DEVELOPING VIRUSES FOR GENE EDITING TO STUDY VIRUS-SPECIFIC MOLECULAR INTERACTIONS IN *NICOTIANA* SPECIES

A Thesis

by

APRIL MICHELLE DEMELL

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Chair of Committee,	Herman B. Scholthof
Committee Members,	Karen-Beth G. Scholthof
	Timothy Devarenne
Head of Department,	Leland S. Pierson III

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ABSTRACT

The CRISPR/Cas9 gene editing platform is capable of inducing double-stranded breaks in a targeted genomic region using a sequence specific single guide RNA (sgRNA) and programmable Cas9 endonuclease. Plant viral vectors offer flexible and efficient methods to deliver these materials. In this study, *Tobacco mosaic virus* (TMV) and *Tomato bushy stunt virus* (TBSV) have been established as biotechnological tools for this purpose. I explored the adaptability of using viral vectors to deliver gene editing materials in multiple diverse *Nicotiana* species as well as to target components of the plant RNA silencing pathway. First, I evaluated a TMV-based system to target the highly conserved *phytoene desaturase 3* gene (*PDS3*) in multiple *Nicotiana* species as a transient screening tool. Second, I effectively and efficiently delivered components of the CRISPR/Cas9 system to induce gene editing to affect the expression of a specific component of the RNA silencing pathway, HUA Enhancer 1 (HEN1). This served as proof-of-concept that it is possible to used virus-mediated gene editing to study the influence of a specific RNA silencing modification of viral infection.

DEDICATION

I dedicate this thesis to my family, both the one I was born to and the extra family I've gained along the way.

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I would like to thank my committee chair, Dr. Herman Scholthof, and my committee members, Drs. Karen-Beth Scholthof and Timothy Devarenne, for their guidance and support throughout this research and in my graduate career.

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CONTRIBUTORS AND FUNDING SOURCES

This work was supported by a thesis committee comprised of Dr. Herman Scholthof [Advisor], Dr. Karen-Beth Scholthof of the Department of Plant Pathology and Microbiology [Home Department], and Dr. Timothy Devarenne from the Department of Biochemistry and Biophysics [Outside Department]. All work for the thesis was completed independently by the student.

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NOMENCLATURE

TMV	*Tobacco mosaic virus*
TBSV	*Tomato bushy stunt virus*
CRISPR/Cas9	Clustered regularly interspaced palindromic repeats/CRISPR associated protein 9
sgRNA	single guide RNA
P19	TBSV protein and suppressor of viral-induced RNA silencing
TRBO-G	TMV-based coat protein replacement vector expressing GFP
p31	TBSV-based coat protein replacement vector expressing GFP
p33	TBSV-based coat protein replacement vector expressing GFP and lacking a functional P19 suppressor protein

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CHAPTER I

INTRODUCTION

GENE EDITING

Current genome modification technologies utilize the creation of a targeted nucleic acid double-stranded break (DSB) produced by an engineered nuclease, which can stimulate local mutagenesis and gene targeting (Jasin and Haber 2016). DSBs produced as a result of DNA damage, or intentionally, such as during meiosis, are repaired by the host cell via homology directed repair (HDR), or non-homologous end joining (NHEJ). HDR uses undamaged homologous sister chromatids as a template, resulting in accurate repair, or specific desired mutations if a repair template is provided (O'Driscoll and Jeggo 2006; Wyman and Kenaar 2006). However, broken ends are more often repaired via NHEJ, which can be error-prone and inaccurate, producing insertions or deletions (indels) of various lengths. Targeted indels can effectively knock out a genomic region of interest.

Currently, three classes of programmable nucleases have been engineered to create DSBs at essentially any desired genomic target: zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced palindromic repeats (CRISPR)-associated protein 9 (Cas9) nuclease (Gaj et al. 2013). ZFNs utilize the fusion of two functional domains, a DNA-binding domain comprised of zinc finger proteins, and a *Fok*I DNA-cleaving domain. Similarly, TALENs contain a *Fok*I restriction nuclease bound to a DNA binding domain called a TAL effector. Both the zinc finger proteins used with ZFNs and the TAL effectors used with TALENs are designed to recognize specific nucleotide sequences.

While ZFNs and TALENs can be useful tools, they are limited by their complex and laborious design, low engineering flexibility, and can result in off-target breaks within the genome.

The recently advanced CRISPR/Cas9 system has been adapted for targeted gene editing using a programmable RNA-guided nuclease complex (Cong et al. 2013). The necessary components of the CRISPR/Cas9 system consist of an engineered 20 nucleotide single guide RNA (sgRNA) complementary to the genomic region of interest and a sgRNA-programmable Cas9 endonuclease (**Figure 1.1**). When complexed, the sgRNA targets the region of interest through Watson-Crick base pairing upstream of a canonical protospace-adjacent motif (PAM) triplet sequence, and Cas9 induces a site-specific DSB (Jinek et al. 2012). The CRISPR/Cas9 system has been widely adopted due to the precision, efficiency, design simplicity, multiplexing capability, and applications in a wide range of research (Hsu et al. 2014; Cong et al. 2013; Cody et al. 2017).

Presently, common delivery methods of the CRISPR/Cas9 components include transgenic techniques and transient delivery to protoplasts. Transgenic methods such as biolistics or *Agrobacterium tumefaciens* mediated T-DNA insertion (agroinfiltration) of Cas9 and sgRNA coding regions into the plant genome can be expensive, inefficient, and time consuming. Additionally, food plants are subject to regulation or require additional breeding to remove unwanted DNA inserts prior to commercialization (Metje-Sprink et al. 2019). Transient protoplast editing requires the successful propagation of edited protoplasts and plant lines, a technique that requires both specialized skills and effort. Therefore, developing alternative versatile tools with the possibility for targeted gene editing could increase the efficiency of CRISPR/Cas9 component delivery, producing non-transgenic transiently edited plants.

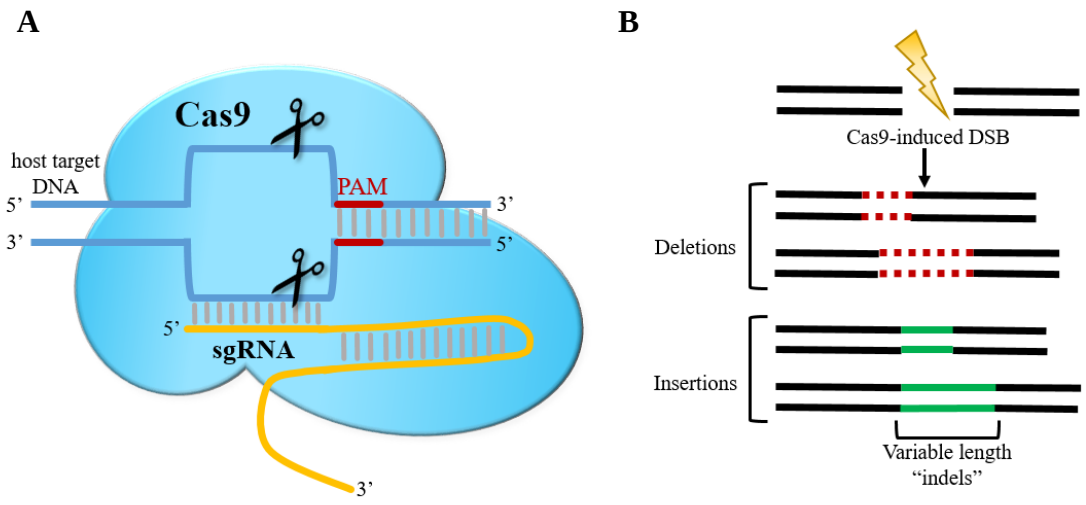

Figure 1.1 A sgRNA and programmable Cas9 can induce DSBs in a target sequence. A, The single guide RNA (sgRNA) and Cas9 endonuclease complex to induce double-stranded breaks (DSBs) in a target sequence complementary to the sgRNA and upstream of a protospacer adjacent motif (PAM) motif (Sander and Joung, 2014). B, One of the two major methods utilized in eukaryotic cells to repair damaged DNA, non-homologous end joining (NHEJ) and homology dependent repair (HDR), is activated. HDR is capable of inserting fragments of sequences, while NHEJ is more common and results in indels within the targeted sequence.

VIRUSES AND THE DEVELOPMENT OF VIRUS-BASED EXPRESSION VECTORS

Since the identification of the first virus, specifically *Tobacco mosaic virus* (TMV) over a century ago, the study of viruses has accomplished significant advancements not only as a namesake field of research, but across a broad variety of fields, including molecular biology, genetics, biochemistry, evolution, nanotechnology, and medicine (Beijerinck 1898; Franklin 1955; Fraile et al. 1997; Gafny et al. 1992; Bruckman et al. 2016). Due to their relatively small size, virus genomes were some of the first to be sequenced, ultimately resulting in the development of full length infectious DNA and cDNA clones, at the dawn of the genomics era of science (Fiers et al. 1976, Gardner et al. 1981; Stanley et al. 1986, Dawson et al. 1986; Ahlquist et al. 1984). Due to the early elucidation of their genetic sequence, most genetic tools such as

promotors, terminators, enhancers, and codon optimization sequences were more established in viruses than in more genetically complex organisms, such as plants and animals. Ultimately this resulted in viral pieces being incorporated into developing expression technology, such as recombinant delivery vectors, including *Agrobacterium* plasmid vectors (Kay et al. 1987). The well-known 35S promotor and terminator from *Cauliflower mosaic virus* (CaMV) have been extensively implemented to drive recombinant gene expression. Additionally, the 5' leader sequence of TMV and the 5' and 3' UTRs from *Tobacco etch virus* (TEV) have been utilized as transcriptional enhancers (Gallie et al. 1987). Likewise, viral proteins shown to suppress host RNA silencing, such as *Tomato bushy stunt virus* (TBSV) P19 and HC-Pro, found natively in potyviruses, have been used to supplement and enhance foreign protein expression (Saxena et al. 2011; Anandalakshmi et al. 1998).

While viral components remain useful in expression technology, the possibility of harnessing whole autonomously replicating viruses developed in the last three decades (Scholthof et al. 1996; Porta and Lomonossoff 1996). The characteristically rapid and robust levels of virus multiplication and concomitant gene expression levels results in efficient transient delivery of the heterologous product of interest. Bacteriophages such as phage λ and M13 have been developed as viral cloning vectors for the incorporation of recombinant DNA into bacteria (Karn et al. 1980; Yanisch-Perron et al. 1985). In biomedicine, viral vectors derived from retroviruses and adenoviruses have been implemented for applications such as gene therapy and the creation of engineered cell lines to study disease (Glorioso et al. 2001). Plant virus-derived vectors have been exploited as biofactories capable of producing biological products such as vaccines and antibodies inexpensively and efficiently for mass production (Scholthof et al. 2002; Matoba et al. 2011, Pogue et al. 2002; Giritch et al. 2006).

Plant viral vectors represent biotechnological tools capable of rapid and efficient delivery of heterologous protein and nucleic acid products to a wide range of plant hosts (Zaidi et al. 2017; Scholthof et al. 1996). Viruses previously exploited for delivery vectors include CaMV (Hohn et al. 1984), *Potato virus X* (PVX) (Chapman et al. 1992), *Bean yellow dwarf virus* (Cermak et al. 2015; Butler et al. 2016), *Wheat dwarf virus* (Wang M., Lu Y. 2017), *Tobacco rattle virus* (Ali et al. 2015), TBSV (Scholthof et al. 1999; Zhang et al. 2000), TMV (Sugiyama et al. 1995; Turpen et al. 1995), and *Citrus tristeza virus* (Folimonov et al. 2007) in both experimental plants, such as *Nicotiana benthamiana*, and crops, such as potato, tomato, rice, wheat, and citrus. The technology enabled the ability to study plant-virus interactions *in vivo* through tracking viral movement in living cells, exposing novel insights into basic aspects of plant physiology such as plasmodesmatal gating and molecular movement between cells, and identification of associated cellular components. Additionally, the transient nature of viral vectors circumvents the 'position effects' of traditional transgenic modifications in the chromosomal DNA, and foreign product delivery can be introduced at various stages to avoid disruption of early development and promote product yield optimization.

While viral vectors can offer significant advantages over traditional transgenic methods, the vector must be built from a virus capable of being cloned, inoculated into the host of interest, and amenable to the application. Production of mechanically introducible infectious clones is limited to viruses with DNA genomes, such as CaMV that can be directly inoculated as plasmid DNA, and viruses with positive-sense RNA-based genomes, such as TMV and TBSV, that can be transcribed from infectious cDNA clones. Alternatively, infectious cloned viral DNA or cDNA can be delivered into the host cell via agroinfiltration. While viral vectors deliverable by

agroinfiltration have been developed for a wide host range of dicotyledonous plants, the development of equivalent vectors for use in monocotyledonous plants are lacking.

Selection of a virus vector is dependent upon the expressed heterologous product for delivery, whether the gene of interest can be integrated into the viral genome without disruption of necessary functions, and if expression can be effectively monitored. A major limitation of foreign products expressible and deliverable via viral vector are size constraints of the modified viral genome. This can sometimes be alleviated by the use of gene replacement vectors, where a dispensable viral gene can be removed and replaced by one encoding the recombinant product. Such nonessential genes include insect transmission factors, or in certain scenarios, the coat protein (CP) gene (Scholthof et al. 1996; Scholthof et al. 1999). While removal of the CP gene would still allow local replication, systemic movement of the virus throughout the plant could be compromised, which may be disadvantageous for certain applications. Alternatively, rod-shaped viruses can manage larger insertion products than their icosahedral or spherical counterparts, and while replication and therefore recombinant product delivery often decrease, viruses such as TMV and TEV have been utilized as insertion vectors, where the foreign gene is an addition to the native genome (Lindbo 2007; Dawson et al. 1989; Dolja et al. 2013). Viral vectors offer rapid and robust heterologous delivery, however, most viruses only support the expression of one foreign gene at a time due to size constraints. Furthermore, using viral vectors built from the same virus to co-express different products results in superinfection exclusion, a phenomenon in which co-infected viruses, such as those utilized for viral vectors, interfere and compete with each other (Folimonova et al. 2010).

RNA SILENCING

RNA silencing is a conserved eukaryotic molecular mechanism of post-transcriptional gene silencing (PTGS) used to regulate gene expression and combat invasive nucleic acids (Wang et al. 2012). Upon infection with a virus, double-stranded RNA (dsRNA) often accumulates in the cell as a result of replication or transcription, activating the viral induced RNA silencing pathway (**Figure 1.2**) (Alvarado and Scholthof 2009). These dsRNAs are recognized and cleaved into 21-26 nucleotide (nt) short interfering RNA (siRNA) duplexes by a Dicer-like protein (DCL) associated with a double stranded RNA binding protein (DRB) (Forstemann et al. 2005; Hiraguri et al. 2005). Cleaved siRNA duplexes are stabilized and methylated by methyltransferase HUA enhancer 1 (HEN1) (Chen et al. 2002) before incorporation into the RNA induced silencing complex (RISC). Lack of HEN1 methylation results in polyuridylation and degradation of the unstable siRNA by the exosome (Li et al. 2005). Members of the AGO protein family form key catalytic units of RISC, and the presence of Piwi Argonaute Zwille (PAZ) domains allows for their direct interaction with siRNAs being loaded into the complex (Song et al. 2003). One strand of the siRNA duplex is selectively loaded by an AGO protein into RISC while the antisense siRNA strand is degraded. After siRNA programming, the RISC-AGO complex surveys for complementary viral RNA, which when identified is subjected to a degradation process.

To counteract the RNA silencing pathway, viruses have developed the ability to express silencing suppressor proteins to protect their genomes from degradation. A suppression mechanism employed by multiple viruses, including P19 of TBSV, involves indirect interference in the siRNA methylation by HEN1, required for incorporation into RISC by AGO (**Figure 1.2**). Specifically, P19 homodimers preferentially sequester 21 nt siRNAs in a non-sequence specific

manner, preventing their incorporation into RISC. This obstructs the endonucleatic cleavage of targeted viral RNA by RISC (Omarov et al. 2007), allowing for systemic viral invasion throughout the plant (Silhavy et al. 2002; Scholthof et al. 1995).

While TBSV P19 is one of the most well-known suppressors in both virology and biotechnology, in fact, most viruses encode suppressors of RNA silencing. Particularly relevant for this study, P21 of *Beet yellows virus* (BYV), P1/HC-Pro of *Turnip mosaic virus* (TuMV), and the 126 kDa replicase subunit of TMV have been shown to interfere with siRNA stabilization by blocking HEN1 methylation (Lozsa et al. 2008; Ding et al. 2004). When the P126 subunit of TMV replicase is produced via an amber stop codon, it includes methyltransferase and helicase domains, as well as a non-conserved region II, all of which are required for RNA silencing suppression activity (Ding et al. 2004; Wang et al. 2012; Harries et al. 2008). P126 physically blocks HEN1-dependent methylation by binding to siRNA duplexes, ultimately preventing further processing and RNA silencing.

The existence of the RNA silencing pathway has been exploited for viral induced gene silencing (VIGS), a method of targeting a host genomic sequence by inserting it into a virus before infection. Plant viruses such as PVX, TRV, and TMV have been adopted for VIGS by combining the plant genomic sequence to be targeted with the modified viral genome, followed by agroinfiltration into the host (Liu et al. 2002; Lange et al. 2013). Within the plant cell, replication of the modified virus results in accumulation of dsRNA, which in defense, is targeted by the virus-induced RNA silencing system. The siRNAs within RISC recognize the targeted genomic sequence within the plant, resulting in targeted degradation. Due to the nature of RNA silencing, target sequence-specific sRNA can also be amplified and transported throughout the plant resulting in systemic gene silencing (Kalantidis et al. 2008).

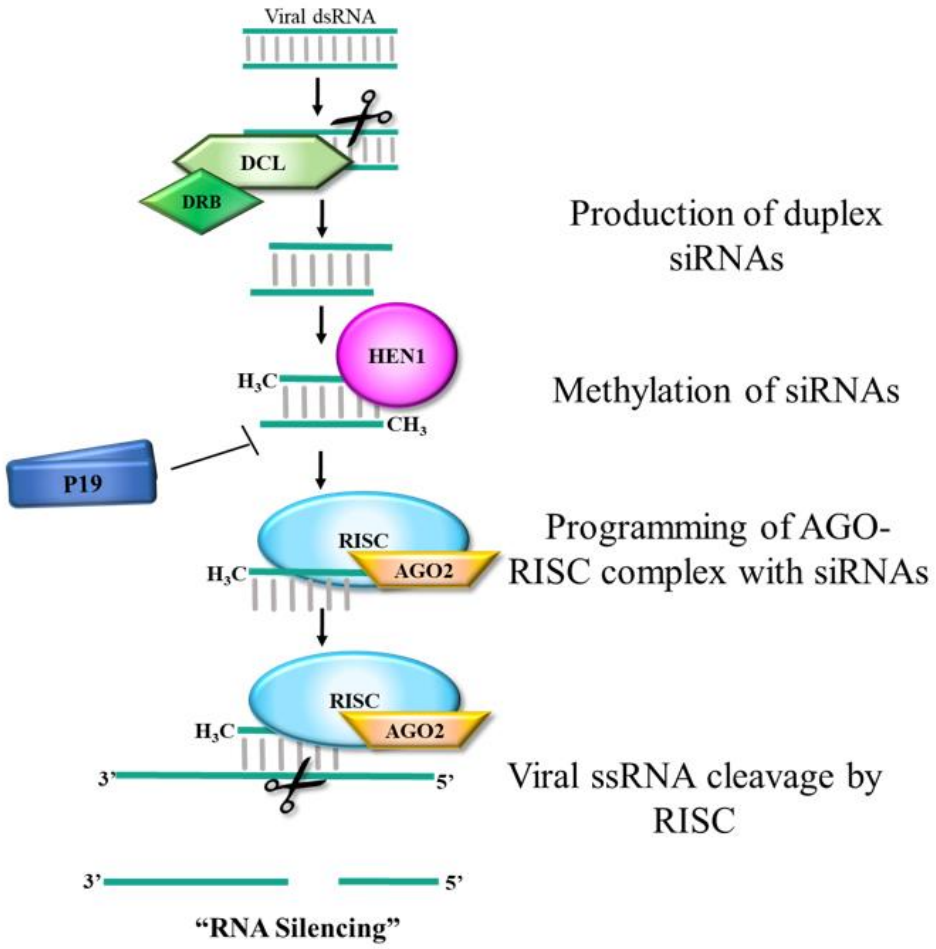

Figure 1.2 Currently understood mechanism for viral-induced RNA silencing. Upon accumulation of double stranded or complex single stranded small RNAs, Dicer-like proteins (DCLs) recognize and process them into short interfering RNAs (siRNAs), which are then methylated by HUA enhancer 1 (HEN1). In the case of certain viruses in *N. benthamiana*, Argonaute 2 (AGO2), interacts with the methylate siRNA and loads it into the RNA induced silencing complex (RISC). This siRNA-loaded complex acts to "search and scan" for complementary viral RNA for targeted cleavage. Functioning as a homodimer, the TBSV P19 suppressor of RNA silencing sequesters siRNAs before their association with AGO2 in RISC.

CHAPTER II

GENE EDITING IN DIVERSE *NICOTIANA* SPECIES USING A VIRAL VECTOR

BACKGROUND

Tobacco mosaic virus

Tobacco mosaic virus (TMV) is a positive sense single-stranded RNA (+ssRNA) *Tobamovirus* that has been historically well established as a model for studies in virology, molecular biology, and plant biotechnology (Holmes 1946; Okada 1999). This is largely due to the ability of TMV to infect a wide range of host plants, enhance heterologous gene expression, and rapidly produce large quantities of recombinant product upon infection (Donson et al. 1991). The TMV genome consists of a two component replicase protein (Ishikawa et al. 1986), a movement protein (MP) required for cell to cell movement via plasmodesmata, and the self-assembling coat protein (CP) that forms rod-shaped virions required for systemic movement throughout the host (**Figure 2.1**) (Scholthof et al. 2004).

While many viruses have been modified as delivery vectors in plants, TMV-based expression vectors have been well established and can provide high levels of foreign protein expression (Pogue et al. 1998). TRBO-G, a CP deletion mutant of the TMV U1 strain contains a GFP coding sequence and was developed as an agroinfiltratable delivery tool for the expression of heterologous proteins in plant cells (Lindbo 2007). Lack of a functional CP prevents systemic movement of the virus throughout the host plant, while a functional MP still allows localized cell-to-cell movement throughout the infiltrated leaf tissue. TRBO-G is capable of expressing GFP in *N. benthamiana* and delivering high concentrations of sgRNAs suitable for Cas9 programming for targeted gene editing with the CRISPR/Cas9 platform (Cody et al. 2017).

Figure 2.1. The ~6.4 kb TMV RNA genome consisting of three open reading frames (boxed regions). The replicase-associated proteins are translated from the genomic RNA, producing a 126 kDa protein and 183 kDa protein expressed by ribosomal read-through of an amber stop codon (asterisk; *). The 30 kDa movement protein (MP) and 17.5 kDa coat protein (CP) are expressed from two separate subgenomic RNAs (arrows) via replicase recognition on individual promoters.

Nicotiana benthamiana: the traditional model organism in virology

While *Arabidopsis thaliana* has traditionally dominated plant genetic research, *Nicotiana benthamiana* has been the preferred model organism for plant virologists, primarily due to its almost universal susceptibility to viruses, in contrast to *Arabidopsis* (Bally et al. 2018). This hyper-susceptibility to plant viruses can be linked to a naturally occurring deletion in an RNA-dependent RNA polymerase (NbRdRP1m) found in all reported laboratory accessions (Bally et al. 2015). The transformation and regeneration efficiency makes *N. benthamiana* useful for studies using VIGS, viral expression vectors, protein localization techniques, and as a plant model for protein accumulation and purification. *N. benthamiana* was further popularized by the development of agroinfiltration.

However, the utility has been somewhat hindered by the genetic complexity of *N. benthamiana*. Genetic analysis has been limited by the 3,100 Mb genome size and allotetraploid nature, compared to the 157 Mbp genome of the diploid, *Arabidopsis* (Bally et al. 2018). An obvious appeal of implementing *Arabidopsis* in genetic experiments is the abundance of high quality and detailed databases and mutant lines available, which are lacking for *N. benthamiana*. The *N. benthamiana* published genome is incomplete, poorly annotated, and the multiple

accessions used in laboratories have not been fully characterized, resulting in a current model with a relatively obscure genetic background. As an allotetraploid evolved from a hybridization of polyploid ancestors, multiple homologs or partial homologs with varying functionality exist for most genes, a product of the two subgenomes that generate alloploidy. This creates a genetic environment that is particularly convoluted during the implementation of sequence-specific technologies, such as the modern gene editing techniques ZFNs, TALENs, and CRISPR/Cas9.

Project description and specific aims

Viral-based delivery vectors represent powerful biotechnological tools capable of flexible, rapid delivery of CRISPR/Cas9 gene editing components in high quantities to a wide variety of hosts. My hypothesis was that these tools could be adapted and further developed to build upon the molecular toolbox capable of interrogating essential cellular and molecular processes and interactions in the plant cell, with an emphasis on virus- specific interactions, and functional in genetically diverse *Nicotiana* species.

Previously, a binary Cas9 expression vector, pHcoCas9 (human codon optimized, Hco) (**Figure 2.2**) combined with a TMV-based vector, TRBO-G was utilized for targeted gene editing that resulted in up to 60% indels within the total sample (Cody et al. 2017; Lindbo 2007). Here, I aim to induce targeted gene editing in multiple genetically diverse *Nicotiana* species using a single set of tools based on the pHcoCas9 and TRBO-G vectors. As proof-of-principle, the sgRNA capable of targeting the highly conserved gene *phytoene desaturase 3* (*PDS3*; sgPDS3) was inserted into the TRBO-G vector (TRBO-gPDS3) (**Figure 2.3**). An additional goal was to examine the flexibility of these tools in genetically diverse species, demonstrated here in diverse *Nicotiana* species as proof-of-principle. As previously mentioned, while *N. benthamiana*

is the currently preferred model for plant virology and is utilized in various other fields, it is limited by a large, complex genome and poor genomic resources, such as an incomplete genome with partial annotation. As a genus, *Nicotiana* encompasses approximately 67 species, most of which are grown natively in the Western hemisphere. In contrast to *N. benthamiana*, most members of the genus possess diploid genomes and some are fully sequenced an annotated. Here, I hypothesized that alternative *Nicotiana* species, including *N. otophora* and *N. attenuata*, can serve as more functional and simple genetic models than the traditional *N. benthamiana*.

Host antiviral RNA silencing mechanisms have the ability to decrease replication and persistence of the viral vector. TBSV P19 is a strong suppressor of RNA silencing, and has been shown to positively impact recombinant product expression from delivery vectors (Scholthof 2006). Therefore, I hypothesized a positive impact on vector expression and gene editing when co-delivering P19 with the aforementioned pHcoCas9 and TRBO-G.

Figure 2.2. The pHcoCas9 binary vector capable of expressing Cas9 in plants. Human codon-optimized Cas9 (HcoCas9) is expressed via pBINPLUS-sel using a *Cauliflower mosaic virus* (CaMV) double 35S promoter; *Tobacco etch virus* (TEV) 5' and 3' UTR; 3X FLAG (grey); nuclear localization signal (NLS, light purple) is present on either side of HcoCas9 coding region in light purple; Term, terminator of nos (nopaline synthase) gene.

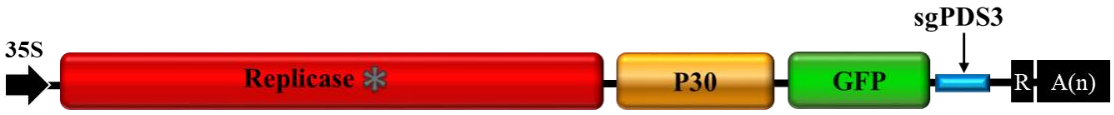

Figure 2.3. The genetic map of the relevant parts of the TRBO-gPDS3 construct. Transcription of the vector is driven by the CaMV 35S promoter. The TMV CP is replaced by GFP, followed by sgPDS3 located 3'-proximal to the GFP protein coding region; R, ribozyme sequence; A(n), polyA tail.

RESULTS

A TMV-based viral vector replicates and expresses recombinant reporter protein in genetically diverse *Nicotiana* species

From a larger screen of nine *Nicotiana* species, three were chosen for further experimentation based on: i) the ability of the TRBO-G viral vector to replicate and express the recombinant GFP reporter protein, ii) the ability of pHcoCas9 to efficiently express Cas9, and iii) a lack of the necrotic hypersensitivity response to P19 upon infiltration (Cody 2018). A primary objective of the research presented in this chapter is to implement a virus-based molecular toolbox in genetically diverse *Nicotiana* species. Therefore, most significantly, species were chosen based on their genetic diversity and nature of the genome compared to *N. benthamiana*. As previously mentioned, *N. benthamiana* is a classic model for plant virology and other fields, but has a genetically complex genome and lacks abundant, high quality genomic resources. The second species selected, *N. otophora*, has a currently unsequenced, but diploid genome of 2,689 Mbp. Lastly, *N. attenuata* possesses a diploid genome of 2,260 Mbp, almost 1,000 Mbp less than that of *N. benthamiana*, and has a well annotated reference genome. Within the genus, *N. benthamiana*, *N. otophora*, and *N. attenuata* occupy three different phylogenic sections,

Suaveolentes, *Tomentosae*, and *Petunioides*, respectively, and are native to extremely varying global regions and climates (Knapp et al. 2004).

Agrobacterium cultures harboring TRBO-gPDS3 were co-infiltrated into leaves of each species with and without pHcoCas9 (Cody et al. 2017) and a P19 expression cassette (Saxena et al. 2011). Green fluorescence under UV light is indicative of replication of the GFP-containing TRBO-gPDS3, imaged at 3, 5, and 7 days post infiltration (dpi) (**Figure 2.4**). In all three experimental species, replication of TRBO-gPDS3 with and without pHcoCas9 began at 3 dpi, and increased until 7 dpi. Additionally, co-expression of P19 with TRBO-gPDS3 and pHcoCas9 resulted in a visually earlier and stronger green fluorescence than without P19. Surprisingly, a noticeable increase in green fluorescence was observed in all three species infiltrated with TRBO-gPDS3 when co-introduced with pHcoCas9, particularly evident in *N. attenuata*. This suggests that the pHcoCas9 delivery plasmid, or possibly the presence of Cas9, allowed for higher proliferation and expression of the viral vector and recombinant products.

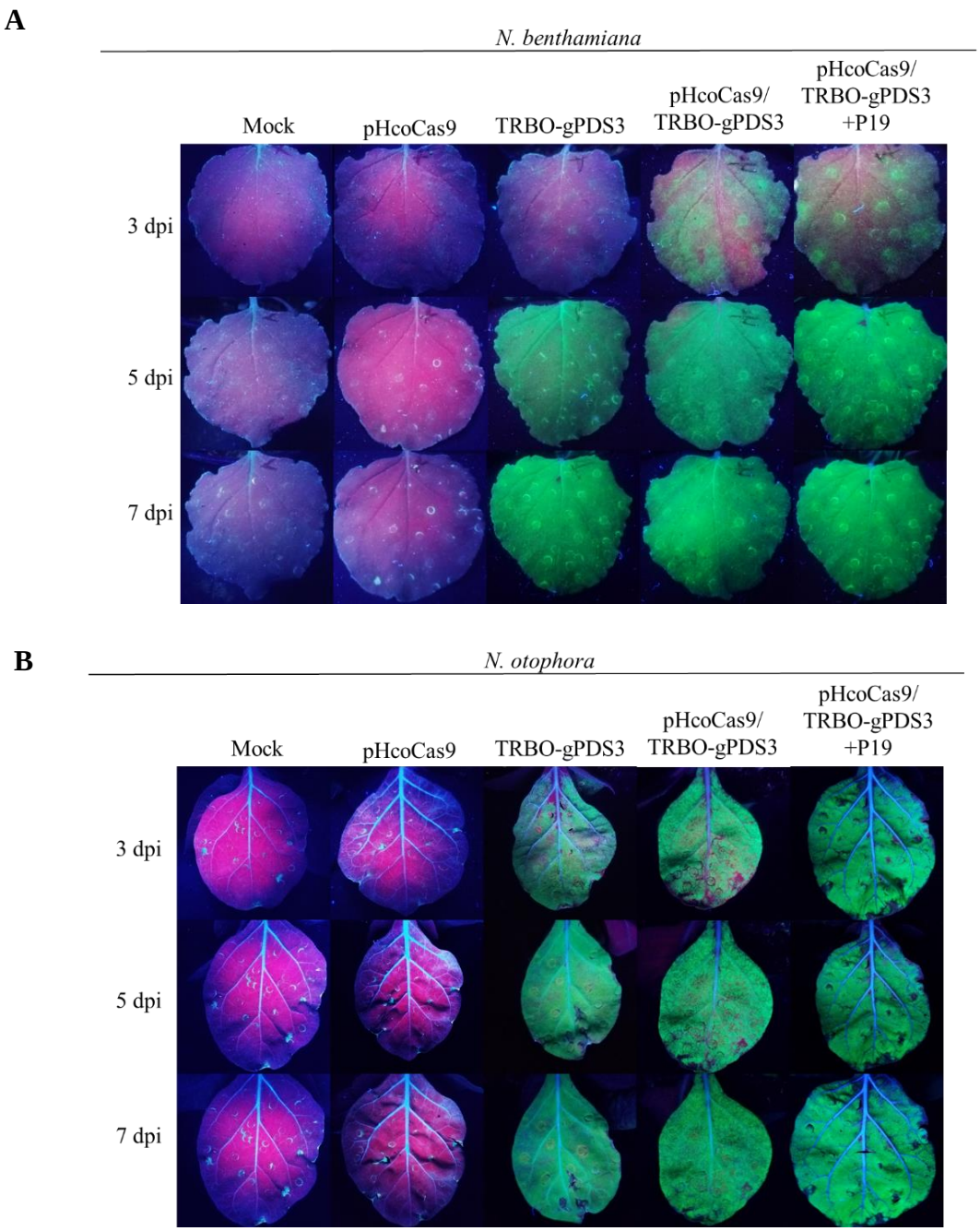

Figure 2.4. Leaves infiltrated with TRBO-gPDS3 with and without pHcoCas9 and P19 visualized under UV light. GFP is representative of TRBO-gPDS3 replication in infiltrated leaves. Leaves were agroinfiltrated with either inoculation buffer (mock, negative control), TRBO-gPDS3 alone, co-infiltrated with pHcoCas9 and TRBO-gPDS3, or co-infiltrated with pHcoCas9, TRBO-gPDS3, and P19. **A)** *N. benthamiana* leaves visualized under UV light at 3, 5, and 7 dpi. **B)** *N. otophora* leaves visualized under UV light at 3, 5, and 7 dpi. **C)** *N. attenuata* leaves visualized under UV light at 3, 5, and 7 dpi.

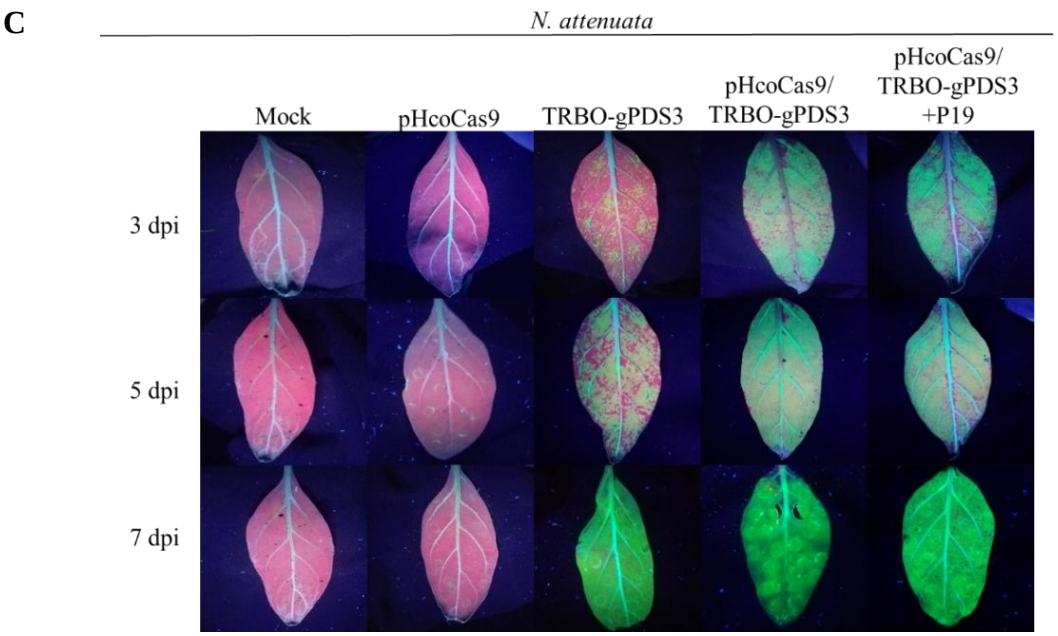

Figure 2.4. Continued.

The binary expression vector efficiently expresses Cas9 in three genetically diverse *Nicotiana* species

As previously mentioned, P19 has been widely used to increase the expression of foreign inserts from both viral and non-viral delivery vectors. Here, I sought to demonstrate the capability of P19 to increase the expression of gene editing delivery tools, such as pHcoCas9, in genetically diverse *Nicotiana* species. Therefore, western blot analysis using an anti-Cas9 antibody was used to detect Cas9 protein in experimental species from leaf tissue collected at 7 dpi (**Figure 2.5**). Upon delivery pHcoCas9 alone, Cas9 could only be faintly detected via western blot in *N. otophora*. However, the addition of P19 resulted in high levels of Cas9 expression in all three species.

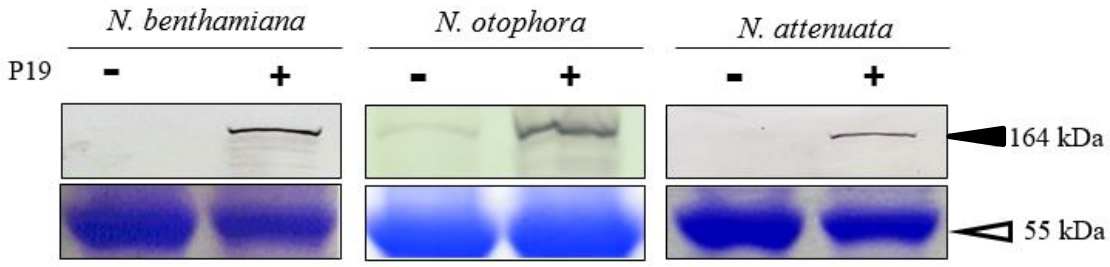

Figure 2.5. pHcoCas9 expression with and without P19 in experimental *Nicotiana* species. Western blot analysis of tissue collected from experimental species at 7 dpi after co-infiltration of pHcoCas9 with (+) and without (-) P19. The top panel shows the western blot using anti-Cas9 antibody (filled arrow) and the bottom panel represents the Coomassie blue staining of RuBisCo (open arrow) to ensure equal protein loading for each sample. Note that P19 increases the expression of Cas9 protein in all three species.

Targeted gene editing in diverse *Nicotiana* species is increased by the presence of P19

While GFP expression was used to observe replication of the viral vector, it is also indicative of sgPDS3 expression from TRBO-gPDS3, which was inserted directly downstream of the GFP stop codon. The *PDS3* coding sequence targeted by sgPDS3 contains an *Avr*II restriction enzyme site, allowing targeted gene editing to be measured via a restriction enzyme assay. Upon successful editing, indels are induced in the targeted region, disrupting the *Avr*II site, and *Avr*II-resistant DNA fragments can be observed upon agarose gel electrophoresis. The percentage of the sample that contained indels can be analyzed as a measure of successful targeted editing using NIH's ImageJ analysis software (ImageJ) based on the intensity of the *Avr*II-resistant DNA fragments compared to the negative control.

In all species, editing of *PDS3* was observed upon delivery of both CRISPR/Cas9 components from pHcoCas9 and TRBO-gPDS3 at 7 dpi, which was increased by the presence of P19 (**Figure 2.6**). Editing in *N. benthamiana* and *N. otophora* approximately doubled in the presence of P19, reaching 40.9 and 33.9%, respectively. Targeted editing in *N. attenuata* was also increased by the presence of P19, from 17.1 to 20.8%.

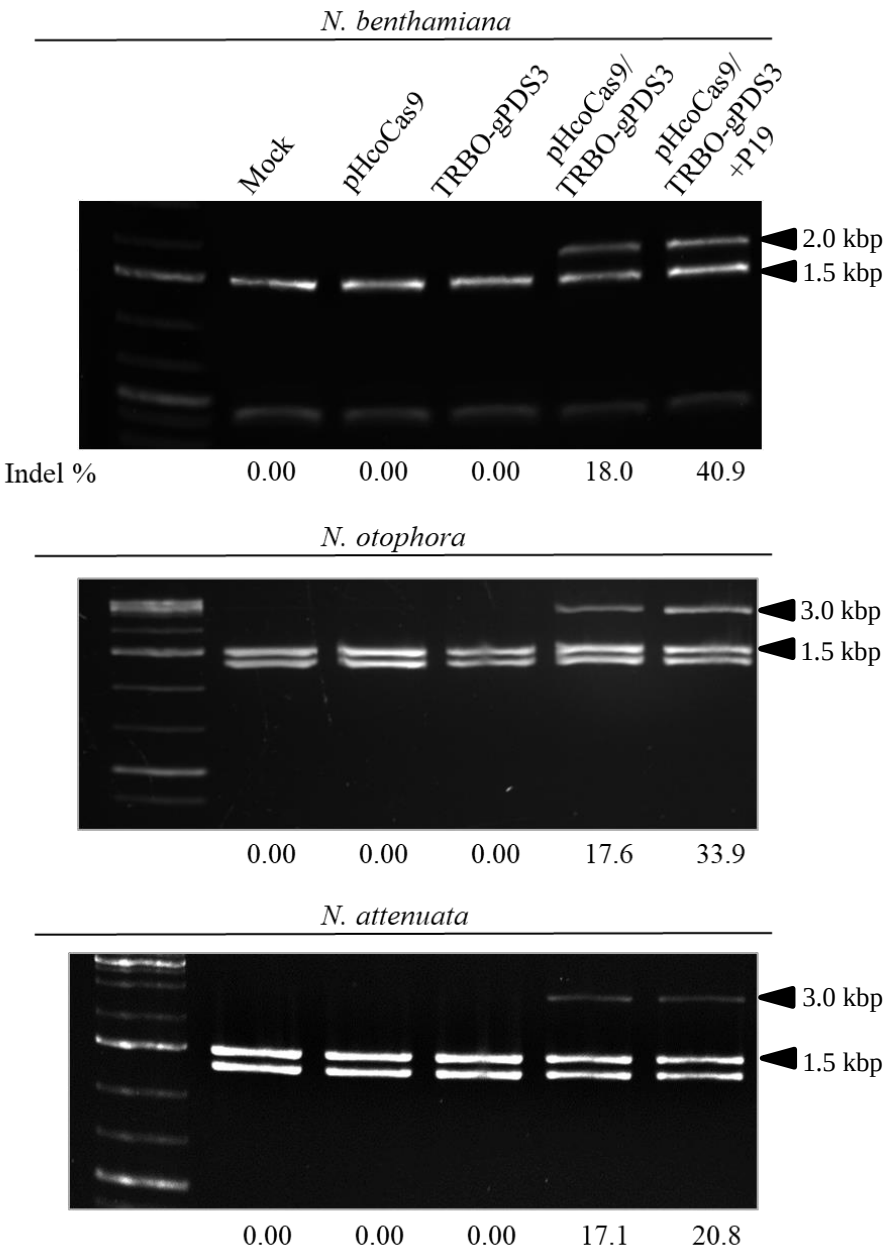

Figure 2.6. Detection and quantification of indels in *Nicotiana PDS3*. Restriction enzyme digest of *Nicotiana* tissue at 7 dpi with infiltration of either infiltration buffer (mock), pHcoCas9, TRBO-gPDS3, or co-infiltration with pHcoCas9 and TRBO-gPDS3 with and without P19. The sgPDS3 was designed complementary to a region of *PDS3* containing the restriction enzyme site for *Avr*II. The presence of indels in that region disrupted the site, resulting in a third, larger, undigested band. ImageJ was used to quantify indel percentages.

DISCUSSION

Despite the availability of transient CRISPR/Cas9 technology for other biological systems such as bacteria and mammalian cell lines, until recently, the equivalent was not convenient for plants (Larson et al. 2013; Qi et al. 2013; Gilbert et al. 2013). Recently, a TMV-based viral vector was used to deliver sgRNAs to locally knockout genes in *N. benthamiana* (Cody et al. 2017). Here, my aim was to demonstrate the functionality of this tool for rapid, transient gene editing, and to show the flexibility and utility by adapting it to multiple genetically diverse *Nicotiana* species.

The three *Nicotiana* species chosen are highly diverse, varying in ecological habitats, phylogeny, and genetics. The TMV-based CRISPR/Cas9 system successfully induced targeted gene editing in all three species after only seven days, and was increased in the presence of P19 (**Figure 2.6**). In *N. attenuata*, editing increased, and in *N. benthamiana* and *N. otophora*, approximately doubled when introduced to P19. Additionally, P19 increased Cas9 expression from a binary plasmid in all three species (**Figure 2.5**). It has been shown that P19 functions as a strong suppressor of RNA silencing, allowing for higher vector viability and heterologous product expression in a variety of biological systems, including plants (Shamekova et al. 2014; Gao et al. 2013 cite me). Here, I demonstrate that P19 mediates suppression of antiviral defense in *N. otophora* and *N. attenuata*, plants with currently little available information about anti-viral RNA silencing. Presumably, P19-mediated suppression of the RNA silencing system allows higher expression from foreign plasmids such as pHcoCas9 in addition to allowing higher replication of the viral vector.

Interestingly, GFP expression was enhanced by the co-delivery of pHcoCas9, most notably in *N. benthamiana* at 3 dpi and *N. attenuata* at 3 and 5 dpi. Therefore, either replication

of the viral vector or expression of recombinant products was increased by either a component of the pHcoCas9 plasmid or the Cas9 protein itself, a previously unreported phenomenon.

MATERIALS AND METHODS

Design of constructs

The binary pHcoCas9 plasmid (addgene plasmid: 42230) (Cong et al. 2013) expresses a Cas9 that was Human codon-optimized (Hco) and expression in plants was mediated in the pBINPLUS-sel plasmid with a CaMV 35S dual promoter and TEV translational enhancer region driving *Cas9* gene expression as previously described (Cody et al. 2007). The P19 expression cassette was described earlier (Saxena et al. 2011). TRBO-gPDS3 was designed by Will Cody (unpublished) via restriction enzyme cloning. The sgRNA targeting *PDS3* was designed from *Arabidopsis* and *N. benthamiana* genomes using sequence alignment (**Figure 2.7**), and the fragment was introduced to the pJL-TRBO-G (Lindbo 2007) backbone using existing *Pac*I and *Not*I restriction sites.

sgPDS3 5′→3′
5′-CTCCGTTCTCTACAGGATCCGTTTTAGAGCTAGAAATAGCAAGTTAAAAT
AAGGCTAGTCCGGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT-3′

Figure 2.7. Sequence of sgPDS3. Sequence of the sgRNA targeting *PDS3* in *N. benthamiana*. Red indicates sequence specific to PDS3. Black indicates sgRNA backbone.

Agroinfiltration of plants

Constructs were transformed into *Agrobacterium* strain pGV3101 via electroporation. *Agrobacterium* cultures harboring constructs were grown in LB liquid media containing 50 mg/L of kanamycin and incubated overnight at 28˚C and shaking at 250 rpm. Bacterial cells were harvested by centrifugation at 3900x*g* for 20 minutes at room temperature, resuspended in infiltration buffer (IB; 10 mM $MgCl_2$, 10 mM MES pH 5.6, and 200 µM acetosyringone), and incubated at room temperature for 1 hour. Cultures were adjusted to a final concentration of OD_{600} 0.5 with IB. Three abaxial leaves per plant of each species were infiltrated with either IB alone (mock, as a negative control), pHcoCas9, or TRBO-gPDS3, or co-infiltrated with pHcoCas9 and TRBO-gPDS3 or pHcoCas9, TRBO-gPDS3 and P19. Infiltrated plants were maintained in a growth chamber (16/8 h light/dark cycle, 25/23°C, and 60% relative humidity).

Protein extraction and western blot analysis

From each experimental plant, 100 mg of infiltrated leaf tissue was collected and proteins were extracted in 500 µl of 5x cracking buffer (645 mM Tris pH 6.8, 10% (w/v) SDS, 715 mM β-mercaptoethanol, 40% (v/v) glycerol, and 0.005% (w/v) bromophenol blue), boiled for 5 minutes, spun down at 10,000x*g* for 2 minutes, and 20 µl of the supernatant was collected for visualization on a 7.5% polyacrylamide-SDS gel. Gels were initially run at 80 V for 20 minutes, then 150 V for 80 minutes in 1x Laemmli running buffer (25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS). The separated proteins were transferred to a nitrocellulose membrane (Biorad, CA) in Tris-glycine transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol, pH 7) at 270 mAmp for 90 minutes. The transferred proteins were blocked in TBST buffer (0.2 M NaCl, 50 mM Tris, and 0.05% (v/v) Tween 20, pH 7.4) containing 5% non-fat milk for 1 hour,

followed by overnight incubation with mouse IgG anti-CRISPR (Cas9) primary antibody (BioLegend) at 1:5,000 dilution at 4°C. After incubation, membranes were subjected to three 5 minute washes with TBST buffer. A secondary IgG anti-mouse (goat) antibody conjugated to alkaline phosphatase (Sigma) is added to the membrane at 1:10,000 dilution and incubated for 1 hour at room temperature, and the membrane was re-washed three times.

Colorimetric detection of Cas9 protein was achieved by submerging the membrane in a solution of 33 µl of 5-bromo-4-chloro-3-indolyl phosphate (100 mg/ml), 66 µl of nitro blue tetrazolium (20 mg/ml) to 10 ml of 1x alkaline phosphatase buffer (100 mM Tris, pH 9.5, 1 M NaCl, and 0.5 M $MgCl_2$). Presence of the Cas9 protein was identified by the characteristic molecular mass of 164 kDa compared to a protein ladder (Thermo Scientific PAGE Ladder Plus Prestained Protein Ladder).

To confirm equal loading of proteins for each sample, rapid staining of an identically loaded and run polyacrylamide-SDS gel in Coomassie Brilliant Blue R-250 was achieved by microwaving the gel twice in water for 2 minutes, followed by microwaving in stain solution (40% methanol, 10% acetic acid, 50% water, and 0.1 % (w/v) Coomassie Brilliant Blue R-250). Gels were incubated with gentle swirling at room temperature for 2 minutes. The gel was submerged in de-staining solution (40% methanol, 10% acetic acid, and 50% water) with gentle rocking until the 55 kDa RuBisCo band could be clearly observed.

Indel analysis via a restriction enzyme digest assay

At 7 dpi, 50 mg of infiltrated leaf tissue was collected and stored at -50˚C until processing. Whole genome DNA was extracted using the GeneCatch Plant Genomic DNA Miniprep Kit (Epoch) according to the manufacturer's instructions, and DNA concentrations

were measured using a Nanodrop spectrophotomer for nucleic acid. The region of interest, including the targeted PDS3 gene was PCR amplified, cleaned using DNA Clean & Concentrator (Zymo Research) kit and resuspended in DNase and RNase-free water. The PCR product was subjected to *Avr*II digestion, then separated on a 0.8% agarose gel at 125 V until the DNA bands were sufficiently separated. The sgPDS3 was designed to target a *PDS3* region containing the restriction enzyme site for *Avr*II. If editing in the region of interest occurred, the *Avr*II restriction site would be disrupted by the presence of indels induced by Cas9 and sgPDS3, and the restriction enzyme would be incapable of cleavage. In contrast, the negative control would exhibit complete digestion. Quantification of gene-specific indel percentages was accomplished using Image J analysis software (NIH).

CHAPTER III

IMPLEMENTING VIRUS-BASED MOLECULAR TOOLS TO STUDY VIRUS-HOST INTERACTIONS WITHIN THE RNA SILENCING PATHWAY

BACKGROUND

Unweaving the RNA silencing pathway and the functionality of its components

As detailed in the previous chapter, the RNA silencing system is a conserved eukaryotic pathway of PTGS essential for development, the regulation of gene expression, and as a defense against invasive nucleic acids and pathogens (Wang et al. 2012). Multiple assorted but essential plant-specific roles exist, spanning from germination to pathogen and herbivore defense (Song et al. 2019). While the general scheme of the biochemical pathway has been established, mostly in *Arabidopsis*, the specific AGOs, DCLs, HENs, and other functional molecules remain questionable, especially in plants such as *Nicotiana* and most crop species, which often have several homologs of conserved genes as a result of polyploidy.

AGO proteins have been identified in numerous plants, including *Arabidopsis* (Vaucheret 2008), *Oryza sativa* (Kapoor et al. 2008), *N. benthamiana* (Alvarado and Scholthof 2011), and *Solanum lycopersicum* (Bai et al. 2012). In *N. benthamiana*, AGO1 (Jones et al. 2016), -2,-3,-5,-7, and -X (Odokonyero 2013) are known to be expressed, but only AGO1 (Ghoshal and Sanfacon 2014) and AGO2 (Scholthof et al. 2011) are shown to contribute substantially to antiviral silencing. In *N. benthamiana*, AGO2 functions as the primary siRNA loading unit of RISC during silencing against certain viruses as shown previously, when AGO2 was targeted to examine its effects on the RNA silencing pathway in *N. benthamiana* using multiple platforms, including VIGS (Scholthof et al. 2011), dsRNA hairpin technology (Odokonyero et al. 2015),

and CRISPR/Cas9 (Ludman et al. 2017). In *Arabidopsis,* five HEN genes have been identified from genetic screens, all with highly diverse roles surrounding interactions with small RNAs (Jali et al. 2014). First identified for its role in floral development, HEN1 has subsequently been shown to be responsible for both siRNA and microRNA (miRNA) processing. At least four DCLs exist in plants, each of which are involved in RNA silencing against different viruses (Fukudome and Fukuhara 2017; Blevins et al. 2006). Other components of the RNA silencing pathway, such as DRBs and RNA-dependent RNA polymerases exist as multiple homologs, and redundancy and overlapping functionality appears throughout the system. Details such as which components of RNA silencing are involved in direct and indirect viral defense, including during recognition and systemic spread of signals, remain largely unknown, particularly in non-*Arabidopsis* plants.

Tomato bushy stunt virus

Tomato bushy stunt virus (TBSV) is the type member of the genus *Tombusvirus* that has a characteristically wide host range, including approximately 120 species in 20 plant families (Yamamura and Scholthof 2005; Panavas and Nagy 2003). The +ssRNA genome encodes two replicase proteins, P33 and P92; a CP, P41 encoded on subgenomic RNA 1, which assembles into icosahedral virions required for systemic movement throughout the host; a MP, P22 required for cell-to-cell movement through plasmodesmata; and P19, a suppressor of the viral-induced plant silencing pathway **(Figure 3.1).** The MP and P19 are encoded on overlapping open reading frames of subgenomic RNA 2 and expressed by context-dependent ribosomal leaking scanning. Upon translation, ribosomal subunits bypass the initial AUG start codon of the MP mRNA,

resulting in downstream translational initiation at the P19 start codon (Johnston and Rochon 1996; Kozak 2002; Scholthof et al. 1999).

In *N. benthamiana*, TBSV-based expression vectors have been exploited to deliver reporter genes and foreign RNA sequences, as well as to study antiviral silencing in plants (Scholthof et al. 1993; Scholthof 1999; Shamekova et al. 2014). The utility of TBSV-based viral vectors lies in the high rate of replication and gene expression with a vast host range, allowing transient product delivery in numerous plant species. Furthermore, the native TBSV protein P19 is a strong suppressor of viral-induced RNA silencing in the host, allowing higher replication of the viral vector, and therefore higher expression of the heterologous payloads to be delivered (Scholthof 2006). TBSV-based viral expression vectors p31 and p33 (referred to also as TG and TGdP19 in the literature, respectively) are TBSV CP replacement mutants capable of expressing GFP, with functional P19 and a non-functional P19 suppressor proteins, respectively. Combined with P19 expression, p31 is capable of yielding high levels of recombinant product delivery, while a lack of P19 expression from p33 allows examination of the viral-induced silencing response in the plant upon infection (Shamekova et al. 2014).

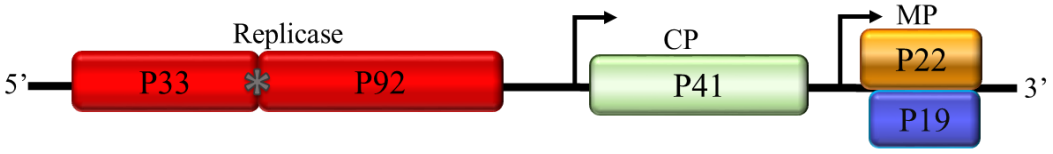

Figure 3.1. The ~4.7 kb TBSV RNA genome consisting of three open reading frames (boxed regions). The P33 and P92 replicase-associated proteins are translated from the genomic RNA using ribosomal read-through of an amber stop codon (asterisk; *). The CP, MP, and P19 suppressor protein are translated from subgenomic RNAs (angled arrows).

Project description and specific aims

Specific components of the RNA silencing system that participate in the viral defense mechanism have yet to be elucidated. Therefore, I sought to implement viral-based tools, such as those utilized in the previous chapter, for targeted editing of a critical component of RNA silencing, HEN1. Transgenically expressed suppressors of RNA silencing from multiple different viruses, including TuMV, BYV, and TMV inhibit methylation of miRNA duplexes by HEN1 in *Arabidopsis*, therefore preventing successful downstream silencing of viral infection (Yu et al. 2005; Vogler et al. 2007).

Tombusvirus P19 has been demonstrated to suppress RNA silencing through the preferential sequestration of 21 nt siRNAs indiscriminate of sequence, preventing their incorporation into RISC (**Figure 1.2**) (Lakatos et al. 2004; Omarov et al. 2007). Perhaps related to its suppressor function, P19 has been shown to influence species-dependent elicitation of a hypersensitivity response, systemic movement past vascular tissue, and systemic necrosis in *N. benthamiana* (Scholthof et al. 1995a; Scholthof et al. 1995b; Scholthof 2006). When transgenically expressed in *Arabidopsis*, P19 was demonstrated to suppress HEN1-mediated methylation of miRNAs (Yu et al. 2006). Therefore, I hypothesized that the siRNA sequestration activity of TBSV P19 inhibits methylation by HEN1 in *N. benthamiana*. To test this hypothesis, I implemented the aforementioned TBSV-based p31 and p33 vectors for transient delivery of a sgRNA capable of targeting *HEN1*.

As described previously, the TBSV-based viral vector p33 lacks a functional P19, resulting in a failure to successfully replicate and proliferate due to host RNA silencing system-mediated defense. However, upon the knockdown of AGO2, an essential component of RNA silencing related to TBSV, p33 was capable of replication, expression of recombinant products,

and cell-to-cell movement (Odokonyero et al. 2015). Based on literature review, TBSV-based viral vectors have not previously been exploited to deliver CRISPR/Cas9 gene editing materials. Therefore, my secondary goal was to implement TBSV-based vectors p31 and p33 to induce CRISPR/Cas9-mediated gene editing.

RESULTS

TBSV-based vectors induce transient, targeted editing of *HEN1*

A sgRNA targeting *HEN1* (sgHEN1) was cloned into p31 (p31gHEN1) and p33 (p33gHEN1) viral vectors (**Figure 3.2)**. In combination with the previously described pHcoCas9, vectors were agroinfiltrated into *N. benthamiana*. DNA from leaf tissue collected at 7 dpi was extracted and the PCR-amplified chromosomal region of interest was sequenced. The presence of indels indicated successful targeted editing of *HEN1* in all experimental samples derived from pHcoCas9 + p31 or pHcoCas9 + p33 infiltrated tissue (**Figure 3.3**).

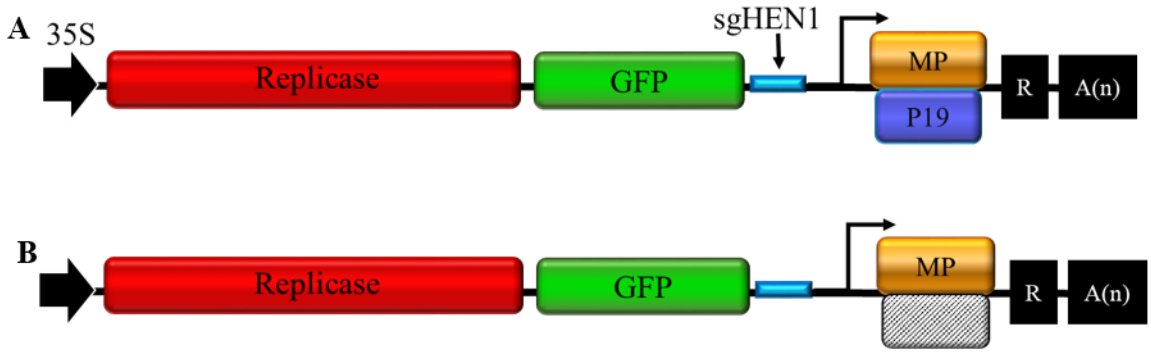

Figure 3.2. The genetic map of the relevant parts of the p31gHEN1 and p33gHEN1A TBSV-based constructs. Driven by a CaMV 35S promotor, the native TBSV CP is replaced with GFP, directly followed by sgHEN1. **A)** p31gHEN1 retains a functional P19 suppressor protein. **B)** p33gHEN1 contains a P19 coding sequence with a premature stop codon, preventing translation of a functional suppressor protein product.

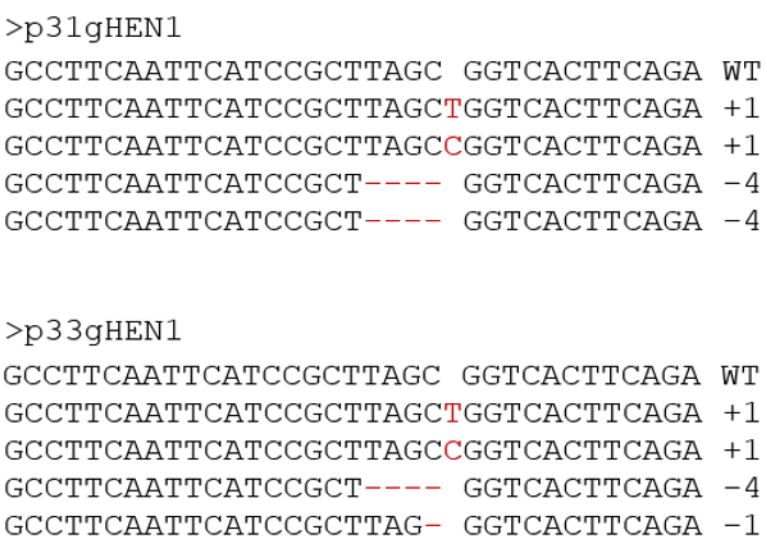

Figure 3.3. Indel sequence analysis of targeted *HEN1* region. The PCR-amplified chromosomal region of interest from plants co-infiltrated with pHcoCas9 and either p31gHEN1 or p33gHEN1 was sequenced at 7 dpi. The shown coding sequence of *HEN1* that was targeted exhibits indels (red) induced by DSBs as a result of CRISPR/Cas9. "WT" indicates the mock negative control. "+" indicates an insertional mutation, and "-" indicates a deletion mutation.

TBSV lacking P19 is capable of replication upon editing of *HEN1*

Visualization of GFP reporter gene expression from p31 (expressing P19) and p33 (not expressing P19) was used to track virus replication and movement at 7 dpi. Plants exhibited strong green fluorescence when co-infiltrated with p31 and pHcoCas9, and decreased, dulled fluorescence when co-infiltrated with p33 and pHcoCas9 (**Figure 3.4**). However, when either p31gHEN1 or p33gHEN1 were co-delivered with pHcoCas9, both vectors were capable of replication. Most notably, p33gHEN1 exhibited significantly increased and intensified green florescence and therefore replication upon successful editing of *HEN1*.

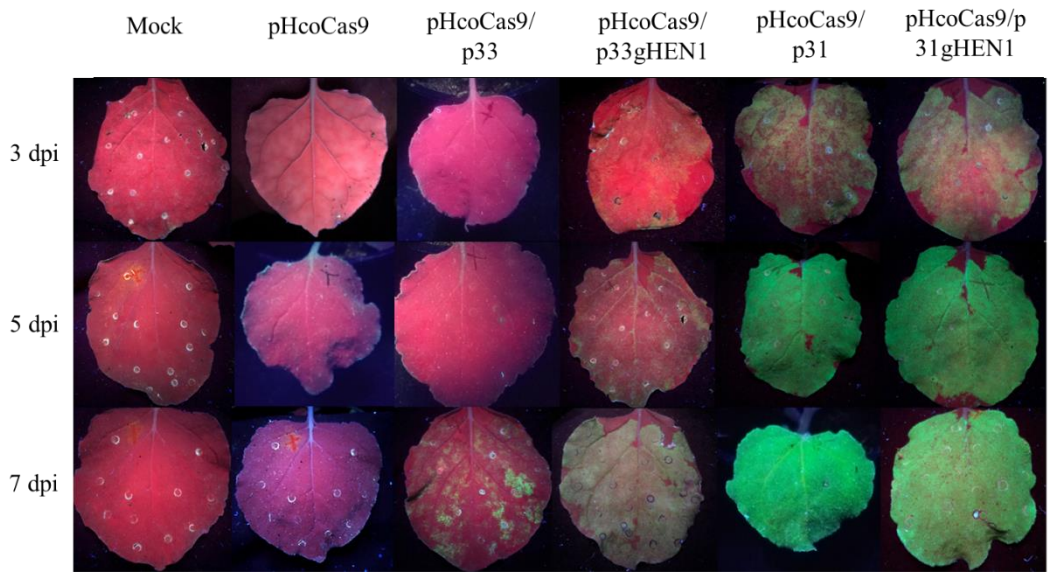

Figure 3.4. Leaves infiltrated with pHcoCas9 and either p31 or p33 with and without sgHEN1 under UV light at 7 dpi. GFP serves as an indication of p31 and p33 replication in infiltrated leaves. Leaves were agroinfiltrated with either IB (mock, negative control), pHcoCas9 alone, co-infiltrated with pHcoCas9 and p31 with and without sgHEN1, or co-infiltrated with pHcoCas9 and p33 with and without sgHEN1.

Targeted editing of *HEN1* allows higher Cas9 expression from pHcoCas9

Previous experiments clearly showed that interfering with RNA silencing by supplying P19 had a beneficial effect on gene expression, for instance from pHcoCas9. I surmised that editing of *HEN1* might similarly interfere with silencing and this could also affect Cas9 accumulation. To examine the potential influence of *HEN1* editing on pHcoCas9 expression, western blot analysis was used to measure Cas9 accumulation. Leaves infiltrated with pHcoCas9 and p31 contained readily observable amounts of Cas9, in contrast to leaves infiltrated with pHcoCas9 alone, presumably due to the effect of P19 expressed by p31 (**Figure 3.5**). In leaves co-infiltrated with pHcoCas9 and p33, no significant Cas9 expression could be observed. However, leaves where pHcoCas9 and p33gHEN1 were co-delivered, and in which successful

editing was shown via sequencing, Cas9 could be detected. This supports the notion that interfering with silencing by editing *HEN1* benefits expression from binary vectors.

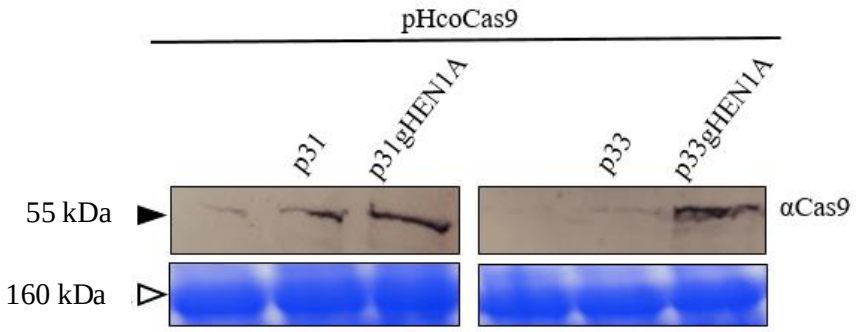

Figure 3.5. pHcoCas9 expression with TBSV-based vectors p31 and p33. Western blot analysis of tissue collected from plants at 7 dpi infiltrated with both pHcoCas9 and either p31 or p33 with and without sgHEN1. The top panel shows the western blot using anti-Cas9 antibody (filled arrow) and the bottom panel represents the Coomassie blue staining of RuBisCo (open arrow) to ensure equal protein loading for each sample.

DISCUSSION

PTGS or RNAi has been the focus of extensive research in multiple biological systems since its serendipitous discovery in plants and *Caenorhabditis elegans* (Napoli et al. 1990; Fire et al. 1998). However, the RNA silencing subset of PTGS has been elucidated to a lesser extent, particularly in plants, and even less so in regards to virus-specific interactions. As intracellular parasites that encode few genes, viruses interact on an intimate molecular level with the host cell throughout their lifecycles, which generally includes transcription, translation, replication, invasion of neighboring cells, and systemic movement. Additionally, viral components have been demonstrated to possess multiple functions that can vary between hosts. Furthermore, the majority of PTGS studies have been conducted in *Arabidopsis*, a diploid with a characteristically small genome, genetically different from the classic model used by virologists, *N. benthamiana*,

and most other crop species (Eamens et al. 2008; Spannagl et al. 2011). Moreover, viral components often have multiple functions, and, such as in the case of AGOs in *Arabidopsis* compared to *N. benthamiana*, can vary by host species. While RNA silencing is a substantial anti-viral defense, significant gaps in knowledge exist, such as the specific components of different viruses that interact in unique and sometimes multiple locations throughout the pathway, their impact on the systemic spread of RNA silencing signals, the cellular locations of these interactions, and alternative functions of both viral components and host RNA silencing machinery.

In this chapter, my research objectives involved synthesizing a TBSV-based viral vector capable of sgRNA delivery, establishing its utility as a rapid, transient screening method for virus-plant interactions, and determining if HEN1 is involved in plant defense against TBSV. Here, I implemented TBSV-derived p31 and p33 expression vectors for rapid, transient sgRNA delivery, which when combined with Cas9 expression, resulted in targeted gene editing of an essential component of the viral-induced RNA silencing pathway, and putative target of P19 suppression, *HEN1*. Editing *HEN1*, and therefore hindering the RNA silencing pathway, increased the expression of Cas9 from the binary delivery plasmid, pHcoCas9. In previous research (Bassam et al. 2009; Cody et al. 2017; Scholthof 2006) and the previous chapter, it was shown that P19 could increase vector expression and recombinant product delivery. Here, I show that the increase in Cas9 expression can also be accomplished by *HEN1*-editing mediated effects on the RNA silencing system. In plants with edited *HEN1*, p33 was able to accumulate despite a lack of functional P19. Therefore, HEN1 must contribute to anti-TBSV defense in *N. benthamiana* and P19 is capable of preventing HEN1-mediated methylation and stabilization of siRNAs.

While this research showed the connection between HEN1 and TBSV defense, a variety of other RNA silencing components, such as DCLs and DRBs, require similar studies to determine their role in TBSV defense, and if they are conserved throughout non-model plant species. Towards this, preliminary evidence from laboratory colleagues (M. R. Mendoza, personal communication) showed that p33-mediated delivery of specific AGO- or DCL-targeting sgRNAs resulted in less antiviral silencing in *N. benthamiana* as evidenced by elevated p33 accumulation. Therefore, the TBSV-mediated gene editing system that I helped to develop appears to be useful in delineating specific homologs of RNA silencing machinery involved in viral defense.

MATERIALS AND METHODS

Design of constructs

The sgRNA sequence was determined from *HEN1* in *Arabidopsis* and *N. benthamiana* using gene sequence alignment by M. R. Mendoza (**Figure 3.6**). Q5 polymerase mutagenesis was used to insert sgHEN1 into the construction vector, and heat shock was used to transform the vector into the *E. coli* competent cell line 5α (New England BioLabs). After antibiotic selection, vector insertion was confirmed using colony PCR and sequencing. Plasmid DNA was isolated from colonies containing the correct insert and used for Gibson Assembly cloning into p31 and p33 viral vectors. Heat shock was used to transform the vectors into *E. coli* competent cell line 5α. After antibiotic selection, colony PCR and sequencing was used to confirm correct insert size, plasmid DNA was isolated and used for transformation into *Agrobacterium* strain pGV3101 via electroporation.

sgHEN1 5′→3′
5′-GCTCTGAAGTGACCGCTAAGGTTTTAGAGCTAGAAATAGCAAGTTAAAAT
AAGGCTAGTCCGGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT-3′

Figure 3.6. Sequence of sgHEN1. Sequence of the sgRNA targeting the antisense *HEN1* in *N. benthamiana*. Red letters indicate sequence specific to *HEN1*. Black letters indicate sgRNA backbone.

Plant infection and analysis

Agrobacterium cultures harboring the constructs were infiltrated into four week old *N. benthamiana* as described in chapter I. At 7 dpi, 50 mg of infiltrated leaf tissue was collected and stored at -50˚C until processing. Whole genome DNA was extracted using the GeneCatch Plant Genomic DNA Miniprep Kit (Epoch) according to the manufacturer's instructions. The region of interest, including the targeted *HEN1* gene was PCR amplified and visualized on an ethidium bromide-stained agarose gel. Bands were gel extracted using the Zymoclean Gel DNA Recovery kit (Zymo Research) and cloned into the pGEM-T Easy vector system (Promega) for sequencing. Also, at 7 dpi, 100 mg of infiltrated leaf tissue was collected and used for protein extraction and western blot analysis as described in chapter I.

CHAPTER IV

CONCLUSION

SUMMARY

Since the development of the CRISPR/Cas9 gene editing system, it has been popularized in multiple biological systems due to the inherent ease of design and specificity for the creation of targeted knockouts (Jinek et al. 2012; Cong et al 2013; Ran et al. 2013). While plant biologists have exploited this system or the creation of plant lines with gene insertions and knock outs, rarely has it been utilized as a transient screening tool for functional genetics as it has been in other research organisms (Schiml et al. 2014; Baltes et al. 2014; Cody et al. 2017). A rapid, transient system for the assay of native gene function through the induction of indels resulting in transcriptional repression or knockdown is a powerful tool for the plant community that warrants further development. Furthermore, while limited previous work has utilized similar tools, the system's utility as a transient screening system for pathogen-host interaction studies should not be overlooked, particularly in regard to virus-host interactions, which can be accomplished, as I have detailed in this work, using virus-based expression vectors (Cody et al. 2017).

Several advancements are detailed in this study, including demonstration of the flexibility and functionality of virus-based molecular tools for rapid targeted gene editing in genetically diverse *Nicotiana* species. This single set of streamlined tools produced editing comparable to previous studies (Cody et al. 2017), and substantially higher than many (Metje-Sprink et al. 2019), in a diversity of plant genomes after only seven days. Using P19 as a suppressor, delivery vectors were able to express more rapidly and robustly, and gene editing at increased or

approximately doubled. Furthermore, here, I present a novel model organism for genetics and genomics, *N. attenuata*, as a genetically superior *Nicotiana* alternative to *N. benthamiana* for plant virus studies.

Other advancements include the implementation of a TBSV-based vector to deliver CRISPR/Cas9 gene editing materials, and utilizing this tool for studying virus-specific components of the critical RNA silencing system. With a characteristically broad host range and robust suppression of plant defense, TBSV represents a powerful candidate as a backbone of a viral vector for the delivery of recombinant gene editing products. Additionally, TBSV-based viral vectors exist with non-functioning P19 suppressors (Shamekova et al. 2014). As demonstrated here, this presents the opportunity to study specific components of the viral-induced RNA silencing system, such as HEN1, which this work found to be involved in defense against TBSV infection. Furthermore, targeting *HEN1*, therefore depressing host RNA silencing capabilities, resulted in higher product expression from the binary delivery plasmid pHcoCas9.

FUTURE STUDIES

While this research presented viral-based molecular tools capable of rapid transient gene editing in genetically diverse *Nicotiana* species, the organisms used in this study remain of the same genus, and were utilized partially as proof-of-principle. Molecular tools such as these could be further developed to combine the rapid and efficient qualities of viral vectors with the design simplicity and specificity of CRISPR/Cas9 in a variety of other plant species, both model and non-model. Particularly when targeting a conserved gene, a single streamlined set of transient tools could be implemented in a variety of species, including crop plants, for studies in functional genetics, pathogen-host relationships, molecular interactions, parallel gene function,

or as a screening tool capable of circumventing traditional issues involved with editing a gene, such as developmental and cellular complications. Additionally, perhaps considering and evaluating "non-model" species, such as was done in this study, could have value and implications more often than one may expect.

As detailed in previous chapters, the RNA silencing system plays a critical role in a variety of functions throughout the cell, and many of its components are multifunctional and redundant. While I demonstrated that HEN1 plays a part in TBSV defense, the roles of other RNA silencing machinery in virus defense remain unknown, such as the multiple AGOs, DCLs and DRBs. However, creating transgenic knockdowns or knockouts of components such as these, which are critical for a variety of necessary cellular processes, could be detrimental to the plant, especially during development. Fortunately, as detailed here, virus-based transient tools exist. I propose the use of tools, such as TBSV-based CRISPR/Cas9 delivery tools implemented here, for the rapid screening of RNA silencing components and their interactions in viral infection, such as during TBSV infection. This toolbox is currently being employed by our laboratory to study the functionality and importance of other components of the RNA silencing system in *N. benthamiana*, such as AGOs and DCLs, as they relate to viral defense.

Combining TMV- and TBSV-based tools for targeted gene editing

In chapter I, I presented possible drawbacks of utilizing viral vectors for CRISPR/Cas9 delivery, such as superinfection exclusion and foreign product size limits, which could represent limitations in utility, depending on the nature of the experiment. Here, I present a system of virus-based tools I am currently implementing to circumvent issues like these, with the ultimate goal of expanding on and diversifying the virus-based toolbox for gene editing.

Superinfection exclusion is a phenomenon wherein the ability of an established or co-infected virus to interfere with a secondary infection by the same or a closely related virus. This mechanism has been documented in a variety of biological systems, including bacteria, animals, and plants (Adams et al. 1985; Bratt and Rubin 1968; Fulton 1978; Wildum et al. 2006; Folimonova 2012). This occurrence is the functional basis behind cross protection, a method that has been employed to protect plants from a pathogenic virus by pre-infecting them with similar, but milder strains of the same virus (Loceq et al. 1991). Evolutionarily, superinfection exclusion functions as a powerful driver of viral genetic population, and protects the existing virus from competition presented by a secondary virus. While the current understanding of this phenomenon is remarkably lacking, many studies in plants have implicated the RNA silencing pathway, hijacked by the primary virus to preferentially target the secondary competing virus, a method systemically spread throughout the host by RNA silencing long distance signals (Ratcliff et al. 1999; Voinnet 2001; Voinnet 2005; Folominova 2012).

In previous chapters, I have reviewed and demonstrated the utility and possibilities of utilizing viral vectors for heterologous product delivery. However, a single viral vector was deployed in any given experiment, while other foreign products, such as the Cas9 protein or the P19 suppressor protein, were deployed via non-viral binary delivery vectors. Depending on the purpose, it may be desirable to express multiple heterologous products in a single cell simultaneously. Due to superinfection exclusion, in order to utilize multiple viral vectors at once, one is limited by the relatedness of the virus backbones. For example, two TMV-based vectors could not be used to deliver different heterologous products, such as each carrying one of the two components of CRISPR/Cas9. Upon infection, the two vectors would compete, with one viral population thriving while the other struggled. Previously, our laboratory established TMV and

TBSV as noncompeting viruses, and demonstrated their ability to deliver two different recombinant reporter proteins simultaneously to a single cell (Mendoza et al. 2017). Currently, I am beginning to implement a system to utilize two noncompeting viral vectors, TMV-based TRBO-G and TBSV-based p31, to deliver both components of the CRISPR/Cas9 gene editing platform. This system utilizes a TRBO-G vector capable of delivering Cas9, currently the largest heterologous payload successfully inserted into a viral vector (Chiong 2018), and p31 carrying the sgAGO2, a component of the viral-induced RNA silencing system, which has been previously successfully targeted by hairpin knockdown technology (Odokonyero et al. 2012). Using anti-Cas9 western blot analysis to detect product delivery from the TMV-based vector and visualization of the GFP reporter contained in the TBSV-based vector p31, I have confirmed recombinant product delivery from vectors containing both components of CRISPR/Cas9. Future experimentation will focus on confirming targeted gene editing of *AGO2*. This set of virus-based tools has the potential to circumvent limitations to recombinant payload size deliverable via viral vector and allow multiple viral vectors to be utilized simultaneously.

Satellite viruses as a molecular tool for systemic sgRNA delivery

Although viruses are in themselves obligate cellular parasites, multiple viruses, including TMV, PMV, and Tobacco necrosis virus, are associated with even smaller molecular parasites termed satellite viruses (Hu et al. 2009). Satellite viruses lack an autonomous replicase system, and therefore cannot replicate in the absence of their helper virus, to which they are evolutionarily unrelated, but can co-evolve upon helper virus encounter (Yassi and Dodds 1998). Additionally, satellite viruses are capable of negatively or positively altering host symptoms normally produced by the helper virus, be commensal, or even beneficial (Hu et al. 2009). TMV

can be associated with and facilitate systemic replication of satellite TMV, or STMV, a 1 kb ssRNA icosahedral virus (Dodds 1998).

As previously discussed, a downfall of viral vectors are the foreign product size limitations, which previously have been overcome by replacing dispensable genes, such as the CP, exemplified in previous chapters, with recombinant payloads of interest. However, the CP is required for systemic movement throughout the host, and therefore removal of the CP would result in solely localized viral replication and product delivery in the leaf where it was introduced. While this characteristic may be desirable for many applications, as detailed in previous chapters, systemic product delivery may be attractive in certain scenarios. Currently, I am developing a system to exploit STMV as a tool for systemic recombinant product delivery when combined with a systemically replicating TMV-based viral vector. This system utilizes a viral vector with a TMV backbone that has retained the native CP, but also expresses a GFP reporter protein, and is therefore capable of systemic movement associated with systemic GFP expression (Lindbo 2007). I have created an STMV-based viral vector containing the previously described sgPDS3 (STMVgPDS3). Green fluorescence has been used to confirm movement of the TMV helper viral vector, and by association, STMVgPDS3. Future experimentation is required for confirmation of systemic STMVgPDS3 co-replication, expression of the heterologous sgRNA, and to confirm targeted editing of PDS3. Previously, no satellite viruses have been developed as delivery platforms. This STMV-based tool has the potential to be utilized for delivery of CRISPR/Cas9 components without sacrificing the opportunity for transient, in vivo systemic gene editing.

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