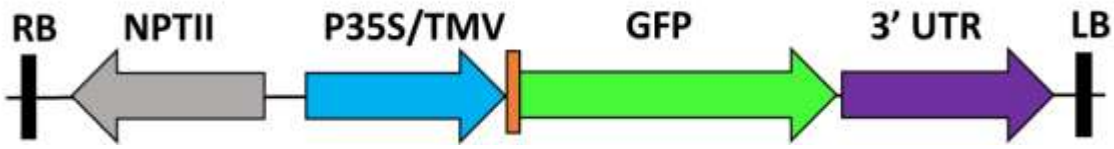
3.6 Figures

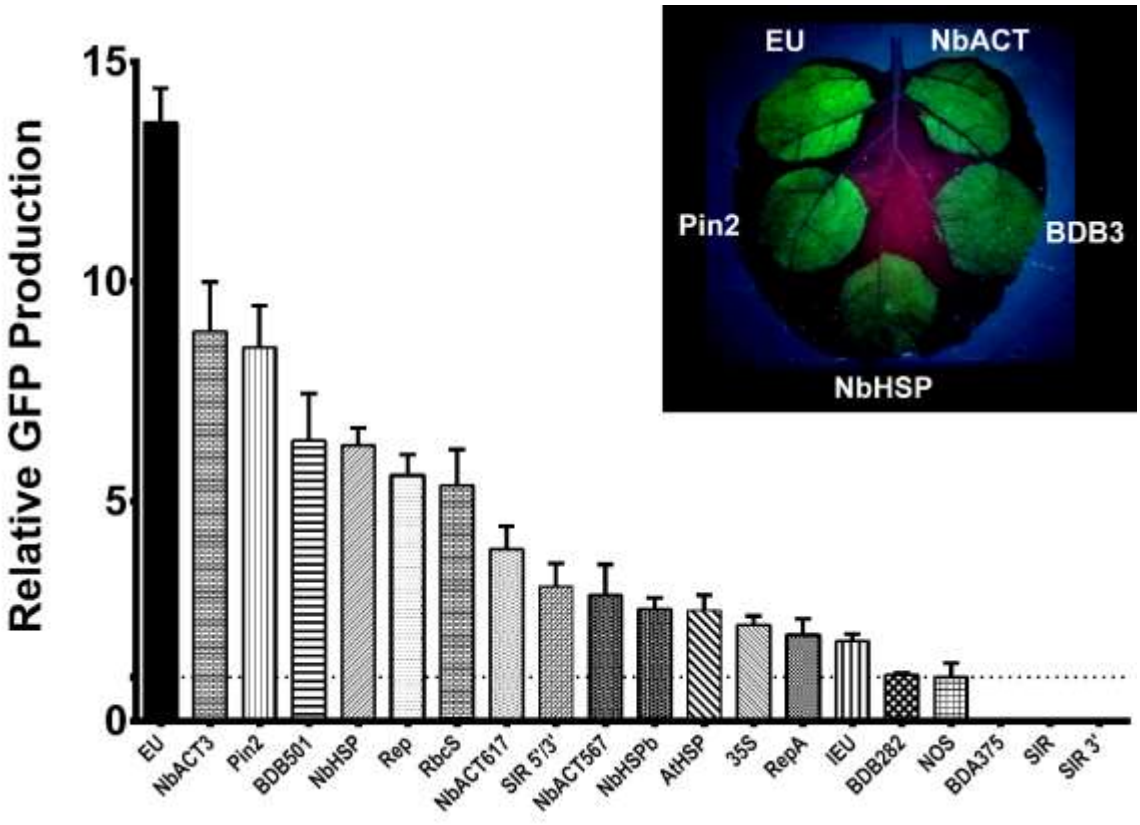
3.6.1 Table 1. Oligonucleotides used in this study

TM6-EcoRI-F	TCCGAATTCTAATATTTAGAAATTTAATTAACATAACCAAGG
TM6-KpnI-R	CTGGTACCGACATCCTAGGTTCAATCAAAT
DsR-Xba-F	GAGTCTAGAACATGGTGCGCTCCTCC
VspHT	TGAATAGTGCATATCAGCATACCTTA
EU-Bsa-F	AGGGTCTCGGCTCAAAGCAGAATGCTG
EU-Bsa-R	AAGGTCTCGGAGCGTCATAACTGTAGAAATGATTCC
BDB-Bsa-F	GGGTCTCGGCTCTGACAACATCAGCAAG
NbHSP-Bsa-F	AGGGTCTCGGCTCACTGAGGAAATATATAGACAAATTAAG
35S-Bsa-R	AAGGTCTCGGAGCGTCACTGGATTTTGGTTTTAGG
NbHSP-Bsa-R	AAGGTCTCAGAGCTCCCAAAGGAAACTATGTGTAC
NOS-Bsa-R	AGGGTCTCGGCTCAGATCGTTCAAACATTTG
Pin2-Bsa-F	GAGGTCTCAGCTCGTACCCTGCAATGTGACC
NbACT-Bsa-F	AGGGTCTCGGCTCATACAGCATTCCCA
BDB501-Sac-F	CCGAGCTCTGACAACATCAGCAAGAACG
BDB501-Eco-R	AAGAATTCAAAGGAAACCCATAAGATGCG
NbHSP-Sac-F	TCGAGCTCACTGAGGAAATATATAGACAAATTAAGTTTGGTTC TATG
NbHSP-Eco-R	GTGAATTCGCTCCCAAAGGAAACTATGTGTACTTC
NbACT-Sac-F	GCGAGCTCATACAGCATTCCCAGAAAGAGAAAC
NbACT-Eco-R	TAGAATTCATGCTAGCTTGTTTACACCTCG
BDA375-Sac-F	GAGAGCTCGGAGAACGCCTTATTATTGTATATGGC
BDA375-Eco-R	AAGAATTCGCTCATCACTGCACTTCAAGC
AtHSP-Sac-F	TAGAGCTCATATGAAGATGAAGATGAAATATTTGGTGTG
AtHSP-Eco-R	ATGAATTCCTTATCTTTAATCATATTCCATAGTCCATACC
AtHSP-Bsa-R	AAGGTCTCGGAGCCTTATCTTTAATCATATTCCATAGTCCATAC C
Rep-Sac-F	AGCGAGCTCTAATAGGTTGCCAGTCTGATTTC
Rep-Eco-R	CTAGAATTCTTGCCATCGTTTTGTGG
RepA-Sac-F	TCGGAGCTCTGAACGTGCCTCTCCTC
SIR-Sac-F	AAGGAGCTCTAAAATGATTATTTTATGAATATATTTCATTGTGC
NbACT617-EcoR	AATGAATTCGAACCCCAATTACTGGAGC
35S-Bsa-F	GCGGTCTCGGCATGGTGGAGCACGA
NOS-Bsa-F	AGGGTCTCGGCTCAGATCGTTCAAACATTTG
TNVD3-Bsr-F	ATTGTACAAGTAATTGCTTTCATAGATCCGTCTTCC
TNVD3-Sac-R	TAGAGCTCGGGTTCCTAGAGAGATCTCTAGG
TMV3-Bsr-F	TATGTACAAGTAAGGTAGTCAAGATGCATAATAAATAACGGAT TGTG

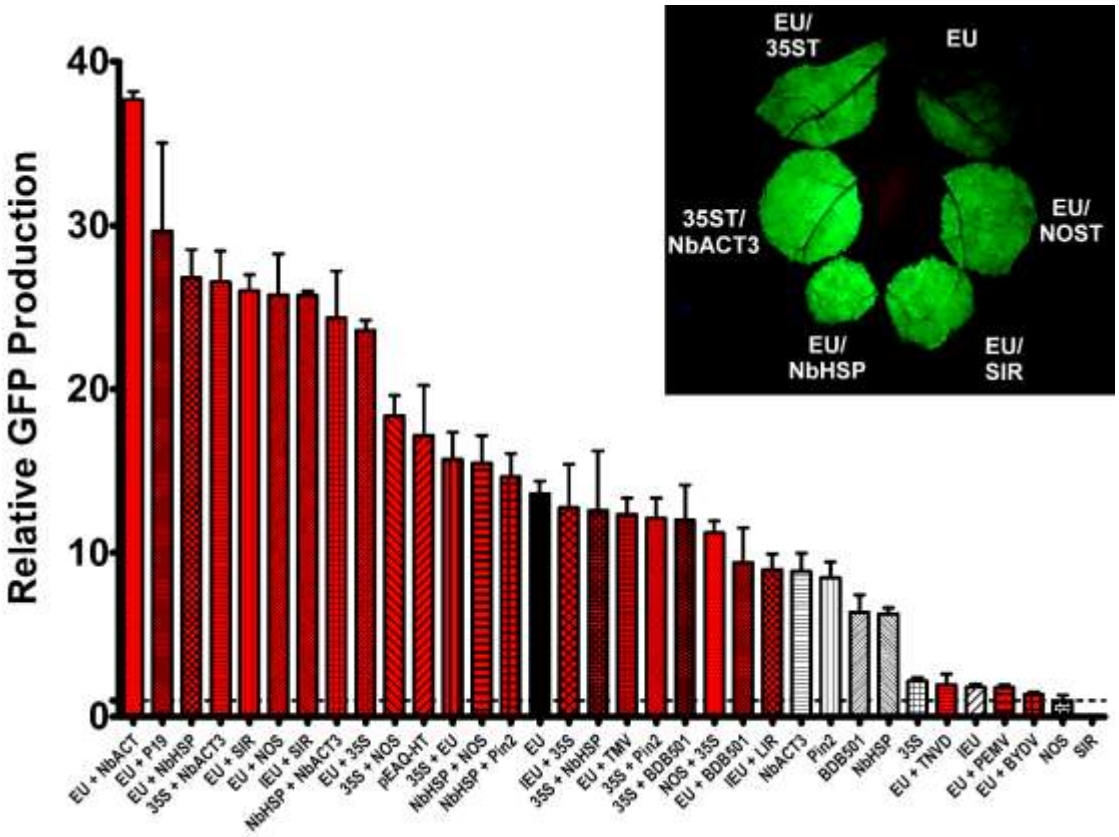
TMV3-Sac-R	TAGAGCTCTGGGCCCCTACCGGGGGTAA
TNVD5-F	TCGAGATACCTAACCAGTGTCTCAGTGATTAAGTAATCAGCT
TNVD5-R	CTAGAGCTGATTACTTAATCACTGAGACACTGGTTAGGTATC
PEMV5-F1	TCGAGGGTATTTATAGAGATCAGTATGAACTGTGTCGCTAGGA TCAAGCGG
PEMV5-F2	TGGTTCACACCTGACTTCACCCCTGGCGAGGGCGTGAAGTCTA C
PEMV5-R1	CATGGTAGACTTCACGCCCTCGCCAGGGGTGAAGTCAGGTGTG AACCACCGC
PEMV5-R2	TTGATCCTAGCGACACAGTTCATACTGATCTCTATAAATACCC
PEMV3-Bsr-F	ATTGTACAAGTAAGGCTTCGCTTCCCGCC
BYDV3-Kpn-F	AAGGTACCAGTGAAGACAACACC
BYDV3-Sac-R	ATGAGCTCGGGTTGCCGAACTGC



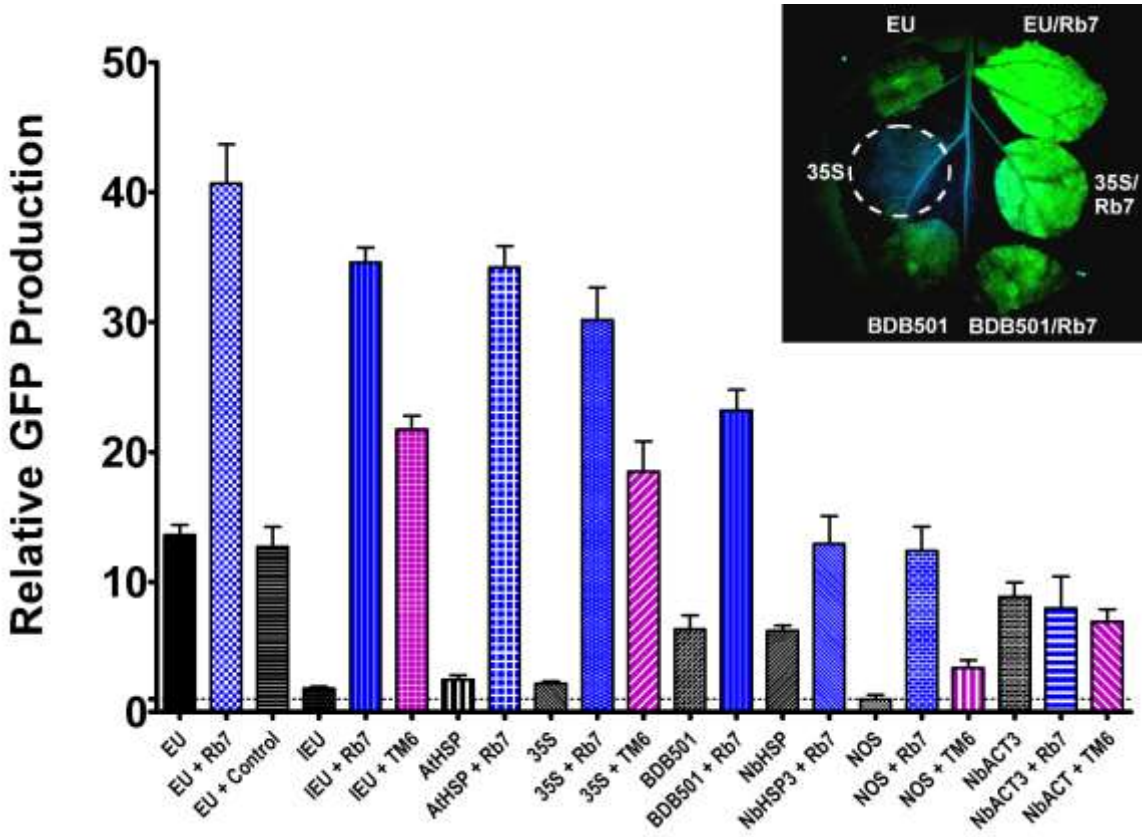
3.6.2 Figure 1. Vector Map
Generalized schematic representation of the T-DNA region of the vectors used in this study. RB and LB, the right and left borders of the T-DNA region; NPTII, kanamycin resistance cassette; P35S, 35S promoter from cauliflower mosaic virus; TMV, 5' UTR from tobacco mosaic virus; 3' UTR is either a single terminator, double terminator, matrix attachment region, or combination of these elements.



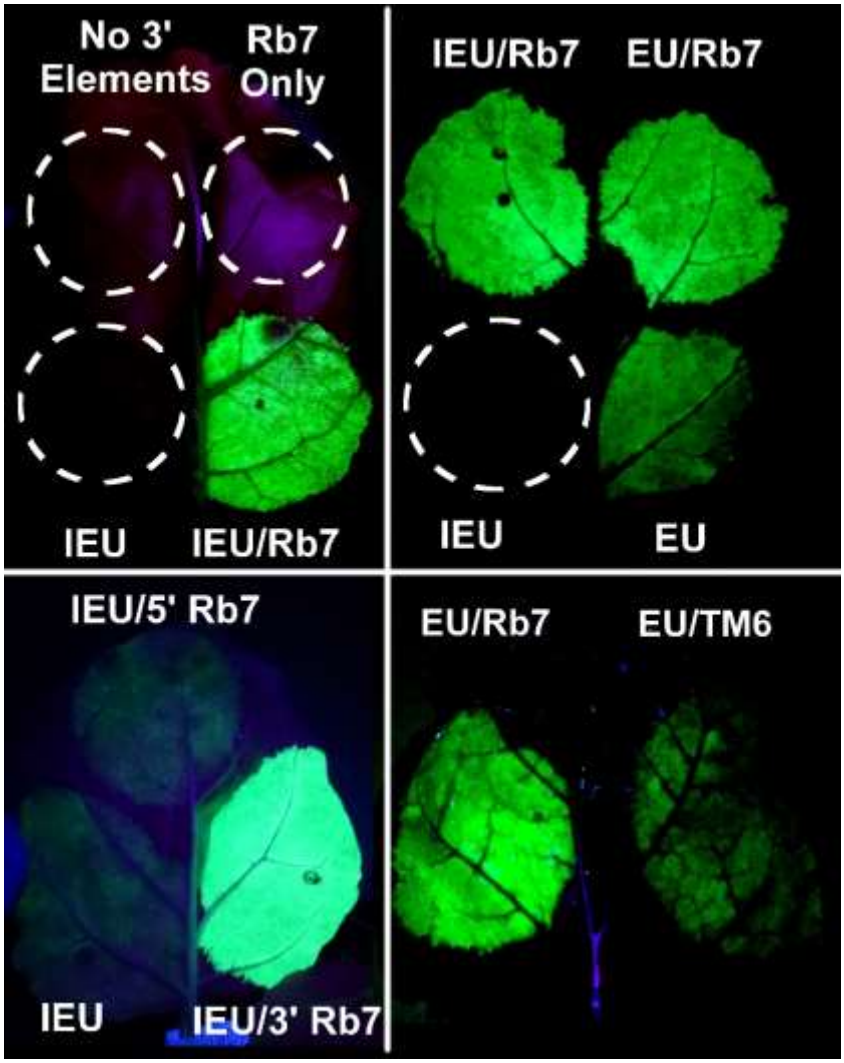
3.6.3 Figure 2. Evaluation of diverse 3' UTRs on GFP production
Nonreplicating vectors containing different 3' UTRs inserted downstream from the GFP gene were agroinfiltrated into *Nicotiana benthamiana* leaves. Leaves were photographed at 5 DPI under UV illumination (365nm). Images are representative of 3-4 independently infiltrated leaves. Agroinfiltrated leaves were harvested between at 5 DPI and extracts were analyzed by SDS-PAGE followed by observation under UV illumination (365nm) and Coomassie staining. GFP band intensity was quantified using ImageJ software, using native plant protein bands as a loading control. Columns represent means ± standard error of independently infiltrated leaves. All leaves were infiltrated with the EU vector in addition to the other vectors as an internal control for leaf and plant variability. Abbreviations: EU, intronless tobacco extensin 3' UTR; IEU, intron-containing tobacco extensin 3' UTR; NbACT3, *Nicotiana benthamiana* actin 3' UTR; NbHSP; *Nicotiana benthamiana* HSP 3' UTR; pinII, potato proteinase inhibitor II 3' UTR; rbcS, pea rubisco small subunit 3' UTR; SIR, short intergenic region of bean yellow dwarf virus; BDB, bean dwarf mosaic virus DNA B nuclear shuttle protein 3' UTR; Rep, bean dwarf mosaic virus rep gene 3' UTR; RepA, bean dwarf mosaic virus repA gene 3' UTR; AtHSP, *A. thaliana* heat shock protein 3' UTR; 35S, cauliflower mosaic virus 35S 3' UTR; NOS, agrobacterium nopaline synthase 3' UTR.



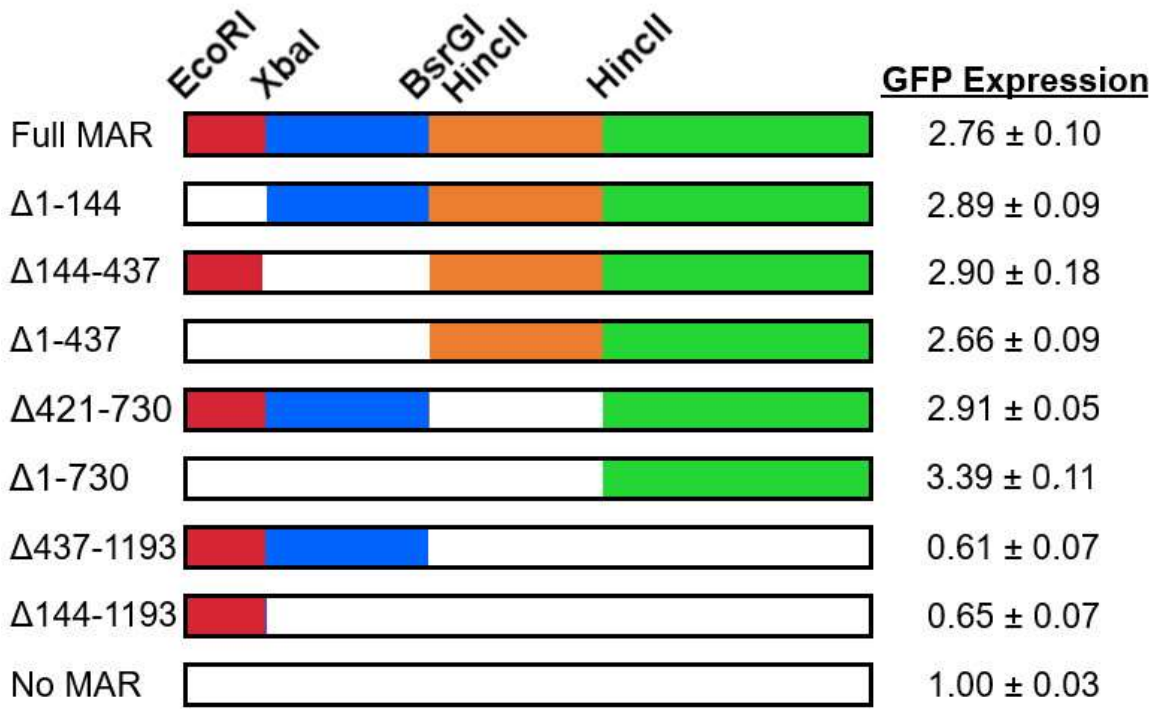
3.6.4 Figure 3. Double Terminators Strongly Enhance GFP Gene Expression Nonreplicating vectors containing different double terminators downstream from the GFP gene were agroinfiltrated into *Nicotiana benthamiana* leaves and analyzed for GFP production at 5 DPI. Red bars indicate double terminators. Abbreviations: BYDV, barley yellow dwarf virus 3' UTR; PEMV, pea enation mosaic virus 3' UTR; TNVD, tobacco necrosis virus-D 3' UTR; TMV, tobacco mosaic virus 3' UTR; LIR, long intergenic region from bean yellow dwarf virus.



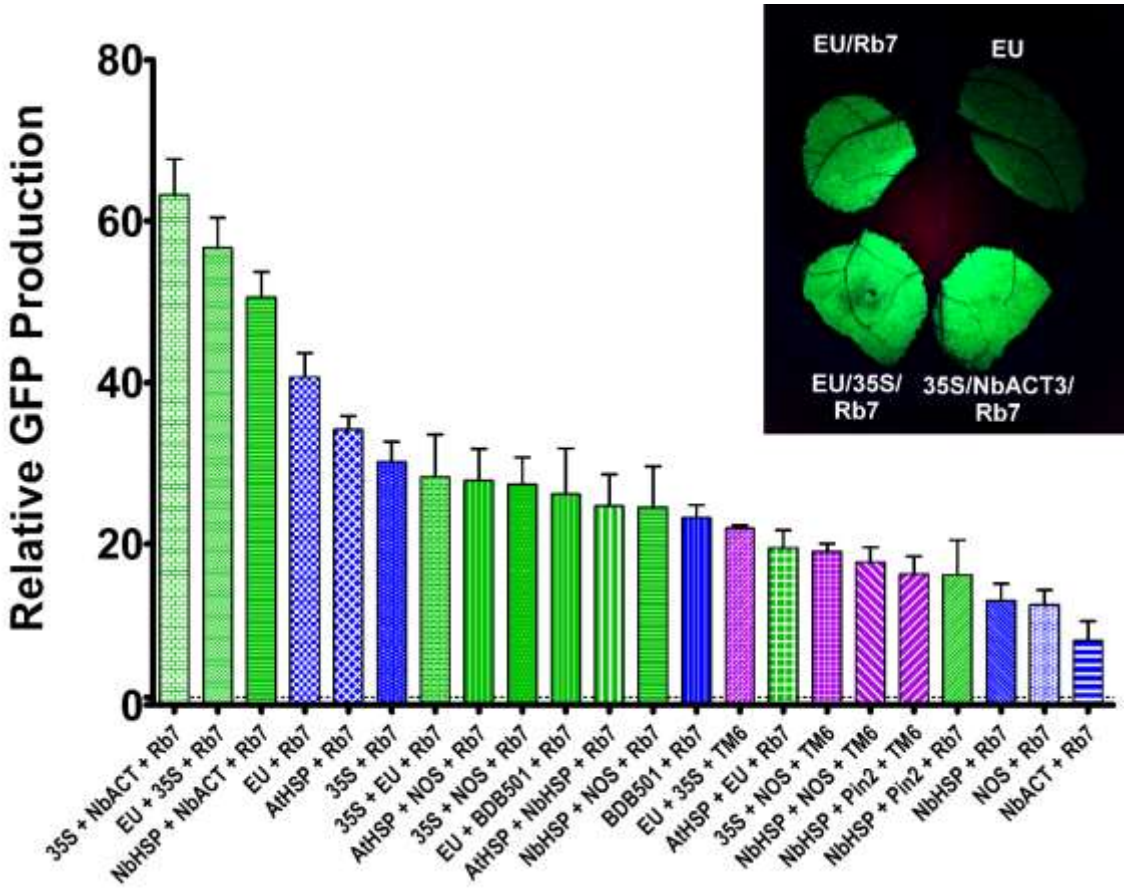
3.6.5 Figure 4. MARs Strongly Enhance GFP Expression
Nonreplicating GFP vectors containing either the tobacco Rb7 or tobacco TM6 MAR sequences inserted 3' of the gene terminator were agroinfiltrated into the leaves of *Nicotiana benthamiana* and evaluated for GFP production at 5 DPI. "EU + Control" indicates DNA sequence obtained from an inverted region of the Norwalk virus capsid protein coding sequence was inserted 3' of the EU gene terminator in place of the Rb7 MAR.



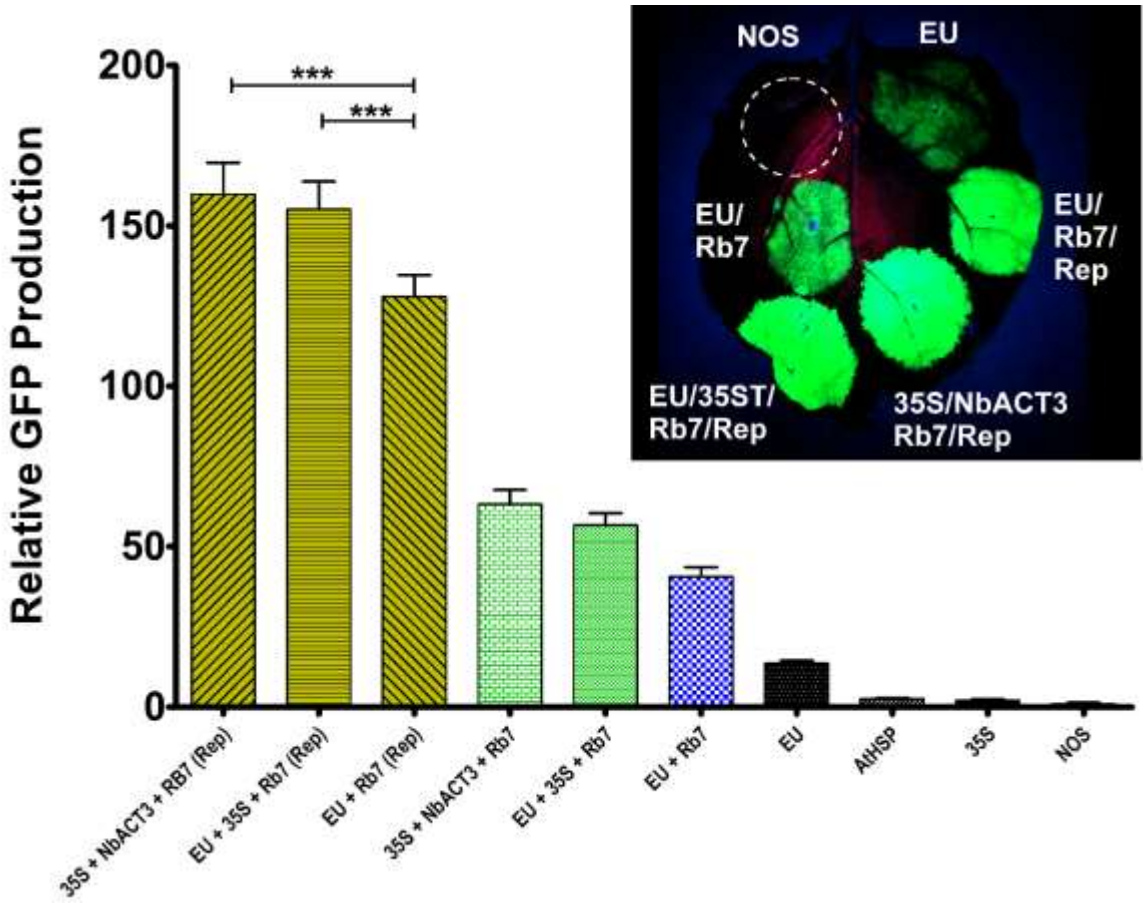
3.6.6 Figure 5. Characterization of Rb7 and TM6 MARs
Leaves of *Nicotiana benthamiana* were agroinfiltrated with nonreplicating GFP vectors containing either the tobacco Rb7 or tobacco TM6 MAR sequences and photographed after 5 DPI under UV illumination (365nm). Abbreviations: No 3' Elements, contains no terminator or MAR; Rb7 Only, contains only the Rb7 MAR with no terminator; 5' Rb7, contains the Rb7 MAR inserted 5' of the promoter; 3' Rb7, Rb7, or TM6, indicates each respective MAR was inserted 3' of the gene terminator.



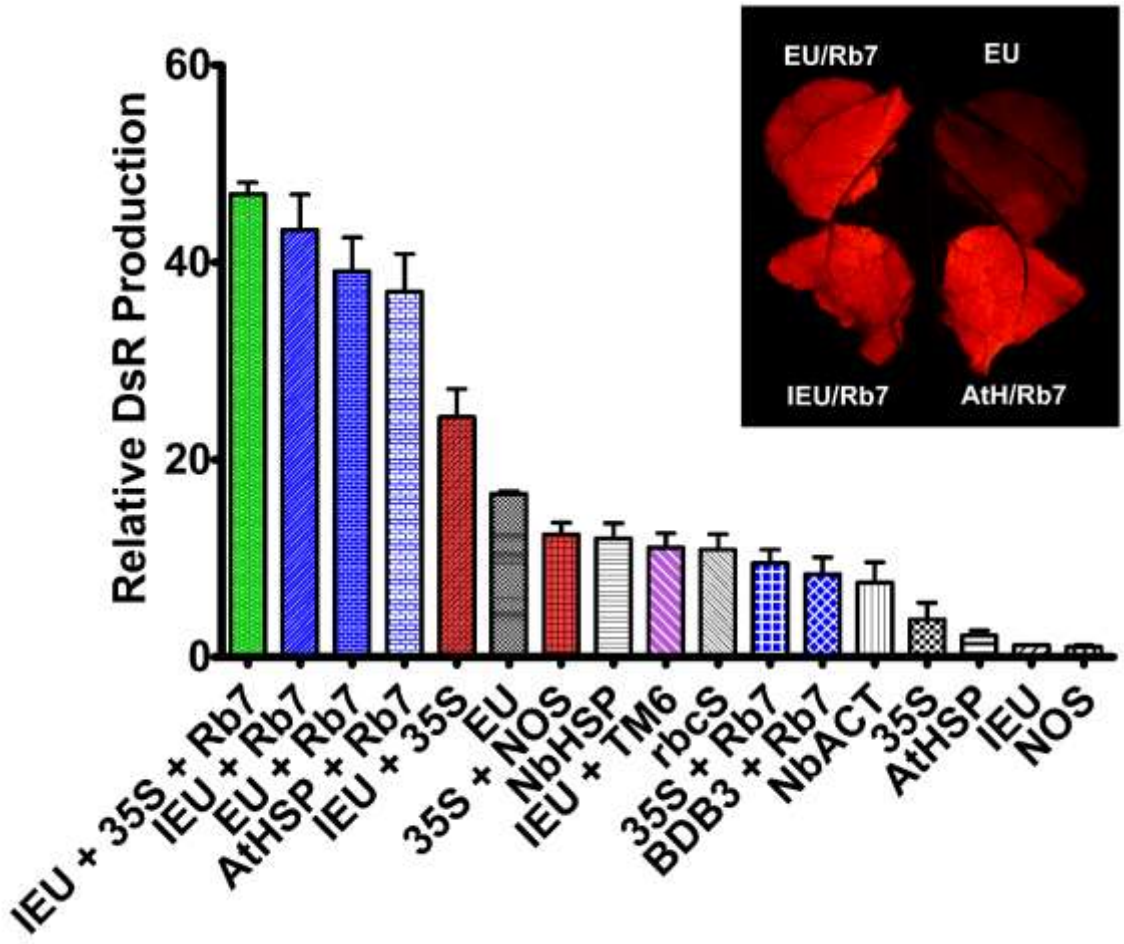
3.6.7 Figure 6. Deletion Analysis of the Rb7 MAR
GFP vectors containing deletions of regions within the Rb7 MAR were generated using the marked restriction sites present in the native sequence, agroinfiltrated into *Nicotiana benthamiana* leaves, and analyzed for GFP production at 5 DPI. Number values represent relative GFP production means ± standard error from 4 independently infiltrated leaf samples.



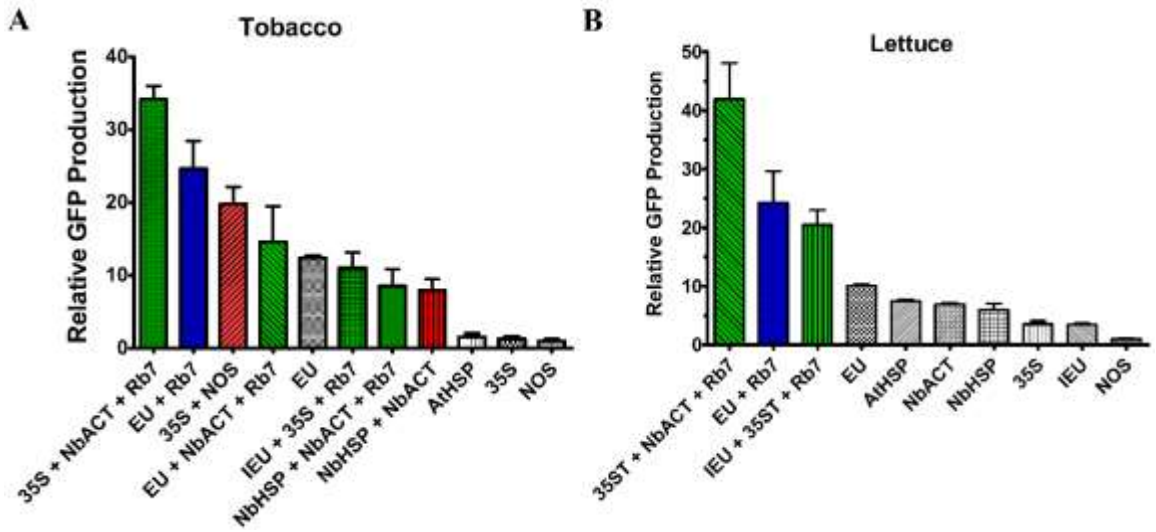
3.6.8 Figure 7. Combined 3' UTRs Strongly Enhance GFP Expression
Nonreplicating GFP vectors with combined terminators were created, agroinfiltrated into the leaves of *Nicotiana benthamiana*, and evaluated for GFP production at 5 DPI. Green bars indicate double terminators combined with Rb7 MAR; purple bars indicate double terminators combined with TM6 MAR; blue bars indicate single terminators combined with Rb7 MAR.



3.6.9 Figure 8. Evaluation of Combined 3' UTRs in Replicating Vectors
Replicating vectors containing elements of bean yellow dwarf virus (Diamos et al, 2016) were constructed with various combined terminators, agroinfiltrated into the leaves of *Nicotiana benthamiana*, and evaluated for GFP production. "Rep" indicates replicating geminiviral vector.



3.6.10 Figure 9. Comparison of Selected 3' UTRs Expressing DsRed
Nonreplicating vectors were constructed with single, double, or MAR-containing terminators downstream from the DsRed gene, and agroinfiltrated into the leaves of *Nicotiana benthamiana*. DsRed production was evaluated at 5 DPI by SDS-PAGE and UV fluorescence.



3.6.11 Figure 10. Comparison of Selected 3' UTRs in Tobacco and Lettuce Nonreplicating vectors were constructed with single, double, or MAR-containing terminators downstream from the GFP gene, and agroinfiltrated into the leaves of either tobacco (A) or lettuce (B) plants. GFP production was evaluated at 5 DPI by UV fluorescence and SDS-PAGE.

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CHAPTER 4

MODIFYING THE REPLICATION OF GEMINIVIRAL VECTORS ALLOWS ROBUST PRODUCTION OF TOXIC BIOPHARMACEUTICAL PROTEINS IN *NICOTIANA BENTHAMIANA* LEAVES

4.1 Abstract

Plants are a promising platform to produce biopharmaceuticals and vaccine antigens, however the toxic nature of some proteins inhibits their accumulation. Previously, we have created a replicating geminiviral expression system based on bean yellow dwarf virus which allows production of very high levels of recombinant proteins. To study the role of replication in this system, we generated vectors that allow separate and controlled expression of BeYDV rep and repA. Using this system, we show that the ratio of rep and repA strongly controls the efficiency of replication. Rep, RepA, and vector replication all elicit the plant hypersensitive response, resulting in cell death. We find that a modest reduction in rep/repA expression reduces plant leaf cell death which, despite reducing the accumulation of viral replicons, increases target protein accumulation. A single nucleotide change in the 5' untranslated region (UTR) of rep/repA is shown to reduce rep/repA expression, reduce cell death, and enhance the production of monoclonal antibodies using mutant BeYDV vectors. We also find that replicating vectors achieve optimal expression with lower agrobacterium concentrations than nonreplicating vectors, further reducing cell death. Viral 5' and 3' UTRs are also shown to contribute to cell death, while a native plant-derived 5' UTR does not. Using these modifications, leaf necrosis from expressing GII.4 norovirus capsids is strongly

reduced, allowing accumulation of GII.4 capsid protein at up to 0.7 mg/g leaf fresh weight (LFW), more than twice the highest level previously reported.

4.2 Introduction

Plant-based expression systems offer many potential advantages over traditional systems, including safety, speed, versatility, scalability, and cost. The demonstration that plant-made pharmaceuticals can be glyco-engineered to have authentic human N-glycans, with greater homogeneity and subsequently greater efficacy than their mammalian-produced counterparts further underscores the potential of plant-based systems for the production of therapeutic proteins (Zeitlin *et al.* 2011, Hiatt *et al.* 2014, Strasser *et al.* 2014). However, the economic feasibility of plant-based systems is often limited by low yields. High accumulation of foreign proteins, especially when ER-targeted, often puts significant stress on the plant cells. In some cases, this may lead to prohibitive levels of tissue necrosis that reduce yields (Hamorsky et al. 2015).

A plant-based transient expression system has been developed which uses the replication machinery from the geminivirus bean yellow dwarf virus (BeYDV) to substantially increase transgene copy number in the plant nucleus, with a subsequent increase in transcription of the target gene (Huang et al. 2009; Huang et al. 2010). This system has been used to produce high levels of vaccine antigens and pharmaceutical proteins in *Nicotiana benthamiana* leaves (Phoolcharoen et al. 2011; Lai et al. 2012a; Moon et al. 2014; Kim et al. 2015; Diamos et al. 2016). High levels of tissue necrosis have been noted when expressing certain proteins using BeYDV vectors, including ebolavirus glycoprotein, hepatitis b core antigen, GII norovirus particles, monoclonal

antibodies and other ER-targeted proteins (Phoolcharoen et al. 2011; Mathew et al. 2014, and unpublished data). The factors contributing to cell death in the BeYDV system have not been thoroughly investigated.

The geminiviruses comprise a family of small (~2.5kb) single-stranded DNA viruses which replicate in the nucleus of host cells, associating with histones to form viral chromosomes (Pilartz and Jeske 2003). BeYDV and other mastreviruses produce only four proteins: a coat protein and movement protein, which are produced by the virion sense DNA strand, and two replication proteins, Rep and RepA, produced on the complementary sense DNA strand. Rep and RepA are produced from a single intron-containing transcript: RepA is the predominant protein product from the unspliced transcript, while a relatively uncommon excision of an intron alters the reading frame to produce Rep. Production of all viral proteins is driven by a single bidirectional promoter in the long intergenic region (LIR) which also contains the viral origin of replication. Both divergent transcripts convert at a short intergenic region (SIR), which has bidirectional transcription terminator signals and is suspected to be the origin of complementary strand synthesis (Liu *et al.*, 1998).

Because geminiviruses produce few gene products, they are heavily reliant on host enzymes. The mastrevirus Rep protein, which is produced early in infection, is a multifunctional protein responsible for initiating rolling circle replication by nicking a conserved stem-loop sequence in the LIR. The majority of replication then occurs using cellular machinery to extend the free 3’ end of the nicked viral replicon, though it is likely that Rep recruits many of the involved cellular factors (Gutierrez 1999). Rep also

plays a role in ligating newly synthesized DNA to create circular viral genomes and possesses helicase activity (Choudhury et al. 2006). In the bipartite begomoviruses, Rep has been shown to form homo-oligomers, or possibly hetero-oligomers with RepA or other proteins, which may play a role in replication (Horváth et al. 1998; Krenz et al. 2011).

A primary function of RepA is thought to be the creation of a cellular environment suitable for replication. Some evidence suggests this occurs by binding retinoblastoma-related proteins, which are involved in cell cycle regulation. With RepA bound, previously sequestered transcription factors are able to initiate S-phase gene production, creating the cellular machinery necessary for viral replication (Gutierrez et al. 2004). An LxCxE motif has been shown to contribute to retinoblastoma-related protein binding (Ruschhaupt et al. 2013). However, other functions of RepA, many of which are still unidentified, have also been shown to enhance viral replication. A set of proteins known as GRAB proteins, which are involved in leaf development and senescence, have also been found to interact with RepA (Lozano-Durán et al. 2011).

Viral proteins are often potent inducers of the plant hypersensitive response, an immune defense mechanism that triggers the release of reactive oxygen species, autophagy, host translation shutoff, and programmed cell death in response to pathogen infection (Dodds and Rathjen 2010; Zhou et al. 2014b; Zorzatto et al. 2015). In the begomoviruses, the bean dwarf mosaic virus nuclear shuttle protein (NSP) was shown to activate the hypersensitive response in bean plants (Garrido-Ramirez et al. 2000), and this activity was mapped to its N-terminus of the NSP (Zhou et al. 2007a). As a

countermeasure, the TrAP protein from tomato leaf curl New Delhi virus prevents the activation of the hypersensitive response generated by its NSP (Hussain et al. 2007). Additionally, the NSP is known to interact with a host immune NB-LRR receptor-like kinase to enhance virus pathogenicity, and is involved in preventing translation shutoff in response to virus infection (Sakamoto et al. 2012; Zhou et al. 2014a). The Rep protein from African cassava mosaic virus also elicited the hypersensitive response in *Nicotiana benthamiana* (Van Wezel et al. 2002), and it was further reported that altering a single amino acid reversed hypersensitive response induction without affecting protein function (Jin et al. 2008). While many studies have focused on the begomoviruses, the role of the hypersensitive response during mastrevirus infection has not been investigated.

In this study, we have created a system that allows separate and controlled expression of BeYDV rep and repA. Using this system, we investigate how rep and repA control replication, and contribute to leaf cell death. By reducing expression of rep and repA, BeYDV-based expression vectors elicit lower levels of cell death, with a corresponding increase in the production of vaccine antigens and monoclonal antibodies. We also explore other factors contributing to cell death in plant expression systems, including the relationship between vector replication and agrobacterium concentration, and the contribution of viral elements to cell death. By combining optimizations designed to reduce cell death, we demonstrate a simplified expression system which allows high-level production of otherwise toxic biopharmaceutical proteins.

4.3 Materials and Methods

4.3.1 Vector Construction

A series of expression vectors containing promoters of varying strengths were created to express rep and repA. The Ubi3 promoter was obtained from pUbi3-GUS (Garbarino and Belknap 1994) by BseRI (T4 blunt) PstI digestion, and ligated into pRep110 (Huang et al. 2009) digested SbfI (T4 blunt) and XhoI, to create pRep107. The Ubi3 promoter with ubiquitin fusion was excised from pUbi3-GUS by PstI-NcoI digestion, and ligated into pRep110 digested SbfI-SacI along with rep/repA excised from pBY036 digested NcoI-SacI to create pRep106. The soybean vspB promoter was obtained from pGUS220 (Mason et al. 1993) by HindIII-NcoI digestion and ligated with pRep110 digested HindIII-SacI and pBY034 digested NcoI-SacI to create pRep108. The agrobacterium NOS promoter was obtained from The NOS promoter was obtained from pGPTV-Kan (Becker et al. 1992) by HindIII-NcoI digestion and ligated into pBI101 (Jefferson et al. 1987) along with rep/repA excised from pBY036 digested NcoI-SacI to create pRep111.

The intron-deleted form of BeYDV rep was previously described (Mor et al. 2003b). For RepA vectors, the sequence following the RepA stop codon was deleted and an additional stop codon was inserted in the Rep reading frame to prevent further translation. To accomplish this, a primer RepA-Sac-R (5'-CGGAGCTCTATGTTAATTGCTTCCACAATGGGAC) designed to insert a stop codon and create a SacI site at the end of the RepA coding sequence was used to amplify RepA from pRep110 along with primer TEV (5'- GCATTCTACTTCTATTGCAGC). The

product was digested ClaI-SacI, and ligated into pRep110 digested likewise to yield pRepA110. XhoI-SacI or NcoI-SacI fragments containing either the deleted intron form of rep excised from pBY037, or repA excised from pRepA110, were ligated into expression vectors containing the promoters Ubi (pRep106), UbiF (pRep107), VspB (pRep108), or NOS (pRep111) to generate rep and repA expressing vectors.

To create BeYDV expression vectors that required rep/repA to be supplied in trans, rep/repA were deleted from the NVCP-expressing vector pBYR2e-sNV or the rituximab-expressing vector pBYR2e-MRtx (Diamos et al. 2016) by BamHI digestion and self-ligation of the backbone vector to yield pBY-2e-sNV and, pBY-2e-MRtx respectively. The empty replicon vector pBY-EMPTY was created by excising the PstI-SacI fragment from pKS-RT38, which contains the potato pinII terminator region derived from pRT38 (Thornburg et al. 1987), and ligating it into pBY-GFP (Huang et al. 2009) digested SbfI-SacI. To introduce a AACATG to CACATG mutation to the 5' UTR of rep/repA, the primer LIRc-Nhe2-R (5'-taGCTAGCAGAAGGCATGTGGTTGTGACTCCGAGGGGTTG) containing the mutation was used to amplify the modified LIR from pBY027 with primer M13F. The PCR product was digested NheI-AgeI and ligated into pBYR2e-GFP digested BspDI-AgeI along with the rep-containing NheI-BspDI fragment from pBYR2e-GFP to create pBY-R2-GFP.

A GII.4 norovirus capsid expression vector combining cell death reducing modifications was created by three fragment ligation: the vector backbone containing the Rb7 3' MAR was obtained by XhoI-SacI digestion of pBYR2e-MrtxGM (Diamos et al.

2016), the NbPsaK truncated 5' UTR was obtained from pGFPe-NbPsaK2T (Diamos et al. 2016) by XhoI-XbaI digestion, and the plant codon-optimized synthetic Minerva norovirus capsid protein (based on Genbank Accession ABL74395.1) was digested XbaI-SacI to create pBYR2eK2M-MinV. The reduced rep/repA expression mutant was introduced to pBYR2eK2M-MinV by ligating the AgeI-BspDI fragment from pBY-R2-GFP into pBYR2eK2M-MinV to create pBYeR2K2M-MinV.

4.3.2 Agroinfiltration of *Nicotiana benthamiana* Leaves

Binary vectors were separately introduced into *Agrobacterium tumefaciens* GV3101 or EHA105 by electroporation. The resulting strains were verified by restriction digestion or PCR, grown overnight at 30°C, and used to infiltrate leaves of 5- to 6-week-old *Nicotiana benthamiana* maintained at 23-25°C. Briefly, the bacteria were pelleted by centrifugation for 5 min at 5,000g and then resuspended in infiltration buffer (10 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.5 and 10 mM MgSO4) to OD600=0.2, unless otherwise described. The resulting bacterial suspensions were injected by using a syringe without needle into leaves through a small puncture (Huang *et al.* 2004). Plant tissue was harvested after 5 DPI, or as stated for each experiment. Leaves producing GFP were photographed under UV illumination generated by a B-100AP lamp (UVP, Upland, CA).

4.3.3 Protein Extraction

Total protein extract was obtained by homogenizing agroinfiltrated leaf samples with 1:5 (*w:v*) ice cold extraction buffer (25mM sodium phosphate, pH 7.4, 100mM NaCl, 1mM EDTA, 0.1% Triton X-100, 10 mg/mLsodium ascorbate, 0.3 mg/mL PMSF)

using a Bullet Blender machine (Next Advance, Averill Park, NY) following the manufacturer's instruction. To enhance solubility, homogenized tissue was rotated at room temperature or 4°C for 30 minutes. The crude plant extract was clarified by centrifugation at 13,000g for 10 min at 4°C. Necrotic leaf tissue has reduced water weight, which can lead to inaccurate measurements based on leaf mass. Therefore, extracts were normalized based on total protein content by Bradford protein assay kit (Bio-Rad) with bovine serum albumin as standard.

4.3.4 SDS-PAGE and Western Blot

Clarified plant protein extract was mixed with sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.02 % bromophenol blue) and separated on 4-15% polyacrylamide gels (Bio-Rad). For reducing conditions, 0.5M DTT was added, and the samples were boiled for 10 min prior to loading. Polyacrylamide gels were either transferred to a PVDF membrane or stained with Coomassie stain (Bio-Rad) following the manufacturer's instructions. For rep/repA detection, the protein transferred membranes were blocked with 5% dry milk in PBST (PBS with 0.05% tween-20) for 1 h at 37˚C and probed in succession with rabbit anti-pRep (antibodies raised against an N-terminal 154 amino acid fragment of rep/repA) diluted 1:2000 and goat anti-rabbit IgG-horseradish peroxidase conjugated (Sigma) diluted 1:10,000 in 1% PBSTM. Bound antibody was detected with ECL reagent (Amersham).4.3.5 Protein Quantification by ELISA

GI and GII norovirus capsid concentration was analyzed by sandwich ELISA. A rabbit polyclonal anti-GI or anti-GII antibody was bound to 96-well high-binding

polystyrene plates (Corning), and the plates were blocked with 5% nonfat dry milk in PBST. After washing the wells with PBST (PBS with 0.05% Tween 20), the plant extracts were added and incubated. The bound norovirus capsids were detected by incubation with guinea pig polyclonal anti-GI or anti-GII antibody followed by goat anti-guinea pig IgG-horseradish peroxidase conjugate. The plate was developed with TMB substrate (Pierce) and the absorbance was read at 450nm. Plant-produced GI or GII capsids were used as the reference standard (Kentucky Bio Processing).

For rituximab quantification, plant protein extracts were analyzed by ELISA designed to detect the assembled form of mAb (with both light and heavy chains) as described previously (Giritch et al. 2006). Briefly, plates were coated with a goat anti-human IgG specific to gamma heavy chain (Southern Biotech, Birmingham, AL, USA). After incubation with plant protein extract, the plate was blocked with 5% non-fat dry milk in PBST, then incubated with a HRP-conjugated anti-human-kappa chain.

4.3.6 Plant DNA Extraction and Replicon Quantification

Total DNA was extracted from 0.1 g plant leaf samples using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. DNA (~1 μg) was separated on 1% agarose gels stained with ethidium bromide. The replicon DNA band intensity was quantified using ImageJ software, using the high molecular weight plant chromosomal DNA band as an internal loading control. Columns represent means ± standard deviation from 3 or more independently infiltrated samples.

4.3.7 RT-PCR

Total RNA was extracted from 0.1 g leaf samples using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Residual DNA was removed using the DNA-Free system (Ambion). First-strand cDNA was synthesized from 1 µg of total RNA primer using the Superscript III First Strand Synthesis System (Invitrogen) according to the manufacturer's instructions using oligo dT_{22} primer. RT-PCR was performed using primers RepF (5'-ACCCCAAGTGCTCATCTC) and RepR1 (5'-GCGACACGTACTGCTCA) to detect rep and repA transcripts.

4.4 Results

4.4.1 Controlled Production of Rep and RepA in Plant Leaves

In the BeYDV expression system, production of Rep/RepA leads to excision, circularization, and replication of any gene expression cassette flanked by the cis-acting LIRs. Previously, we showed that a rep/repA-supplying vector could be delivered in trans to amplify a replication-deficient BeYDV containing the LIRs but lacking rep/repA (Huang et al. 2009). However, this system was only capable of producing rep and repA together, at constant high levels under the control of the strong 35S promoter from cauliflower mosaic virus. To create a modular system to study vector replication, a series of agrobacterium T-DNA expression vectors were constructed that separately expressed either rep or repA under the control of five different promoters: the 35S promoter, the nopaline synthase promoter from agrobacterium (NOS), the vegetative storage protein B promoter from soybean (vspB), or the ubiquitin-3 promoter from potato with (UbiF) or without (Ubi) ubiquitin fusion (Fig. 1A). To characterize the expression of rep and repA

by these vectors, they were infiltrated into the leaves of *Nicotiana benthamiana* and analyzed by western blot and RT-PCR. Rep and RepA from the related wheat dwarf virus are known to form oligomeric complexes (Missich et al. 2000). Antibodies targeting both rep and repA produced together in their native wildtype configuration reacted strongly with nonreduced protein extracts, revealing large complexes near 250kDa in size. RepA produced two distinct high molecular weight bands, whereas rep produced only a single resolvable band (Fig. 1B, nonreduced). However, when rep and repA were expressed together, only a single band at the size of rep alone was observed (Fig 1B, right panel). Under reducing conditions, rep (predicted 39kDa) produced predominately monomeric 35-40kDa bands, while repA (predicted 33kDa) showed 65-75kDa bands suggestive of oligomeric forms. Interestingly, when both rep and repA were coexpressed, a slightly larger 45-50kDa band of unknown origin also appeared (Fig, 1B). RT-PCR and western analysis both showed that the 35S construct far exceeded the other expression vectors, followed by the NOS, vspB, UbiF constructs, with the unfused Ubi construct providing the weakest expression (Fig. 1B).

4.4.2 The Ratio of Rep and RepA Is Important for Efficient BeYDV Replication

To determine the effects of altered rep and repA expression on replicon amplification, a replicon vector pBY-2e-sNV encoding a synthetic GI norovirus capsid protein was coinfiltrated with rep and repA supplying vectors. For simplicity, further experiments were performed with either UbiF vectors for low expression or 35S vectors for high expression, as no major notable differences were observed among the lower expressing constructs. The vector pBYR2e-sNV, which contains the wildtype rep/repA

configuration driven by the native LIR promoter, was used as a control. In agreement with previous data on mastrevirus replication (Huang et al. 2009; Ruschhaupt et al. 2013), no replication was detected when repA alone was supplied, and very low replication was detected when rep was supplied alone with either a weak or strong promoter (Fig. 2). However, coinfiltration of both rep and repA resulted in robust replication (Fig. 2). Interestingly, overproduction of either rep or repA relative to the other resulted in impaired replication, suggesting that the relative abundance of each protein is important for efficient replication (Fig. 2). Although expression of rep and repA by the strong 35S promoter was comparable to or exceeded wildtype expression levels (Fig. 1B), the wildtype configuration resulted in a consistent increase in replicon accumulation, possibly due to differing to ratios of rep/repA expression (Fig. 2). These results show that the level of vector replication can be controlled by differential expression of rep and repA.

4.4.3 Reducing Vector Replication Reduces Cell Death and Increases Transgene Expression

Previously, we have shown that coinfiltration of a replicon vector and a rep/repA-supplying vector encoding both rep and repA together in the native configuration enhances the production of target proteins (Mor et al. 2003b; Huang et al. 2009). To further characterize the relationship between replicon amplification and target protein accumulation, the production of NVCP from replicons amplified with variable levels of rep and repA was measured by ELISA. The control vector psNV120e contains no BeYDV elements and thus cannot replicate, whereas pBY-2e-sNV contains the intergenic

regions from BeYDV necessary for replication. Interestingly, even in the absence of rep and repA, pBY-2e-sNV substantially increased NVCP expression by 3.1-fold compared to psNV120e, accumulating NVCP at 0.57 mg/g LFW (Fig. 3A). NVCP expression was further enhanced by an additional 2.7-fold when pBY-2e-sNV was coinfiltrated with 35S-driven rep/repA or when rep/repa were supplied by the wildtype LIR promoter, yielding NVCP at approximately 1.5 mg/g LFW (Fig. 3A). Unexpectedly, coinfiltration with vectors supplying rep and repA at lower than wildtype levels produced the highest yield of NVCP, reaching 2.0 mg/g LFW. The increase in NVCP expression was notably associated with a reduction in plant cell death (Fig. 3B). Among replicating vectors, NVCP expression was lowest when there was a mismatch in rep and repA production, consistent with our data showing that these combinations have impaired replication (Fig. 3B).

4.4.4 Rep and RepA Induce Leaf Cell Death

As viral proteins are often contributors to cell death, the individual contribution of BeYDV proteins to plant leaf necrosis was investigated. Vectors using the strong 35S promoter to express either rep, repA, the movement and coat proteins from BeYDV, or GFP were individually agroinfiltrated into *Nicotiana benthamiana* leaves and monitored for leaf tissue health. Both rep and repA produced chlorotic leaf tissue by 3-5 DPI which developed signs of leaf browning and eventually progressed to necrotic lesions by 6-10 DPI, whereas the movement protein, coat protein, and GFP did not produce any notable symptoms (Fig. 4A). The progression of leaf necrosis was greater for Rep than RepA, and the development of necrosis was quick in older leaves than in younger leaves (data

not shown). We also investigated whether replicon amplification itself might contribute to leaf necrosis. The vector pRep110, which expresses rep/repA together in the native configuration and is insufficient to cause significant cell death on its own, was coinfiltrated with either pBY-EMPTY, which contains the cis-elements necessary for replication but with gene coding sequences replaced with a terminator, or pPS1, which contains no replication elements. Leaf spots infiltrated with pBY-EMPTY and pRep110 produced chlorotic leaf tissue after 3-4 DPI, and necrotic leaf tissue after 6-8 DPI, whereas leaf spots infiltrated with pPS1 and rep/repA did not produce necrotic tissue up to 10 DPI (Fig. 4B).

4.4.5 Expression of Toxic Proteins is Enhanced by Reducing rep/repA Expression

To determine whether a modest reduction in rep/repA would also benefit the expression of other transgenes, reduced rep/repA vectors were coinfiltrated with either pBY-2e-GFP, encoding GFP, or with pBY-2e-MRtx encoding the heavy and light chains of the monoclonal antibody rituximab. These vectors were compared to replicating vectors containing rep/repA in the wildtype configuration driven by the native LIR promoter: pBYR2e-GFP and pBYR2e-MRtx. GFP is known to be well tolerated even when produced at very high levels in *Nicotiana benthamiana* leaves, whereas we have previously observed rituximab to induce a strong cell death response. A small but statistically insignificant decrease was observed in GFP expression when low rep/repA were supplied, compared to high rep/repA or wildtype, and no cell death was observed with any vector (Fig. 5A, and data not shown). By contrast, heavy cell death was observed when rituximab was expressed with wildtype or high rep/repA, but not when

rep/repA were reduced, and this reduction in cell death was correlated with a notable increase in antibody accumulation (Fig. 5B/C). These results suggest that reducing rep/repA from the wildtype level enhances the production of otherwise toxic proteins.

4.4.6 A Single Nucleotide Mutation Reduces Replication, Reduces Cell Death, and Increases Antibody Production

Reducing replication by supplying rep and repA on separate vectors requires simultaneous delivery of three different agrobacterium cultures. To construct a simplified vector with reduced expression of rep and repA, single nucleotide mutations were created in the native 5' UTR of rep/repA at the -3 position from the rep/repA start codon. These mutations were designed to provide a less favorable sequence context for translation initiation, which has been shown to favor A or G in the -3 position for dicot plants (Sugio et al. 2010). We found that a AACATG to CACATG mutation (where ATG indicates the rep start codon) reduced both Rep/RepA accumulation (Fig. 6A) and replicon amplification (Fig. 6B) by approximately 40%, similar to the results observed with low-expressing separated rep/repA vectors. To characterize expression and cell death with this vector, rituximab was produced with or without the mutation. As expected, the rep/repA mutant had reduced cell death (Fig. 6C), and increased antibody production (Fig. 6D). These results indicate that vector replication can be reduced with a single change from the wildtype rep/repA gene.

4.4.7 Replicating Vectors Require Lower Agrobacterium Concentrations Than Nonreplicating Vectors

In *Nicotiana benthamiana* leaves, we have previously found that agrobacterium strain EHA105 reduces leaf necrosis relative to other commonly used agrobacterium strains when used to deliver replicating BeYDV vectors (Diamos et al. 2016). Many nonreplicating vector systems use high agrobacterium concentrations of around an OD_{600} of 1.2 (Sainsbury et al. 2009). To investigate the relationship between agrobacterium concentration and vector replication, a replicating BeYDV vector expressing GFP was infiltrated at various agrobacterium concentrations. No significant differences in GFP expression were observed until the OD_{600} was reduced below 0.2 (Fig. 7A). By contrast, GFP expression with pEAQ-HT-GFP (Sainsbury et al. 2009) was reduced by nearly half when the agrobacterium OD_{600} was decreased from 1.2 to 0.2 (Fig. 7B). Similar results were found with other nonreplicating vectors (data not shown). While GFP was well tolerated at all agrobacterium concentrations tested, we reasoned that the added agrobacterium load may be less tolerable with more toxic proteins. To further evaluate the relationship between agrobacterium concentration and cell death, replicating BeYDV vectors expressing hepatitis B core antigen tandem-linked heterodimers (Peyret et al. 2015) were infiltrated at decreasing agrobacterium concentrations. Agrobacterium OD_{600} concentrations of 1.6 and 0.8 produced visible leaf necrosis, while 0.4 and 0.2 did not (Fig. 7C). Taken together, these data show that replicating BeYDV vectors provide optimal expression with lower agrobacterium concentrations than nonreplicating vectors, allowing further reductions in cell death.

4.4.8 Viral Flanking Regions Contribute to Cell Death

While no substantial necrosis developed with either BeYDV or pEAQ vectors expressing GFP, leaf chlorosis appeared only with pEAQ-HT-GFP, an effect which was more pronounced at higher agrobacterium concentrations (Fig. 8A). As pEAQ vectors contain the 5' and 3' UTRs from cowpea mosaic virus, we assessed whether other viral UTRs may contribute to cell death. The 5' UTR from tobacco mosaic virus was found to increase the cell death response compared to the native *Nicotiana benthamiana* NbPsaK 5' UTR, despite the TMV 5' UTR producing less recombinant protein (Fig. 8B and Diamos et al. 2016). The 5' and 3' UTRs from pea enation mosaic virus also substantially increased cell death, while those from barley yellow dwarf virus did not (Fig. 8C). These data show that viral untranslated regions can increase the cell death response.

4.4.9 Combined Cell Death-Reducing Modifications Allow Robust Production of Highly Toxic Proteins

Previously, expression of the GII.4 norovirus capsid using the TMV-based magnICON system resulted in the rapid onset of cell death, and correspondingly low yields of 0.3 mg/g LFW (Mathew et al. 2014). To attempt production of GII.4 norovirus capsid protein using vectors designed to reduce cell death, previously identified modifications (Diamos et al. 2016) were combined with those identified in this study. To this end, BeYDV vectors containing the Rb7 matrix attachment region, and the NbPsaK 5' UTR with or without reduced rep/repA expression were infiltrated using agrobacterium strain EHA105 OD_{600} of 0.2 into the leaves of *Nicotiana benthamiana* and monitored for cell death. An unmodified vector without these changes was used as a

control. Neither of the modified vectors produced substantial cell death by 4-5 DPI, indicating that cell death reduction was substantial without rep/repA reduction, whereas the unmodified vector developed necrotic patches (Fig. 9A and data not shown). No statistically significant difference was observed between the two modified vectors, though the mean expression was lower with reduced rep/repA (Fig. 9B). By contrast, the modified vector with wildtype rep/repA produced GII.4 capsids at 0.7 mg/g LFW, compared to 0.4 mg/g LFW for the unmodified control. These data show that BeYDV vectors containing cell death reduction modifications can produce high levels of GII.4 norovirus capsids without the development of leaf tissue necrosis (Fig. 9C).

4.5 Discussion

Transient expression systems have become the most commonly used systems to produce recombinant proteins in plants (Gleba et al., 2014). While extensive work has been done to optimize the gene expression cassette and other aspects of the BeYDV system (Diamos et al. 2016), vector replication has not been thoroughly investigated. To study replication, a modular system was created using promoters of varying strengths to express rep and repA at controlled levels. As geminiviruses encode few proteins, they are heavily on host enzymes for replication. The mastrevirus wheat dwarf virus repA preferentially forms octamers while rep forms 6-8 subunit oligomers, which assemble at the initiation site and are thought to recruit host replication machinery (Gutierrez et al. 2004). Among the begomoviruses, tomato yellow leaf curl Sardinia virus rep was found to form dodecamers with helicase activity (Clérot and Bernardi 2006), and the self-interaction of Abutilon mosaic virus Rep was demonstrated *in planta* (Krenz et al. 2011).

We found BeYDV rep and repA form high molecular weight bands consistent with the formation of oligomers comprised of 6-8 monomers (Fig. 1B).

There is discrepancy in the necessity of repA for mastreviral rolling circle replication. In cell culture experiments with wheat dwarf virus (Collin et al. 1996) or BeYDV (Liu et al. 1998; Hefferon and Dugdale 2003), intron-deleted rep has been reported to support high levels of replication. In contrast, maize streak virus only supported very low levels of replication in the absence of repA (Ruschhaupt et al. 2013). In agreement with the results of Ruschhapt et al, we observed only low levels of replication when expressing rep alone in *Nicotiana benthamiana* leaves, even in the presence of high levels of rep (Fig. 2B). Despite the small increase in NVCP-expressing replicon accumulation by supplying rep alone, a small decrease in NVCP expression was observed, perhaps indicating that replicons generated this way are less available for transcription, or that some other function of repA increases transgene expression. Notably, expression of repA alone also had a small negative effect on NVCP expression, indicating that both rep and repA are indeed required for productive enhancement of transgene expression (Fig. 3A). Furthermore, we also find that the relative ratio of rep and repA is essential for replication. Expression of both rep and repA from a relatively weak promoter still resulted in robust replicon production, but this did not occur if either rep or repA were overexpressed relative to the other (Fig. 3). Rep and RepA share the same N-terminus, including DNA binding and oligomerization domains, which may permit hetero-oligomerization (Horváth et al. 1998; Missich et al. 2000). Proper hetero-

oligomerization of Rep and RepA may be disrupted when either monomer is overexpressed.

In their native configuration, production of either Rep or RepA is controlled by the excision of an intron and thus the frequency of intron removal controls the relative abundance of each protein. For maize streak virus in infected maize, it has been reported that approximately 80% of transcripts produce repA, and only 20% produce rep (Wright et al. 1997). We observed that 35S-driven rep and repA produced as much or more combined Rep/RepA than the wildtype gene (Fig. 1B), yet had reduced replicon amplification (Fig. 2A). By reducing western blot it was possible to distinguish the 39kDa rep, which forms a single ~35-40kDa band when expressed alone, from the 33kDa repA, which ran as a 65-75kDa band when expressed alone, perhaps suggestive of dimer formation (Fig. 1B). We repeatedly observed that 35S-driven rep/repA overproduced the rep monomer-sized band and underproduced the repA dimer-sized band compared to the wildtype configuration (Fig. 1B), suggesting that 35S-driven rep/repA may not produce the proper ratio of each protein, and thus leading to reduced replication. However, when rep and repA were produced together, an additional 40-45kDa band consistently appeared regardless of promoter (Fig. 1B). As our antibody probe reacts with both rep and repA, it was not possible to conclusively determine the origin of this band. Interestingly, under nonreducing conditions, repA forms two distinct high molecular weight bands when expressed alone. However, when coexpressed with rep, only a single band was observed. This may be suggestive of different compositions present in homo-oligomers and hetero-

oligomers. Further studies, especially that can conclusively distinguish between rep and repA, are needed to address these questions.

Plants employ the hypersensitive response as a mechanism to combat viral infection. The hypersensitive response is characterized by a burst of reactive oxygen species and the formation of necrotic lesions resulting from programmed cell death. In this study, we find that BeYDV Rep and RepA both contribute to leaf cell death, while the BeYDV MP and CP did not produce notable symptoms (Fig. 4A). Furthermore, our data is suggestive of vector replication itself as a further contributor to cell death. Viral DNA sensors are well studied components of the innate immune system in animal cells (Takeuchi and Akira 2009), however similar sensors have not thus far been identified in plants (Zvereva and Pooggin 2012). Alternatively, many DNA viruses have been shown to activate the DNA damage response during replication (Luftig 2014). Here, we show that when rep/repA are supplied to an empty vector that has had all gene products removed but is still capable of accumulating viral replicons, the cell death response is enhanced compared to when rep/repA are supplied to a vector incapable of replicating (Fig. 4B). However, we cannot exclude the possibility that some other cryptic component of the vector contributed to the observed cell death.

Using a controlled reduction in rep/repA expression, leaf cell death caused by geminiviral replicons is alleviated (Figs. 3B and 5C). Despite reducing the number of available DNA templates for transcription, we found minimal reduction in the total yield of recombinant protein with nontoxic proteins (Fig. 5A), and an increased accumulation of otherwise toxic proteins (Figs. 3A, 5B, and 6D). Several hypotheses may explain this

observation. BeYDV vectors have replaced the viral movement and coat proteins with an expression cassette containing the gene of interest. During native BeYDV infection, the coat protein results in the accumulation of single-stranded viral DNA, which is packaged into virions, shuttled out of the nucleus, and, in concert with the movement protein, facilitates cell-to-cell movement and systemic spread of viral DNA (Liu et al. 2001). These interactions reduce the amount of double-stranded viral DNA available for transcription. As modified BeYDV expression vectors do not contain the movement and coat proteins, the amount of double-stranded DNA available in the nucleus to serve as a transcription template may exceed wildtype levels. Furthermore, BeYDV vectors also contain the RNA silencing suppressor P19, which likely increases the expression of rep and repA relative to wildtype levels. Taken together, these data suggest that more viral replicons are produced than are needed to saturate the plant transcription machinery. Therefore, we suspect reducing rep and/or repA expression allows a reduction in the plant hypersensitive response while still producing enough DNA templates to drive maximal transcription. By alleviating the hypersensitive response, further protein accumulation is possible for genes that otherwise would have had their production limited by cell death. Additionally, as RNA silencing and the hypersensitive response are interrelated pathways that act in concert against invading viruses, reducing the onset of hypersensitive response may also prevent premature silencing of BeYDV vectors (Zvereva and Pooggin 2012).

The sequence context around the initiation site plays a critical role in translation (Kozak 1999). Experiments with tobacco cells found that altering the initiation context from CAUAUGC to AAUAUGG (start codon underlined) resulted in a 4-fold increase in

gene expression (Ayre et al. 2002). While we were able to reduce cell death and increase yield of antibodies by reducing rep/repA expression, it required coinfiltration of three separate agrobacterium vectors. As the native rep gene also controls the optimum ratio of rep/repA by intron splicing, we reasoned that a mutation in the 5' UTR of rep/repA would be a simple modification to simultaneously reduce expression of both genes while maintaining the native mechanism of controlling the relative production of rep/repA. The resulting vector, containing an AAUAUG to CAUAUG mutation, reduced rep/repA expression, reduced cell death, and provided enhanced expression of toxic proteins (Fig. 6C/6D). As multiple BeYDV replicons can be placed in tandem on the same T-DNA (Huang et al. 2010), this strategy can be used to produce heteromultimeric proteins from a single vector.

Agrobacterium contributes to the plant cell death response in a complex manner (Hwang et al. 2015), though infiltration with higher agrobacterium concentrations has often been found to contribute to cell death (Wroblewski et al. 2005). While an agrobacterium OD_{600} of ~0.2 is sufficient to deliver T-DNA to the majority of plant cells, nonreplicating vector systems often use much higher concentrations of agrobacterium to achieve optimum expression. This may be due to the delivery of multiple DNA copies to each cell, which serve as additional transcription templates. As replicating systems greatly amplify the input T-DNA, additional copies would be unnecessary. Sainsbury et al. (2009) reported that, when using a nonreplicating vector, target protein accumulation decreased if the agrobacterium concentration was reduced below an OD_{600} of 1.2, which agrees with our findings (Fig. 7B). By contrast, we found no reduction in yield by

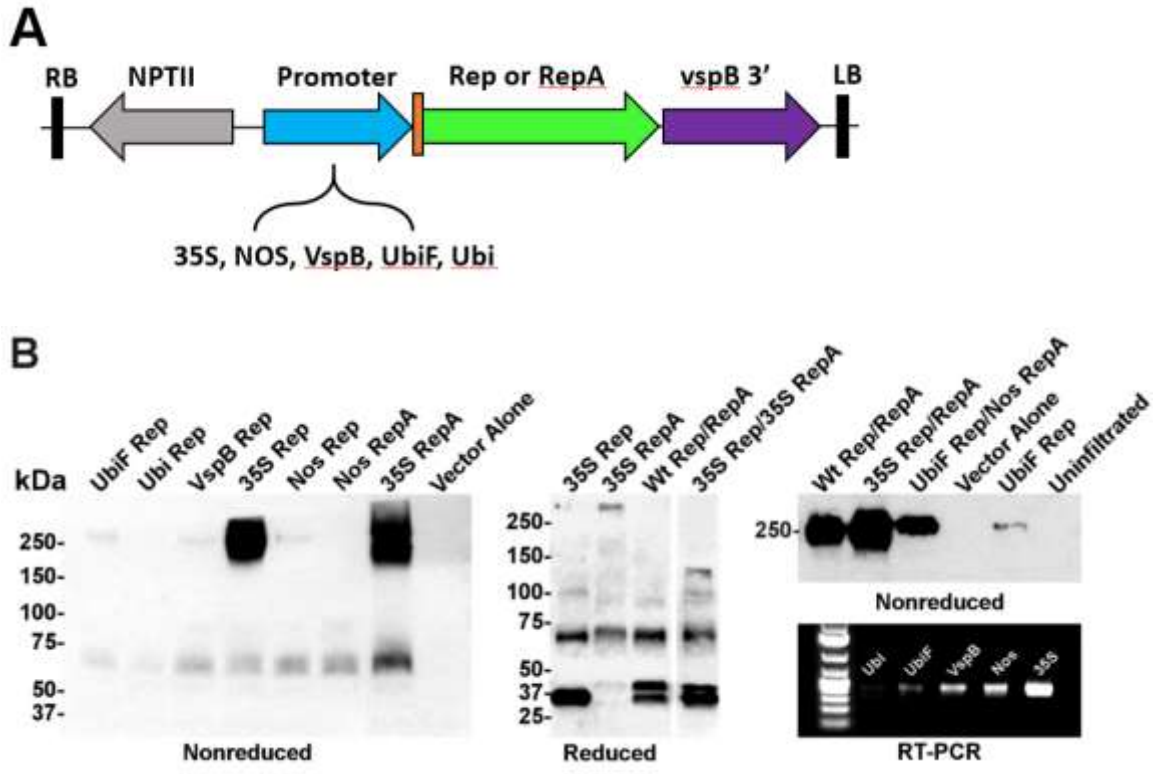
reducing the agrobacterium concentration from 1.2 to 0.2 using replicating BeYDV vectors (Fig. 7A/7B). For the expression of toxic proteins, we observed that necrosis developed when using higher agrobacterium concentrations, but not with lower concentrations (Fig. 7D). That this relationship was observed only with certain proteins suggests that cell death only occurs when a combination of factors cross a certain threshold. For the production of recombinant proteins with DNA-based systems, the development of cell death depends on the individual composition of the protein being produced, subcellular localization of the target protein (Howell 2013), glycosylation of the target protein (Hamorsky et al. 2015), target protein expression level, agrobacterium strain (Diamos et al. 2016) and concentration (Wroblewski et al. 2005, Fig. 7D), DNA elements like matrix attachment regions (Diamos et al. 2016), 5' and 3' UTR elements (Fig. 7C/7E), viral replication elements (Fig. 3B/4B/5C), and plant health and growth conditions (Qian et al. 2015; Matsuda et al. 2017). Modifying these factors allows enhanced accumulation of proteins that may, under less favorable conditions, elicit a cell death response.

Norovirus infection is the leading cause of acute gastroenteritis worldwide (Lopman et al. 2008), with GII.4 norovirus strains responsible for most outbreaks (Siebenga et al. 2007). While TMV-based systems have proven to be highly effective at producing small proteins (Gleba et al. 2014), the expression of some larger proteins have resulted in substantially lower yields. Production of the 59kDa HPV L1 protein was reported at 0.24 mg/g LFW using the DNA-based pEAQ system, a 15-fold increase compared to the RNA-based magnICON system (Matić et al. 2012). We found the 58kDa

Norwalk virus capsid protein accumulates at up to 2 mg/g LFW using BeYDV vectors (Fig. 3A), compared to 0.8 mg/g with TMV-based vectors (Santi et al. 2008). Previous attempts using a TMV-based system to produce the 59kDa GII.4 norovirus capsids were severely limited by the rapid onset of cell death after 4 DPI, with correspondingly low yields of 0.3 mg/g LFW corresponding to <2% total soluble protein (Mathew et al. 2014a). Infection of *Nicotiana benthamiana* with wildtype TMV causes severe leaf necrosis, stem collapse, and plant death. While modified TMV vectors lack the native coat protein, which contributes to symptom developments, coat protein deletion mutants can still develop mosaic symptoms (Lindbeck et al. 1992; Dawson 1999). The helicase component of the TMV replicase has been shown to elicit the hypersensitive response in *Nicotiana benthamiana* (Brendolise et al. 2017) and the TMV movement protein also affects carbohydrate metabolism (Almon et al. 1997). To circumvent the necrosis associated with using TMV-based vectors to produce GII.4 norovirus capsids, we combined several different strategies, including incorporating matrix attachment regions into the BeYDV replicon (Diamos et al. 2016), altering the agrobacterium strain (Diamos et al. 2016), using a modified native *Nicotiana benthamiana* psaK 5' UTR (Diamos et al. 2016, and Fig. 7D), and reducing rep/repA expression (Fig. 6C). Using these modifications, we produced GII.4 norovirus capsids at 0.7 mg/g LFW, over twice the highest level previously reported, with no development of leaf tissue necrosis (Fig. 9). Interestingly, no substantial cell death was observed even when wildtype rep/repA expression was preserved (Fig. 9A), indicating the 5' UTR, 3' MAR, and *Agrobacterium* strain modifications were effective enough to allow GII.4 norovirus capsid production without eliciting the hypersensitive response. We have previously observed that larger

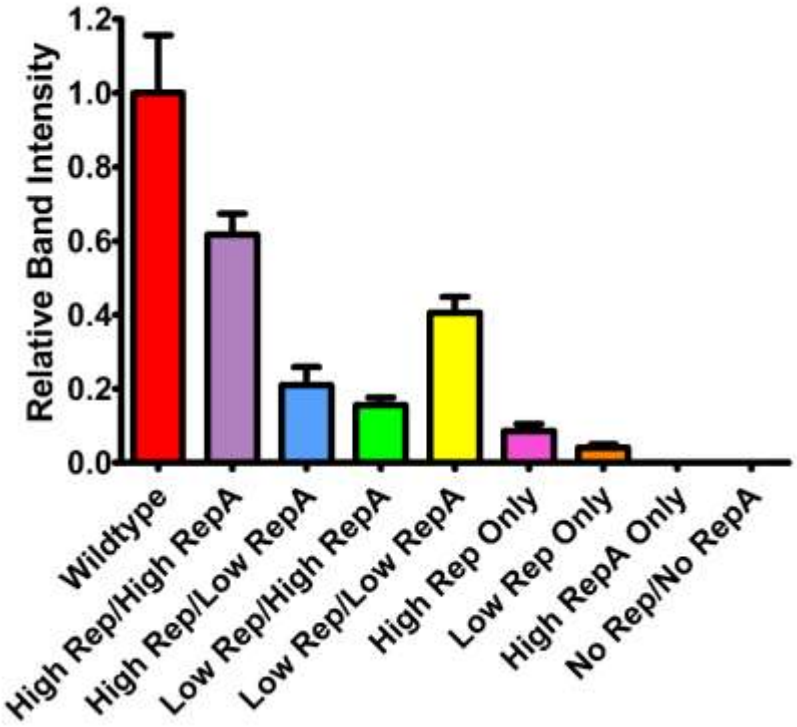
replicons accumulate to lower amounts than smaller replicons (unpublished data), and thus incorporation of the long 1.2kb Rb7 MAR may also reduce replicon accumulation, potentially leading to cell death reduction. We anticipate these modifications will allow high-level production of other toxic biopharmaceutical proteins.

4.6 Figures

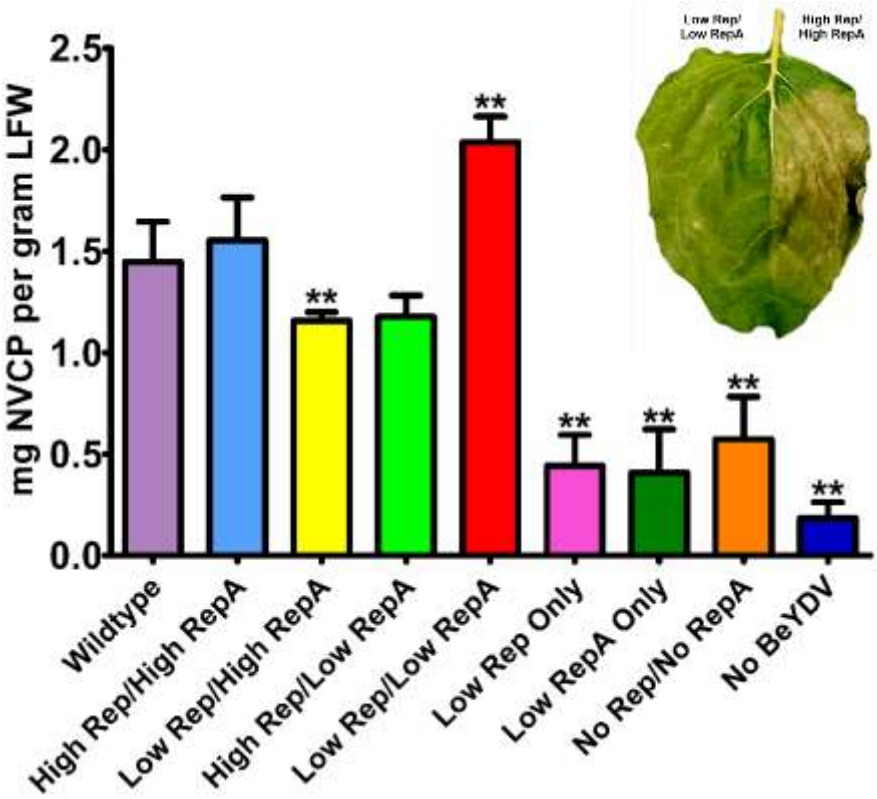


4.6.1 Figure 1. Controlled Expression of Rep and RepA in *Nicotiana benthamiana* Leaves

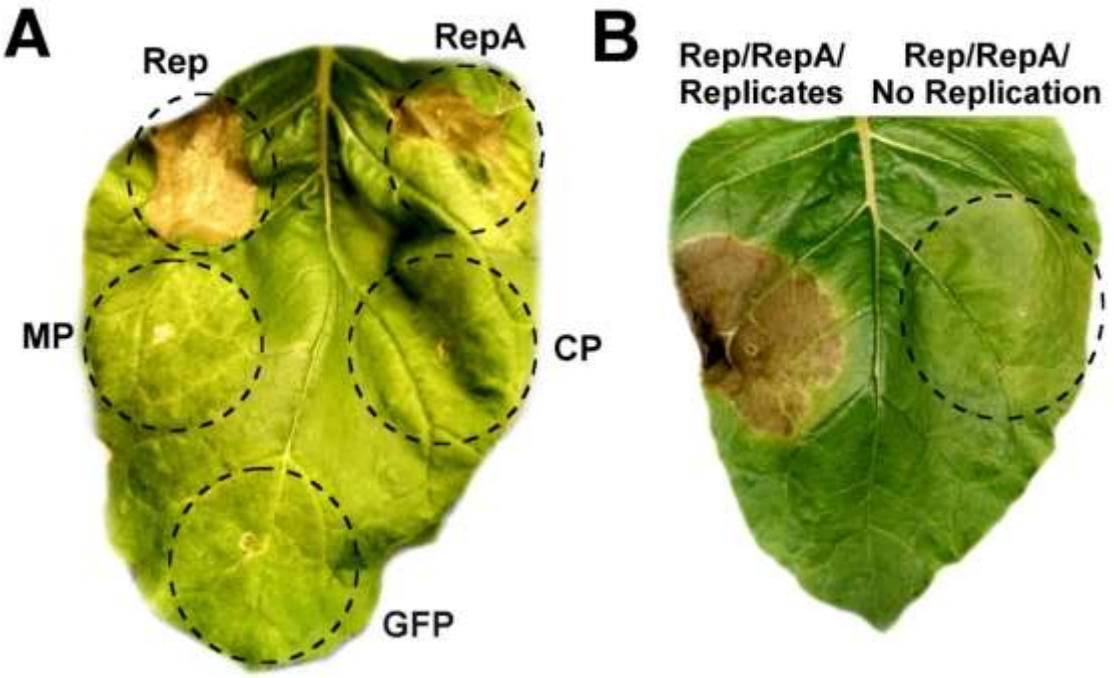
(A) Generalized schematic representation of the T-DNA region of the rep/repA vectors used in this study. RB and LB, the right and left borders of the T-DNA region from agrobacterium; NPTII, kanamycin resistance cassette; VspB 3', vegetative storage protein B gene terminator from soybean; Promoter, various promoters as described with 5' UTR from tobacco etch virus; 35S, the 35S promoter from cauliflower mosaic virus; NOS, the nopaline synthase promoter from agrobacterium; VspB, the vegetative storage protein B promoter from soybean; Ubi, the ubiquitin-3 promoter from potato; UbiF, Ubi with ubiquitin fusion. (B) Agrobacterium carrying the indicated T-DNA vectors were infiltrated into the leaves of *Nicotiana benthamiana*. After 4 days post infiltration (DPI), leaf tissue samples were harvested, and protein extracts were analyzed by reducing or nonreducing western blot. For RT-PCR, RNA was extracted from leaf samples and 50ng of converted cDNA were PCR amplified with rep-specific primers.



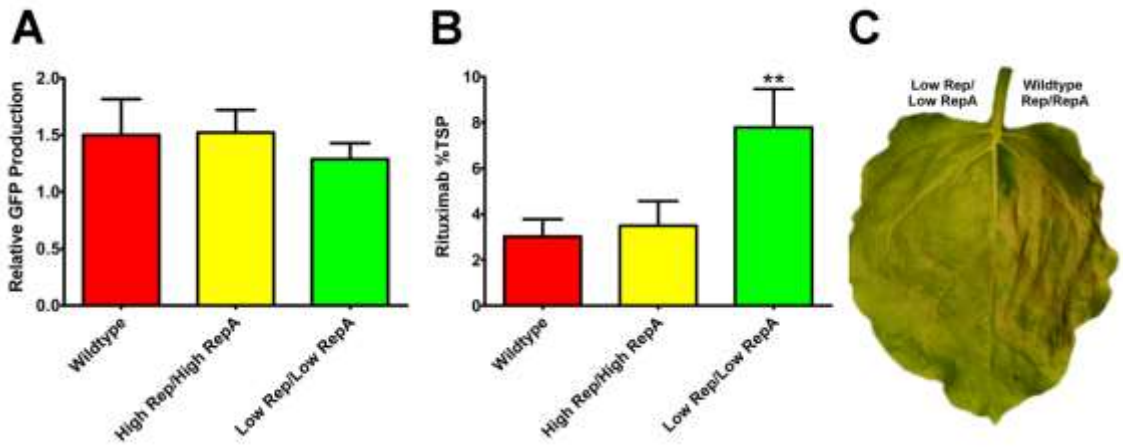
4.6.2 Figure 2. Replicon Accumulation by Differential rep/repA Expression
Leaves of *Nicotiana benthamiana* were agroinfiltrated with either low (UbiF) or high (35S) expression vectors producing combinations of rep and/or repA, along with the replicon vector pBY-2e-NVCP. Leaf tissue samples were harvested at 4 DPI, and 1 µg of extracted total DNA was separated and visualized by ethidium bromide stained agarose gel electrophoresis. The relative intensity of replicon bands was quantified with ImageJ software. Error bars are means ± standard deviation of 3 or more independently infiltrated samples.



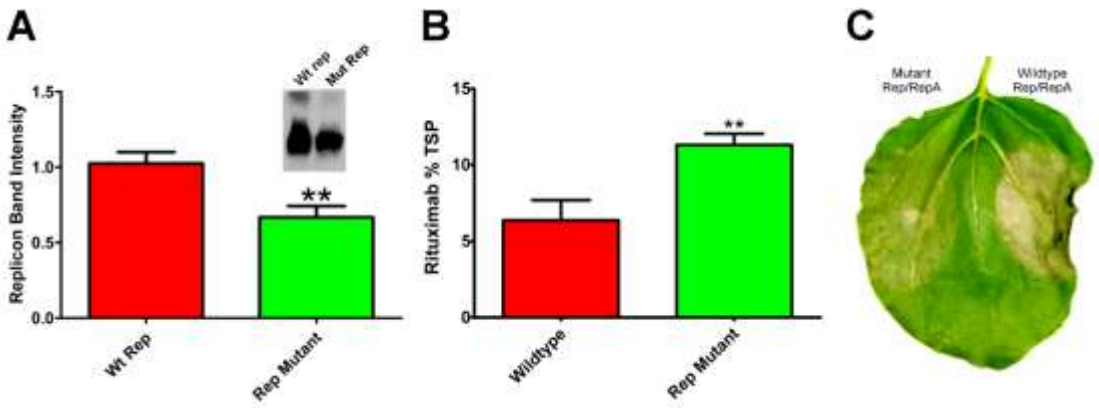
4.6.3 Figure 3. NVCP Production by Differential rep/repA Expression
Leaves were agroinfiltrated with expression vectors producing either low (UbiF/NOS promoters) or high (35S promoter) amounts of rep and/or repA, along with the replicon vector pBY-2e-NVCP. Leaf tissue samples were harvested at 4-5 DPI, and protein extracts were analyzed for NVCP production by ELISA. Bars represent means ± standard deviation from 3 or more independently infiltrated leaf samples. Two stars (**) indicates $p < 0.05$ by student's t-test compared to wildtype rep/repA. Representative leaves were imaged at 4-5 DPI under visible light to monitor the development of necrosis.



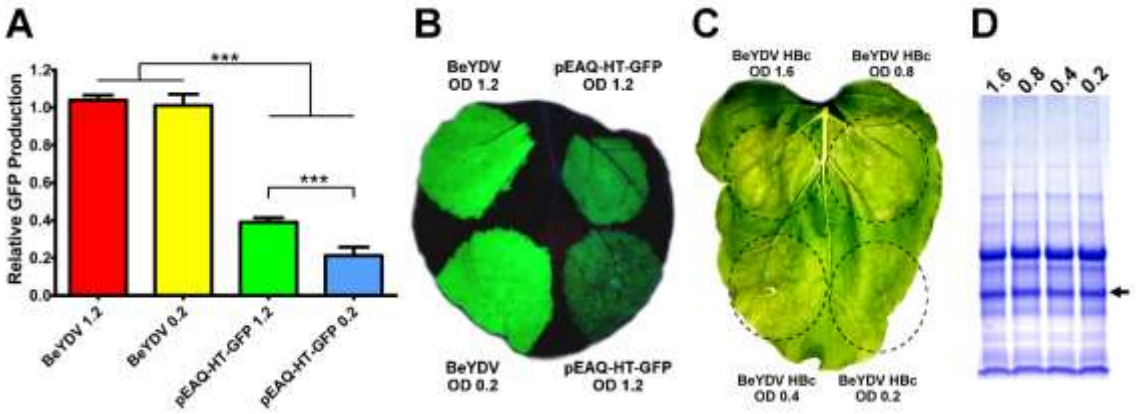
4.6.4 Figure 4. rep/repA Expression Induces Chlorosis and Cell Death
(A) Leaves were agroinfiltrated with vectors supplying high levels of rep, repA, GFP, or an empty vector with coding sequences removed. Leaves were monitored for tissue necrosis, and representative images were taken at 8 DPI (B) Leaves were agroinfiltrated with either rep/repA (pRep110) alone, or both pRep110 and the empty replicon vector pBY-EMPTY. Image was taken at 8 DPI.



4.6.5 Figure 5. Expression of GFP and Rituximab with Modified rep/repA Vectors. Leaves were coinfiltrated with modified rep/repA vectors and replicon vectors expressing either (A) GFP or (B) rituximab. For GFP analysis, protein extracts were separated on SDS-PAGE gels, and the GFP band intensity was quantified using ImageJ software. Columns are means ± standard deviation of three or more independently infiltrated samples. For rituximab, antibody production was quantified by IgG ELISA. Total soluble protein was determined by Bradford assay using bovine serum albumin and standard. Columns represent means ± standard deviation from three or more independently infiltrated leaf samples. Two stars (**) indicates $p < 0.05$ by student's t-test compared to wildtype rep/repA. (C) Representative leaves were imaged at 4-5 DPI under visible light to monitor the development of necrosis.

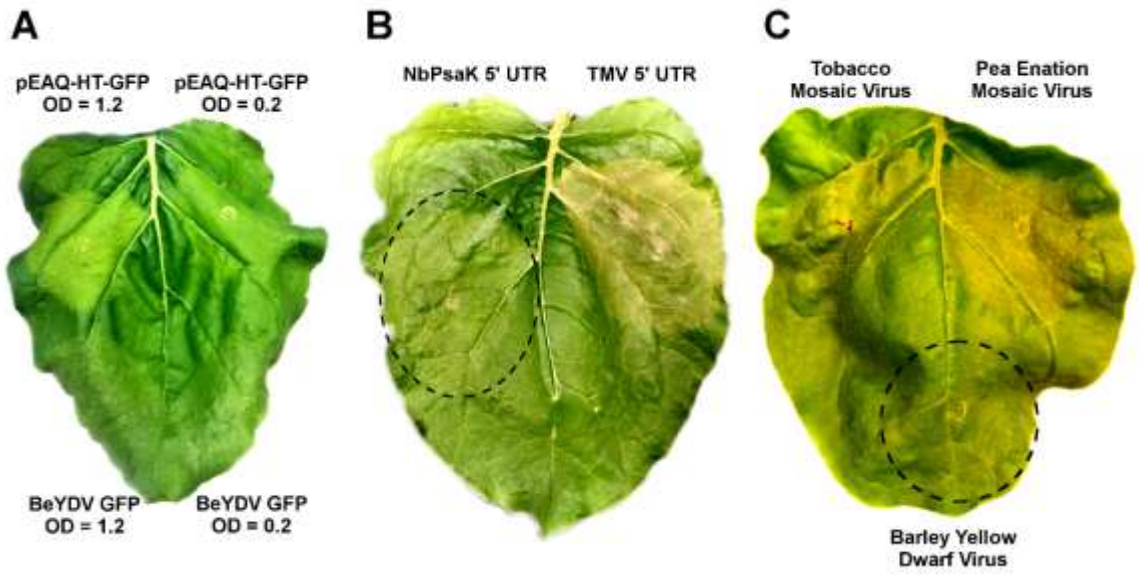


4.6.6 Figure 6. Characterization of rep/repA 5' UTR Mutant
Leaves of *Nicotiana benthamiana* were agroinfiltrated with the rituximab-producing replicon vector with (pBYe-R2-MRtx) or without (pBYR2e-MRtx) a mutated rep/repA 5' UTR and analyzed after 4-5 DPI for (A) replicon band intensity quantified from 500 ng total DNA by ethidium bromide stained agarose gel or (A, inset) western blot. (C) Rituximab production by IgG ELISA, and (D) leaf necrosis photographed at 5 DPI.

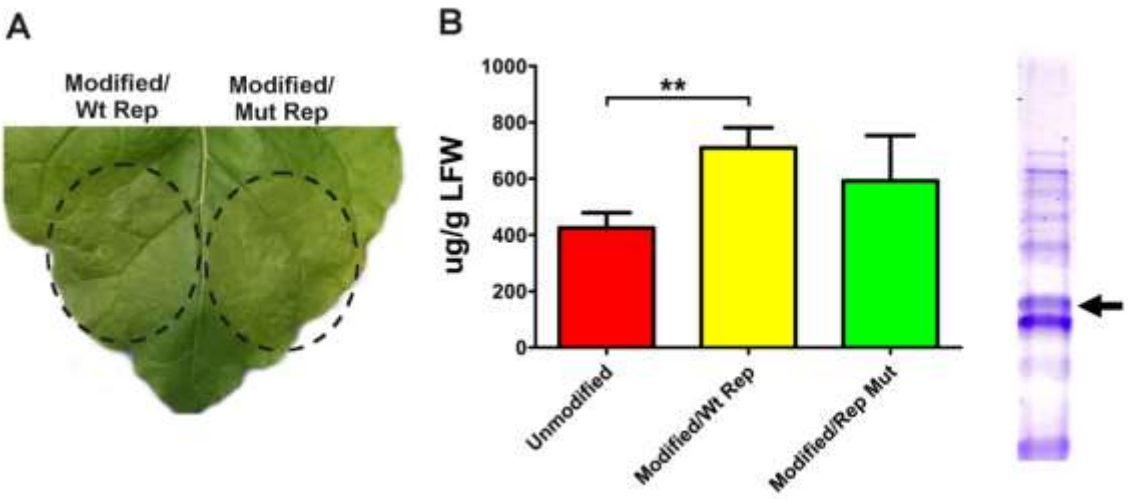


4.6.7 Figure 7. Replicating Vectors Require Lower Agrobacterium Concentration For Optimal Expression

Leaves of *Nicotiana benthamiana* were agroinfiltrated with the GFP-expressing BeYDV vectors or the nonreplicating vector pEAQ-HT-GFP at the indicated OD_{600} values. (A) Leaf spots were assayed for GFP production by SDS-PAGE followed by quantification of fluorescence band intensity by ImageJ software. (B) Leaf images under UV light or (C) visible light. (D) Protein extractions from leaf spots agroinfiltrated at the indicated OD_{600} values with a BeYDV vector expressing an HBc heterodimer were visualized by Coomassie staining. Arrow indicates HBc heterodimer band.



4.6.8 Figure 8. Virus-Derived 5’ and 3’ Untranslated Regions Induce Cell Death
(A) Leaves of *Nicotiana benthamiana* were agroinfiltrated with pEAQ-HT-GFP, which contains the CPMV 5’ and 3’ UTRs, or the BeYDV GFP vector pBYR2eK2Mc-GFP, at the indicated OD_{600} values and imaged under visible light at 5 DPI. (B) Leaves were agroinfiltrated with a BeYDV GFP vector containing either the NbPsaK 5’ UTR or TMV 5’ UTR and imaged at 6 DPI. (C) BeYDV GFP vectors containing the 5’ and 3’ UTRs from tobacco mosaic virus, pea enation mosaic virus, and barley yellow dwarf virus were agroinfiltrated and imaged under visible light at 5 DPI.



4.6.9 Figure 9. Production of GII.4 Norovirus Capsids with Cell Death-Reducing Modifications

Agroinfiltrated leaf tissue was (A) photographed at 4-5 DPI, and (B) analyzed for GII norovirus capsid production by ELISA with plant-produced GII norovirus capsid as standard. Data are means ± standard deviation from three independently infiltrated samples. Unmodified, vector pBYR2e-MinV with TMV 5' UTR and no 3' MAR; modified/Wt rep, vector pBYR2eK2M-MinV with wildtype rep, NbPsaK 5' UTR, and Rb7 3' MAR,; modified/rep mut, vector pBYeR2K2M-MinV with rep/repA 5' UTR mutation, NbPsaK 5' UTR, and Rb7 3' MAR. Arrow shows Coomassie stained gel with GII norovirus capsid band indicated.

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CHAPTER 5

THE MOVEMENT PROTEIN AND NUCLEAR SHUTTLE PROTEIN FROM A BIPARTITE BEGOMOVIRUS CONFER CELL-TO-CELL MOVEMENT TO MASTREVIRAL REPLICONS IN *NICOTIANA BENTHAMIANA* LEAVES

5.1 Abstract

Geminiviruses are a group of small plant viruses responsible for devastating crop damage worldwide. The emergence of agricultural diseases caused by geminiviruses is attributed in part to their high rates of recombination, leading to complementary function between viral components across species and genera. We have developed a GFP-based mastreviral reporter system that replicates to high levels in the plant nucleus, expressing very high levels of GFP, but is deficient in cell-to-cell movement. To investigate the potential for complementation of movement function by different geminivirus genera, the movement protein (MP) and nuclear shuttle protein (NSP) from the bipartite begomovirus bean dwarf mosaic virus (BDMV) were produced and characterized in *Nicotiana benthamiana* leaves. While overexpression of MP and NSP strongly inhibited GFP expression from the mastreviral reporter and caused adverse plant symptoms, optimizing the expression levels of MP and NSP allowed functional cell-to-cell movement. Hybrid virus vectors were created that express BDMV MP and NSP from mastreviral replicons using a variety of genetic configurations, allowing efficient cell-to-cell movement comparable to the movement observed in native BDMV replicons. We show that the expression levels of MP and NSP must be fine-tuned to provide a balance of MP/NSP sufficient for movement without eliciting the plant hypersensitive response or adversely impacting gene expression from viral replicons. The ability to confer cell-to-

cell movement to mastrevirus replicons depended strongly on replicon size: 2.1-2.7kb replicons were efficiently moved, while 3kb replicons were significantly inhibited, and 3.9kb replicons were very strongly inhibited. Although the MP and NSP from the mesophyll-invading BDMV functionally moved mastrevirus replicons, we found the MP and NSP from the phloem-limited AbMV were unable to do so in plant leaves.

5.2 Introduction

Geminiviruses are vector-borne plant viruses with ssDNA genomes between 2.5-3.0 kb which cause severe diseases in many economically important crops in tropical and sub-tropical areas (Scholthof *et al.* 2011). As geminiviruses replicate in plant nuclei, they must cross two barriers to transit between cells: the pores of the nuclear envelope to exit to the cytoplasm, and the intercellular channels, known as plasmodesmata, to spread cell-to-cell (Waigaman *et al.* 2004; Krichevsky *et al.* 2006; Lucas 2006; Jeske 2009). Though these general barriers must be overcome all geminiviruses, the specific mechanisms of doing so differ between genera. The begomoviruses are the largest, most well-studied geminivirus genus, and are divided into the monopartite begomoviruses, which contain only one single-stranded circular DNA component, and the bipartite begomoviruses, which contain both a DNA-A and DNA-B component. Both DNA-A and DNA-B are small, having genomes strictly between 2.5 and 2.8 kilobases, which is thought to be required for cell-to-cell movement (Gilbertson et al. 2003). The DNA-A component produces the replication protein Rep, the coat protein (CP), and auxiliary proteins that regulate transcription, suppress gene silencing, and enhance replication, while the DNA-

B component is responsible for virus movement by encoding a nuclear shuttle protein (NSP) and a movement protein (MP).

Both the MP and NSP have been shown to bind DNA in a sequence non-specific manner that instead depends on form and size. Though some differences have been reported between species, in general, genome-sized DNA molecules are efficiently transported (Rojas et al. 1998; Hehnle et al. 2004). Interestingly, the NSP is not required for virus infectivity with several begomoviruses, likely due to overlapping function with the CP (Zhou et al. 2007a). Conversely, CP mutants are still able to spread cell-to-cell, but lack systemic movement (Sudarshana et al. 1998). The DNA-A component of the bipartite begomoviruses can spread systemically in the absence of DNA-B, though in a very limited capacity (Hou et al. 1998). Inside the nucleus, viral genomes have been shown to interact with NSP and histone H3 (Zhou et al. 2011). The current model of intracellular movement is that a complex of histone H3, NSP, and viral genomic DNA is exported out of the nucleus, where it interacts with the MP. The MP-NSP-genome-histone H3 complex is then trafficked to the cell periphery and through the plasmodesmata to neighboring cells (Krenz et al. 2010). Though the exact mechanism of transport is not fully understood, heat shock cognate 70kDa protein has been implicated in the movement of viral replicons through a network of stromules which are formed upon infection and connect the nucleus, plastids, and plasmodesmata (Krenz et al. 2012). The NSP is also responsible for nuclear import of replicated viral genomes in neighboring cells (Krichevsky et al. 2006).

Mastreviruses are less well-studied than the begomoviruses, though they result in significant crop damage, especially in sub-Saharan Africa (Shepherd et al. 2007). The mastreviruses have only one small circular single-stranded DNA genome (2.6-2.8kb) comprising four genes: two replication association proteins, a CP, and a MP. The CP is thought to replace the function of the NSP by shuttling viral replicons out of the nucleus (Liu et al. 1999a). Additionally, the CP is required for ssDNA production for genome packaging (Azzam et al. 1994). After nuclear export, the MP localizes the NSP-DNA complex to the cell periphery for cell-to-cell transport (Jeffrey et al. 1996). The MP from mastreviruses does not seem to bind to DNA directly, instead interacting with the CP-DNA complex. Thus, mastreviruses require the CP for all forms of movement.

Exchange of genetic components can alter the host range or tissue specificity of a virus. The susceptibility of plant host species differs drastically between even closely related plant viruses, likely due to the ongoing arms race between plant defenses and virus counter-defenses (Rojas et al. 2005). Cassava mosaic geminivirus, formerly a relatively benign pathogen, underwent recombination to produce a much more severe strain that greatly impeded cassava production in Africa (Legg and Fauquet 2004). In Spain, a tomato-infecting geminivirus isolated in a wild reservoir was shown to be a recent genetic recombinant between Tomato yellow leaf curl Sardinia virus and Tomato yellow leaf curl virus, and had a broader host range than either of the two parent viruses (García-Andrés et al. 2006). Briddon et al. showed that exchanging the coat protein from African cassava mosaic begomovirus, which is transmitted by white fly, with the CP of beet curly top curtovirus, which is transmitted by leafhopper, allowed the generation of a

chimeric virus that had acquired the ability to be transmitted by the beet curly top curtovirus leafhopper vector. This work illustrates the powerful complementary potential of geminivirus recombination (Briddon et al. 1990).

There is substantial evidence for intra- and inter-species recombination between mastreviruses (Kraberger et al. 2013). Nevertheless, in contrast to the many examples of successful complementation experiments reported among members of the begomoviruses that extend cross-genus to the curtoviruses and topocuviruses, there are almost no examples of successful complementation reported for the mastreviruses. Multiple chimeras created between BeYDV and maize streak virus were unable to replicate or infect either host (Liu et al. 1999b). The only report of viable genetic complementation with a mastrevirus was carried out by swapping the coat and movement protein from similar strains of maize streak virus, which shared high levels of sequence homology (van der Walt et al. 2008).

Previous attempts to construct recombinant mastrevirus replicons capable of cell-to-cell or systemic movement with the native CP and MP have not been successful (Palmer and Rybicki 2001), suggesting some selectivity exists beyond genome size. Therefore, we investigated the potential for movement proteins from other geminiviruses to function with mastreviral replicons. A reporter system was devised that allows high-level GFP expression from wildtype-sized mastreviral replicons delivered by agroinfiltration. Expression of BDMV MP and NSP separately at controlled levels allowed functional cell-to-cell movement of recombinant mastreviral replicons in *Nicotiana benthamiana* leaves. Using fluorescence microscopy, the movement dynamics

of different genetic configurations of chimeric geminiviral/begomoviral genomes were studied. Optimizing the vector size and expression level of MP and NSP improved the efficiency of cell-to-cell movement. We also investigated the function of MP and NSP from a phloem-limited geminivirus.

5.3 Materials and Methods

5.3.1 Vector construction

A mastreviral vector with GFP replacing the BeYDV CP was created by overlap extension PCR. Primers LIRAscI (5'-TGGCGCGCCGCTCTAGCAGAAGGCATGTTG) and GFP-V2-R (5'-TCGCCCTTGCTCACCATGATGCACCCCGCCTA) were used to amplify the LIR-MP from pBY002 (Mor et al. 2003) and combined by overlapping GFP segments amplified with primers GFP-V2-F (5'-GTAGGCGGGGTGCATCATGGTGAGCAAGGGCGA) and VSPHT (5'-TGAATAGTGCATATCAGCATACCTTA) from pBY-GFP (Huang et al. 2009). The final vector pBYR-V1SGFP was assembled by three fragment ligation: the vector backbone from pBY-GFP digested AscI-EcoRI, the overlap PCR fragment digested AscI-SacI, and the truncated extensin terminator from pBY027.Eu2 (manuscript in preparation) digested SacI-EcoRI. A construct pBYR-V1GFP fusing GFP directly downstream from MP without the intervening sequence upstream from the CP was created similarly, using overlapping primers GFP-V1-R (5'-TCGCCCTTGCTCACCATCTACGGTCCTGGATGATCC) and its complementary primer. To mutate the start codon of MP, overlapping mutagenic primer V1-Mut-R (5'-CAATATACGCTTTATCAAATACCATCAC) and its complement were used with

LIRascI and Ext3-R (5’-CTTCTTCTTCTTCTTTTCTCATTGTC) to amplify an LIR-V1mut-GFP segment from pBYR-V1SGFP. The product was digested AscI-SacI and ligated into pBYR-V1SGFP digested likewise to yield pBYR-V1mutSGFP. A mastreviral replicon fusing GFP directly downstream from the LIR promoter was created by PCR amplifying the LIR from pBY-GFP with primer LIR-Xho (5’-AACTCGAGCAAATACCATCACATCG), designed to insert a XhoI site upstream from the MP start codon, and primer PNOS-Xho-R (5’-GGCTCGAGTTTGGATTGAGAGTGAATATGAGAC). The final vector pBYR-L5GFP was assembled by three fragment ligation: the vector backbone pBYR-V1GFP digested AscI-SacI, the PCR product digested AscI-XhoI, and the XhoI-SacI GFP fragment with TMV 5’ UTR from pBYR2e-GFP (Diamos et al. 2016). The construct pBYR-LGFP, which has the TMV 5’ UTR deleted, was created by XhoI-XbaI digestion of pBYR-L5GFP, blunting the ends with Klenow fragment of DNA polymerase, and self-ligating the backbone vector.

Vectors pBDA1.5-GFP containing the BDMV DNA-A with coat protein replaced by GFP (Sudarshana et al. 1998) and pBDB1.5 containing BDMV DNA-B were kind gifts from R. Gilbertson. NSP was amplified from pBDB1.5 with primers BV1-Xba-F (5’- gaTCTAGAATGTATGGTTTGCGGAATAAAC) and BV1-Sac-R (5’-ccGAGCTCTCAACC GATATAATCAAGGTCAAAC) designed to insert XbaI and SacI sites flanking the NSP gene. The amplification product was digested XbaI-SacI, and ligated into pGFPe-TMV (Diamos et al. 2016), an agrobacterium T-DNA expression vector containing the strong 35S promoter and the P19 suppressor of RNA silencing,

digested with likewise to yield pBV1e. The BDMV MP was amplified from pBDB1.5 with primers BC1-Xba-F (5'- ggTCTAGAATGGATTCTCAATTGG TCAATC) and BC1-Sac-R (5'-gcGAGCTCTTATTGCAACGATTTGGGCT), digested XbaI-SacI, and ligated into pGFPe-TMV digested likewise to create pBC1e.

To create chimeric mastrevirus replicons containing the BDMV MP and NSP, a preliminary cloning vector was created by four fragment ligation: from pBYRL5-GFP, the AscI-XhoI fragment (LIR-TMV-GFP) and EcoRI-FseI fragment (SIR-Rep-LIR) were excised and, along with the SacI-EcoRI potato pinII terminator fragment from pHB114 (Richter et al. 2000), ligated into pBY027 (Mor et al. 2003) digested AscI-FseI. To create a vector with only the BeYDV bidirectional SIR terminator but with available SacI-EcoRI sites for further terminator insertions, the potato pinII terminator was deleted by digestion with SbfI-SmaI, blunting the ends with Klenow fragment of DNA polymerase, and self-ligating the backbone. Then, BDMV NSP was excised from pBV1e by XhoI-SacI digestion and ligated into this new vector digested likewise to yield pBYR0-LBV1. The initial MP/NSP hybrid vector pBY0BDa was created by four fragment ligation: the vector backbone from pBYR0-LBV1 digested AgeI-SacI, the SIR-containing SacI-AvaI fragment from pBYR0-LBV1, the BDMV MP amplified from pBC1e with primers BC1-Xba-F and BC1-Ava-R (5'- CACCCGAGTTATTGCAACGATTTGGGCT) digested XbaI-AvaI, and the BeYDV LIR amplified from pBY027 with primers LIR-C-Xba-R (5'-GGTCTAGAGTTGTTGTGACTCCGAGG) and M13RHT (5'-GGAAACAGCTATGAC CATG) digested AgeI-XbaI. A T-DNA dual replicon vector pBYBDa-L5GFP (referred to as pBYBDx in Table 1, were "x" denotes the indicated

letter) containing both the mastreviral GFP replicon and BDMV MP/NSP hybrid replicon was created by four fragment ligation: the AscI-SacI backbone fragment and SacI-ApaI (GFP-TMV-LIR) fragment from pBYR-L5GFP were ligated with the ApaI-NsiI (LIR-MP) and NsiI-AscI (MP-SIR-NSP-LIR) fragments from pBY0BDa. Details about the construction of other vector derivatives are available upon request.

MP and NSP from AbMV were kind gifts from H. Jeske. AbMV NSP was amplified from pMDC7-HA-NSP (Kleinow et al. 2009) with primers AbBV1-Xba-F (5'-cgTCTAGAAT GTACCCGTCTAGGAATAAA) and AbBV1-Sac-R (5'-agGAGCTCTTAACCAATATAGTC AAGGTCAAAC), digested XbaI-SacI, and ligated into either pBV1e digested likewise to yield pABV1e, or into pBYR0-LBV1 digested likewise to create pBYR0-LABV1. AbMV MP was amplified from CpMDC7-c-Myc-MP (Kleinow et al. 2009) with primers AbBC1-Bsr-F (5'-ggTGTACAATGGATTCTCAGTTAGTAAATCCTC) and AbBC1-Kpn-R (5'-gcGGTACCT TATTTCAATGATTTGGCTTGAGAAG), digested BsrGI-KpnI, and ligated into pBD209 digested likewise to yield pBD209-AbBC1. The AbMV TMV 5' UTR-AbMV MP-35S terminator fragment was excised from pBD209-AbBC1 by XhoI-EcoRI digestion and ligated with the backbone fragment from pBC1e digested XhoI-ApaLI and the small ApaLI-EcoRI fragment from pBC1e to yield pABC1e. The hybrid vector pBY0ABa was created by four fragment ligation: the vector backbone from pBYR0-LABV1 digested AgeI-SacI; the SIR-containing SacI-AvaI fragment from pBYR0-LABV1; the AbMV MP amplified from pBD209-ABC1 with primers AbBC1-Bsr-F and AbBC1-Ava-R (5'-gcCCCGAGTTATTTCAATGATTT GCTTGAGAAG)

digested BsrGI, blunted, then AvaI; and the BeYDV LIR amplified from pBY027 with primers LIR-C-Xba-R (5'-GGTCTAGAGTTGTTGTGACTCCGAGG) and M13RHT (5'- GGAAACAGCTATGACCAT G) digested XbaI, blunted, then AgeI. pBY0ABa was used to create dual replicon hybrid vectors with pBYR-L5GFP as described for pBY0BDa. Further details about vector construction are available upon request.

5.3.2 Agroinfiltration of *Nicotiana benthamiana* leaves

Binary vectors were separately introduced into *Agrobacterium tumefaciens* EHA105 by electroporation. The resulting strains were verified by restriction digestion or PCR, grown overnight at 30°C, and used to infiltrate leaves of 5- to 6-week-old *Nicotiana benthamiana* maintained at 23-25°C. Briefly, the bacteria were pelleted by centrifugation for 5 min at 5,000g and then resuspended in infiltration buffer (10 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.5 and 10 mM MgSO4) to OD600=0.2. The resulting bacterial suspensions were injected by using a syringe without needle into leaves through a small puncture (Huang *et al.* 2004). Plant tissue was harvested after 5. Leaves producing GFP were photographed under UV illumination generated by a B-100AP lamp (UVP, Upland, CA). GFP fluorescent cells were observed with a Zeiss Axioskop A.1.

5.3.3 Protein extraction

Total protein extract was obtained by homogenizing agroinfiltrated leaf samples with 1:5 (*w:v*) ice cold extraction buffer (25mM sodium phosphate, pH 7.4, 100mM NaCl, 1mM EDTA, 0.1% Triton X-100, 10 mg/mLsodium ascorbate, 0.3 mg/mL PMSF) using a Bullet Blender machine (Next Advance, Averill Park, NY) following the

manufacturer's instruction. To enhance protein solubility, homogenized tissue was rotated at room temperature for 30 minutes. The crude plant extract was clarified by centrifugation at 13,000g for 10 min at 4°C. Protein concentration of clarified leaf extracts was measured using a Bradford protein assay kit (Bio-Rad) with bovine serum albumin as standard.

5.3.4 SDS-PAGE and Western Blot

Clarified plant protein extract was mixed with sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.02 % bromophenol blue) and separated on 4-15% polyacrylamide gels (Bio-Rad). For GFP fluorescence, PAGE gels were visualized under UV illumination (365nm). PAGE gels were stained with Coomassie stain (Bio-Rad) following the manufacturer's instructions. The 26kDa band corresponding to GFP was analyzed using ImageJ software to quantify the band intensity. For western blot, polyacrylamide gels were transferred to PVDF membranes, blocked with 5% dry milk in PBST (PBS with 0.05% tween-20) for 1 h at 37˚C and probed in succession with rabbit anti-HA (1:2000) and goat anti-rabbit IgG-horseradish peroxidase conjugated (Sigma) diluted 1:10,000 in 1% PBSTM. Bound antibody was detected with ECL reagent (Amersham).

5.3.5 Plant DNA Extraction and Replicon Visualization

100 mg plant leaf samples were harvested and ground to a fine powder in liquid nitrogen. Total DNA was extracted from ground leaf samples using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. DNA (~1 µg) was separated on 1% agarose gel stained with ethidium bromide. Band intensities were

quantified using ImageJ software using the native plant chromosomal DNA as an internal loading control.

5.4 Results

5.4.1 Construction of a Replication-Competent, Movement-Deficient Mastreviral Reporter System

To create a system to study geminivirus movement in *Nicotiana benthamiana*, a mastreviral GFP expression vector based on bean yellow dwarf virus (BeYDV) was constructed for delivery by agroinfiltration. The vector was designed to contain the BeYDV rep and repA genes for vector replication, with the MP and CP replaced by GFP (Fig. 1A). Both sets of genes were placed under the control of the bidirectional LIR promoter, which was positioned on both ends of the vector to allow replicational release of the viral replicon in the plant nucleus (Grimsley et al. 1987; Mor et al. 2003b). We found that fusion of the complete GFP coding sequence directly downstream from the BeYDV MP coding sequence resulted in no detectable GFP expression (Fig. 1B). However, if a small native intervening region between the movement and coat protein ORFs was preserved, GFP was produced at low levels (Fig. 1B), indicating that this intervening sequence is likely involved in CP expression. To verify that MP translation is necessary for CP production, the MP start codon was mutated, which abolished GFP production despite the presence of an intact start codon in the GFP gene (Fig. 1B). These results suggest that CP is not produced by leaky scanning past the MP start codon, but instead requires both V1 initiation and the intervening sequence upstream of the CP for

translation to occur. Replacement of both the movement and coat proteins by directly fusing GFP to the LIR in an optimal translation initiation context resulted in substantially higher levels of GFP fluorescence (Fig. 1B). To further enhance the utility of this modified BeYDV vector as a reporter system, the tobacco mosaic virus 5' UTR was inserted upstream from GFP. The resulting 2,587bp vector (pBYR-L5GFP), which is very close in size to the authentic 2,561 bp genome of BeYDV, produced intense green fluorescence when infiltrated into plant leaves, and was found to replicate to a very high copy number, accumulating large quantities of replicon DNA which were visible on ethidium bromide stained gel, characteristic of robust mastreviral replication (Fig 1C). As the movement and coat proteins were removed, no cell-to-cell or systemic movement was detected (data not shown).

5.4.2 Coexpression of BDMV and AbMV MP and NSP with BeYDV GFP Replicons

Previous attempts to create a recombinant mastreviral system capable of cell-to-cell movement using the maize streak virus CP and MP were unsuccessful (Palmer and Rybicki 2001). In agreement with these results, we produced the BeYDV MP and CP either from separate expression vectors, on BeYDV replicons, or from wildtype genomes, but in all cases failed to achieve cell-to-cell movement of recombinant genomes (data not shown). Instead, the potential for begomovirus movement proteins to interact with recombinant mastreviral genomes was investigated as they were reported to interact with DNA molecules in a sequence nonspecific manner. The BDMV MP and NSP were each separately cloned with an HA epitope tag into an agrobacterium T-DNA binary vector under the control of the strong 35S promoter, tobacco etch virus 5' UTR, and soybean

vspB terminator. We have previously used this vector to efficiently produce BeYDV rep and repA in *Nicotiana benthamiana* leaves (Huang 2009). Each vector was separately agroinfiltrated into the leaves of *Nicotiana benthamiana*, and harvested leaf tissue was found to produce readily detectable MP and NSP (Fig. 2A). To ensure that the HA tag did not interfere with protein function, unmodified MP and NSP were also produced and used for subsequent experiments.

To assess the interaction between BeYDV replicons and the BDMV MP and NSP, the replicating vector pBYR-L5GFP was compared with or without coinfiltration of 35S-MP and 35S-NSP. While the vector alone produced intense green fluorescence, GFP expression was strongly inhibited by addition of MP and NSP together (Fig. 2B). To determine whether the MP or NSP, or both, were responsible for this inhibition, each gene was infiltrated separately with the replicating GFP vector. While coinfiltration of 35S-MP had no substantial effect on GFP expression, 35S-NSP strongly inhibited GFP expression (Fig. 2C). As the NSP has been shown to interact with genome-sized DNA molecules, and 35S-NSP produced high levels of NSP, we hypothesized overproduction of the NSP may sequester or otherwise interfere with BeYDV replicon utilization, preventing GFP production. Consistent with this hypothesis, coexpression of pBYR-L5GFP with 35S-NSP strongly inhibited replicon accumulation (Fig. S1A) and coexpression of the coat protein from BeYDV with pBYR-L5GFP, which has nuclear shuttling functions, also similarly inhibited GFP expression (data not shown). Therefore, expression vectors were constructed with the much weaker nopaline synthase (NOS) promoter from agrobacterium to reduce production of NSP and/or MP. Unlike with 35S-

MP/NSP, separate infiltration of either NOS-MP or NOS-NSP alone with the BeYDV vector had no detectable effect on GFP production (Fig. 2D). While infiltration of 35S-MP and 35S-NSP alone caused leaf chlorosis that progressed to necrotic lesions for NSP, NOS-MP and NOS-NSP produced no negative leaf symptoms (Fig. 2E, Fig. S1B). To test whether MP/NSP would cause spreading of GFP replicons, BeYDV GFP replicons were delivered to only a fraction of all the leaf cells using a low agrobacterium OD_{600} of 0.002 while MP/NSP vectors were infiltrated with a much higher agrobacterium OD_{600} of 0.1 to ensure they were supplied to most cells. While coinfiltration of either NOS-MP or NOS-NSP separately with pBYR-L5GFP had no notable effects (data not shown), coinfiltration of both NOS-MP and NOS-NSP simultaneously with the replicating vector under these conditions resulted in a detectable increase in GFP fluorescent area by 5 DPI (Fig. 2F).

5.4.3 Creation of Chimeric BeYDV/BDMV Dual Replicon Vectors

In native begomovirus infection, MP/NSP are expressed from replicating DNAs, which, along with the rep-supplying replicon, spread together from cell-to-cell. To more authentically model native replicon movement, a chimeric BeYDV/BDMV vector was created which expresses BDMV MP and NSP from the BeYDV bidirectional LIR promoter. The native BeYDV replication proteins were replaced by the MP, and the BeYDV coat and movement proteins were replaced by the BDMV MP. Importantly, while this vector is incapable of replicating on its own, it contains all of the necessary cis-elements for replication, and thus is capable of replication when Rep and RepA are supplied in trans from a replication-competent BeYDV vector (Mor et al. 2003b). To

optimize expression of MP and NSP, and to modify the size of the vector, various vector configurations were constructed which the reversed the relative position of the MP and NSP, and modified 5' UTR, terminator, and promoter elements (Table 1). Each of these modified hybrid vectors was then combined with the replication-competent GFP vector pBYR-L5GFP to create a single vector containing both replicons placed in tandem and separated by a LIR (Fig. 3A). This arrangement allows simultaneous delivery of both replicons on a single agrobacterium T-DNA, which are individually released and replicated in a noncompeting manner (Huang et al. 2010).

Hybrid BDMV/BeYDV vectors were compared to the original vector pBYR-L5GFP, which lacks MP/NSP, at a low agrobacterium OD600 of 0.002 to allow monitoring of individual GFP foci for movement. Numerous MP/NSP vector iterations strongly inhibited GFP expression, likely due to improper expression levels of MP/NSP. By contrast, several vector configurations produced GFP spots which strongly increased in fluorescent size between 4-7 DPI, whereas GFP spots infiltrated with pBYR-L5GFP reached peak fluorescence around 4 DPI and remained unchanged thereafter (Fig. 3B). To quantitatively assess GFP expression by these vectors, protein was extracted from agroinfiltrated leaf material and analyzed by SDS-PAGE. When hybrid or MP/NSP deficient vectors were assayed for GFP expression at an agrobacterium OD600 of 0.2, which is sufficient to deliver GFP replicons to the majority of plant cells, no differences in GFP expression were observed (Fig. 3C). By contrast, GFP expression by the hybrid vector pBYBDr was 3-fold greater at an agrobacterium OD600 of 0.02 (Fig. 3C).

Different configurations designed to alter the relative expression levels of MP and NSP in the BeYDV replicon strongly impacted vector performance (Fig. 3D).

5.4.4 BDMV MP and NSP Provide Cell-to-Cell Movement to Mastreviral Replicons

To obtain more direct evidence of cell-to-cell movement, fluorescence microscopy of agroinfiltrated leaf tissue was performed. Leaves of *Nicotiana benthamiana* were agroinfiltrated with either hybrid BeYDV/BDMV vectors containing MP/NSP, or pBYR-L5GFP lacking MP/NSP, using a low agrobacterium OD600 of 0.0002 to produce predominately isolated infected cells. As a control for authentic geminiviral movement, the T-DNA vector pBDA-GFP, which contains BDMV DNA-A modified to produce GFP in place of the coat protein (Sudarshana et al. 1998), was agroinfiltrated either alone or in combination with pBDB, a T-DNA vector containing BDMV DNA-B to supply MP and NSP in their native configuration. Leaf spots agroinfiltrated with only pBDA-GFP contained mostly isolated fluorescent cells, with an average fluorescent cluster size of 1.2 ± 0.1. By contrast, leaf spots coinfiltrated with pBDA-GFP and pBDB produced predominately clusters of fluorescent cells, with an average fluorescent cluster size of 2.4 ± 0.2 (Fig. 4A/B). Both constructs produced similar levels of intense fluorescence in individual leaf cell cytoplasm and nuclei (Fig. 4A). Similar to the results observed with BDMV DNA-A/B supplying vectors, leaf spots agroinfiltrated with pBYR-L5GFP alone showed mostly isolated fluorescent cells, whereas leaf spots infiltrated with chimeric BDMV/BeYDV vectors were found to contain larger aggregates of fluorescent cells similar in size to those observed for native BDMV (Fig. 4A/B). The average cluster size of fluorescent cells in the hybrid vector

pBYBDr was 3.1 ± 0.3 compared to 1.2 ± 0.04 for pBYR-L5GFP. Interestingly, vector configurations differed both in the extent of movement, and the proportion of cells showing movement. The larger 2,733 bp replicon from pBYBDf produced fewer cells displaying cell-to-cell movement, and a reduced number of adjacent fluorescing cells compared to pBYBDr (Fig. 4C/4C). Together, these results indicate that the BDMV MP and NSP allow cell-to-cell spread of mastreviral replicons at efficiencies similar to native begomoviral replicons when the expression levels of MP and NSP are optimized.

5.4.5 BeYDV and BDMV Replicon Size Restricts Movement

The BDMV MP and NSP have been reported to bind DNA in a size-dependent manner. To test whether chimeric BeYDV/BDMV vectors were size-restricted, a dual-replicon vector (pBYBDf-3.9) was constructed which contains an unmodified MP/NSP replicon, but has a substantially larger 3.9kb GFP replicon. The larger replicon was constructed by insertion of a 35S promoter driving GFP, as well as insertion of a longer form of the extensin 3' UTR. These modifications were designed to increase replicon size while minimally altering the activity of the vector, as the LIR and 35S promoters are both strong, and the short and long forms of the extensin terminator have not been found to vary substantially in activity in BeYDV vectors (data not shown). While the original vector containing a 2.5kb GFP replicon was found to substantially increase in GFP fluorescence between 4 and 8 DPI, the vector containing the larger 3.9kb replicon had only minimal changes in GFP fluorescence after 4 DPI (Fig. 5). A 3.1kb vector containing an intermediately sized 3' UTR also inhibited GFP fluorescence after 4 DPI compared to the 2.5kb vector (data not shown), in agreement with previous findings that

the BDMV MP/NSP function primarily with genome-sized DNA molecules (Gilbertson et al. 2003). However, pBYBDb, which contains a 2,183 bp MP/NSP replicon, had strong evidence of movement (Fig. 3D/4B), indicating that reductions from the native genome size are also functional.

5.4.6 Characterization of AbMV MP and NSP Expression with BeYDV Replicons

The AbMV MP and NSP have shown similar DNA-Binding properties to BDMV (Hehnle et al. 2004), however AbMV is phloem-limited while BDMV is not. To explore whether AbMV MP and NSP can also function with masterviral replicons *in planta*, we produced AbMV MP and NSP in *Nicotiana benthamiana* leaves. Similar to the results found for BDMV NSP, overexpression of AbMV NSP strongly inhibited expression from BeYDV GFP replicons (Fig. 6A) whereas MP did not. When expressed at high levels, AbMV NSP elicited a stronger cell death response than that observed for BDMV, whereas AbMV MP was no more toxic than BDMV NSP (Fig. 6B). Chimeric AbMV/BeYDV dual replicon vectors modeled after functional BDMV/BeYDV vectors were constructed and tested for their capacity to provide functional cell-to-cell movement. Similar to the results observed with BDMV, significant increases in leaf fluorescent area were observed with the AbMV MP and NSP, which corresponded to increased numbers of adjacent fluorescent cells (Fig. 6C/6D). We further created a chimeric AbMV/BDMV/BeYDV replicon expressing the BDMV NSP and AbMV MP, which also performed similarly (Fig. 6C/6D). These data suggest that AbMV MP and NSP also provided cell-to-cell movement to BeYDV replicons, and that AbMV MP can function with BDMV NSP.

5.5 Discussion

Genetic recombination is a major driving force of virus evolution and has been very well documented for geminiviruses (Varsani et al. 2008). Unlike the RNA viruses, which contain error-prone polymerases, geminiviruses replicating using host machinery, which has much higher fidelity. Despite this, they maintain error rates similar to those of RNA viruses (Duffy et al. 2008). It seems likely that the high mutation rate coupled with the high propensity for recombination has resulted in the emergence of many devastating agricultural diseases caused by geminivirus infection (Lefeuvre and Moriones 2015).

As would be expected of viruses that arose through inter-species and inter-strain recombination, there are many examples of the capacity for components of related geminiviruses to functionally complement each other (Briddon et al. 1990; Comai et al. 1990; Levy and Czosnek 2003; van der Walt et al. 2008; Varsani et al. 2008; Monjane et al. 2012; Monjane et al. 2014). However, previous attempts to make a mastrevirus-based expression vector capable of cell-to-cell movement have failed. Inserting an herbicide resistance expression cassette into the wildtype sequence of maize streak virus did not hinder replication, but abolished systemic movement, presumably due to increased genome size (Shen and Hohn 1995). Further studies retained the wildtype genome size by using two separate replicons with movement functions on one, and replication functions on the other, each of proper size. Yet, recombination quickly produced wildtype virus, which predominated. As an alternate strategy, wildtype virus was coinfected with a replication-competent vector of proper size. These efforts still lead to preferentially spreading of only the wildtype genome, demonstrating some selectivity in DNA-binding

capacity of the MP and CP exists (Palmer and Rybicki 2001; van der Walt et al. 2008). In agreement with these data on maize streak virus, we found expression of BeYDV MP and CP separately or from wildtype BeYDV genomes failed to provide movement function to recombinant BeYDV genomes, even when appropriately sized (data not shown), strongly suggesting mastreviral CP and MP function recognizes elements of the wildtype genome. Therefore, we evaluated the movement machinery from bipartite begomoviruses to function with mastreviral replicons.

To create a suitable reporter system capable of generating appropriately sized GFP-expressing mastreviral replicons, we fused GFP to the BeYDV LIR virion-sense promoter which natively drives expression of the MP and CP. The precise mechanism by which the BeYDV MP and CP are produced is unknown. As the LIR is thought to be the only functional mastrevirus promoter element, the MP and CP are suspected to be mainly produced from a single dicistronic transcript. While an intron was shown to be active in the MP gene of maize streak virus (Wright et al. 1997), none has been detected in BeYDV, though it has been suggested that the CP in BeYDV may be produced by translational frameshifting (Dekker et al. 1991). When the CP was replaced with GFP, we found that mutation of the MP start codon prevented GFP expression. These data support a mechanism such as translational frameshifting, which would require initiation to first occur at MP for later CP translation (Fig. 1B). We further found that a small intervening sequence between the MP and CP coding sequences was required for GFP expression, though GFP expression was still low in this configuration, suggesting the native CP coding sequence may play a role in efficient CP production. Fusion of GFP directly

downstream from the LIR resulted in robust GFP production, indicating that the low levels of observed GFP expression with CP replacements likely did not result from a lack of transcriptional capacity by the LIR promoter, providing further support for translation as the key determining factor in MP/CP production. When GFP was placed in an optimal translation initiation context (Sugio et al. 2010), especially with the TMV 5' UTR, a known translational enhancer (Gallie 2002), very high levels of GFP were produced from the LIR promoter (Fig. 1B).

The MP and NSP from BDMV have been reported to bind DNA in a sequence nonspecific manner, allowing cell-to-cell movement of recombinant BDMV genomes (Sudarshana et al. 1998). MP was also shown to facilitate cell-to-cell movement of appropriately sized plasmid DNA (Gilbertson et al. 2003). We found that BDMV NSP strongly inhibited GFP expression from genome-sized BeYDV replicons when produced from the strong 35S promoter (Fig. 2B/C), suggesting that NSP efficiently interacts with mastreviral replicons. We suspect high levels of NSP may reduce the availability of transcription templates, either by transport out of the nucleus or interfering with replication, as NSP binds most DNA forms at high concentrations, forming collapsed structures (Rojas et al. 1998; Hehnle et al. 2004). Increased cell death was also observed with overexpression of NSP (Fig. 2E/S1), however GFP inhibition occurred many days prior to the development of leaf necrosis. Expression of NSP from a weaker promoter did not appreciably impact GFP expression (Fig. 2D) and in support of the general sequence-nonspecific transport capabilities of the BDMV MP and NSP to move genome-sized

DNA molecules, coexpression of each by the NOS promoter caused an increase in GFP fluorescent area produced by recombinant mastreviral replicons (Fig. 2F).

In our initial studies, MP and NSP were supplied on nonreplicating T-DNA vectors and thus would be unable to efficiently spread cell-to-cell, necessitating delivery with high agrobacterium concentrations that may contribute to plant cell death. Therefore, we constructed chimeric BeYDV/BDMV genomes using the replication and transcription elements from BeYDV to produce MP and NSP from BeYDV replicons. These vectors provided very high levels of GFP expression even when the agrobacterium concentration was reduced by a factor of 10, unlike vectors lacking the BDMV MP and NSP (Fig. 3C). By fluorescence microscopy, MP and NSP expressing replicons produced fluorescent cell clusters similar in size to those reported for BDMV replicons (Levy and Czosnek 2003; Gilbertson et al. 2003), which agreed with our results coinfiltrating BDMV DNA-B and DNA-A modified with GFP replacing the CP (Fig. 4C). While geminivirus NSP has been shown to be involved in preventing host translation shut-off (Zorzatto et al. 2015), we found no difference in individual cell fluorescence (Fig. 4A), only in the number of fluorescent cells (Fig. 4C), which correlated with the increased GFP fluorescent area observed in leaves and the measured GFP expression data (Fig. 3B/D). Furthermore, increased GFP expression required that replicons not be delivered to all cells (Fig. 3C) and no increased expression or evidence of movement was seen when MP or NSP were delivered separately (Fig. 2, and data not shown). These data strongly suggest increases in GFP fluorescence result from cell-to-cell movement, not from increased expression of GFP per cell.

Geminivirus gene products are temporally regulated: in the early stages of infection, complementary-sense nonstructural gene products predominate, which then activate virion-sense gene products late in infection (Muñoz-Martín et al. 2003). Native NSP production requires transactivation late in infection by AC2 (Berger and Sunter 2013), so we inserted NSP under the control of the late-acting virion-sense LIR promoter, mimicking the native configuration of DNA-B. Attempts at reversing the positions of MP and NSP strongly inhibited GFP expression (Fig. 3D, Table 1), probably due to rapid accumulation of NSP. Furthermore, efficient cell-to-cell movement was highly sensitive to the precise levels of MP and NSP being produced. Increasing NSP expression by addition of a stronger 5' UTR or additional terminator, which we and others have previously shown to be a general enhancer of gene expression (Beyene et al. 2010, and data to be presented elsewhere) severely inhibited GFP replicon expression. However, decreases in NSP expression also strongly limited function. Mutation of the LIR v-sense TATA-box (TATAAA to TATAAG) decreased promoter activity by an estimated 50% without impacting replication (Fig. S2A and S2B). While the hybrid vector pBYBDb-L5GFP produced efficient GFP spreading, introduction of the v-sense TATA-box mutation to reduce NSP expression also reduced GFP fluorescent area by 65% (Fig. 3D).

Geminiviruses contain bidirectional terminator elements at the ends of the complementary-sense and virion-sense genes. For BeYDV and BDMV DNA-B, we have shown that efficient termination requires the upstream coding sequence of the gene being terminated, the SIR in the case of BeYDV, and also the downstream reverse coding sequence of the opposite-sense gene (data to be presented elsewhere). Interestingly, when

the native 3' ends of BDMV DNA-B, including the wildtype intergenic region, were used in the chimeric vector pBYBDp, GFP fluorescent area was strongly reduced (Fig. 3D). Since the BeYDV LIR was the only difference between this vector and the BDMV DNA-B, and further since DNA-B supplied levels of MP and NSP adequate for efficient movement (Fig. 3D/4B), this suggests intrinsic differences in promoter activity between BDMV and BeYDV produced improper levels of MP and/or NSP. However, it cannot be ruled out that the small replicon size of this vector (2,017 bp) affected its performance, though the 2,183 bp MP/NSP replicon of pBYBDb was highly functional.

The BDMV and Tomato Leaf Curl New Delhi Virus NSP have been shown to induce the plant hypersensitive response in *Phaseulus vulgaris* and *Nicotiana benthamiana* respectively (Hussain et al. 2005; Zhou et al. 2007a). However, the experiments done with *Nicotiana benthamiana* involved overexpression of NSP with viral vectors. We found both MP and NSP produced leaf chlorosis when expressed from the strong 35S promoter (Fig. 2E), and NSP leaf spots eventually developed necrotic lesions, especially for AbMV (Fig. S1/6B). However, expression from the weaker NOS promoter produced no visible symptom development with either protein (Fig. 2E). Replicating vectors expressing MP and NSP at levels that produced efficient cell-to-cell movement had no notable differences in leaf toxicity compared to pBYR-L5GFP alone, nor did native BDMV DNA-B with pBDA-GFP. However, replicating hybrid vectors overexpressing NSP that had impaired GFP expression produced higher levels of tissue necrosis (data not shown). Together with our expression results, our data suggest efficient cell-to-cell movement *in planta* strongly depends on the relative expression levels of MP

and NSP. MP and NSP expression may be fine-tuned to strike a balance between eliciting the hypersensitive response, providing efficient cell-to-cell movement capabilities, and allowing replicon availability for transcription. Disruption of this balance may play a role in determining host susceptibility to geminivirus infection. Further studies which can precisely quantify MP/NSP expression levels are needed to better address these questions.

Using size-increased mastrevirus replicons, movement function by MP and NSP was inhibited by even a small increase from a 2.5kb to a 3.0 kb replicon, and severely inhibited with a 3.9 kb replicon (Fig. 5A/B). Our data agrees with the findings of Gilbertson et al. (2003), who found that MP-mediated movement of 3.4kb DNA molecules was moderately limited and 5.5kb DNA molecules were severely limited in *Nicotiana benthamiana* leaves. Function movement was observed only with 2.1-2.7 kb replicons (Fig. 3D/4B), supporting the notion that MP and NSP primarily function with genome-sized DNA molecules. NSP strongly inhibited production from replicons in excess of 4 kb (data not shown), in agreement with previous results that MP is primarily responsible for the size limitation (Gilbertson et al. 2003; Hehnle et al. 2004). For biotechnology purposes, this limits the utility of geminiviral movement-based vectors, as larger recombinant proteins would exceed the size restrictions for efficient cell-to-cell movement. Furthermore, many DNA-based systems achieve maximal expression using long genetic elements to enhance gene expression that require larger replicon sizes (Diamos et al. 2016).

Abutilon mosaic virus (AbMV) is restricted to the phloem, whereas the related bean dwarf mosaic virus (BDMV) can spread through epidermal, cortical and phloem cells in *Phaseolus vulgaris* (Wang et al. 1996; Wege et al. 2000). Prior studies have found that complementing the DNA-A from AbMV with the DNA-B of BDMV confered enhanced tissue invasion to the AbMV DNA-A, successfully moving AbMV out of the phloem. Interestingly, the DNA-B from AbMV did not limit the movement capacity of BDMV DNA-A, despite its phloem limitation when paired with its own DNA-A (Levy and Czosnek 2003). Additionally, a begomovirus lacking DNA-B entirely was complemented in movement function by both a curtovirus and a topocuvirus mixed infection (Briddon and Markham 2001). Despite the phloem limitation of the native AbMV, we found AbMV NSP interacts with BeYDV replicons similarly to BDMV (Fig. 6A), and AbMV MP can move BeYDV replicons cell-to-cell with similar or greater efficiencies compared to BDMV MP and NSP (Fig. 6C/6D). These data further support the hypothesis that the AbVM phloem limitation does not arise from the intrinsic function of the MP and NSP themselves, as no cell-to-cell movement limitations were observed when AbMV MP and NSP were expressed at appropriate levels in non-phloem cells.

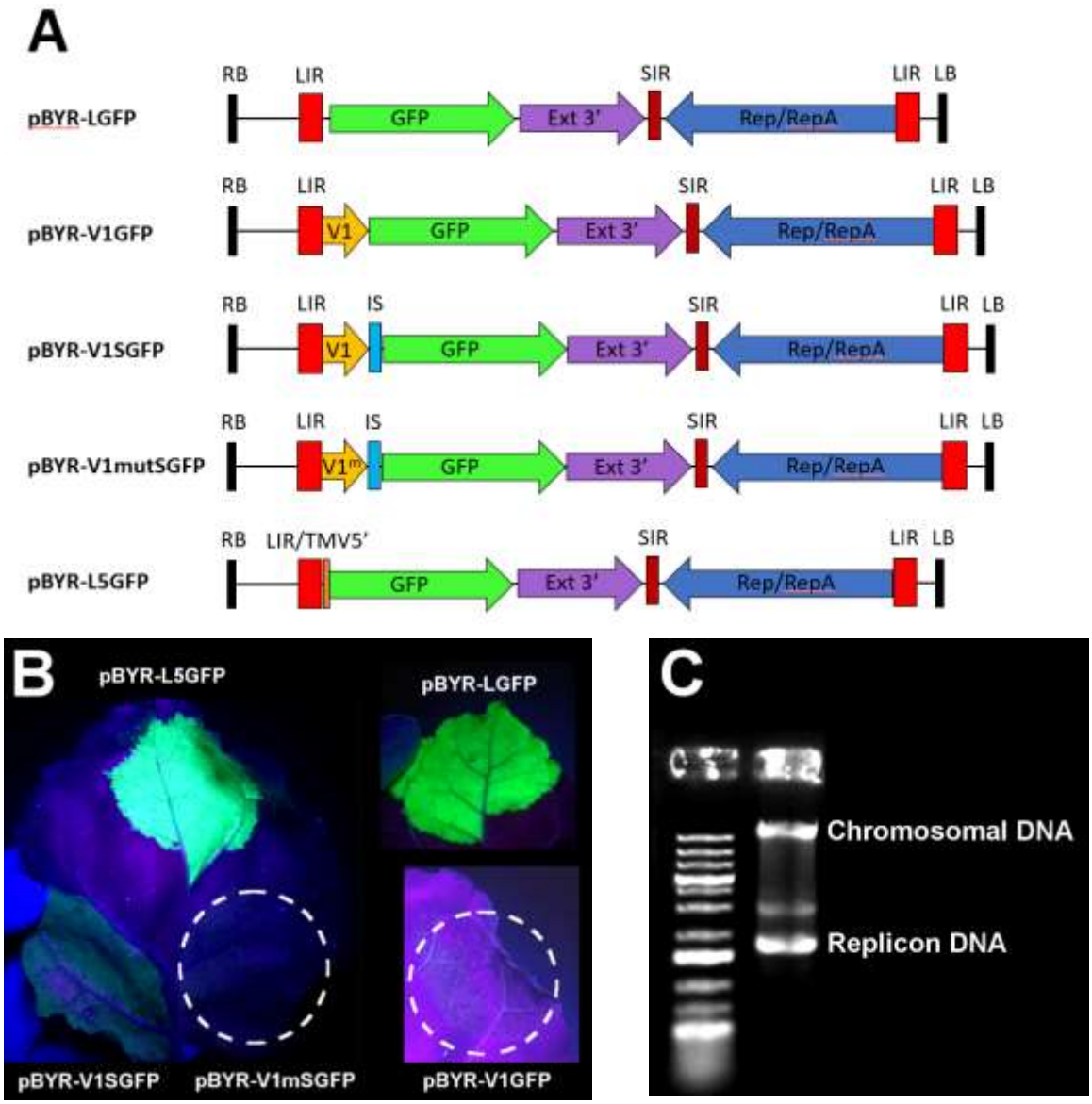
In summary, we have characterized production of MP and NSP from the bipartite begomoviruses BDMV and AbMV in *Nicotiana benthamiana* leaves, showing that the MP and NSP from either confer functional cell-to-cell movement to recombinant mastreviral replicons. Overexpression of MP and especially NSP caused plant toxicity, however production of functionally relevant levels of MP and NSP did not cause notable symptoms with BeYDV replicons. A chimeric vector containing a BeYDV replicon

supplying BDMV or AbMV MP and NSP from the LIR promoter provided cell-to-cell movement to replication-competent BeYDV GFP vectors, comparable or exceeding the movement efficiency of the DNA-B movement of native BDMV replicons. These vectors result in rapid accumulation of very high levels of GFP even when typical agrobacterium concentrations are substantially reduced. We find that efficient cell-to-cell movement strongly depends on the size of the replicons and on the expression dynamics of MP and NSP, which may have implications for the host range and tissue limitations of geminiviruses.

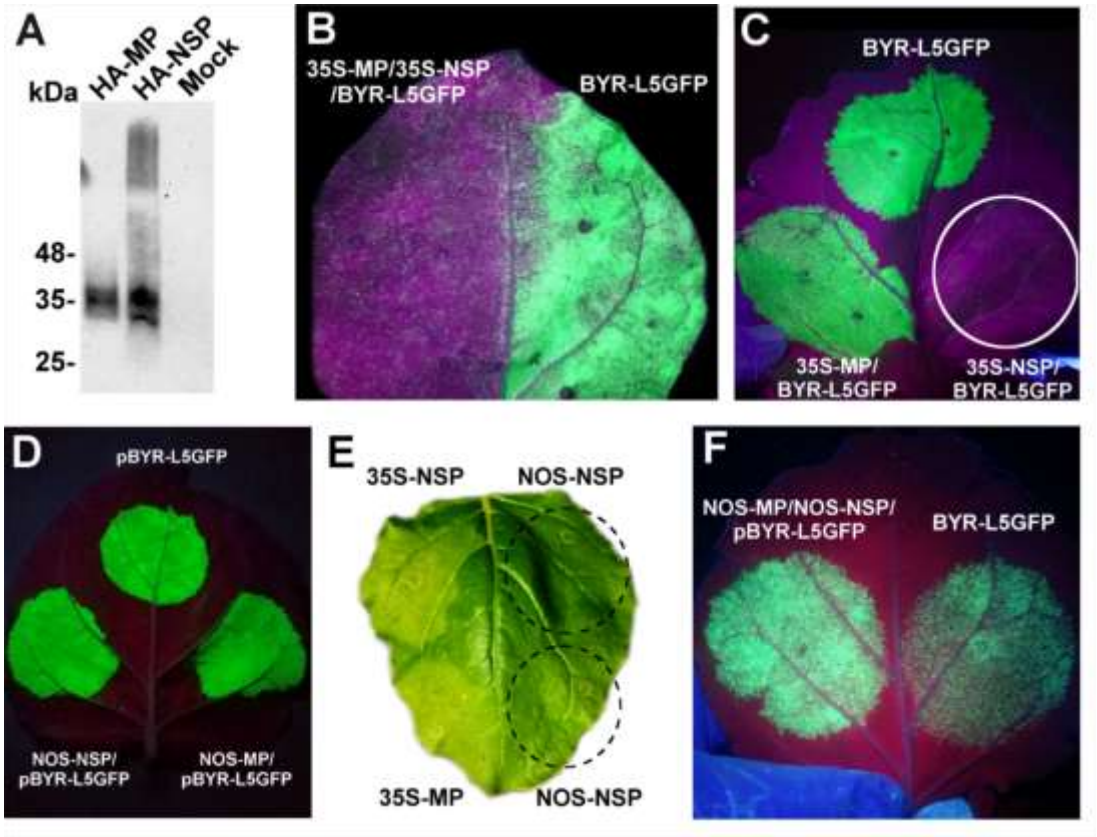
5.6 Figures

Vector	F1	LIRv	T1	SIR	T2	LIRc	F2	Size	Activity
BYBDa	TMV 5’	NSP	None	SIR	-	MP	-	2,247	-
BYBDb	-	NSP	None	SIR	-	MP	-	2,183	++
BYBDc	LIR Mut1	NSP	PinII	SIR	-	MP	-	2,491	++
BYBDd	LIR Mut2	NSP	None	SIR	-	MP	-	2,183	+
BYBDf	-	NSP	VspB	SIR	-	MP	-	2,733	+
BYBDh	-	MP	VspB	SIR	-	NSP	-	2,733	---
BYBDi	-	NSP	Ext	SIR	-	MP	-	2,658	---
BYBDk	TEV 5’	NSP	35S	SIR	-	MP	-	2,533	---
BYBDl	-	NSP	None	SIR	VSP	MP	-	2,748	-
BYBDm	-	NSP	PinII	SIR	-	MP	-	2,492	+
BYBDp	-	NSP	None	BD	-	MP	-	2,017	+
BYBDq	-	NSP	None	SIR	-	MP	TMV 5’	2,243	++
BYBDr	LIR Mut1	NSP	PinII	SIR	-	MP	TMV 5’	2,562	+++

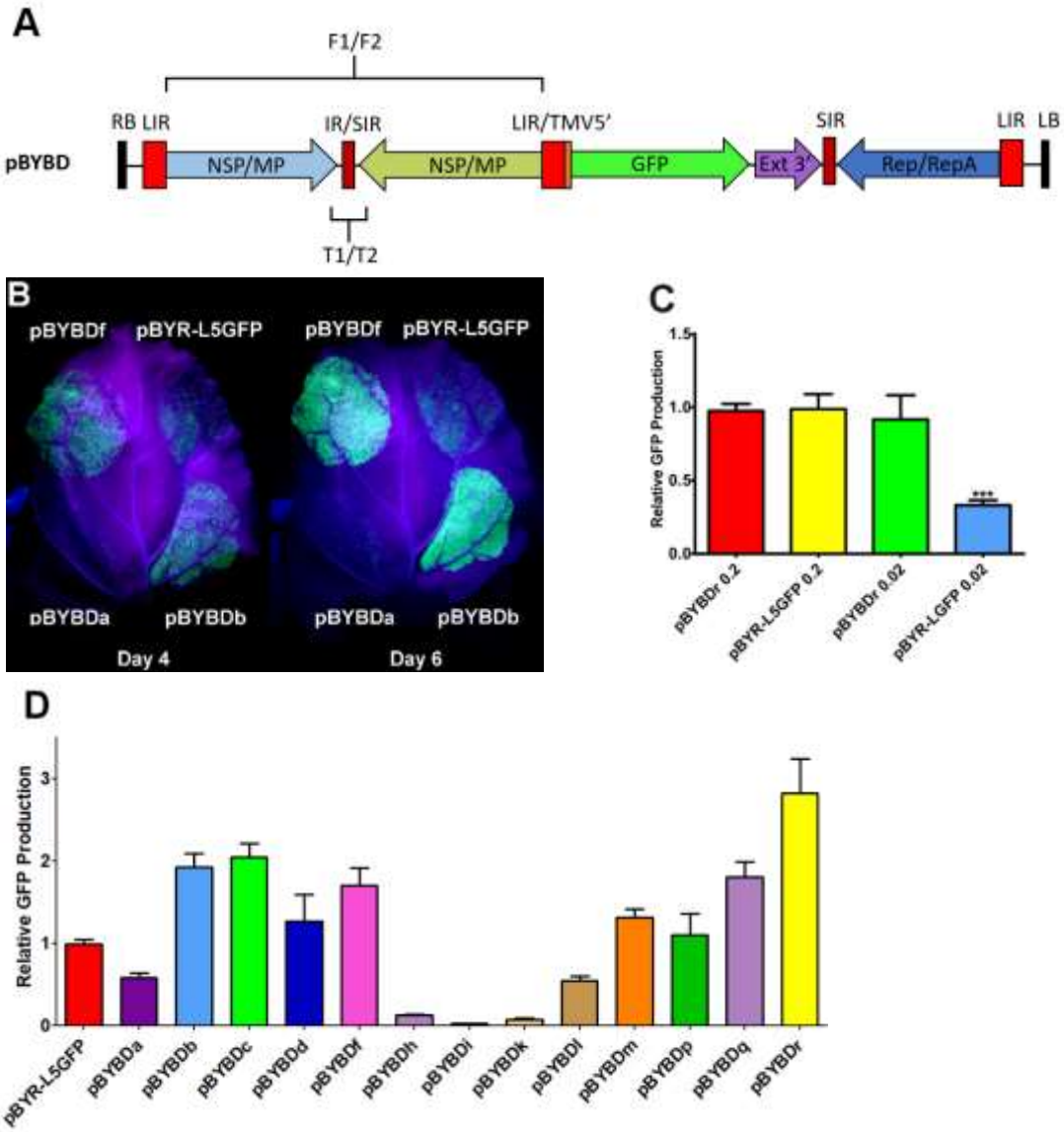
5.6.1 Table 1. Chimeric BeYDV/BDMV Dual Replicon Vector Components
Dual replicon vectors containing the indicates genetic components for expression of BDMV MP and NSP. A GFP replicon vector identical to pBYR-L5GFP is also present in all constructs. Flanking 1, upstream modifications from MP/NSP driven by v-sense LIR promoter; LIRv, the gene driven by the v-sense LIR promoter; T1, additional terminator for LIRv in addition to SIR; SIR, bidirectional terminator region from either BeYDV (SIR) or BDMV (BD); T2, additional terminator for LIRc in addition to SIR; LIRc, gene driven by the c-sense LIR promoter; F2, upstream modifications from the gene driven by LIRc; TMV 5’, 5’ UTR from tobacco mosaic virus; LIR Mut1; the start codon sequence context contains (GGAATG) mutation to reduce gene expression, with mutation in bold; LIR Mut2; the LIR TATA-box contains (TATAAG) mutation to reduce gene expression; TEV 5’, 5’ UTR from tobacco etch virus; PinII, the potato proteinase inhibitor II terminator; VspB, the soybean vegetative storage protein B terminator; Ext, the tobacco intronless extensin terminator; 35S, the 35S terminator from cauliflower mosaic virus.



5.6.2 Figure 1. Construction of Mastreviral Reporter System
(A) Generalized schematic representation of the T-DNA region of recombinant mastreviral genomes expressing GFP. RB and LB, the right and left borders of the Agrobacterium T-DNA region; LIR, the long intergenic region from BeYDV; V1, the movement protein gene from BeYDV; V1mut, V1 with mutated start codon; IS, the small intergenic sequence between the BeYDV movement and coat protein genes; GFP; the plant-optimized coding sequence of green fluorescent protein; Ext 3'; a truncated form of the gene terminator from tobacco extensin; SIR, the short intergenic region from BeYDV; Rep/RepA, the replication associated genes from BeYDV; TMV5', the 5' UTR from tobacco mosaic virus. (B) Leaves of *Nicotiana benthamiana* were agroinfiltrated with the indicated vectors and representative images showing GFP fluorescence are shown.

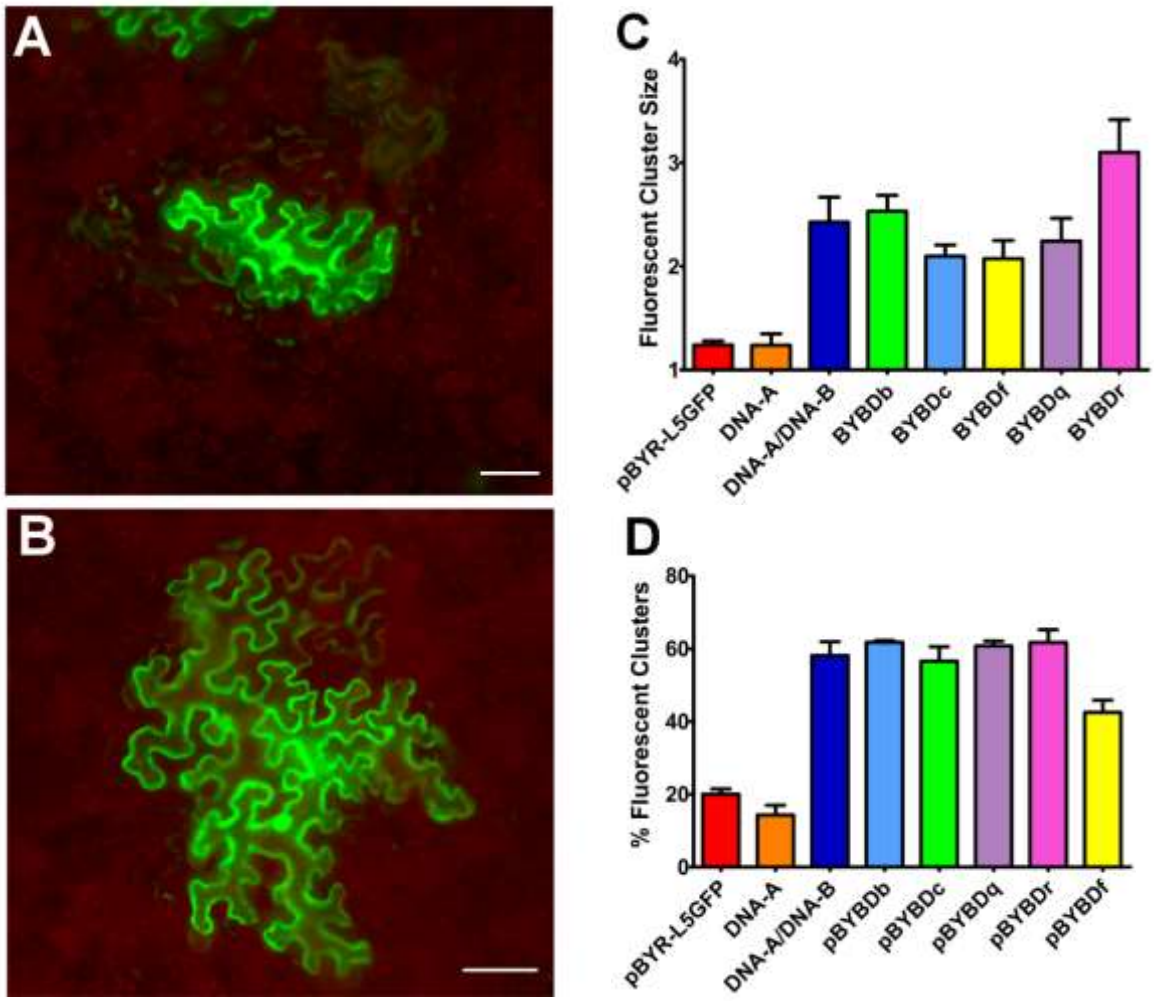


5.6.3 Figure 2. Coexpression of BDMV MP and NSP with Mastreviral Replicons
(A) Leaves of *Nicotiana benthamiana* were agroinfiltrated with HA-tagged 35S-MP or 35S-NSP detected by western blot with HA-specific antibodies. Mock samples were infiltrated with the empty vector pPS1. (B-F) Leaf spots were coinfiltrated with the mastreviral replicon vector pBYR-L5GFP and either pPS1 or the indicated vectors. Representative leaves were photographed after 3-5 DPI under UV light to monitor GFP fluorescence or visible light to monitor leaf health.

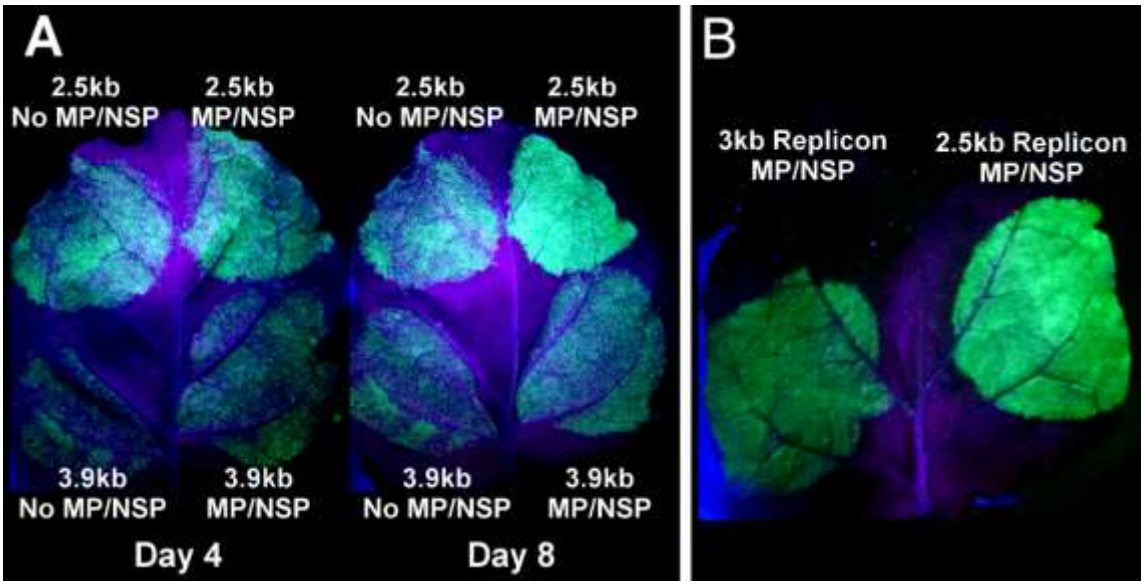


5.6.4 Figure 3. Characterization of GFP Expression by Chimeric BDMV/BeYDV Dual Replicon Vectors

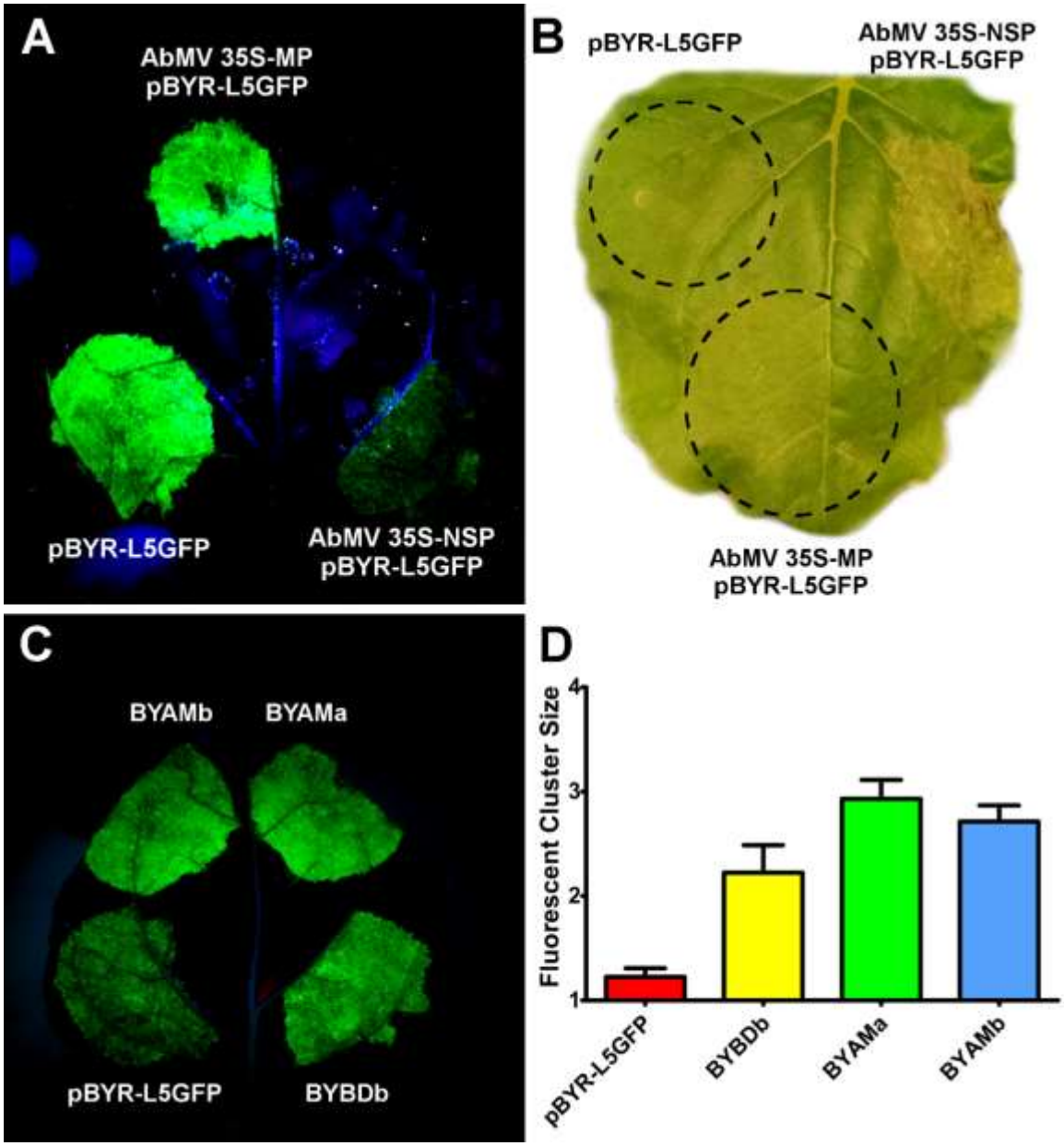
(A) Schematic of the general arrangement of chimeric BDMV/BeYDV dual replicon vectors (pBYBDx), allowing replicational release of a MP/NSP-containing replicon and a separate GFP/Rep replicon identical to pBYR-L5GFP. The components F1/F2 and T1/T2 refer to flanking region modifications and terminator insertions respectively as described in Table 1. (B) Leaves agroinfiltrated at the OD_{600} values of 0.2 or 0.02 as indicated with either chimeric vectors or pBYR-L5GFP as control were imaged at 4 DPI (left) or 6 DPI (right). (C,D) Protein extracts from agroinfiltrated leaves were analyzed for GFP expression by SDS-PAGE and quantification of the fluorescent band using ImageJ software. Columns represent means ± standard deviation of 3 independently infiltrated samples.



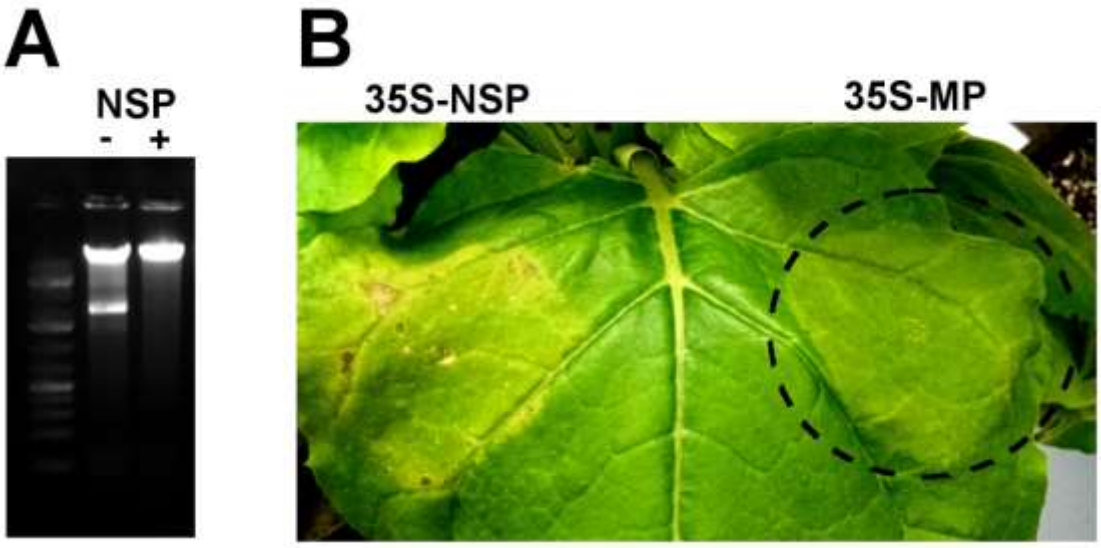
5.6.5 Figure 4. Fluorescence Microscopy of Chimeric BeYDV/BDMV Replicons
Leaves of *Nicotiana benthamiana* were agroinfiltrated with chimeric BeYDV/BDMV replicon vectors (pBYBD- shown in Table 1) or T-DNA vectors supplying BDMV DNA-A and DNA-B and analyzed by fluorescence microscopy after 5 DPI. Representative images were taken showing (A) individual fluorescent cells seen predominately without MP/NSP expression or (B) clusters of fluorescent cells seen predominately with MP/NSP expression. (C) Average sizes of fluorescent cell clusters or (D) the proportion of individual fluorescent cells to clustered fluorescent cells were counted. Columns represent means ± standard deviation of 50 or more cell counts from two independent experiments.



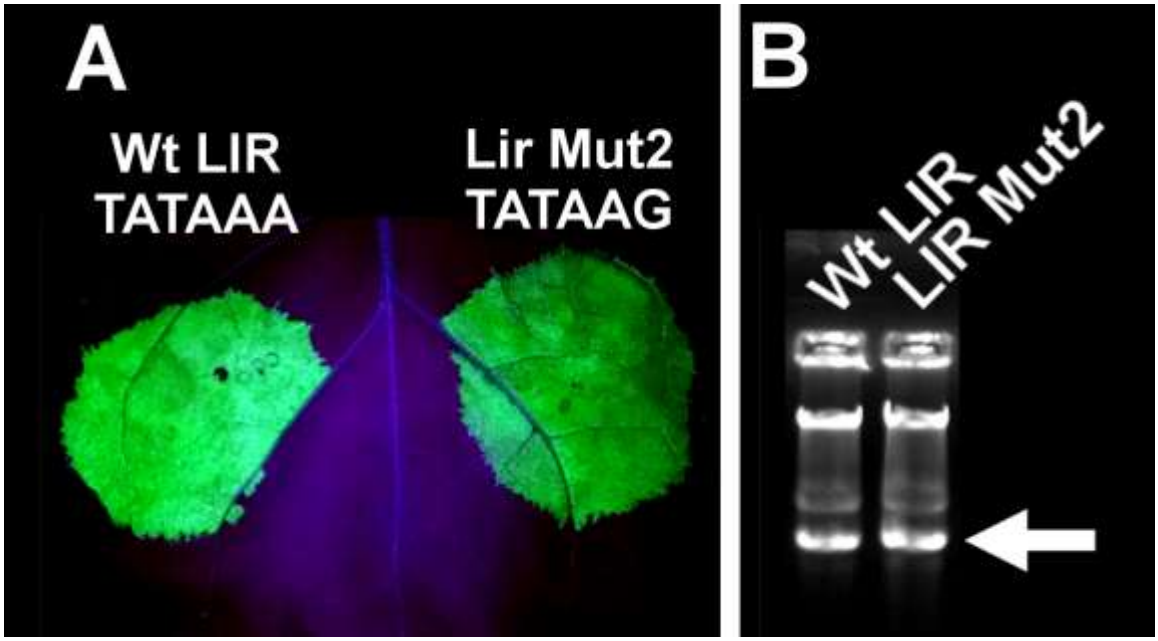
5.6.6 Figure 5. Replicon Size Limitations of BDMV MP and NSP
(A) Leaves were agroinfiltrated with either pBYBDf, containing the 2.5kb GFP replicon from pBYR-L5GFP, or pBYBDf3.9, containing a 3.9kb GFP replicon with two insertions: (1) the 35S promoter with duplicated enhance regions driving GFP, and (2) the intronless tobacco extensin terminator. Both vectors contain identical 2.7kb MP/NSP replicons. Images were taken under UV light at 4 DPI (left leaf) and 8 DPI (right leaf).
(B) Leaves were agroinfiltrated with either pBYBDf3.0 (3kb GFP replicon), which contains an inserted 35S promoter driving GFP, or pBYBDf (2.5kb GFP replicon). Images were taken under UV light at 6 DPI.



5.6.7 Figure 6. Characterization of AbMV MP and NSP with BeYDV Replicons (A,B) The strong 35S promoter was used to produce AbMV NSP (pABV1e) and MP (pABC1e) in plant leaves. Coinfiltration of AbMV MP and NSP with pBYR-L5GFP is shown (A) under UV light or (B) in visible light after 4 DPI. (C) Agroinfiltrated leaf spots imaged at 5 DPI under UV light with hybrid vectors modeled after pBYBDb containing both AbMV MP and NSP (pBYAMa) or AbMV MP and BDMV NSP (pBYAMb).



5.6.8 Figure S1. NSP Inhibits Replicon Accumulation and Induces Necrosis
(A) Replicon DNA extracted from leaves of *Nicotiana benthamiana* agroinfiltrated with pBYR-L5GFP either with or without 35S-NSP (pBV1e). (B) Leaf spots agroinfiltrated with 35S-NSP (pBV1e) or 35S-MP (pBC1e) and imaged at 8 DPI to show leaf cell death.



5.6.9 Figure S2. Mutation of LIR TATA-Box Reduces Gene Expression Not Replicon Accumulation

Leaves were agroinfiltrated with either pBYR-L5GFP containing the wildtype LIR TATA-box or with a vector containing a single nucleotide change (TATAAA to TATAAG) and characterized for (A) GFP expression and (B) replicon accumulation.

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CHAPTER 6

CONCLUSIONS AND FUTURE WORK

6.1 Viral Vector Choice and Cell-to-Cell Movement

While many of the improvements identified here show the benefits of optimizing the genetic elements of DNA-based expression systems, there are many questions left unanswered by this work. One of the most fundamental questions left unaddressed is whether bean yellow dwarf virus is an optimal plant virus upon which to base a gene expression system, especially due to its cell-to-cell movement limitations. Many more mastreviruses have been discovered since the initial work done on BeYDV, and many more types of plant viruses in general as well. Nanoviruses consist of 6-8 genome compartments, each essentially carrying a single gene that each undergo rolling circle replication similarly to geminiviruses. The genome compartments are individually encapsidated and spread throughout infected plants. Could it be possible to construct a DNA vector system using nanoviruses or other plant viruses capable of efficient cell-to-cell or even systemic movement of one or more recombinant proteins? While cell-to-cell movement was achieved with BDMV MP and NSP, the extent of movement was far more limited than that observed with RNA virus expression systems, and only functional with small proteins. Furthermore, preliminary attempts to produce three replicons: one for MP/NSP, one for rep and an antibody light chain, and one for an antibody heavy chain, were unsuccessful. Whether this represents inefficient replicon production or inefficient cell-to-cell movement remains to be determined. An additional strategy could be to incorporate geminiviral replicons, or some other component of an expression

system, into transgenic plants. Using an inducible system that allows activation only at a desired timepoint, the advantages of transgenic systems, such as the production of recombinant protein by every cell without the need for vector movement, could be combined with the rapid, high yield of transient expression systems.

However, many of these questions depend whether cell-to-cell movement capabilities are actually necessary or useful. Bean yellow dwarf virus appears to produce saturating levels of target gene transcripts, and is relatively benign to plant cells while doing so, at least compared to other viral expression systems, offering tremendous potential for high yields of recombinant protein. Furthermore, replicating vectors already achieve 6-fold reductions in *Agrobacterium* concentration compared to nonreplicating vectors, and there seems to be excellent potential for the optimization of *Agrobacterium* itself as a gene-delivery vehicle. It was shown over 15 years ago that incorporation of mutant forms of *Agrobacterium vir* genes, even when expressed separately from the chromosome on the T-DNA binary vector, can strongly affect the T-DNA transfer process. Future work may explore these and other modifications of EHA105 or other *Agrobacterium* strains.

One of the unique advantages of DNA-based systems is the ability to simultaneously express 2 or more genes. BeYDV vectors produce two proteins, such as monoclonal antibodies, from a single replicon with two expression cassettes, or from tandem-linked replicons. Placing three replicons in tandem has produced variable results, however using larger replicons with multiple expression cassettes has yielded very promising results. This creates an interesting niche for the production of heterooligomeric

proteins, such as IgAs, rotavirus VLPs, bluetongue VLPs, and other vaccine components or biopharmaceuticals that cannot be efficiently produced with other systems. Using the studies presented here, individual genes could be expressed at controlled levels by changing the 5' and 3' flanking regions. Such fine-tuning of expression may be useful to produce certain heterooligomeric proteins that do not have equal ratios of each individual component. Further work is needed to attempt production of these complex heterooligomeric proteins using BeYDV vectors.

6.2 Circumventing the Plant Immune System

Unlike the RNA viruses which carry out their life cycle in the cytoplasm, DNA viruses like the geminiviruses replicate in plant cell nuclei. Studies on Abutilon mosaic virus have shown that geminivirus DNA associates with cellular histones to form viral minichromosomes (Pilartz and Jeske 2003; Paprotka et al. 2015). As a result, geminiviruses are susceptible to repressive DNA and histone methylation as a defense mechanism by plants (Raja et al. 2010; Raja et al. 2014). Heavily methylated genomes are associated with plant recovery from infection (Rodríguez-Negrete et al. 2009). Due to this unique property, geminivirus-based vectors must overcome host silencing at both the transcriptional level and post-transcriptional level. Future work might investigate whether silencing suppressors that predominately target components of the immune system like DCL3, which are involved in transcriptional gene silencing, may benefit geminiviral expression systems. For instance, the begomoviruses produce several auxiliary proteins not seen in mastreviruses with silencing suppressor activity.

Previous studies with BeYDV vectors have found that coexpression of the P19 silencing suppressor from tomato bushy stunt virus has the capacity to enhance mRNA accumulation and protein production (Huang et al. 2009). P19 interferes with RISC formation by sequestering 20-22 nt siRNA (Vargason et al. 2003). Additionally, by a separate mechanism, P19 induces miRNA168, which downregulates AGO1, inhibiting RISC formation (Várallyay et al. 2014). However, even with P19 coexpression, transgene RNA declines after 4-5 DPI, possibly indicating that post-transcriptional gene silencing overwhelms P19s anti-silencing capacity, leading to transcript degradation. This suggests further silencing reduction may be possible. Alternatively, disruption of normal cellular processes by accumulation of the target protein, or viral replicons, may lead to translation shutoff (Zorzatto et al. 2015) and/or eventual cell death. Surely, a theoretical limit exists as to how much recombinant protein a cell can produce before no longer being able to function normally. However, it may be possible to silence some of the surveillance mechanisms that lead to translation shutoff, or to increase production of molecular chaperones or other gene products that may allow maximal devotion of plant resources to the production of recombinant proteins. To this end, the generation of transgenic plants tailored towards a particular gene expression system could be explored. The benefits of transgenic plants capable of producing human-type glycosylation are a powerful demonstration of the ability to modify the plant hosts themselves to improve plant-based expression systems. Especially with the advent of new gene editing technologies, it seems exciting potential exists in this area. Using hairpin RNAs, the silencing machinery can be turned against itself: dsRNA targeting endogenous plant genes allows even components of the silencing machinery to themselves be silenced. This sort of targeted gene silencing

can be employed either transgenically, or delivered by agrobacterium to effect silencing at the time of agroinfiltration. It may be possible to design multi-targeted hairpin RNAs that allow silencing of numerous plant genes simultaneously from a single hairpin transcript.

6.3 Mechanisms Controlling Genetic Elements

In many cases, the work here presents only an initial investigation into the many genetic processes that control gene expression. In the case of 5' UTRs, different sequences, especially those viral in origin, appear to either disrupt cellular processes or are otherwise detected by immune surveillance mechanisms, leading to cell death. Further work is needed to better understand these mechanisms, which may also present opportunities to avoid, disable, or otherwise subvert them. Another unanswered question is the mechanism by which the Rb7 MAR improve gene expression and reduces cell death. Perhaps replicon DNA is processed to appear more "plant-like" with the incorporation of native plant elements? Or perhaps replicon accumulation is simply reduced by the production of larger replicons, similar to the cell death reductions observed with rep/repA reduction? As limited examples of MARs were explored in these studies, it would be interested to see if other similar genetic regions have similar effects, or to further investigate the precise regions and mechanisms involved in enhancement of gene expression.

While this work clearly demonstrates the broad potential for combined gene terminators to improve gene expression, the mechanisms underlying this observation are relatively unexplored. For instance, reversing the positions of each individual terminator

in a double terminator combination resulted in major differences in performance. This suggests the elements within each terminator can synergistically work together, if appropriately positioned. It would be interesting to determine which regions of the terminator are cooperating to produce this effect. It is also conceivable to combine individual elements from different terminators to create synthetic gene terminators. Many of the individual terminators and other genetic regions identified in this work could be studied in greater detail to better understand the mechanisms underlying its function. Additionally, gene-specific differences clearly affect the performance of individual terminators. Therefore, the optimal 3' flanking regions determined for GFP may not be optimal for the expression of other genes. Especially in the case of multi-subunit proteins like antibodies, optimization may require using different 5' and 3' regions on each protein subunit. Further work into this area, such as the development of a series of geminiviral vectors with different components, may allow improved expression of a range of recombinant proteins.

6.4 Cell Death Reduction and Vector Replication

While rep/repA reduction with earlier geminiviral vectors produced clear cell death and expression benefits, incorporation of other necrosis-reducing optimizations appears to have obviated the need for replication reduction, at least with many previously toxic proteins. Further work is needed to determine whether especially toxic proteins that exceed the cell-death inducing nature of those tested in these studies would be benefited by rep/repA-reducing vectors. By contrast, perhaps increased vector replication would be tolerable with vectors already optimized to reduce cell death. This depends strongly on

whether saturating levels of transcription are reached by a given vector. As increasing replicon size has been noted to reduce replicon accumulation, the addition of long components like MARs to vectors, or when producing especially large proteins, may lead to insufficient replicon production for transcriptional saturation. In these cases, increasing rep/repA production could conceivably be beneficial.

Substantial evidence implicates animal DNA viruses in causing the DNA-damage response. However, the effect of geminiviruses on these pathways in plants has not been thoroughly investigated. As cell death was noted as a consequence of viral replicon accumulation, it is conceivable that geminivirus Rep/RepA or replication itself may trigger the DNA damage surveillance pathways in plants. Further work is needed to determine whether plant factors involved in these pathways are upregulated during geminivirus infection, or individual expression of Rep/RepA.

It has often been noted that proteins targeted to the ER result in lower accumulation and increased cell death. This likely occurs due to the unfolded protein response and ER-stress (Howell 2013; Duwi Fanata et al. 2013). It may be possible to modify plants, either transiently or with transgenic modifications, to allow increased production of molecular chaperones to properly fold proteins more rapidly. This might better allow plant cells to meet the massive demands placed on the ER by recombinant protein production.

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